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Nuclear Export Signal-Interacting Protein Forms Complexes with Lamin A/C-Nups To Mediate the CRM1-Independent Nuclear Export of Large Hepatitis Delta Antigen

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Nuclear export is an important process that not only regulates the functions of cellular factors but also facilitates the assembly of viral nucleoprotein complexes. Chromosome region maintenance 1 (CRM1) that mediates the transport of proteins bearing the classical leucine-rich nuclear export signal (NES) is the best-characterized nuclear export receptor. Recently, several CRM1-independent nuclear export pathways were also identified. The nuclear export of the large form of hepatitis delta antigen (HDAg-L), a nucleocapsid protein of hepatitis delta virus (HDV), which contains a CRM1-independent proline-rich NES, is mediated by the host NES-interacting protein (NESI). The mechanism of the NESI protein in mediating nuclear export is still unknown. In this study, NESI was characterized as a highly glycosylated membrane protein. It interacted and colocalized well in the nuclear envelope with lamin A/C and nucleoporins. Importantly, HDAg-L could be coimmunoprecipitated with lamin A/C and nucleoporins. In addition, binding of the cargo HDAg-L to the C terminus of NESI was detected for the wild-type protein but not for the nuclear export-defective HDAg-L carrying a P205A mutation [HDAg-L(P205A)]. Knockdown of lamin A/C effectively reduced the nuclear export of HDAg-L and the assembly of HDV. These data indicate that by forming complexes with lamin A/C and nucleoporins, NESI facilitates the CRM1-independent nuclear export of HDAg-L.

Hepatitis delta virus (HDV) is a human pathogen associated with fulminant hepatitis and progressive chronic liver cirrhosis upon superinfection or coinfection with hepatitis B virus (HBV) (1). The HDV RNA genome contains only one open reading frame that encodes the viral structural protein, hepatitis delta antigen (HDAg). HDAg has two isoforms, small HDAg (HDAg-S; 195 amino acid [aa] residues) and large HDAg (HDAg-L; 214 amino acid residues). HDAg-S functions as a *trans*-activator in HDV replication (2, 3), whereas HDAg-L contains a 19-amino-acid C-terminal extension which is necessary for virion assembly (4–6). Both HDAg-S and HDAg-L possess nuclear localization signals (NLSs) and mainly localize in the nucleus. Nevertheless, the proline-rich nuclear export signal (NES), 198-ILFPADPPFSPQS-210, which is located at the C terminus of HDAg-L, triggers the nuclear export of HDAg-L essential for HDV morphogenesis (7).

The nuclear-cytoplasmic transport of proteins and RNAs across the nuclear envelope occurs through channels formed by macromolecular structures known as nuclear pore complexes (NPCs) (8). The NPC in vertebrates contains about 50, or even more, different proteins. The NPC spans the dual membrane of the nuclear envelope and acts as a gateway for macromolecular trafficking. A general theme in the nuclear export of proteins is that specialized export receptors recognize cargoes harboring specific export signals. A well-known example is the recognition of the classical leucine-rich NES by the chromosome region maintenance 1 (CRM1) exportin (9–11).

There are several NESs that contribute to CRM1-independent nuclear export pathways. The heterogeneous nuclear ribonucleoprotein (hnRNP) A1 and K proteins contain serine and acidic amino acid-rich NESs (12, 13). The HDAg-L protein (7) and the human cytomegalovirus transactivator protein pUL69 (14) both contain proline-rich NESs. However, the mechanisms of the involvement of these proteins in the CRM1-independent nuclear export pathways remain unclear.

In our earlier study, a novel human NES-interacting protein, designated NESI, was identified. NESI protein interacts with the proline-rich NES of HDAg-L and plays a critical role in the assembly of HDV (15). NESI protein consists of 467 amino acid residues and possesses a putative bipartite NLS. NESI shares significant sequence homology with a membrane protein family to which the limb region 1 (LMBR1) protein (16) and the lipocalin-1-interacting membrane receptor (LIMR) belong (17). Although NESI protein plays a critical role in the CRM1-independent nuclear export of HDAg-L, it remains unknown thus far how NESI participates in this process. Here, we set out to identify nuclear binding partners of the NESI protein as a key toward understanding the mechanisms of NESI involved in the regulation of the CRM1-independent nuclear export pathway, particularly for the cargo protein HDAg-L which contains a proline-rich NES.

MATERIALS AND METHODS

Plasmids. (i) pcDNA-NESI-V5HisTopo. Plasmid pcDNA-NESI-V5HisTopo contains cDNA encoding the NESI protein of 467 amino acid residues as previously described (15).

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(ii) pGEX-NESI(1–185), pGEX-NESI(213–310), and pGEX-NESI(310–467). To generate plasmids pGEX-NESI(1–185) and pGEX-NESI(310–467) (where the numbers in parentheses indicate amino acid regions of the truncated NESI proteins), a KpnI/SalI fragment (591 bp) and an EcoRI/EcoRV fragment (495 bp) were independently obtained from pcDNA-NESI-V5HisTopo and cloned into the SmaI site of plasmid pGEX-6P-1 following a blunt-end reaction. To generate plasmid pGEX-NESI(213-310), an EcoRI fragment (292 bp) obtained from pcDNA-NESI-V5HisTopo was subcloned into the EcoRI site of plasmid pGEX-6P-1.

(iii) pGEX-NESI(1–30), pGEX-NESI(1–116), and pGEX-NESI(147–227). For construction of plasmids pGEX-NESI(1–30), pGEX-NESI(1–116), and pGEX-NESI(147–227), cDNA fragments representing NESI protein from amino acid residues 1 to 30, 1 to 116, and 147 to 227 were first generated by PCR from pcDNA-NESI-V5HisTopo with the following primer sets: NESI(1)F (5′-ATGAAAAATCAAAATGGTACATTTAAGG ACTGGG-3′) and NESI(30)R (5′-AGTATAGTAACCGTATAATACAG TGTCCTC-3′); NESI(1)F and NESI(116)R (5′-ACCATGACTACTTCC AAGTTCTTCAAATAGGG-3′); and NESI(147)F (5′-CCCATATGTTAA ATCTGATAAAAGGC-3′) and NESI(227)R (5′-GCGGATCCAGCGCC ACAAAATTTTGT-3′). The fragments were independently cloned into plasmid pCRII-TOPO (Invitrogen) to generate pCRII-NESI(1–30), pCRII-NESI(1–116), and pCRII-NESI(147–227). The NESI-encoding DNA fragments obtained from a further treatment of the resultant plasmids with EcoRI restriction endonuclease were then cloned into the EcoRI site of plasmid pGEX-6P-1 to generate plasmids pGEX-NESI(1-30), pGEX-NESI(1-116), and pGEX-NESI(147-227), respectively. All expression constructs were verified by DNA sequencing.

(iv) pECE-d-BE, pECE-d-SM, pECE-d-BE(P205A), pECE-HDAg-L(L199A), pECE-HDAg-L(D203A), and pECE-C-ES. Plasmids pECE-d-BE and pECE-d-SM contain cDNAs encoding HDAg-L and HDAg-S, respectively (5). Plasmids pECE-d-BE(P205A), pECE-HDAg-L(L199A), and pECE-HDAg-L(D203A) encode mutant HDAg-L with amino acid residues Pro-205, Leu-199 and Asp-203, respectively, replaced by Ala (18). Plasmid pECE-C-ES encodes the small form of hepatitis B surface antigen (HBsAg) (5).

(v) pAD4-Flag-NESI. For construction of plasmid pAD4-Flag-NESI, a DNA fragment containing a Flag epitope and the full-length NESI was generated by PCR from pGEMTeasy-HA-NESI (15) with primers 5′-GC CACCATGGATTACAAGGATGACGACGATAAGTACCCATACGATG TTCCAG-3′ and 5′-GCTCTAGAGCTCGTGAACTTGCGGGGTTTTT C-3′ and inserted into the HindIII and SacI sites of plasmid pAS2-1 (BD Biosciences). pAD4-Flag-NESI encodes a Flag-tagged NESI protein.

(vi) Plasmids encoding GST-lamin fusion proteins. Plasmids encoding glutathione *S*-transferase (GST) fusion proteins that consist of various domains of the lamin A protein (head, amino acid residues 1 to 129; rod 1, amino acid residues 117 to 239; rod 2, amino acid residues 216 to 384; tail 1, amino acid residues 369 to 519; and tail 2, amino acid residues 490 to 660) were constructed as described previously (19; kindly provided by Joel D. Baines, Cornell University, NY).

(vii) pLKO.1-shLam and pLKO.1-shLuc. Plasmids pLKO.1-shLam and pLKO.1-shLuc were obtained from National RNAi Core Facility (Academia Sinica, Taiwan). pLKO.1-shLam transcribed short hairpin RNA (shRNA) (5′-CUGGACUUCCAGAAGAACAUC-3′) for lamin A/C RNA interference, whereas pLKO.1-shLuc transcribed shRNA 5′-CAAAUCAC AGAAUCGUCGUAU-3′ for luciferase RNA interference.

Antibodies. Rabbit polyclonal antibodies specific to HDAg were generated as described previously (20). Mouse monoclonal antibody specific to lamin A/C was purchased from Upstate Biotechnology. Mouse monoclonal antibodies specific to GST and Flag tag were from Sigma. Goat polyclonal antibodies specific to HBsAg were from Dako. Mouse monoclonal antibody to nucleoporins (Mab414) was from Abcam. Rabbit polyclonal antibodies specific to His tag and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were from Santa Cruz Biotechnology. Alexa 488- and Alexa 594-conjugated secondary antibodies were from Invitrogen. Secondary horseradish peroxidase-conjugated antibodies were purchased from the Jackson Laboratory.

Cell line and DNA transfection. Huh7 cells were maintained at 37°C with 5% CO_2 in Dulbecco's modified Eagle's medium (Gibco) supplemented with 10% heat-inactivated fetal bovine serum plus 100 units of penicillin and 100 μg of streptomycin per ml. For transient DNA transfection, cells were cultured and seeded at 15 to 20% confluence 18 h prior to transfection. DNA transfection was performed with cationic liposomes (Invitrogen) according to the manufacturer's procedures.

Immunofluorescence staining, coimmunoprecipitation, and Western blot analysis. Immunofluorescence staining, coimmunoprecipitation, and Western blot analysis were performed as previously described (7).

Cellular protein fractionation. For membrane protein fractionation, cultured cells were resuspended in homogenization buffer (20 mM HEPES, pH 7.5, and 0.25 M sucrose) supplemented with protease inhibitor cocktail (1 mM phenylmethylsulfonyl fluoride, 10 μg aprotinin/ml, 1 μg of pepstatin A/ml, and 1 μg of leupeptin/ml [Roche]) and sonicated for 10 s at 4°C three times. Unbroken cells were removed by centrifugation at 800 × *g* for 8 min. The supernatant was then centrifuged at 15,500 × *g* for 1 h at 4°C to separate the cytosolic fraction from the membrane fraction. SDS sample buffer (2% SDS, 2% β-mercaptoethanol, 10% glycerol, 50 mM Tris-HCl, pH 6.8, 0.2% bromophenol blue) was added to the membrane fraction for further analysis. For examination of protein solubility by nonionic detergent, cells were lysed on ice with buffer containing 1% Triton X-100 in phosphate-buffered saline (PBST) and subjected to three freeze-thaw cycles and centrifugation at 10,000 × *g* for 20 min at 4°C to separate the cell lysate into soluble and insoluble fractions.

PNGase F and Endo H treatment. Total protein lysates obtained from transfected cells treated with PBST in the presence of protease inhibitor cocktail were subjected to immunoprecipitation with antibodies specific to the His tag of the NESI-V5His protein. The immunoprecipitates in which the NESI-V5His protein had been enriched were then incubated for 16 h at 37°C with 1,000 units of peptide *N*-glycosidase F (PNGase F) or 1,000 units of endoglycosidase H (Endo H; New England BioLabs) in 50 mM sodium phosphate buffer (pH 7.5) containing 0.5% SDS, 0.04 M dithiothreitol (DTT), and 1% NP-40.

***In vitro* transcription/translation-coupled reaction and posttranslational modifications.** Plasmid pcDNA-NESI-V5HisTopo encoding the full-length NESI protein with a V5His tag was subjected to an *in vitro* transcription/translation-coupled reaction according to the manufacturer's specifications (Promega). In brief, the reaction mixtures consisting of T7 RNA polymerase, rabbit reticulocyte lysate, amino acid mixture with 1 mM methionine, RNasin RNase inhibitor, and the circular plasmid DNA template were incubated at 30°C for 90 min in reaction buffer and then chilled on ice prior to SDS-PAGE and Western blot analysis. For posttranscriptional modifications, the reaction was performed in the presence of canine pancreatic microsomal membranes (rough endoplasmic reticulum [ER] membranes [RMs]) (Promega).

Expression and purification of recombinant fusion proteins and GST pulldown assay. Expression of GST-tagged fusion proteins in *Escherichia coli* BL21 and partial purification of His-tagged fusion proteins have been described previously (6). To perform a GST pulldown assay, GST fusion proteins precoupled to glutathione-Sepharose 4B beads (GE Healthcare Bio-Sciences) were incubated at 4°C overnight with partially purified His-tagged fusion proteins or total protein lysates prepared from cultured cells treated with PBST. The protein-bound beads were then washed five times with PBST and eluted with the SDS sample buffer prior to Western blot analysis.

Harvest of HDV virus-like particles (VLPs) and determination of package activity. To determine the package activity of HDAg-L and HBsAg under various conditions, HDV VLPs were collected from culture medium at 4 days posttransfection as described previously (7). Protein lysates prepared from the VLPs were then subjected to SDS-PAGE and Western blot analysis.

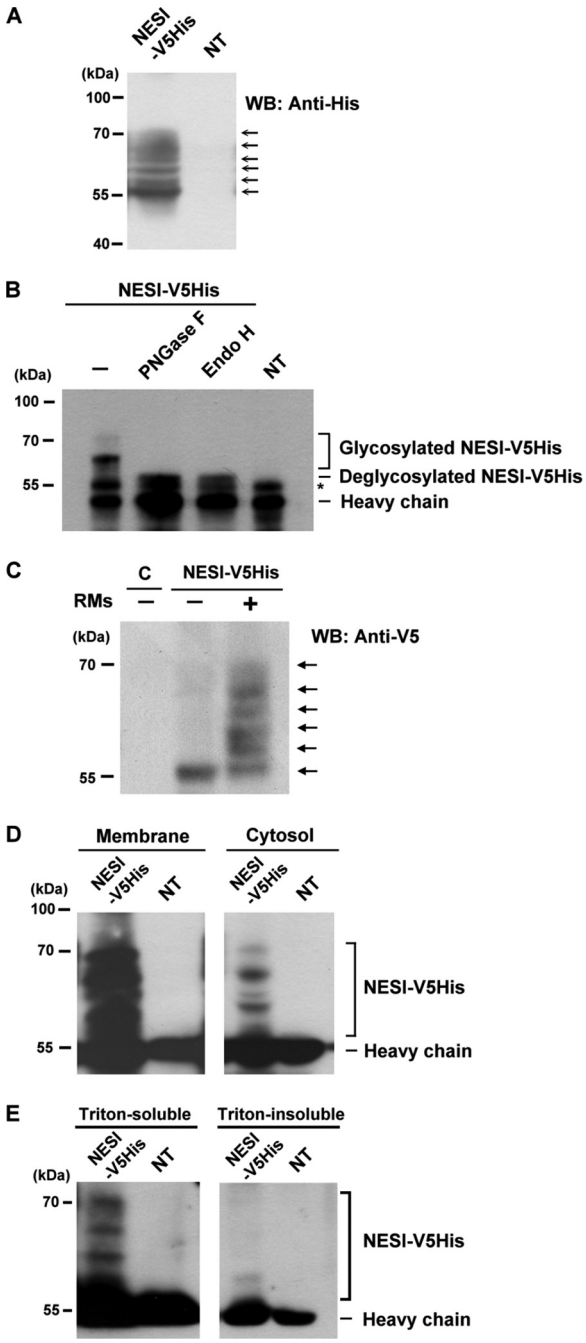

FIG 1 Characterization of NESI protein. (A) Transient expression of NESI in Huh7 cells. Huh7 cells were transfected with plasmid pcDNA-NESI-V5HisTopo encoding an NESI protein with a V5His tag. Forty-eight hours posttransfection, cells were harvested and subjected to Western blot (WB) analysis with antibodies specific to the His tag. The NT represents nontransfected cells as a negative control. Arrows mark the major 55-kDa NESI-V5His protein and its modification forms. Molecular mass standards are shown in kDa on the left. (B) Glycosylation of NESI in Huh7 cells. Cell lysates prepared from Huh7 cells transiently expressing NESI-V5His proteins were subjected to immunoprecipitation with antibodies against the His tag. The immunoprecipitates were treated with PNGase F and Endo H as indicated, followed by Western blotting with the anti-His antibodies. The star indicates a nonspecific band. (C) NESI glycosylation *in vitro*. Plasmid pcDNA-NESI-V5HisTopo was subjected to an *in vitro* transcription/translation reaction in the absence or presence of canine pancreatic rough microsomes (RMs). The products were detected by Western blotting with the anti-V5 antibody following SDS-PAGE. The C represents a vector control analyzed in parallel. (D) Subcellular distribution of NESI. Cell lysates prepared from Huh7 cells transiently expressing NESI-V5His were fractionated into membrane and cytosol fractions. The fractions were subjected to immunoprecipitation and Western blot analysis with antibodies against the His tag. (E) Solubility of NESI in nonionic detergent. Huh7 cells transiently expressing the NESI-V5His proteins were lysed with PBST. The cell lysates were subjected to centrifugation to separate supernatant (Triton-soluble) and pellet (Triton-insoluble) fractions, followed by immunoprecipitation and Western blot analysis with antibodies against the His tag.

RESULTS

NESI is a glycosylated membrane protein. Amino acid sequence analysis revealed the existence of putative N-glycosylation sites and transmembrane helices in the NESI protein. To understand the structure-function relationship of the NESI protein, biochemical characterization was performed. Western blot analysis detected a ladder of signals ranging from 55 kDa to 70 kDa when a V5His-tagged NESI protein with the expected molecular size of 55 kDa was transiently expressed in Huh7 cells (Fig. 1A). The characteristics of the signals with molecular masses higher than the major 55-kDa protein were analyzed by endoglycosidase digestion following an immunoprecipitation that enriched the NESI protein in the cell lysates. As shown in Fig. 1B, the larger molecules were no longer detected after the treatment of peptide-*N*-glycosidase F (PNGase F) and endoglycosidase H (Endo H) which removes the N-linked sugar moieties. NESI protein has a hydrophobic nature and is assumed to be membrane inserted. To monitor the properties of the NESI protein in ER targeting and protein modifications, we carried out cell-free transcription/translation and posttranslational reactions in the presence of canine pancreatic microsomal membranes (rough ER membranes [RMs]). As shown in Fig. 1C, in the absence of RMs, a prominent 55-kDa NESI-V5His protein was produced from plasmid pcDNA-NESI-V5HisTopo, whereas in the presence of RMs five additional proteins ranging from 58 to 70 kDa appeared in accordance with a reduced level of the 55-kDa protein. Since no potential signal peptide has been predicted for NESI, these results indicate that there are five glycosylation events, which alter the molecular mass by approximately 3 kDa each. Taken together, our findings suggest that the NESI protein is synthesized on free ribosomes and targets to ER-derived membranes for further glycosylation.

To understand the subcellular distribution of the NESI protein, whole-cell lysates prepared from Huh7 cells transiently expressing the NESI protein were subjected to differential centrifugation that separates the membrane fraction from the cytosol. The results demonstrated that the NESI protein was distributed in both the membrane and cytosol fractions, with a relatively higher proportion being detected in the membrane fraction (Fig. 1D), and none was detected in the culture medium (data not shown). In addition, the membrane-associated NESI protein could be solubilized by Triton X-100 (Fig. 1E). These results indicate that NESI is a membrane protein with different glycosylation forms.

Identification of lamin A/C as an NESI-interacting protein. To identify potential cellular proteins that specifically interact with NESI, a coimmunoprecipitation assay was performed with antibodies that recognized the V5His-tagged NESI. Two cellular proteins with molecular masses of approximately 70 kDa and 57 kDa were specifically coimmunoprecipitated with NESI. Mass spectrometry analysis identified the NESI-associated proteins to be lamin A/C and vimentin (Fig. 2A). Lamin A/C is known to

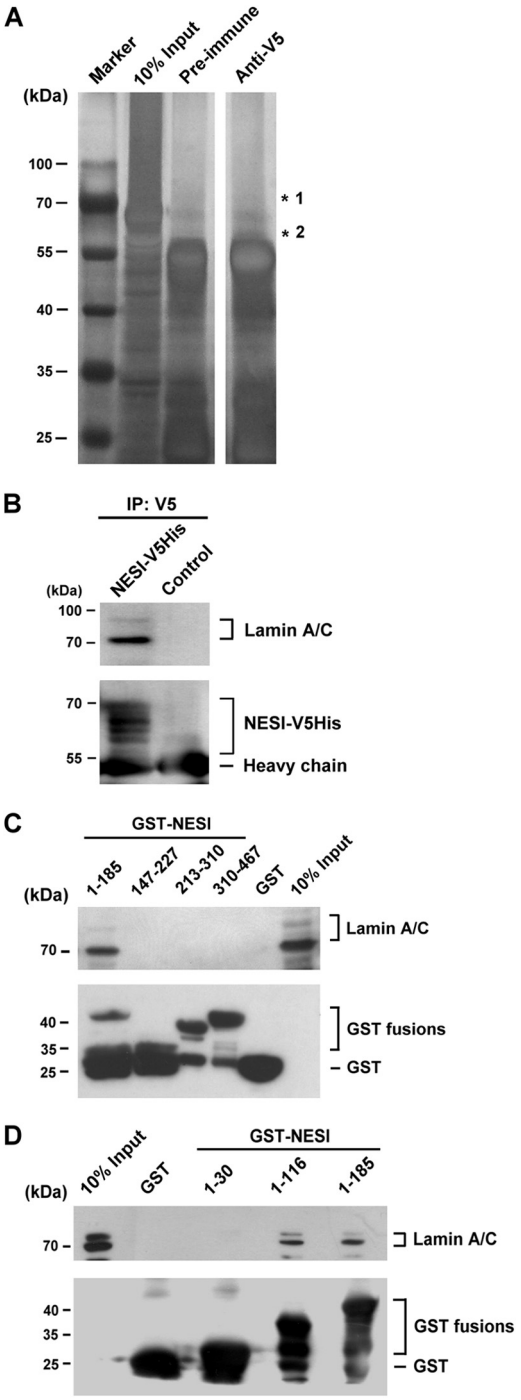

FIG 2 Identification of lamin A/C as an NESI-interacting protein. (A) Coimmunoprecipitation of cellular factors with the NESI-V5His protein. Cell lysates prepared from Huh7 cells transiently transfected with pcDNA-NESI-V5HisTopo encoding an NESI protein with a V5His tag (NESI-V5His) were subjected to a coimmunoprecipitation assay with the anti-V5 antibody and the control preimmune serum. Silver staining is shown. The stars mark the bands of cellular protein 1 and 2 that were coimmunoprecipitated with the NESI-V5His protein. Molecular mass markers are indicated on the left. (B) Coimmunoprecipitation of lamin A/C with NESI-V5His. Cell lysates prepared from Huh7 cells transiently transfected with pcDNA-NESI-V5HisTopo (NESI-V5His) or with pcDNA3.1 vector (Control) were subjected to a coimmunoprecipitation (IP) assay with anti-V5 antibodies and Western blot analysis with anti-lamin A/C (top) and anti-His tag (bottom) antibodies. (C and D) GST pulldown assays with GST-NESI fusion proteins and Huh7 cell lysates. A GST proteins consisting of truncated NESI proteins (indicated at the top by amino acid regions) bound to glutathione-Sepharose 4B beads were incubated independently with Huh7 cell lysates. Following the GST pulldown reaction, Western blot analysis was performed with antibodies specific to lamin A/C (top) and GST (bottom).

target to the nuclear membrane to maintain the integration of the nuclear envelope (21), whereas vimentin is mainly localized in the cytosol and involves in the position of cellular organelles. Since NESI is a nuclear protein (15), the association between lamin A/C and NESI was further elucidated. By performing Western blot analysis with an antibody against lamin A/C following a coimmunoprecipitation assay with the V5 antibody to NESI-V5His, the identity of lamin A/C as an NESI-interacting protein was established (Fig. 2B). To map specific domains responsible for the interaction of the NESI protein with lamin A/C, GST fusion proteins consisting of various domains of the NESI protein were generated and subjected to GST pulldown assays with Huh7 cell lysates. Results demonstrated a specific interaction between a fusion of GST with amino acids 1 to 185 of the NESI protein [GST-NESI(1–185)] and lamin A/C (Fig. 2C). No indication of the lamin A/C binding domain is present in the C terminus of the NESI protein. Further experiments narrowed down the lamin A/C-interacting domain of NESI to the N-terminal 116 amino acid residues (Fig. 2D). The data clearly demonstrated a specific association of the NESI protein with lamin A/C in mammalian cells.

The N-terminal domain of NESI interacts directly with the tail domain of lamin A/C. To determine specific domains of the lamin A/C that interact with the NESI protein, a series of GST-lamin A fusion proteins (Fig. 3A) was subjected to analysis for their abilities to pull down the full-length NESI expressed in Huh7 cells. As shown in Fig. 3B, both lamin A tail 1 (aa 369 to 519) and tail 2 (aa 490 to 660) had the ability to pull down NESI-V5His, whereas none of the other lamin A domains (head, aa 1 to 129; rod 1, aa 117 to 239; and rod 2, aa 216 to 384) showed the ability to interact with NESI-V5His. Nevertheless, only GST-lamin A(490–660) was capable of pulling down His-NESI(1–185) that was produced in the *E. coli* system and partially purified through a nickel column (Fig. 3C). These results indicate that the N-terminal domain of NESI interacts directly with the tail 2 domain of lamin A. Together with the results shown in Fig. 2C, these data indicate a direct interaction of the N-terminal domain of NESI [NESI(1–185)] with the tail 2 domain of lamin A [lamin A(490–660)] and a possible indirect association of the NESI protein with GST-lamin A(369–519). Alternatively, the full-length NESI may, through its N-terminal domain, bind to two sites of the lamin A tail domain from amino acid residues 389 to 661. Failure of the NESI(1–185) protein to interact with GST-lamin A(369–519) could be due to an inappropriate conformation. Furthermore, since there is no evidence indicating an involvement of the tail 2 region in the interactions between lamin molecules and between lamin and lamin receptor, these results suggest that NESI interacts with lamin A to facilitate a unique function of NESI rather than to involve in the rearrangement of lamin A.

Colocalization of NESI with lamin A/C and nucleoporins at the nuclear envelope. The nuclear lamina is a meshwork of lamins and lamin-associated proteins through which NPC is anchored to the nuclear envelope (21). The NPC acts as a gateway in controlling the transport of macromolecules between the nucleus and the

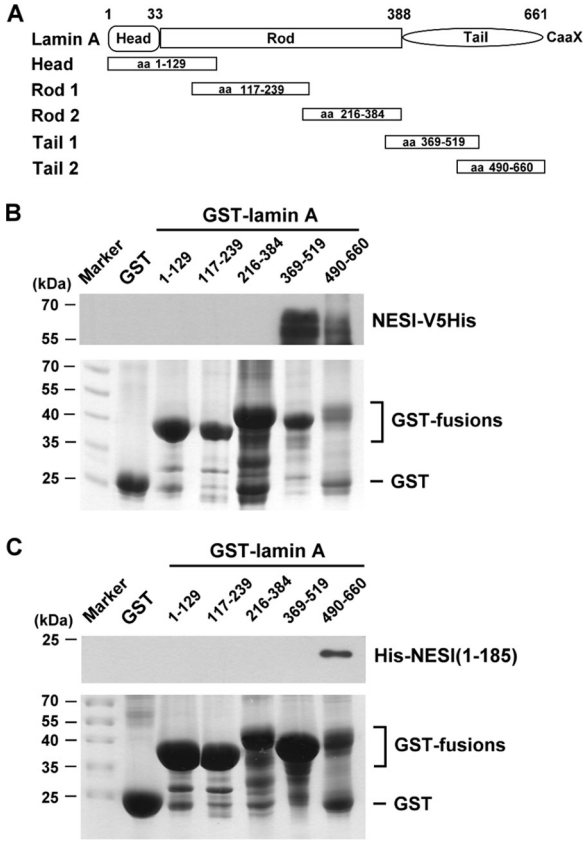

FIG 3 A direct interaction between NESI(1–185) and lamin A/C tail 2 domain. (A) Schematic representation of lamin A and its subdomains designated head, rod 1, rod 2, tail 1, and tail 2. (B and C) GST pulldown assay with the NESI protein and GST fusion proteins containing various domains of the lamin A (indicated at the top by amino acid regions). GST pulldown assays were performed with GST-lamin A fusion proteins precoupled to glutathione-Sepharose beads and lysates prepared from Huh7 cells expressing NESI-V5His (B) or purified His-NESI(1–185) protein (C). Following the GST pulldown reaction, Western blot analysis was performed with antibodies against the His tag of the NESI-V5His and His-NESI(1–185) proteins (top). GST fusion proteins that serve as loading controls were detected by Coomassie blue staining (bottom). Positions of molecular mass markers are indicated on the left.

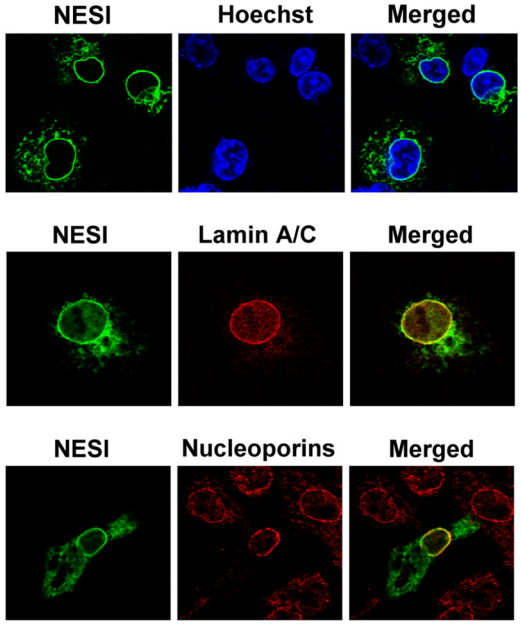

FIG 4 Colocalization of NESI with lamin A/C and nucleoporins in Huh7 cells. Huh7 cells transiently expressing NESI-V5His were subjected to immunofluorescence staining with antibodies against the His tag followed by Alexa 488-conjugated goat IgG (green) and with anti-lamin A/C and anti-nucleoporins antibodies followed by Alexa 594-conjugated goat IgG (red) under a Leica TCS SP2 confocal microscope. Merged signals appear in yellow. In the top panel, Hoechst 33258 was used along with the secondary antibody to detect the nucleus.

cytoplasm. A number of proteins called nucleoporins localize to discrete regions of the NPC. The association between lamin A/C and nucleoporins at the nuclear envelope has been clearly demonstrated (21). Therefore, we set out to understand the localization of NESI in association with lamin A/C and nucleoporins. Huh7 cells transiently expressing the NESI protein were subjected to immunofluorescence staining. The images from confocal microscopy demonstrated that the NESI protein was distributed mainly to the nuclear envelope and colocalized with lamin A/C and nucleoporins (Fig. 4), suggesting interactions between NESI and lamin-nucleoporin complexes and a tight association of the NESI protein with nucleoskeleton.

NESI forms complexes with nucleoporins. For further examination of the potential interactions between NESI and nucleoporins, a GST pulldown assay was performed with Huh7 cell lysates and GST fusion proteins consisting of various domains of the NESI protein. Western blot analysis with monoclonal antibody Mab414 that specifically recognizes the FXFG repeats present in a series of nucleoporins detected specific interactions of the GST-NESI(147–227) protein with Nup62, Nup98, and Nup153 (Fig. 5A). The results indicate that, different from the lamin A/C-interacting region from amino acid residue 1 to 116, the subdomain spanning from amino acid residue 147 to 227 is sufficient for NESI to form complexes with nucleoporins. Further coimmunoprecipitation-Western blot analysis clearly demonstrated a specific association of the NESI protein with Nup153 but none with either Nup98 or Nup62 (Fig. 5B). Taken together, these data suggest specific binding of NESI to the nucleoporin at the nuclear envelope.

Association of HDAg-L with NESI, lamin A/C, and nucleoporins. Upon infection, viruses utilize host machineries for protein synthesis, assembly, and release. Viral genomes and proteins localized to the host cell nucleus are thought to form NES-protein export complexes and bind to various nucleoporins as they transit from the nucleus to the cytoplasm (21). We have previously demonstrated an association of the NESI protein with the HDAg-L protein of HDV (15). For further identification of the interacting domains of the NESI protein with HDAg-L, a GST pulldown assay was performed with GST fusion proteins consisting of various domains of the NESI protein and the full-length HDAg-L overexpressed in Huh7 cells. As shown in Fig. 6A, GST-NESI(310–467) pulled down the HDAg-L protein, whereas GST-NESI(1–185), GST-NESI(147–227), GST-NESI(213–310), and the control GST failed to pull down HDAg-L. These results indicated that the domain spanning amino acid residues 310 to 467 is sufficient for the NESI protein to interact with HDAg-L. Further studies with GST-NESI(310–467) and amino acid substitution mutants of the HDAg-L demonstrated a critical role of Pro-205 for the interac-

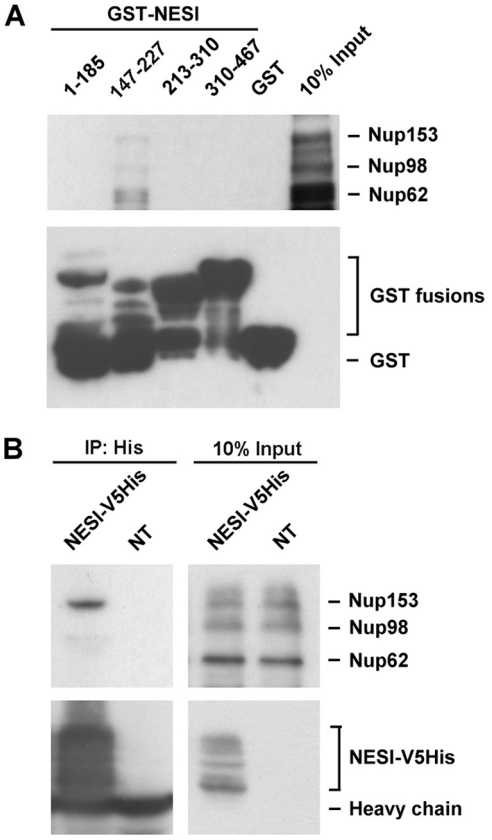

FIG 5 NESI forms complexes with nucleoporins. (A) GST pulldown assay. GST pulldown assays were performed with Huh7 cell lysates and GST fusion proteins containing various domains of the NESI protein, indicated at the top, followed by Western blotting with antibodies specific to nucleoporins (top) and GST (bottom). (B) Coimmunoprecipitation analysis. Cell lysates prepared from Huh7 cells transiently expressing the NESI-V5His protein were subjected to immunoprecipitation assay with anti-His antibodies followed by Western blotting with antibodies specific to nucleoporins (top) and the V5 tag of NESI-V5His protein (bottom). NT, nontransfected cells used as negative controls.

tion (Fig. 6B). On the other hand, Leu-199 and Asp-203, the conserved residues in the clathrin box of the HDAg-L (18), had no effect on the binding of HDAg-L to NESI (Fig. 6B). In addition, coimmunoprecipitation assays showed complex formation between lamin A/C and HDAg-L but not HDAg-S, which lacks the NES of the HDAg-L (Fig. 6C). Furthermore, Pro-205 amino acid substitution significantly diminished the specific interaction of HDAg-L with lamin A/C (Fig. 6D). Similarly, HDAg-L coimmunoprecipitated with nucleoporins bearing FXFG repeats, but no association was detected between HDAg-S and nucleoporins (Fig. 6E). Taken together, these results strongly suggest the association of HDAg-L with NESI, lamin A/C, and nucleoporins at the nuclear envelope.

Knockdown of lamin A/C inhibits the nuclear export of HDAg-L and the assembly of HDV. We have previously demonstrated an essential role of HDAg-L in the nuclear export and sequential assembly of the HDV ribonucleoprotein complex comprising the HDV RNA genome and of HDAg-S with the cytoplasm-localized HBsAg to form intact HDV particles (22). In addition, in the absence of HBsAg, HDAg-L was restricted to the nucleolus and nucleoplasm, the type I and II patterns, whereas in

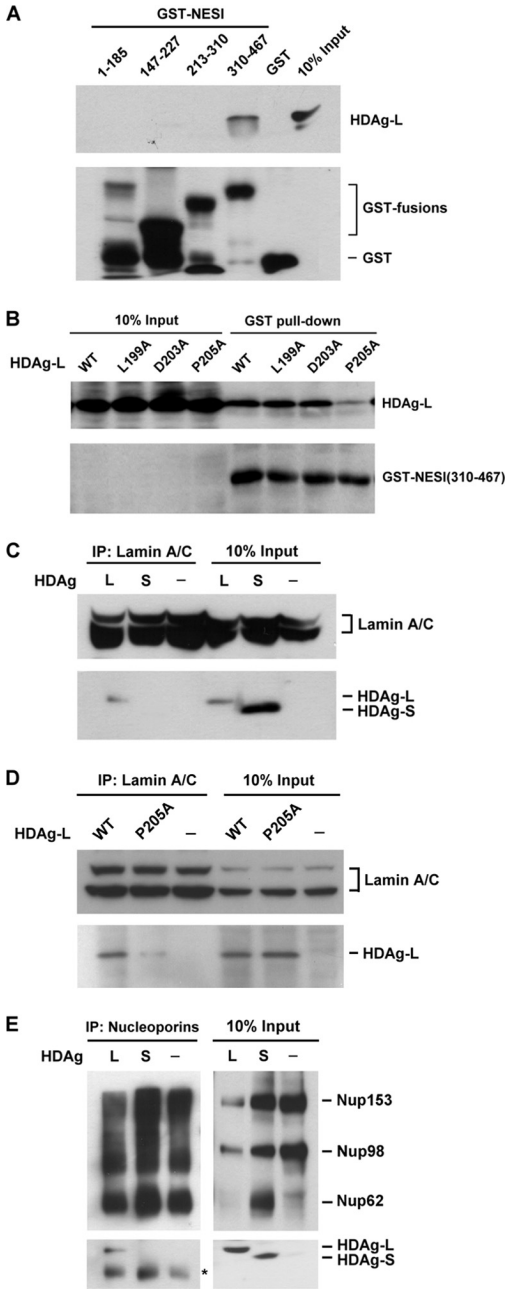

FIG 6 Interactions of HDAg-L with NESI, lamin A/C, and nucleoporins. (A) GST pulldown assay. Various GST-NESI fusion proteins and a GST control protein bound to glutathione-Sepharose 4B beads were incubated with the cell lysates prepared from Huh7 cells transiently expressing HDAg-L. Following the GST pulldown reaction, Western blot analysis was performed with antibodies specific to HDAg (top) and GST (bottom). (B) Pro-205 is critical for HDAg-L to interact with NESI. GST pulldown assays were performed with GST-NESI(310–467) and total lysates prepared from Huh7 cells expressing wild-type (WT) HDAg-L or its mutants, HDAg-L(L199A), HDAg-L(D203A), and HDAg-L(P205A), as indicated, followed by Western blotting with antibodies against HDAg-L (top) and GST (bottom). (C to E) Association of HDAg-L with lamin A/C and nucleoporins. Huh7 cells were transfected with plasmids pECE-d-BE, pECE-d-BE(P205A), and pECE-d-SM encoding HDAg-L, HDAg-L(P205A), and HDAg-S, respectively, as indicated. At 2 days posttransfection, cells were harvested and subjected to immunoprecipitation with antibodies specific to lamin A/C (C and D) and nucleoporins (E), followed by Western blotting with antibodies against lamin A/C, nucleoporins, and HDAgs. The star marks the signal of the light chain.

the presence of HBsAg, HDAg-L appeared to be distributed to the cytoplasm, the type III pattern, in approximately 39% of the transfected Huh7 cells (Fig. 7B) (7). To examine whether the association of HDAg-L with lamin A/C contributes to HDV assembly, a specific shRNA directed against lamin A/C was applied. Efficiency and specificity of the shRNA to knock down the expression of endogenous lamin A/C protein were demonstrated by Western blotting (Fig. 7A). With the treatment of shRNA specific to lamin A/C, the level of HDAg-L transported to the cytoplasm dropped from 39% to 17%, as analyzed by immunofluorescence staining, whereas the control luciferase shRNA had little effect (Fig. 7B). In addition, with the shRNA specific to lamin A/C, there was a significant decrease of HDV VLPs released into the culture medium, as detected by the presence of HDAg-L (Fig. 7C), whereas no effect was detected for the control small HBsAg. Taken together, these results indicate a critical role of lamin A/C in the nuclear export of HDAg-L and the assembly of HDV.

DISCUSSION

In this study, we have demonstrated that NESI is a nuclear membrane protein with potential glycosylation sites even though it does not carry a potential signal peptide for docking to the ER, where glycosylation takes place. Our findings using *in vitro* and *in vivo* expression systems suggest that NESI, similar to opsin (23, 24), is synthesized on free ribosomes and targets to the ER for glycosylation via its transmembrane domain(s). As the outer membrane of the nucleus is continuous with the ER and as NESI protein possesses a putative bipartite nuclear localization signal, it is possible that the NESI protein migrates along the membrane to the nucleus. We have identified and characterized NESI-interacting proteins that may serve to regulate protein export from the nucleus to the cytoplasm. The NESI protein interacts possibly simultaneously with lamin A/C and nucleoporins at the nuclear envelope. Domain mapping indicates that the NESI protein consists of the N-terminal lamin A/C-binding domain, the middle Nup153-binding domain, and the C-terminal HDAg-L-binding domain (Fig. 8). Nevertheless, the orientation of NESI, Nup153, lamin A/C and HDAg-L in the nuclear membrane needs to be further examined. In addition, results from analysis using different glycosylation prediction programs (NetNGlyc 1.0 Server [http://www.cbs.dtu.dk/services/NetNGlyc/], EnsembleGly [http://turing.cs.iastate.edu/EnsembleGly/predict.html], and N-GlycoSite [http://www.hiv.lanl.gov/content/sequence/GLYCOSITE/glycosite.html]) pointed to the same glycosylation profile, indicating that there are six glycosylation sites in the NESI protein. However, only five glycosylation forms were detected in this study (Fig. 1A and C). These glycosylation sites are expected to be situated in the membrane-spanning NESI exposed to the lumen of the ER. The exact membrane topology and the glycosylation sites of the NESI in the ER and nuclear membrane need to be further elucidated. Furthermore, glycosylation is not necessary for the binding of NESI to lamin A/C, nucleoporins, and HDAg-L proteins in the *in vitro* expression system (Fig. 2, 3, 5, and 6) but may be required for its targeting to the nuclear membrane.

Previous studies have provided evidence that NPC on the nuclear envelope plays a critical role in the process of nuclear transport. The basic framework of the NPC consists of a central core with a ring-spoke structure exhibiting 50- to 100-nm fibrils extended into the nucleoplasm and the cytoplasm (21). The classical

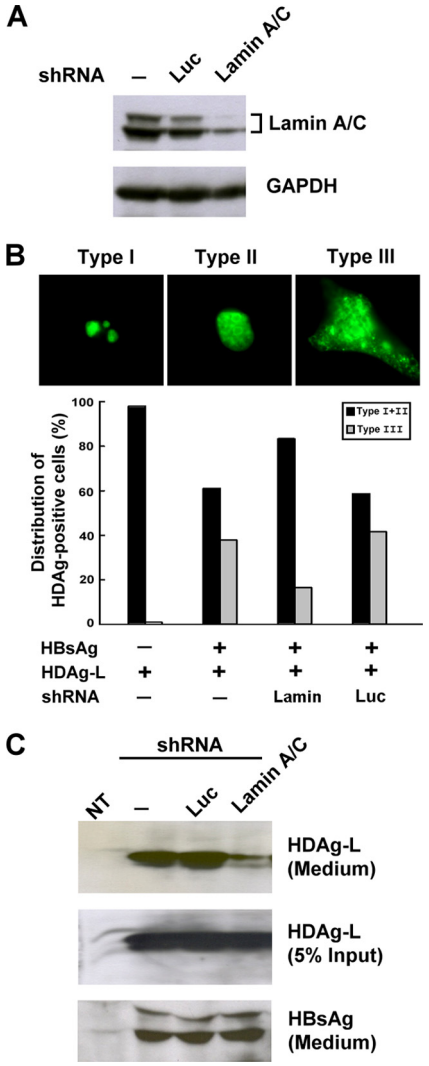

FIG 7 Interference of HDAg-L nuclear export and HDV assembly in cells with downregulated lamin A/C expression. (A) shRNA knockdown of lamin A/C. Huh7 cells transfected with the plasmid pLKO.1-shLam expressing shRNA specific to lamin A/C were harvested at 2 days posttransfection for Western blot analysis with antibodies against lamin A/C. Cells without transfection (−) and cells expressing shRNA against luciferase served as controls. GAPDH was used as an internal control. (B) Effects of lamin A/C knockdown on the nuclear export of HDAg-L. Huh7 cells were cotransfected with plasmids pECE-d-BE, pECE-C-ES, and pLKO.1-shLam or pLKO.1-shLuc expressing HDAg-L, small HBsAg, and the shRNAs specific to lamin A/C or to luciferase, respectively. At 3 days posttransfection, cells were subjected to immunofluorescence staining with antibodies against HDAg. Three representative staining patterns of the HDAgs are shown: type I, nucleolus distribution; type II, both nucleolus and nucleoplasm distribution; type III, nucleolus, nucleoplasm, and cytoplasm distribution. For statistic analysis, fields containing at least 200 HDAg-positive cells each were randomly selected. Cell numbers bearing each type of the defined staining patterns of HDAg were counted and plotted as the percentage of the total number of the HDAg-positive cells. (C) Effects of lamin A/C knockdown on HDV assembly. Huh7 cells were cotransfected with plasmids expressing HDAg-L, small HBsAg, and shRNAs as described in the legend to panel B. Cells without coexpressing shRNA (−) served as a control. At 4 days posttransfection, VLPs released into the culture medium were harvested for Western blot analysis with antibodies specific to HDAg (top) and HBsAg (bottom). HDAg-L present in cell lysates (5% Input) was used as a control. NT, nontransfected cells.

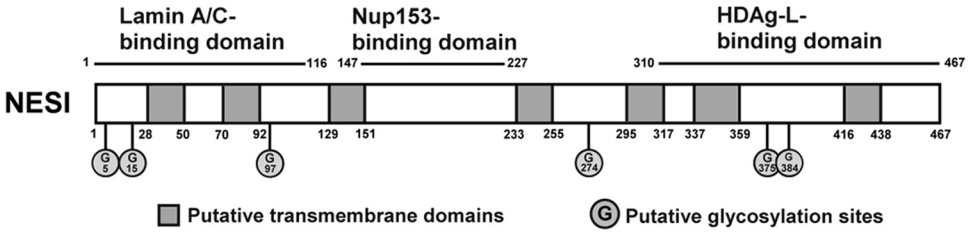

FIG 8 A schematic diagram for the interacting domains of the NESI protein with lamin A/C, nucleoporin 153 (Nup153), and HDAg-L that bears a proline-rich NES. The putative glycosylation sites and transmembrane domains of the NESI protein are indicated.

NLS-mediated nuclear import has been well demonstrated (8), but detailed mechanisms concerning signal-mediated export pathways are beginning to emerge. Nuclear export signals have been identified in many cellular and viral proteins. Viral genomes coated with multiple different transport proteins thread their way through the nuclear pores, and the nuclear export of viral proteins facilitates virus morphogenesis. CRM1 is the most common receptor that mediates the nuclear export of proteins containing leucine-rich NESs originally identified in the Rev protein of HIV-1 (22). On the other hand, the M9 domain of the hnRNP A1 protein contains an NES that is rich in serine and acidic amino acid residues; it directs the nuclear export of the hnRNP A1 protein via a transportin-mediated pathway (13). Unlike the CRM1-dependent transport of the Rev protein and the transportin-mediated CRM1-independent transport of the hnRNP A1, the nuclear export of HDAg-L is mediated by binding of its proline-rich NES to the NESI protein in a CRM1-independent manner (7, 15). In addition, Pro-205 is critical for the NES function of the genotype I HDAg-L. The association of HDAg-L with NESI in the nucleus facilitates the transport of HDAg-L from the nucleus to the cytoplasm for subsequent HDV morphogenesis. Here, we have further demonstrated that the wild-type HDAg-L specifically bound to the C-terminal domain of NESI, whereas the HDAg-L(P205A) mutant lost the ability to interact with NESI (Fig. 6), explaining why HDAg-L(P205A) is a nuclear export-defective mutant. We hypothesize that NESI functions as a nuclear export mediator that interacts with the cargo protein HDAg-L carrying a proline-rich NES and shuttles from the nucleus to the cytoplasm. Whether additional cellular proteins within the nucleus are required for a proper function of nuclear export needs to be further elucidated. Nevertheless, our results lead to a potential utilization of the NESI protein by more diverse cellular cargoes.

The NPC is anchored in the nuclear membrane through the nuclear lamina, a meshwork of lamins and lamin-associated proteins (21). Our previous studies demonstrated that overexpression of antisense NESI RNAs inhibited the expression of NESI and meanwhile abolished the HDAg-L-mediated nuclear export and assembly of HDV genomic RNA (15). In addition, the CXXX isoprenylated motif at the extreme C-terminal domain may direct HDAg-L to the nuclear membrane (25). Results from this study suggest an NESI-mediated association of the HDAg-L with lamin A/C-nucleoporin complexes. These results suggest a prerequisite role of the isoprenylation modification for HDAg-L to target to the nuclear envelope where, through interacting with NESI, HDAg-L associates with lamin A/C and forms nuclear export complexes with nucleoporins and various transport proteins. The complexes then thread through the nuclear pore and transit to the cytoplasm.

Although there is a considerable geographical variation, it has been estimated that about 5% of HBV carriers are coinfected with HDV, leading to a total of 15 million persons infected worldwide (26). Because there is no specific therapy, HDV infection causes more severe liver disease than HBV infection alone (27–29). This study uncovered the molecular mechanism of NESI in shuttling HDV and host proteins from the nucleus to the cytoplasm. A new therapeutic approach following a screening of chemicals that inhibit the interaction between HDAg-L and NESI may be established for fighting HDV.

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