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Suppression of hepatitis B viral gene expression by protein-tyrosine phosphatase PTPN3

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Summary

Protein-tyrosine phosphatase PTPN3 is a membrane-associated non-receptor protein-tyrosine phosphatase. PTPN3 contains a N-terminal FERM domain, a middle PDZ domain, and a C-terminal phosphatase domain. Upon co-expression of PTPN3, the level of human hepatitis B viral (HBV) RNAs, 3.5 kb, 2.4/2.1 kb, and 0.7 kb transcribed from a replicating HBV expression plasmid is significantly reduced in human hepatoma HuH-7 cells. When the expression of endogenous PTPN3 protein is diminished by specific small interfering RNA, the expression of HBV genes is enhanced, indicating that the endogenous PTPN3 indeed plays a suppressive role on HBV gene expression. PTPN3 can interact with HBV core protein. The interaction is mediated via the PDZ domain of PTPN3 and the carboxyl-terminal last four amino acids of core. Either deletion of PDZ domain of PTPN3 or substitution of PDZ ligand in core has no effect on PTPN3-mediated suppression. These results clearly show that the interaction of PTPN3 with core is not required for PTPN3 suppressive effect. Mutation of ^{359}serine and ^{835}serine of 14-3-3β binding sites to alanine, which slightly reduces the interaction with 14-3-3β, does not influence the PTPN3 effect. In contrast, mutation of the invariant ^{842}cysteine residue in phosphatase domain to serine, which makes the phosphatase activity inactive, does not change its subcellular localization and interaction with core or 14-3-3β, but completely abolishes PTPN3-mediated suppression. Furthermore, deletion of FERM domain does not affect the phosphatase activity or interaction with 14-3-3β, but changes the subcellular localization from cytoskeleton-membrane interface to cytoplasm and nucleus, abolishes binding to core, and diminishes the PTPN3 effect on HBV gene expression. Taken together, these results demonstrate that the phosphatase activity and FERM domain of PTPN3 are essential for its suppression of HBV gene expression.

Introduction

Protein-tyrosine phosphatases (PTPs) play vital roles in numerous cellular processes including differentiation, cell activation, cell-cycle progression, apoptosis, cytoskeletal rearrangement and cell movement, cell–matrix adhesion, cell–cell adhesion, and metabolic homeostasis, and are implicated in a growing number of human diseases, ranging from cancer to cardiovascular, neurological, metabolic, immunological, and infectious diseases [1–5]. Protein-tyrosine phosphatase PTPN3 (also named PTPH1) belongs to a family

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of membrane-associated non-receptor PTPs characterized by containing a FERM (four point-1, ezrin, radixin and moesin) domain, which directs proteins at the interface between cell membrane and cytoskeleton [6]. Consistent with possessing FERM domain, PTPN3 has been shown to be cytosolic, but clearly enriched at or near the plasma membrane [7]. In addition to N-terminal FERM domain, PTPN3 contains a middle PDZ domain, and a C-terminal phosphatase domain [8, 9]. PDZ domain, which is a globular 80–100 amino acid module, consists of six beta-strands and two alpha-helices and is involved in protein–protein interaction. Most of the PDZ-mediated interactions occur through recognition of short carboxyl-terminal peptide motif. Recognition is achieved through an extended binding groove that terminates in a conserved carboxylate binding loop. Alternatively, some PDZ domains interact with internal motifs if presented within a secondary structure that is sterically compatible with the PDZ binding groove. PDZ domain can also interact with other PDZ domain or lipid [10–12]. Phosphatase domain is a conserved catalytic domain that is 240–250 amino acid residues in length and contains the signature motif HCXAGXXR. The invariant cysteine residue in the phosphatase domain is essential for phosphatase activity and functions in nucleophilic attack upon the phosphate group of the substrate [13]. Mutations of PTPN3 have been found in different types of cancers, suggesting that it may be a tumor suppressor gene [5, 14].

Infection of human hepatitis B virus (HBV), which can cause both acute and chronic hepatitis, is a major health problem worldwide. A significant percentage of patients with chronic HBV infection will develop liver cirrhosis and, most dreadfully, primary hepatocellular carcinoma [15]. HBV is a small enveloped DNA virus with a partially double-stranded 3.2-kb genome. Two 3.5-kb precore and pregenomic RNAs, 2.4- and 2.1-kb surface RNAs, 0.7-kb X RNA, and splicing RNAs are produced in viral replication. The 3.5-kb pregenomic RNA serves as mRNA that encodes the core and polymerase proteins. It can also be packaged into core particles along with viral polymerase and serve as the template for viral DNA synthesis [16, 17]. The 21-kDa core protein contains the N-terminal structural domain and C-terminal regulatory domain. The former directs the core particle assembly, whereas the latter mediates the binding of viral RNA and DNA [18, 19].

In this report, we find that PTPN3 suppresses the HBV gene expression. Although PTPN3 interacts with HBV core protein, this interaction is not essential for PTPN3-mediated suppression. In consistence, the PDZ domain, which is responsible for interaction of PTPN3 with core, is not essential for PTPN3-suppressive effect. On the contrary, the PTPase activity and FERM domain of PTPN3 is essential for its suppression of HBV gene expression.

Materials and methods

Vectors, reagents and antibodies

Vector pSilencer^{TM}3.1-H1hygro was purchased from Ambion. Vector pGEX-3X, Ready-to-Go^{TM} kit, [^{35}S]methionine (1000 Ci/mmol), Rediprime^{TM} II random prime labeling system, Hybond ECL nitrocellulose paper for Western, GFX^{TM} PCR DNA and gel band purification kit were from Amersham. Antibody against HBV core protein was from Dako. HYB-9 hybridization solution was from Gentra. Fluorescein isothiocyanate-conjugated goat anti-mouse IgG and Anti-mouse IgG-TRITC conjugated secondary antibody were from Jackson. TRI reagent was from Molecular research center. Immobilon^{TM}-Ny^{+} transfer membrane was from Millipore. GeneJuice transfection reagent was from Novagen. High range prestained protein marker was from Oncogene. Western Lightning Chemiluminescence Reagent Plus was from PerkinElmer. jetPEI^{TM} transfection reagent was from Polyplus. TNT-quick coupled transcription/translation system, tyrosine phosphatase assay system, and horseradish peroxidase-conjugated anti-mouse and anti-rabbit IgG were from Promega. Complete^{TM} protein inhibitor cocktail, antibody against HA, and Expand^{TM} high fidelity PCR system were from Roche. Antibody against 14-3-3β was from Santa Cruz. Vector pCMV2-FLAG, *n*-propyl gallate, hoechst H33258, glutathione-conjugated agarose beads, M2 beads, and antibody against FLAG were from Sigma. pCMV, pCMV-GST, pCMV-HA, pGEM-HA, and antibody against GST were generated by our laboratory [20].

Expression plasmids

For bacterial expression, the 119–185 (2/5C) of core protein was fused downstream of a GST tag of a pGEX-3X vector. For *in vitro*-transcription and translation reaction, the PDZ domain of PTPN3 was inserted downstream of an influenza viral hemagglutinin (HA) epitope in a pGEM-HA vector, respectively. For expression in mammalian cells, a full-length core protein (C), amino acid 31–185 (9/10C), 119–185 (2/5C), and 144–185 (1/5C) of core protein, respectively, were fused downstream of a GST tag of a pCMV-GST vector as previously described [20, 21]. The sequence of the most C-terminal four amino acids of 2/5C was substituted from ESQC to GNSIVTD in GST-2/5Cs4. A full-length core protein (C) was fused downstream of CMV promoter of a pCMV plasmid to express the full-length core without tag. The C-terminal ESQC residues of intact core protein were substituted with EAQK in CoreΔPDZL. The same substitution was in core of an expression plasmid pHBV3.6 [22] to generate pHBV3.6ΔPDZL. The full-length PTPN3 was fused down of an HA tag of a pCMV-HA vector and an FLAG tag of pCMV2–FLAG. The PDZ domain of PTPN3 was fused down of an HA tag of a pCMV-HA vector to express HA-PDZ. Expression plasmids of FLAG-PTPN3 mutants were generated by PCR.

Cell culture and transfection

Human embryonic kidney 293T (HEK293T) cells and human hepatoma HuH-7 cells were cultured and transfected with plasmid DNA as previously described [20, 21].

Northern blot analysis

Total cellular RNA were prepared from transfected HuH-7 cells with TRI reagent. Northern blot analysis was performed as previously described [23] using 1.9-kb *Pst*I fragment of HBV DNA or 1-kb *Eco*RI fragment of GAPDH as probe.

Reverse transcriptase-PCR

The Ready-to-Go™ kit was used to measure the expression of PTPN3 and FLAG-PTPN3 mRNAs in HuH-7 cells as previously described [21]. PCR primers used for analyses were the following: PTPN3 (NM_002829.2), forward primer corresponding to nt 937–958: 5′-ATACGTTCTTT CAGGCAAAGAA-3′ (22 mer); reverse primer corresponding to nt 1542–1521: 5′-AG-CTGTCACCATTATCATTCTT-3′ (22 mer); RT-PCR product: 606 bps. FLAG-PTPN3, forward primer corresponding to FLAG sequence: 5′-ATGGACTACAAAGACGATGACGAC-3′ (24 mer); reverse primer corresponding to nt 557–537 of PTPN3: 5′-GGGTATAAAGTGACTATCG-GA (21 mer); RT-PCR product: 612 bps. β-actin, forward primer: 5′-TGACGGGGGTCACCCA-CACTGTGCCCATCTA-3′ (31 mer); reverse primer: 5′-CTAGAAGCATTTGCGGTGGACGAT GGAGGG-3′ (30 mer); RT-PCR product: 661 bps.

RNA interference technique and selection of stable transfectants

For expression of PTPN3-specific siRNA#1 and #2, corresponding to nt 382–400 and nt 2697–2715 of PTPN3, the synthesized primers 5′-GATCCC-CAACAAACCAGGCACTTGTATTCAAGAG ATACAAGTGCCTGGTTTGTTTTTTTGGAA A-3′ and 5′-AGCTTTTCCAAAAAAACAAAC-CAGGCACTTGTATCTCTTGAATACAAGTG CCTGGTTTGTTGGG-3′ for siRNA#1, and 5′-GATCCCCAAGTTTGTGTGTGAAGCGATT-CAAGAGATCGCTTCACACACAAACTTTTT TTGGAAA-3′ and 5′-AGCTTTTCCAAAAAAA GTTTGTGTGTGAAGCGATCTCTTGAATCG CTTCACACACAAACTTGGG-3′ for siRNA#2, respectively, were annealed and cloned downstream of H1 promoter of pSilencer™3.1-H1hygro vector. After transfection of HuH-7 cells with siRNA#2 expression plasmid, stable transfectants were selected and cloned by medium containing 50 μg/ml hygromycin. Fifteen hygromysin-resistant stable transfectants were obtained. For transient transfection of HuH-7 cells with siRNA#2 and HBV expression plasmids, 1.5×10^6 HuH-7 cells in 6-well plate were transfected with 0.6 μg of pHBV3.6 plus different amount of siRNA#2 expression plasmid (0, 0.6, 1.2, or 2.4 μg) and of RNAi vector (2.4, 1.8, 1.2, or 0 μg) using jetPEI™ transfection reagent for 24 h according to manufacturer's procedure.

GST pulldown, immunoprecipitation and Western blot

Recombinant glutathione *S*-transferase (GST) fusion proteins were prepared, pulled down with glutathione-conjugated agarose beads, and immunoprecipitated with anti-FLAG M2 beads as previously described [20, 21]. Western blots were performed as previously described [20, 21].

In vitro interaction

In vitro-transcription and translation reactions were performed with TNT-quick coupled transcription/translation system in the presence of [^{35}S]methionine as previously described [20, 21].

In vitro phosphatase activity

Transfected HEK293T cells in 10-cm dish were lysed by phosphate-free 1% NP-40 lysis buffer and immunoprecipitated with anti-FLAG M2 beads. Beads were washed three times with phosphate-free 1% NP-40 lysis buffer, and resuspended in the same buffer. Lysates were used for *in vitro* phosphatase activity assay according to manufacturer's recommendation and Western blot.

Indirect immunofluorescence and confocal microscopy

HuH-7 cells were cultured in 2.2 cm × 2.2 cm cover glasses (or cover slips) coated with poly-L-lysine 18–24 h, and transfected expression plasmids with GeneJuice transfection reagent for 6 h. About 24–36 h posttransfection, cells were washed with PBS, fixed with 3.7% formaldehyde in PBS for 20 min at room temperature (RT), and permeabilized by cold acetone for 3 min followed by washing with PBS. The permeabilized cells were incubated with BSA (5 mg/ml) at RT for 1 h and detected by antibody against FLAG for 1 h at RT and then with fluorescein isothiocyanate-conjugated goat anti-mouse IgG for 1 h at RT. Hoechst 33258 was used to stain nucleus as the counter stain. Cover glasses (or cover slips) were mounted using 20 mM *n*-propylgallete in 80% glycerol in PBS and visualized by confocal microscopy.

Results

Effect of PTPN3 expression on HBV gene expression

We have previously identified a partial segment of protein-tyrosine phosphatase PTPN3 to interact with the C-terminal portion of HBV core protein by a yeast two-hybrid system. To address the possible role of PTPN3 in regulating HBV gene expression, we examine the effect of overexpressing PTPN3 on the level of HBV RNAs. A construct containing more than a unit length of HBV genome, pHBV3.6, was transiently transfected into human hepatoma HuH-7 cells with the FLAG-tagged full-length PTPN3 expression plasmid. Viral gene expression closely mimics viral infection *in vivo* after transfection [22]. The amount of the HBV RNAs was measured by Northern blotting analysis, using GAPDH as a loading control (Figure 1). The 3.5-kb RNAs are precore and pregenomic

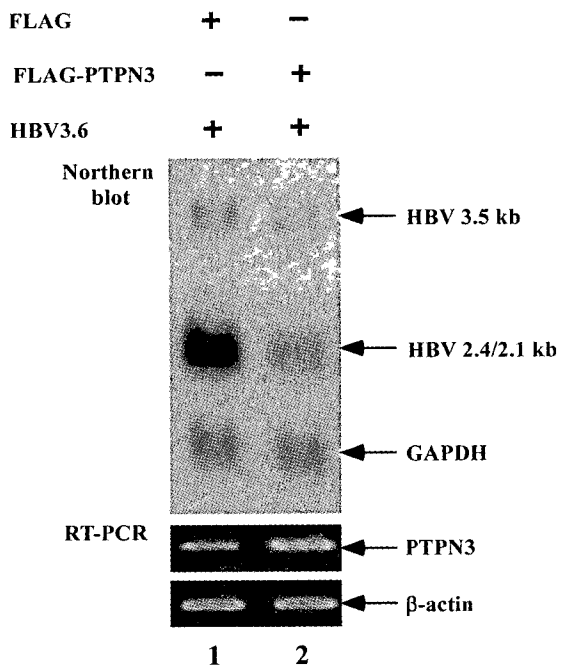

Figure 1. Effect of PTPN3 on HBV gene expression. HuH-7 cells were transiently cotransfected pHBV3.6 with pFLAG-PTPN3 or pFLAG-CMV2. Total RNA was harvested for expression of HBV RNAs detected by Northern blot analysis, using GAPDH as a control. The combined expression of endogenous PTPN3 and overexpressed FLAG-PTPN3 RNAs in total RNA was analyzed by RT-PCR, using β-actin as a control.

RNAs. The 2.4/2.1-kb RNAs include the 2.4-kb large surface, 2.1-kb middle and major surface, as well as some splicing RNAs. The amount of endogenous PTPN3 and FLAG-PTPN3 RNAs was measured by RT-PCR, using β-actin as a loading control. Overexpression of PTPN3 resulted in a dramatic reduction in the level of HBV RNAs, demonstrating the suppression of HBV gene expression by PTPN3.

Enhancement of HBV gene expression by silencing endogenous PTPN3

To further determine the role of endogenous PTPN3 on HBV expression, two expression plasmids which overexpress PTPN3-specific siRNA#1 and siRNA#2 (Figure 2A) by histone H1 promoter were constructed. These plasmids were cotransfected with FLAG-PTPN3 expression plasmid into human embryonic kidney 293T (HEK293T) cells. The expression of FLAG-PTPN3 was detected by immunoprecipitation with anti-FLAG antibody M2 beads followed by Western blot using the anti-FLAG antibody. The expression of FLAG-PTPN3 was greatly reduced by siRNA#2, but only slightly reduced by siRNA#1 (Figure 2B). HuH-7 cells were transiently cotransfected pHBV3.6 with different amount of siRNA#2 expression plasmid. Level of HBV RNAs increased with the increasing amount of siRNA#2 expression plasmid, suggesting that the silencing of endogenous PTPN3 expression enhanced the HBV gene expression (Figure 2C, D). Stable HuH-7 transfectants which overexpress siRNA#2 were selected and cloned. Expression of endogenous PTPN3 RNA of 15 stable transfectants was detected by RT-PCR. Stable transfectants #1 and #20 expressed much lower level of endogenous PTPN3 (Figure 2E). Level of HBV RNAs was much higher in stable transfectants #1 and #20 compared to parental HuH-7 cells, suggesting that the HBV RNA level was enhanced when the endogenous PTPN3 expression was reduced (Figure 2F). PTPN3 overexpression in stable transfectants #1 and #20 led to a dramatic reduction in the level of HBV RNAs (Figure 2G). Taken together, these results clearly demonstrate that the endogenous PTPN3 plays a suppressive role in the HBV gene expression.

Interaction between PTPN3 and HBV core protein

One possible mechanism for PTPN3-mediated suppression of HBV gene expression is that PTPN3 interacts with HBV core protein. To determine whether PTPN3 interacts with HBV core, the HA-tagged full-length PTPN3 (HA-PTPN3) was overexpressed in HEK293T cells together with the GST-tagged full-length core (GST-C). The GST-C protein was bound to glutathione-beads, and its ability to pull down HA-PTPN3 was determined by Western blot using the anti-HA antibody (Figure 3A, lane 3, top panel). The expression of GST-C in the pull-down (middle panel) and HA-PTPN3 in the total lysates (bottom panel) was analyzed by Western blot using the anti-GST and the anti-HA antibodies, respectively. HA-PTPN3 indeed bound to GST-C. Their interaction was further confirmed by coimmunoprecipitation experiments. Cell lysates from 293T cells cotransfected with FLAG-tagged full-length PTPN3 (FLAG-PTPN3) and the full-length core without tag were immunoprecipitated with anti-FLAG antibody M2 beads for FLAG-PTPN3, and anti-core antibody was used to detect core in the immunoprecipitated complex by Western blot (Figure 3B, lane 2, 1st panel). The expression of FLAG-PTPN3 in the immunoprecipitated product (3rd panel) and core in the whole cell extract (4th panel) was analyzed by Western blot using the anti-FLAG and anti-core antibodies, respectively. PTPN3 has previously been shown to interact with the chaperone protein 14-3-3β [24]. Interaction between FLAG-PTPN3 and 14-3-3β was indeed observed by immunoprecipitation with anti-FLAG antibody M2 beads followed by Western blot using the anti-14-3-3β antibody (2nd panel). The expression of 14-3-3β in the whole cell extract (5th panel) was analyzed by Western blot using the anti-14-3-3β antibody.

PTPN3 contains a PDZ domain, which is generally involved in protein–protein interaction. To test if the PDZ domain of PTPN3 is responsible for binding to core, the HA-tagged PDZ was co-expressed with GST-core. HA-PDZ could indeed interact with GST-C (Figure 3C, lane 6). To further confirm that the PDZ domain of PTPN3 is responsible for binding to core, FLAG-PTPN3 with deletion of the PDZ domain, FLAG-PTPN3ΔPDZ, was overexpressed with core. As wild type FLAG-PTPN3 (Figure 4, 1st

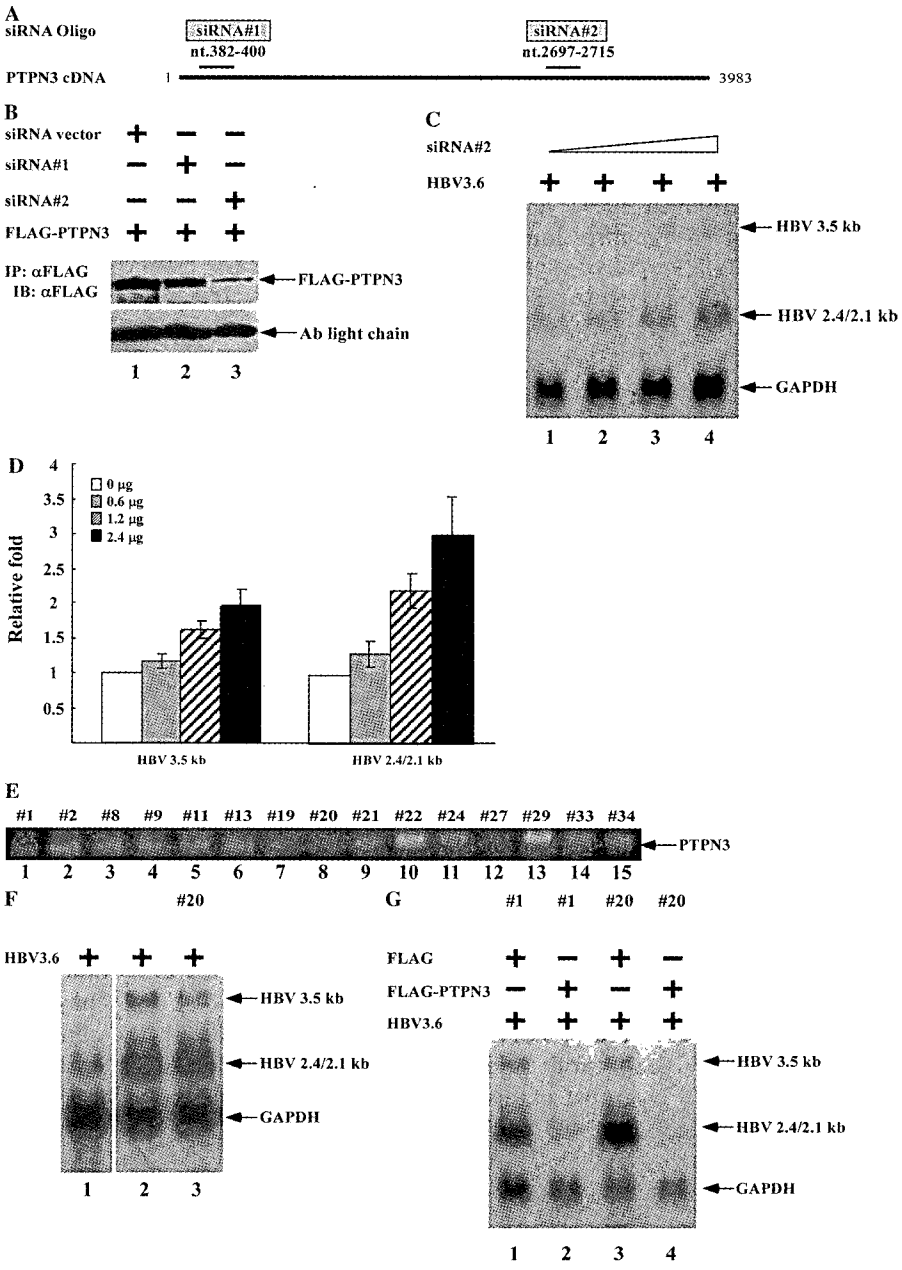

Figure 2. Role of endogenous PTPN3 on HBV gene expression. (A) Depiction of PTPN3 cDNA and position of two PTPN3-specific siRNAs. (B) Effect of PTPN3-specific siRNAs on expression of FLAG-PTPN3 protein. HEK293T cells were cotransfected pFLAG-PTPN3 with pPTPN3siRNA#1, pPTPN3siRNA#2 or empty vector for 72 h. Expression of FLAG-PTPN3 protein was detected by immunoprecipitation with anti-FLAG antibody M2 beads followed by Western blot using anti-FLAG antibody, using antibody light chain as control. (C, D) Effect of increasing amount of PTPN3-specific siRNA#2 on level of HBV RNAs. HuH-7 cells were cotransfected with pHBV3.6 and increasing amount (0, 0.6, 1.2, and 2.4 μg) of pPTPN3siRNA#2 for 24 h. Amount of HBV RNAs was detected by Northern blot, using GAPDH as control. Representative Northern blot and relative fold suppression of HBV 3.5-kb and 2.4/2.1-kb RNAs as means ± standard deviation of four independent experiments are shown in (C) and (D). (E) Expression of endogenous PTPN3 RNA in stable transfectants of PTPN3-specific siRNA#2. HuH-7 cells were transfected with pPTPN3siRNA#2. The expression of endogenous PTPN3 of 15 stable transfectants was examined by RT-PCR. (F) Amount of HBV RNAs in stable transfectants detected by Northern blot. Parental HuH-7 (lane 1) and stable transfectants #1 (lane 2) and #20 (lane 3) were transfected with pHBV3.6. (G) Effect of PTPN3 overexpression on HBV RNA level in stable transfectants detected by Northern blot. Stable transfectants #1 (lanes 1 & 2) and #20 (lanes 3 & 4) were cotransfected pHBV3.6 with pFLAG-PTPN3 or empty vector.

panel), the localization of FLAG-PTPN3ΔPDZ was cytosolic, but clearly enriched at or near the plasma membrane, indicating a submembrane localization (Figure 4, 2nd panel). However, its interaction with core was abolished (Figure 3B, lane 3). In contrast, deletion of PDZ domain did not affect its interaction with 14-3-3β. It also did not affect its *in vitro* phosphatase activity (data not shown). These results indicate that the PDZ domain of PTPN3 does not play a role in its subcellular localization, *in vitro* phosphotase activity, and interaction with 14-3-3β, but affects its interaction with core.

To determine which region within core is essential in mediating its interaction with PTPN3, HA-PTPN3 or HA-PDZ was co-expressed with several GST-tagged forms of core. Among them, 9/10C is nearly the full-length core with the deletion of N-terminal 30 amino acids to prevent the formation of core particle, since this region is essential for the assembly of core particle [25]. GST-2/5C and -1/5C contain the C-terminal 67- (amino acid 119–185) and 36-amino acid regions of core (amino acid 150–185), respectively [20, 21]. As shown in Figure 3A and C, the C-terminal 36-amino acid region still contains the essential sequence. Since the PDZ-mediated interactions occur through recognition of short C-terminal peptide motif or internal motif [10–12], we test whether the interaction of core with PTPN3 is through the short C-terminal peptide. Replacing C-terminal ESQC residues of GST-2/5C with seven unrelated amino acids (GST-2/5Cs4) abolished its binding to PDZ domain (Figure 3C, lane 3). Furthermore, substitution of C-terminal ESQC residues of intact core with EAQK (CoreΔPDZL) abolished its interaction with FLAG-PTPN3 (Figure 3D, lane 3).

In vitro-translated, [^{35}S]labeled HA-PDZ or HA was incubated with glutathione bead-bound GST or GST-2/5C fusion protein. HA-PDZ was pulled down by GST-2/5C, but not by GST, demonstrating the *in vitro* interaction between HA-PDZ and GST-2/5C (Figure 3E).

Together, these results demonstrate that the PDZ domain of PTPN3 binds to the carboxyl-terminal last four amino acids of core protein.

Effect of interaction of PTPN3 with HBV core on HBV gene expression

It is interesting to know whether the interaction with core is responsible for PTPN3-mediated suppression of HBV gene expression. Although FLAG-PTPN3ΔPDZ could not interact with core, it still suppressed the level of HBV RNAs. These results suggest that interaction between PTPN3 and core is not essential for this suppression (Figure 5A). HBV expression plasmid HBV3.6ΔPDZL is able to express all HBV RNAs and proteins except the last four amino acids of core were substituted as that in CoreΔPDZL. FLAG-PTPN3 could suppress the level of HBV RNAs produced by HBV3.6ΔPDZL, despite that it could not interact with mutated core made from HBV3.6ΔPDZL (Figure 5B). These results together clearly show that the interaction with core does not play a role in the PTPN3-mediated suppression.

Effect of different domains of PTPN3 on HBV gene expression

Since the interaction of PTPN3 with HBV core does not affect its suppressive effect on HBV gene expression, the role of two novel motifs RSL^{359}SVE and RVD^{835}SEP of PTPN3 is studied. PTPN3 interacts with 14-3-3β in a serine phosphorylation-dependent manner. These two motifs RSL^{359}SVE and RVD^{835}SEP were identified as major 14-3-3β binding sites, although the integrity of PTPN3 is important for its optimal interaction with 14-3-3β [24]. Mutation of ^{359}serine and ^{835}serine to alanine (FLAG-PTPN3-SA1+2) indeed slightly reduced its interaction with 14-3-3β as previously reported, but did not affect its interaction with core (Figure 6A, lane 2). It also did not change its *in vitro* phosphatase activity (Figure 6B, lane 4). This mutant enriched in submembrane (Figure 4, 3rd panel), similar to the wild type FLAG-PTPN3. It also suppressed the HBV gene expression (Figure 6C, lane 4). Therefore, these two serine residues as well as their phosphorylation, and interaction with 14-3-3β do not affect the subcellular localization and *in vitro* phosphatase activity of PTPN3, its interaction with core and its ability to suppress the HBV gene expression.

The invariant ^{842}cysteine residue in PTPN3 phosphatase domain is essential for its phosphatase activity [13]. Mutation of ^{842}cysteine to serine (FLAG-PTPN3-CS) indeed abolished *in vitro* phosphatase activity of PTPN3 (Figure 6B, lane 3). This catalytically inactive mutant

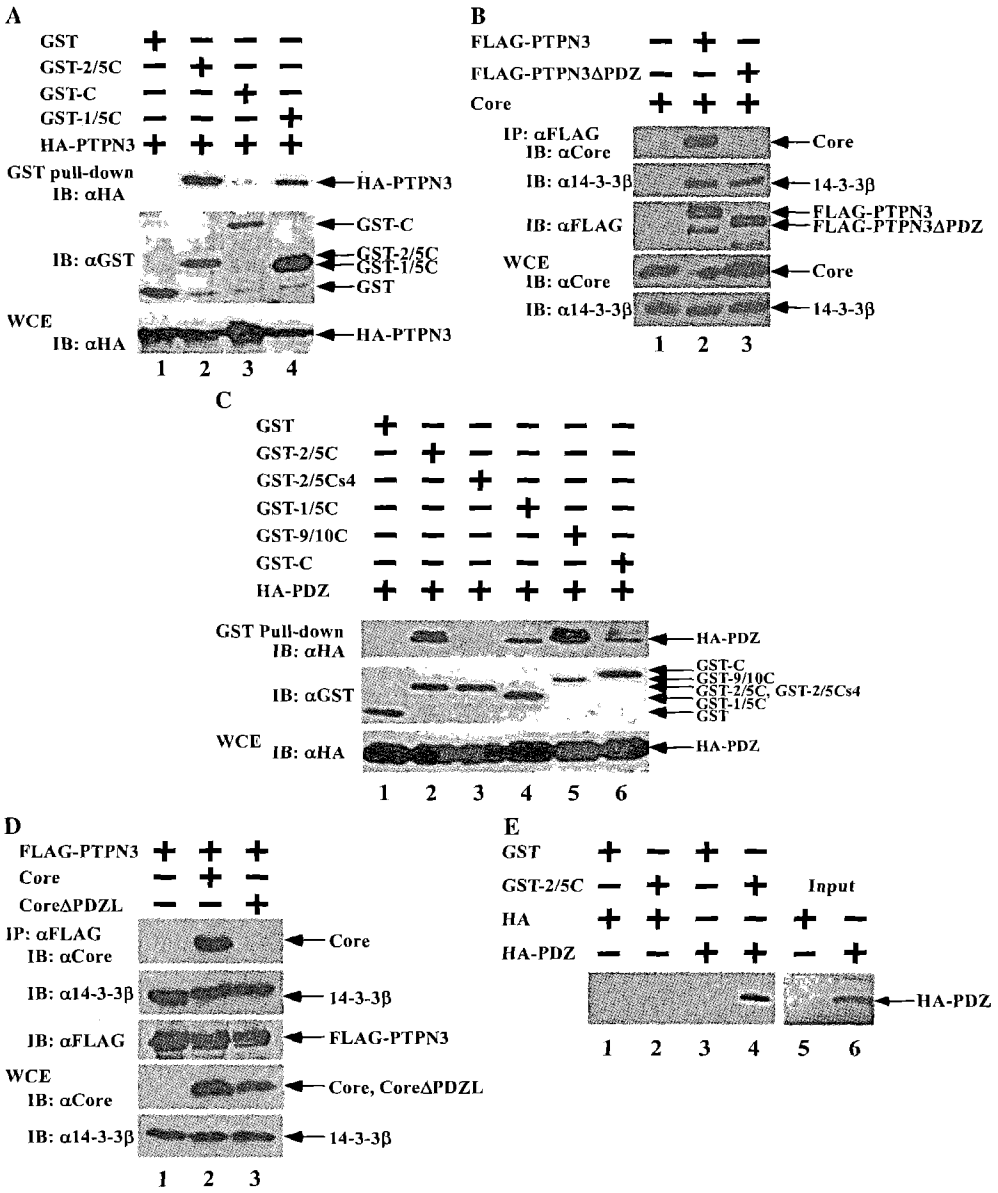

Figure 3. Interaction of core with PTPN3. (A–D) *In vivo* interaction. Pull-down experiments of HEK293T cell lysates coexpressing GST, GST-2/5C, GST-1/5C, GST-2/5Cs4, GST-9/10C, GST-C, Core, or CoreΔPDZL with HA-PTPN3, HA-PDZ, FLAG-PTPN3, or FLAG-PTPN3ΔPDZ were performed with glutathione beads or anti-FLAG antibody M2 beads. Proteins in pull down and in whole cell extracts were analyzed by Western blot with anti-HA, -GST, -core, -FLAG, or –14-3-3β antibody. (E) *In vitro* interaction. *In vitro*-transcribed/translated [^{35}S]methionine-labeled HA or HA-PDZ was incubated with glutathione bead-bound GST or GST-2/5C. After extensive wash, bead-bound proteins were analyzed by autoradiography.

has the same results as catalytically active wild type in interaction with core or 14-3-3β (Figure 6A, lane 3), and in subcellular localization (Figure 4, 4th panel). However, this mutation completely abolished its suppressive effect on HBV gene expression (Figure 6C, lane 3). These results demonstrate that the phosphatase activity of PTPN3 has no effect on its localization and interaction with core or 14-3-3β, but plays an essential role in suppression of HBV gene expression.

Deletion of FERM domain of PTPN3, FLAG-PTPN3ΔFERM, did not affect either its *in vitro* phosphatase activity (data not shown) or interaction

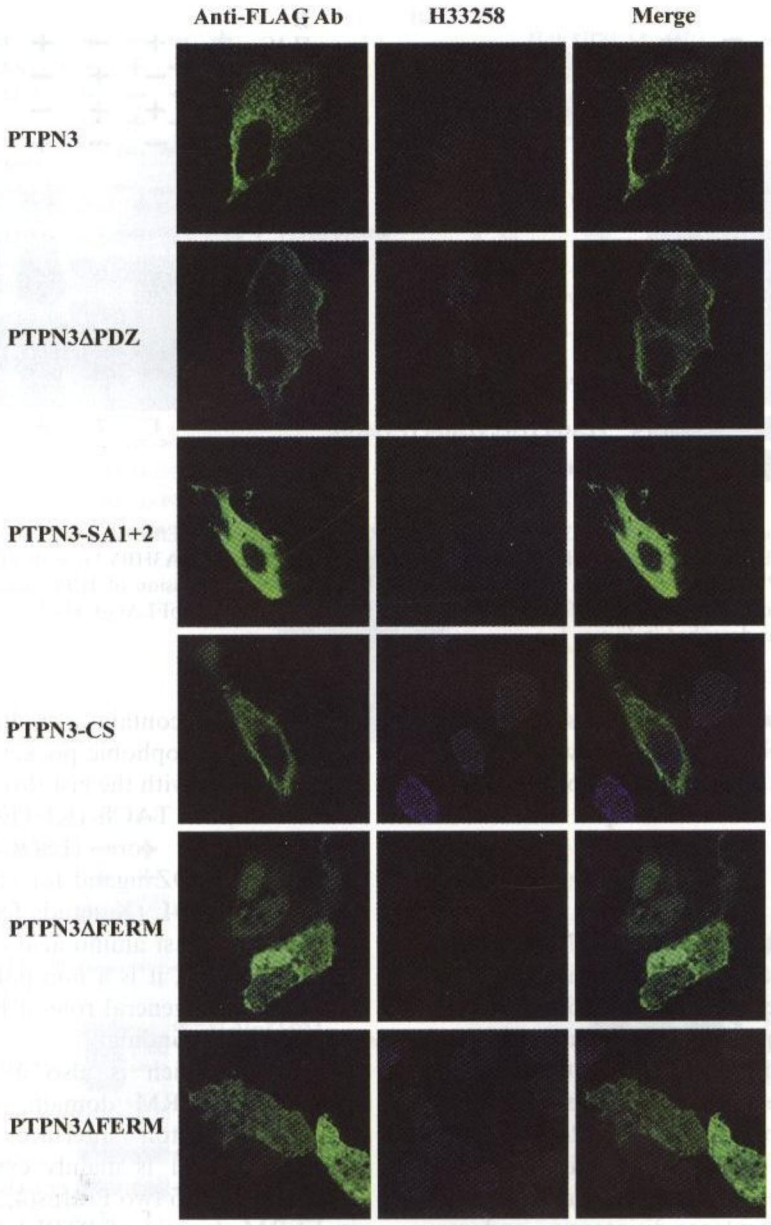

Figure 4. Localization of wild type PTPN3 and its mutants in HuH-7. HuH-7 cells were transiently transfected with FLAG-PTPN3 (1st panel), FLAG-PTPN3ΔPDZ (2nd panel), FLAG-PTPN3-SA1+2 (3rd panel), FLAG-PTPN3-CS (4th panel), and FLAG-PTPN3ΔFERM (5th and 6th panels) expression plasmids, respectively. The localization of PTPN3 wild type and its mutants was detected by anti-FLAG antibody and anti-mouse IgG-FITC conjugated secondary antibody, and analyzed by laser confocal microscopy. Hoechst 33258 was used as a counter stain for nucleus.

with 14-3-3β (Figure 6D). However, its interaction with core was lost. Furthermore, this mutant did not localize in submembrane, which is consistent with the expected role of FERM domain [6, 7]. Surprisingly, in addition to cytoplasm localization, it also localized in nucleus (Figure 4, 5th and 6th panels). Three distribution patterns, much more nucleus distribution than cytoplasm ($N \gg C$), about equal distribution in nucleus and cytoplasm ($N = C$), and more cytoplasm distribution than nucleus ($N < C$), were observed. The percentage of cells with these three patterns was about the same. The PTPN3-suppressive effect was abolished by deletion of FERM domain (Figure 6E, lane 3). Therefore, FERM

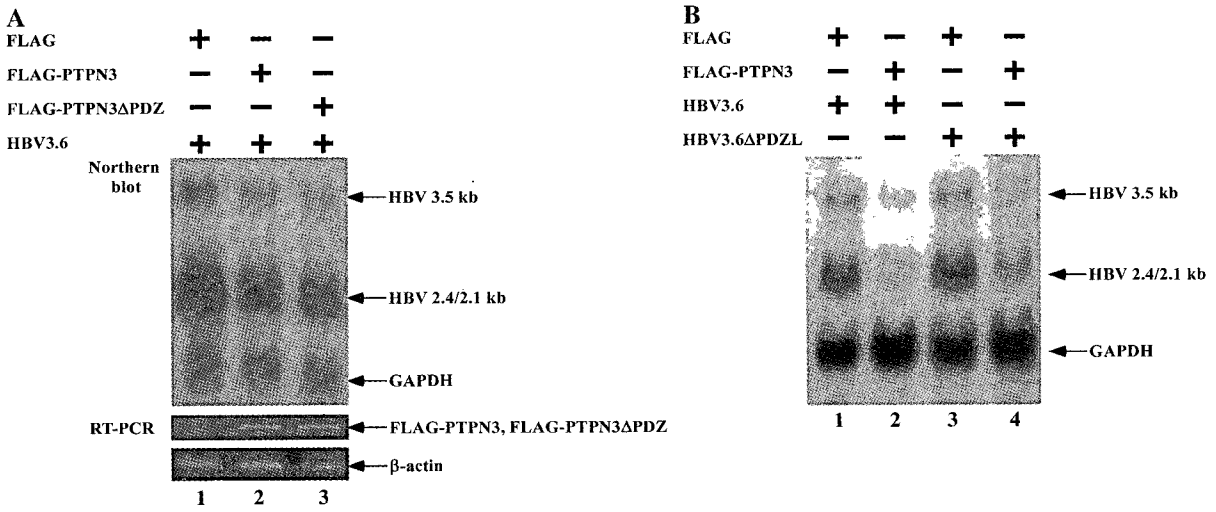

Figure 5. Effect of interaction between PTPN3 and core on HBV RNA expression. (A) Effect of overexpressed PTPN3 without the PDZ domain on HBV RNA expression. HuH-7 cells were transiently cotransfected pA3HBV3.6 with pFLAG-CMV2, pFLAG-PTPN3 or pFLAG-PTPN3ΔPDZ. (B) Effect of overexpressed PTPN3 on RNA expression of HBV lacking the interaction with PTPN3. HuH-7 cells were transiently cotransfected pHBV3.6 or pHBV3.6ΔPDZL with pFLAG-CMV2 or pFLAG-PTPN3. Experiments were performed as described in Figure 1.

domain plays an essential role in the submembrane localization of PTPN3, its interaction with core, and its suppression on HBV gene expression.

Discussion

Protein-tyrosine phosphatase PTPN3 interacts with many cellular and viral proteins. It interacts with the chaperone protein 14-3-3β in a serine phosphorylation-dependent manner. Although two RSL^{359}SVE and RVD^{853}SEP motifs are major 14-3-3β binding sites of PTPN3, the entire PTPN3 is essential for the best interaction [24]. PTPN3 is also able to bind to the cytoplasmic domain of TACE (tumor necrosis factor alpha-convertase), which is a metalloprotease-disintegrin, and may act as a negative regulator of TACE levels and function [26]. Na(v)1.5 is the pore-forming alpha-subunit protein of the cardiac sodium channel and is crucial for the conduction of the cardiac impulse. PTPN3 interacts with Na(v)1.5 and shifts the Na(v)1.5 availability toward hyperpolarized potentials [27]. Recently, human papillomavirus (HPV) E6 oncoprotein has been shown to recruit PTPN3 to E6AP and results in the proteosome dependent degradation of PTPN3 [28]. In this report, we show that PTPN3 interacts with the HBV core protein. PTPN3 through its class I PDZ domain, which contains a cradle of β barrels with a conserved hydrophobic pocket and a buried arginine, interacts with the last three to five C-terminal amino acids of TACE (KETEC), Na(v)1.5, HPV E6 and HBV core (ESQC). The consensus sequence of PDZ ligand for class I PDZ domain is X-T/S-X-V/I/L (X stands for any amino acid). Although the last amino acid of TACE and HBV core is cysteine, it is a non-polar amino acid and still fits in the general role of hydrophobic amino acid for PDZ binding.

PTPL1, which is also a non-receptor PTP containing FERM domain, localizes in membrane-cytoskeleton interface. Without FERM domain, PTPL1 is mainly cytosolic. Binding of PtdIns(4,5)P2 to two PtdIns(4,5)P2 binding motifs in FERM domain of PTPL1 has been shown to play an essential role in its targeting to interface between membrane and cytoskeleton. Both PtdIns(4,5)P2 binding motifs seems to cooperate for proper intracellular targeting of the PTPL1 FERM domain [29]. PTPN3 also contains two PtdIns(4,5)P2 binding motifs [29]. Our result shows that without FERM domain, PTPN3 no longer localizes in submembrane. Surprisingly, in addition to cytosolic localization, it also localizes in nucleus. More nuclear distribution than cytoplasm distribution (N > C), about equal nuclear and cytosolic distribution (N = C), and more

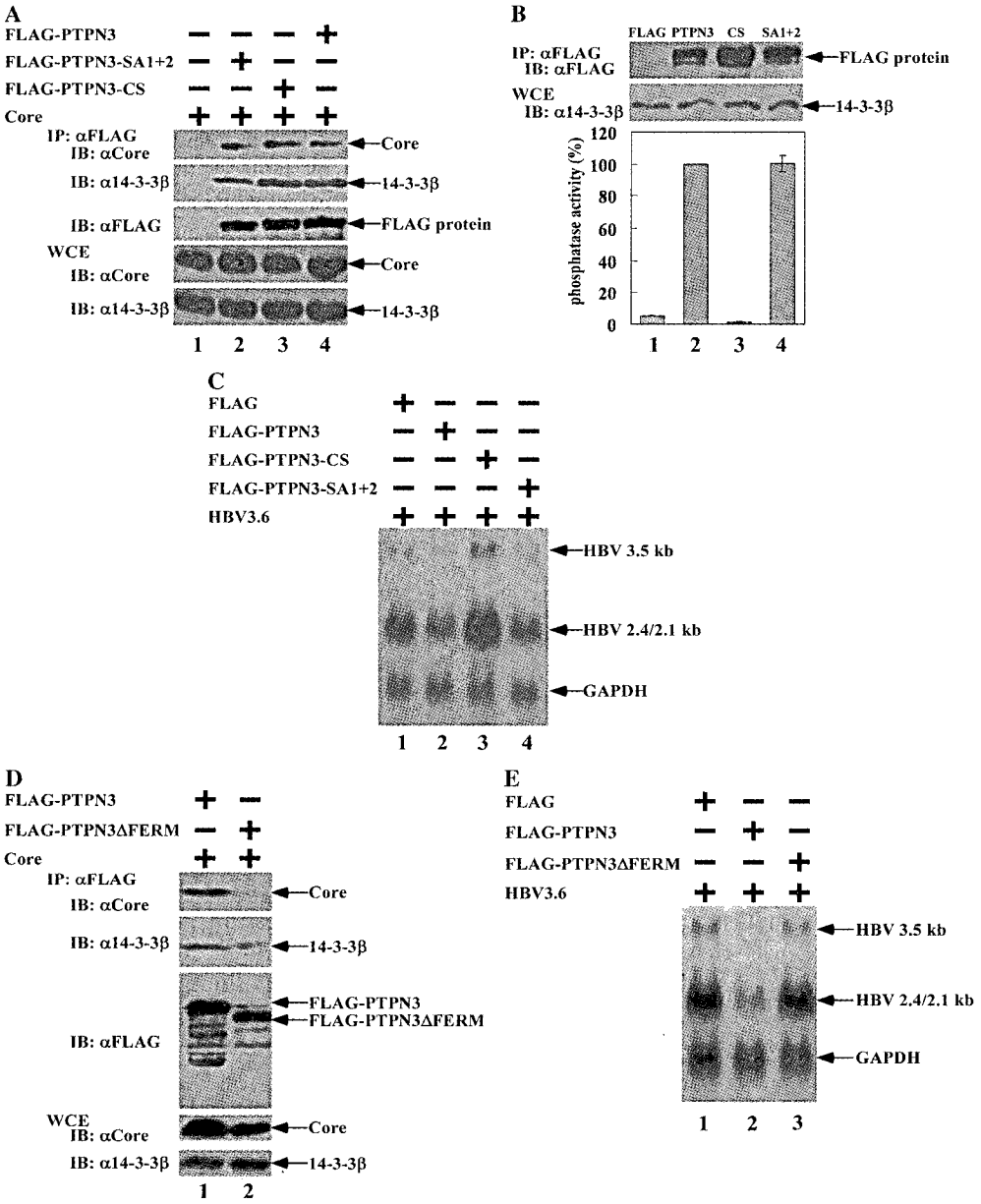

Figure 6. Effect of PTPN3 mutants on its interaction with core and 14-3-3β, and suppression of HBV RNA expression. (A, D) *In vivo* interaction. HEK293T cells were cotransfected core with FLAG, FLAG-PTPN3, FLAG-PTPN3-SA1+2, FLAG-PTPN3-CS, or FLAG-PTPN3ΔFERM. (B) *In vitro* phosphatase activity. HEK293T cells were transfected with FLAG, FLAG-PTPN3, FLAG-PTPN3-SA1+2, or FLAG-PTPN3-CS. Cell lysates were immunoprecipitated with anti-FLAG antibody M2 beads followed by *in vitro* phosphatase assay and Western blot using anti-FLAG antibody, respectively. *In vitro* phosphatase activity (bottom panel) expressed as % of wild type FLAG-PTPN3 activity is shown as means ± standard deviation of three independent experiments. Representative expression of FLAG fusion proteins in immunoprecipitated products (top panel) and protein control 14-3-3β in whole cell extracts (middle panel) is selected from these experiments. $p < 0.001$. (C, E) Suppression on HBV RNA expression. HuH-7 cells were cotransfected pHBV3.6 with pFLAG-CMV2, pFLAG-PTPN3, pFLAG-PTPN3-SA1+2, pFLAG-PTPN3-CS, or pFLAG-PTPN3ΔFERM. *In vivo* interaction, Northern blot analysis, and Western blot were performed as described in Figures 1 and 3.

cytoplasm distribution than nucleus distribution (N < C) are three observed patterns. By functional domain analysis, three nuclear localization signals (NLSs) at amino acid 264–267, 389–392, and 655–658, respectively, are identified in PTPN3. When these three NLSs are mutated in PTPN3 lacking

the FERM domain, distribution in nucleus is greatly reduced, with majority cells showing a N≪C distribution (data not shown). This result indicates that these three NLSs are indeed functional. In contrast, when these three NLSs are mutated in wild type PTPN3, the distribution is not changed and still in submembrane (data not shown). These results, together, suggest that FERM domain plays a dominant role in the localization of PTPN3. Only when FERM domain is absent, NLSs will lead PTPN3 into nucleus.

Although PTPN3 interacts with HBV core through the PDZ domain of PTPN3 and the PDZ ligand of core, two lines of evidence indicate that suppression of HBV gene expression mediated by PTPN3 does not depend on its interaction with HBV core. The first evidence is that the deletion of PDZ domain of PTPN3 has no effect on PTPN3-suppressive effect. The second is that PTPN3 still suppresses the gene expression of HBV whose core lacks the PDZ ligand. Although PTPN3 interacts with 14-3-3β, this interaction is not required for suppression of HBV gene expression. On the other hand, the PTPN3 effect is abolished by deletion of FERM domain. Deletion of FERM domain leads to abolished interaction with core. However, it has no effect on PTPN3-mediated suppression. Since deletion of FERM domain loses the submembrane localization, this result suggests that it is the FERM domain itself or the submembrane localization is crucial. T-cell receptor (TCR) ζ-subunit is the substrate of PTPN3. PTPN3 inhibits the signal transduction of TCR by dephosphorylation of ITAM tyrosine residues of ζ-subunit. Like FERM domain is essential for PTPN3 effect on HBV gene expression, deletion of FERM domain of PTPN3 blocks its inhibitory effect on TCR signaling [30, 31]. In addition, without protein-tyrosine phosphatase activity, PTPN3 loses its suppressive effect on HBV gene expression. Protein-tyrosine phosphatase activity of PTPN3 is not needed for its localization and interaction with either core or 14-3-3β. It therefore strongly indicates that protein-tyrosine phosphatase activity itself is crucial for PTPN3-mediated suppression.

The PDZ domain of PTPN3 is not essential for its suppression of HBV gene expression, suggesting that its interacting partners, which binds to its PDZ domain, are not involved in this suppression. In contrast, its phosphatase activity is needed for suppression, indicating that substrates of PTPN3 may be responsible for its suppression. As described above, TCR ζ-subunit is the substrate of PTPN3 [30, 31]. The suppression of HBV gene expression by PTPN3 through TCR ζ-subunit is supported by that PTPN3 effect on both TCR signaling and HBV gene expression depends on its FERM domain and PTPase activity. However, it is not supported by the fact in which TCR ζ-subunit is only present in T and NK cells, not in liver or hepatoma cells [32, 33]. PTPN3 has also been shown to bind to GH-induced GHR and dephosphorylate the GHR, leading to specific down-regulation of GHR signaling [34, 35]. GHR has multiple signaling pathways. The possibility that GHR is involved in the PTPN3 effect on HBV gene expression cannot be ruled out [36]. Valosin-containing protein (VCP), an abundant and a highly conserved member of AAA (ATPase associated with different cellular activities) family, is a direct substrate of PTPN3 [37, 38]. Specific dephosphorylation of VCP is noted when PTPN3 expression is induced. It is clear now that VCP is a multi-ubiquitin chain-targeting factor that is required in the degradation of many Ub-Pr pathway substrates, for example, IκBα [39, 40]. IκBα is a potent inhibitor of the NF-κB activation pathway [41]. We have found that NF-κB can suppress the HBV gene expression [42]. VCP dephosphorylation by PTPN3 may lead to the IκBα degradation and NF-κB activation, which then suppress the expression of HBV genes. However, the effect of FERM domain on PTPN3-mediated VCP dephosphorylation is unclear. Effect of dephosphorylation of VCP on IκBα degradation is also not clear. Finally, it is possible that other unidentified PTPN3 substrate(s) is involved in the PTPN3-mediated suppression of HBV gene expression.

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