A Novel Chromosome Region Maintenance 1-independent Nuclear Export Signal of the Large Form of Hepatitis Delta Antigen That Is Required for the Viral Assembly*

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Chia-Huei Lee‡, Shin C. Chang¶, C. H. Herbert Wu§, and Ming-Fu Chang‡

From the Institutes of ‡Biochemistry, ¶Microbiology, and §Molecular Medicine, College of Medicine, National Taiwan University, No. 1, Jen-Ai Rd., First Section, Taipei, Taiwan

Hepatitis delta virus (HDV) is a satellite virus of hepatitis B virus, as it requires hepatitis B virus for virion production and transmission. We have previously demonstrated that sequences within the C-terminal 19-amino acid domain flanking the isoprenylation motif of the large hepatitis delta antigen (HDAg-L) are important for virion assembly. In this study, site-directed mutagenesis and immunofluorescence staining demonstrated that in the absence of hepatitis B virus surface antigen (HBsAg), the wild-type HDAg-L was localized in the nuclei of transfected COS7 cells. Nevertheless, in the presence of HBsAg, the HDAg-L became both nuclei- and cytoplasm-distributed in about half of the cells. An HDAg-L mutant with a substitution of Pro-205 to alanine could neither form HDV-like particles nor shift the subcellular localization in the presence of HBsAg. In addition, nuclear trafficking of HDAg-L in heterokaryons indicated that HDAg-L is a nucleocytoplasmic shuttling protein. A proline-rich HDAg peptide spanning amino acid residues 198 to 210, designated NES(HDAg-L), can function as a nuclear export signal (NES) in Xenopus oocytes. Pro-205 is critical for the NES function. Furthermore, assembly of HDV is insensitive to leptomycin B, indicating that the NES(HDAg-L) directs nuclear export of HDAg-L to the cytoplasm via a chromosome region maintenance 1-independent pathway.

Hepatitis delta virus (HDV) consists of a single-stranded circular RNA genome of ~1.7 kilobases and the only known HDV-encoded protein, hepatitis delta antigen (HDAg), enveloped by the hepatitis B virus surface antigen (HBsAg) (1–3). In the livers and sera of HDV-infected patients, there are two forms of HDAG, small HDAG (HDAg-S) and large HDAG (HDAg-L). The two HDAgs are identical in sequence except for an additional 19-amino acid extension at the C terminus of the HDAg-L (1, 2, 4). The HDAgs share identical functional domains within the common region, but exhibit very distinct functions in viral multiplication. The HDAg-S is required for the replication of HDV RNA, whereas the HDAg-L is essential for virion assembly and functions as a potent trans-dominant inhibitor in viral replication (5–10).

Previous studies have demonstrated that the HDAg-L is capable of copackaging with the small HBsAg to form virus-like particles in both transfected Huh-7 and COS7 cells (7–11). HDAg-L is a nuclear phosphoprotein (12), whereas HBsAg confines to the cytoplasm. Extensive deletion analysis of the HDAg-L revealed several functional motifs, including nuclear localization signals and oligomerization domains that are dispensable for the formation of virus-like particles (10, 13). In addition, the unique domain spanning amino acid residues 198–214 of the HDAg-L was demonstrated to contain signals sufficient for HDV assembly (10, 13). These results implied that the HDV assembly occurs in the cytoplasm. Analysis within the isoprenylation motif of the HDAg-L (211-CRPQ-214) revealed a good correlation between package activity and the status of isoprenylation (14), but attachment of an isoprenylation motif did not render the HDAg-S a structure capable of copackaging with HBsAg (10). These results further suggested that the 13-amino acid stretch proceeding the isoprenylation motif of the HDAg-L plays a critical role on HDV assembly. In this study, we have further elucidated the molecular mechanisms by which the 13-amino acid stretch contributes to the assembly of HDV. Site-directed mutagenesis within the 13-amino acid stretch indicated that Pro-205 is critical for the relocalization of HDAg-L from the nucleus to the cytoplasm and is involved in the assembly of HDV. An interspecies heterokaryon assay clearly demonstrated that the HDAg-L is a nucleocytoplasmic shuttling protein. In addition, the C-terminal domain spanning amino acid residues 198–210 of the HDAg-L could function as a nuclear export signal, which directs nuclear export of the HDAg-L to the HBsAg-localized cytoplasm via a CRM1-independent pathway.

EXPERIMENTAL PROCEDURES

Plasmids

Plasmids pECE-d-BE, pECE-d-SM, pECE-C-ES, and pCrev-GFP—Plasmids pECE-d-BE and pECE-d-SM contain cDNAs encoding the HDAg-L and HDAg-S, respectively (12, 15). Plasmid pECE-C-ES encodes the small forms of the HBsAg, p24 and gp27 (10). Plasmid pCrev-GFP (a gift from G. N. Pavlakis, NCI-Frederick Cancer Research and Development Center) encodes a fusion protein of the human immunodeficiency virus (HIV) Rev and the green fluorescent protein (GFP) (16). These plasmids were used in transfection experiments.

Plasmid pB1–3(NdeI)—Plasmid pB1–3(NdeI) was derived from pT7-d-BP (12) by removing the cDNA domain representing the 5’ untranslated region of the HDAg-L. This plasmid was generated for further construction of HDAg-L mutant plasmids.

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‡ To whom correspondence should be addressed. Tel.: 886-2-23123456, ext. 8217; Fax: 886-2-23915295; E-mail: mfchang@ha.mc.ntu.edu.tw.

¶ The abbreviations used are: HDV, hepatitis delta virus; CRM, chromosome region maintenance; GFP, green fluorescent protein; HBsAg, hepatitis B virus surface antigen; HDAg, hepatitis delta antigen; HDAg-L, large HDAg; HDAg-S, small HDAg; HIV, human immunodeficiency virus; IgG(m), mouse IgG; IgG(r), rabbit IgG; NES, nuclear export signal; PBS, phosphate-buffered saline; RNP, ribonucleoprotein.
**Nuclear Export Signal of Delta Antigen**

**HDAG-L Mutant Plasmids**—HDAG-L mutant plasmids encode large HDAGs with point mutations in the region spanning acidic amino acid residues 198–210. The strategy that we used, with modifications, for constructing the mutant plasmids has been described previously (17, 18). In brief, a degenerate 65-mer oligonucleotide C244 was chemically synthesized (5′-TCATCGGGGGGGCAGACATTGGGGGAtAcGGAgGA-GTGCACGGGaGGAGTATTCAATCCAGGA-3′; lowercase letters represent degenerate nucleotides consisting of 91% of the indicated wild-type nucleotide sequences of the corresponding cDNA of the HDAG and 3% each of the other three nucleotides; underlining indicates the extra sequences, including SacI and NcoI recognition sites for cloning purposes). The C244 oligonucleotide was converted into a double-stranded DNA by base pairing two molecules around the NcoI site and mutually primed synthesis with the Klenow fragment of DNA polymerase I. The annealing reaction was performed in a buffer containing 140 mM Tris-HCl, pH 7.6, 15 mM MgCl₂, 2.5 mM dithiothreitol at 65 °C for 30 min and cooled to room temperature over a period of 2 h. The extension reaction was carried out in the presence of 10 mM each of the dNTP for at least 3 h at 37 °C. Following digestions with SacI and NcoI restriction endonuclease, the resultant 49-base pair SacI-NcoI fragments were purified from a 2% agarose gel and used to replace the cognate fragment of plasmid pB1–3(19).

**Heterokaryon Assay**

The heterokaryon assay was performed as described previously (23, 24) with modifications. Briefly, HeLa cells were seeded onto coverslips at 20% confluency and transfected with HDAG-L-encoding plasmid pECE-d-BE on the next day. At 9 h posttransfection, NIH3T3 cells were added to yield 40% confluency, and cell fusion was carried out at 30 h posttransfection to promote interspecies heterokaryon formation. Cycloheximide (100 μg/ml) (Sigma) was added 1 h prior to cell fusion, and cell fusion was performed for 2 min with 50% polyethylene glycol 6000 (Mallinckrodt) in DMEM containing 5% glucose. Following fusion, and an additional incubation for 5 h in the culture medium containing cycloheximide, the cells were fixed and immunofluorescence staining was performed as described earlier. Cell nuclei were detected by Hoechst 33258 (1 μg/ml) (Sigma). The dye gives differential staining patterns of the nuclei of mouse NIH3T3 cells and human HeLa cells. Cells were viewed using a Zeiss Axioskop 2 fluorescence microscope.

**Conjugation of Synthetic Peptides to Rabbit Immunoglobulin**

Peptides NES(HDAG-L) and NES(HDAG-L) that represent HDAG-L from amino acid residues 198–210 (ILFPADPPFSPQS) and the peptide with Pro-205 replaced by Ala (ILFPADFPSQPS, underlining indicates the mutation), respectively, were synthesized by peptide synthesizer (model 431, Applied BioSystems Inc.). Both peptides possess an additional cysteine residue at the N terminus of the NES, allowing conjugation of a single affinity-purified rabbit IgG (IgG(r)) (Sigma). Activation of IgG(r) with the bifunctional cross-linking reagent sulfo-N-succinimidyl 4-(N-maleimidomethyl) cyclohexane-1-carboxylate and conjugation to the synthetic peptides were carried out as described previously (25) with modifications. In brief, 8 mg of sulfo-N-succinimidyl 4-(N-maleimidomethyl) cyclohexane-1-carboxylate were added to 5 mg of IgG(r) in 1 ml of PBS, pH 7.4, and incubated for 1 h at 20 °C. The activated IgG(r) was subsequently separated from excess sulfo-N-succinimidyl 4-(N-maleimidomethyl) cyclohexane-1-carboxylate by passing through a Sephadex P-6 column (Amersham Pharmacia Biotech) equilibrated with PBS, pH 7.4, and conjugated to equal amount of the synthetic peptides NES(HDAG-L) and NES(HDAG-L). The reaction was carried out in PBS, pH 6.5, at room temperature for 3 h. Free peptides were removed by passing the mixture through a Sephadex P-6 column. The coupling ratio of both conjugates, IgG(r)-NES(HDAG-L) and IgG(r)-NES(HDAG-L), was estimated to be 10 peptides per IgG(r) molecule as analyzed by SDS-10% polyacrylamide gel electrophoresis (data not shown). For microinjection, the conjugates were concentrated to 20 mg/ml using a Centricron 30 (Amicon).

**Microinjection and Nuclear Export Assay**

For the study of nuclear export of HDAG peptides, stage VI oocytes from ovaries of X. laevis females were manually defolliculated, maintained at 20 °C in OR2 medium (825 mM NaCl, 2.5 mM KCl, 1.0 mM CaCl₂, 1.0 mM MgCl₂, 1.0 mM Na₂HPO₄, 5.0 mM HEPES, pH 7.8), and used for microinjection within 3 days. For direct injection of protein samples into nuclei, the oocytes were carefully spun at 3,000 rpm for 10 min in an HS-4 rotor (Sorvall) prior to injection. Microinjection needles were prepared on a Flaming/Brown micropipette puller (model P-97, Sutter Instrument Co.) using borosilicate glass capillaries (0.75–1.0 mm, Sutter Instrument Co.). Rabbit IgG conjugated peptides IgG(r)-NES(HDAG-L) and IgG(r)-NES(HDAG-L) were injected at a final concentration of 10 mg/ml, and an affinity-purified mouse IgG was co-injected as an internal control. The protein samples were centrifuged at 15,000 × g for 10 min at 4 °C immediately before injection. Following injections, the oocytes were incubated at room temperature for 1.5 h and then dissected manually in OR2 medium to separate the nuclei from cytoplasmic fraction. The cytoplasmic and nuclear fractions were homogenized by sonication four times for 15 s each and centrifuged at 12,000 × g for 10 min to remove insoluble pellets. The resulting nuclear fractions were subjected to SDS-10% polyacrylamide gel electrophoresis and immunoblot analysis as described earlier.

**Leptomycin B Treatment**

For analysis of the effect of leptomycin B on the subcellular distribution of Rev-GFP fusion protein, leptomycin B (provided by Norvatis) was added to the culture media at a final concentration of 2 μM at 24 h posttransfection, and actinomycin D (1 μg/ml) was added 2 h prior to cell fixation. For evaluation of the effect of leptomycin B on HDV

**Immunoblot Analysis**

To perform immunoblot analysis, protein lysates separated by SDS-polyacrylamide gel electrophoresis were electrotransferred onto an Immobilon-P membrane (Millipore) at 800 V for 2 h at 4 °C. The membrane was blocked with 5% nonfat dried milk in phosphate-buffered saline (PBS) for 1 h at room temperature in a shaker bath and then incubated at 4 °C overnight with specific antibodies as follows. For determination of the package activity of HDAG-L mutants, virus-like particles were collected from culture media as described previously (10), except that the media were harvested 4 days posttransfection. In addition, the viral pellets were resuspended in 2× sample buffer (12.5 mM Tris-HCl, pH 6.8, 2% SDS, 20% glycerol, 0.25% bromphenol blue, 5% β-mercaptoethanol), boiled for 3 min, and subjected to SDS-10% polyacrylamide gel electrophoresis and immunoblot analysis to examine the presence of HBsAg and HDAG mutants.

**Cell Lines, DNA Transfection, and Indirect Immunofluorescence Staining**

Monkey kidney cells (COS7), human cervical carcinoma cells (HeLa), and mouse embryonic fibroblast cells (NIH3T3) were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum plus 100 units of penicillin and 100 μg of streptomycin/ml. DNA transfection (19) and indirect immunofluorescence staining (20) was performed as described previously, except that cells were seeded at 15–20% confluency 16 h prior to transfection.

**Harvest of Virus-like Particles and Determination of Package Activity**

To determine the package activity of HDAG-L mutants, virus-like particles were collected from culture media as described previously (10), except that the media were harvested 4 days posttransfection. In addition, the viral pellets were resuspended in 2× sample buffer (12.5 mM Tris-HCl, pH 6.8, 2% SDS, 20% glycerol, 0.25% bromphenol blue, 5% β-mercaptoethanol), boiled for 3 min, and subjected to SDS-12% polyacrylamide gel electrophoresis and immunoblot analysis to examine the presence of HBsAg and HDAG mutants.

**Microinjection and Nuclear Export Assay**

For the study of nuclear export of HDAG peptides, stage VI oocytes from ovaries of X. laevis females were manually defolliculated, maintained at 20 °C in OR2 medium (825 mM NaCl, 2.5 mM KCl, 1.0 mM CaCl₂, 1.0 mM MgCl₂, 1.0 mM Na₂HPO₄, 5.0 mM HEPES, pH 7.8), and used for microinjection within 3 days. For direct injection of protein samples into nuclei, the oocytes were carefully spun at 3,000 rpm for 10 min in an HS-4 rotor (Sorvall) prior to injection. Microinjection needles were prepared on a Flaming/Brown micropipette puller (model P-97, Sutter Instrument Co.) using borosilicate glass capillaries (0.75–1.0 mm, Sutter Instrument Co.). Rabbit IgG conjugated peptides IgG(r)-NES(HDAG-L) and IgG(r)-NES(HDAG-L) were injected at a final concentration of 10 mg/ml, and an affinity-purified mouse IgG was co-injected as an internal control. The protein samples were centrifuged at 15,000 × g for 10 min at 4 °C immediately before injection. Following injections, the oocytes were incubated at room temperature for 1.5 h and then dissected manually in OR2 medium to separate the nuclei from cytoplasmic fraction. The cytoplasmic and nuclear fractions were homogenized by sonication four times for 15 s each and centrifuged at 12,000 × g for 10 min to remove insoluble pellets. The resulting nuclear fractions were subjected to SDS-10% polyacrylamide gel electrophoresis and immunoblot analysis as described earlier.

**Leptomycin B Treatment**

For analysis of the effect of leptomycin B on the subcellular distribution of Rev-GFP fusion protein, leptomycin B (provided by Norvatis) was added to the culture media at a final concentration of 2 μM at 24 h posttransfection, and actinomycin D (1 μg/ml) was added 2 h prior to cell fixation. For evaluation of the effect of leptomycin B on HDV

The specific interactions between antigens and antibodies were detected by the enhanced chemiluminescence system (Amersham Pharmacia Biotech.).
assembly, leptomycin B was added at 24 h posttransfection at a final concentration of 2 or 20 nm for various time periods prior to immunoblot analysis.

RESULTS

A Mutation at Pro-205 of the HDAg-L Affects the Assembly of HDV—Previous studies have demonstrated that the unique C-terminal domain of the HDAg-L, encompassing a Pro-rich region and an isoprenylation motif 211-CRPQ-214, possesses signals sufficient for the assembly of HDV (10, 13, 14). To determine specific amino acid residues that contribute to HDV assembly, site-directed mutagenesis in the domain composed of amino acid residues 198–210 of the HDAg-L was performed as described under “Experimental Procedures.” Following transformation, 120 recombinant DNA clones were picked for DNA sequencing, from which 41 substitution mutants of the HDAg-L were identified (data not shown). Eight mutant constructs harboring single amino acid substitution were selected (Fig. 1A) for virion package assay. As shown in Fig. 1B, all of the eight constructs expressed mutant proteins in COS7 cells at levels similar to that of the wild-type HDAg-L (top panel). When virus-like particles in the culture media were harvested and analyzed for the presence of HBSAg and HDAg-L, both forms of the small HBsAg, p24 and gp27, were detected at comparable levels among the wild-type control and mutants (Fig. 1B, bottom panel). Nevertheless, the HDAg-L with single substitution of Pro-205 to Ala, designated HDAg(P205A), was not detected in the culture medium (Fig. 1B, middle panel). These results indicated that Pro-205 is critical for the package activity of HDAg-L and the assembly of HDV. It is noteworthy that the amino acid substitution at the adjacent Pro-204 residue had little effect on the package activity. The variation of the package activity of the various HDAg-L mutants should reflect the intrinsic properties of individual amino acid residues. As expected, the HDAg-S that lacks the unique C-terminal sequence of HDAg-L failed to form virus-like particles with the small HBsAg (Fig. 1B, lane HDAg-S).

Cytoplasmic Localization of HDAg-L in the Presence of Small HBsAg—A previous study demonstrated that isoprenylation of HDAg-L mediates the interaction between HDAg-L and HBsAg in vitro and proposed that the interaction is the molecular basis of HDV assembly (26, 27). However, this hypothesis does not explain where and how these two proteins come to direct contact in vivo because HBsAg is localized in the cytoplasm and HDAg-L is a nuclear phosphoprotein (12). To understand how the interaction occurs, COS7 cells were transfected with plasmids encoding HDAg-L and HBsAg, and immunofluorescence staining was performed at 12 h intervals 2 days posttransfection. At 48 h posttransfection, HDAg-L was detected exclusively in the nucleus of transfected cells as the Type I pattern shown in Fig. 2A. At 60 h posttransfection, about 25–35% of the transfected cells retained the Type I nucleolus staining pattern, but 65–75% of the cells exhibited both nucleolus and nucleoplasm staining pattern (Type II pattern) (Fig. 2A). The relocalization of HDAg-L from the Type I to the Type II pattern at 60 h posttransfection was also observed in the absence of HBsAg (Fig. 2B). A striking change was observed at 72 h posttransfection; the HDAg-L became both nuclei- and cytoplasm-distributed (Fig. 2A, Type III) in about 43–47% of the transfected cells in the presence of small HBsAg but was restricted to the nucleolus and nucleoplasm in up to 92–95% of the cells in the absence of HBsAg (Fig. 2B). In addition, when COS7 cells were cotransfected with plasmids encoding HDAg-S and small HBsAg, the HDAg-S confined to the nuclei similar to that of cells transfected with HDAg-L-encoding plasmid alone (data not shown).

The Mutation at Pro-205 Abolished the Cytoplasmic Relocalization of HDAg-L in the Presence of Small HBsAg—The unique property of HDAg-L to relocalize to the cytoplasm in the presence of small HBsAg implied that the intracellular interaction between HDAg-L and HBsAg occurs in the cytoplasm and is mediated by the unique C terminus of the HDAg-L. We therefore made an assumption that the relocalization of HDAg-L to the cytoplasm is an essential step for HDV assembly. To examine this hypothesis, immunofluorescence staining was performed at 72 h posttransfection with the package-defective HDAg(P205A) and the package–competent HDAg(P204A) mutant proteins in the presence of small HBsAg. Interestingly, we observed a similar subcellular distribution pattern for the wild-type HDAg-L and HDAg(P204A) mutant protein, but the package-defective HDAg(P205A) mutant protein failed to relocalize from the nucleus to the cytoplasm. The statistical results are summarized in Fig. 3. The correlation between relocalization and package activity of the HDAg-L suggested that the cytoplasmic relocalization of HDAg-L is facilitated by the unique C terminus of the HDAg-L and is essential for the viral assembly;
the mutation at Pro-205 abolished cytoplasmic relocalization and rendered HDAG-L package-defective.

**HDAG-L Is a Nucleocytoplasmic Shuttling Protein.**—The cytoplasmic relocalization of HDAG-L and its assembly with the cytoplasm-localized HBsAg suggested that HDAG-L bears an NES located in the unique C-terminal domain to facilitate the translocation. To examine this possibility, an interspecies heterokaryon assay was performed in the absence or presence of small HBsAg. Following immunofluorescence staining at various time points posttransfection, fields each containing at least 150 HDAG-positive cells were randomly selected. Cell numbers bearing each type of the defined staining patterns of HDAG-L were counted and plotted as the percentage of the total number of the HDAG-positive cells in the same field. The statistical results represent the average of two independent experiments.

**FIG. 2.** Cytoplasmic relocalization of HDAG-L in the presence of small HBsAg. COS7 cells were transfected with the HDAG-L-encoding plasmid pECE-d-BE alone or together with the HBsAg-encoding plasmid pECE-C-ES. At 48, 60, and 72 h posttransfection, indirect immunofluorescence staining was performed with rabbit antibodies specific to HDAG. A, representative patterns of the subcellular distribution of HDAG-L. The staining patterns were classified into three types: Type I, nucleoli staining; Type II, both nucleoli and nucleoplasm staining; Type III, nucleoli, nucleoplasm, and cytoplasm staining. B, distribution of HDAG-L in the absence or presence of small HBsAg. Following immunofluorescence staining at various time points posttransfection, fields each containing at least 150 HDAG-positive cells were randomly selected. Cell numbers bearing each type of the defined staining patterns of HDAG-L were counted and plotted as the percentage of the total number of the HDAG-positive cells in the same field. The statistical results represent the average of two independent experiments.

**FIG. 3.** The mutation at Pro-205 abolished the cytoplasmic relocalization of HDAG-L. COS7 cells were co-transfected with the HBsAg-encoding plasmid and a plasmid encoding the wild-type (WT) or mutant HDAG-L as indicated. Indirect immunofluorescence staining was performed 72 h posttransfection. Representative cells with the classified distribution patterns of HDAG-L are essentially as those shown in Fig. 2A. The results are presented statistically as described in Fig. 2B.

NIIH3T3 nuclei have a speckled staining pattern, whereas the HeLa cell nuclei were evenly stained (Fig. 4, B and D). When the interspecies heterokaryons were analyzed with antibodies to HDAG-L, the HDAG-L was detected in both nuclei of the transfected HeLa cells and the untransfected NIH3T3 cells (Fig. 4, A and C). Because the new protein synthesis was blocked by cycloheximide, the nuclear trafficking of HDAG-L in heterokaryons indicated that HDAG-L possesses an NES and is a nucleocytoplasmic shuttling protein.

**The Subdomain Composed of Amino Acid Residues 198–210 of the HDAG-L Can Function as a Nuclear Export Signal in a Heterologous Context.**—To further examine whether the NES of
HDAg-L is located in the unique C-terminal domain, an HDAg peptide, NES(HDAg-L), representing amino acid residues 198–210 of HDAg-L, was synthesized and chemically conjugated to rabbit IgG (Fig. 5A). The resultant IgG(r)-NES(HDAg-L) was co-injected with a control mouse IgG (IgG(m)) into the nuclei of Xenopus oocytes. At 1.5 h postinjection, the oocytes were dissected manually into nuclear and cytoplasmic fractions, and immunoblot analysis was performed to detect the subcellular localization of the IgG(r)-NES(HDAg-L) and IgG(m). The images demonstrated that a majority of the IgG(r)-NES(HDAg-L) transported from the nucleus to the cytoplasm, whereas the control IgG(m) remained retained to be nucleus-localized (Fig. 5B, left panel). These results indicated that the HDAg-L peptide spanning amino acid residues 198–210 acted as an NES to escort IgG(r) to the cytoplasm. To correlate the nuclear export function of HDAg-L to the assembly of HDV, an HDAg-L mutant peptide with Pro-205 replaced by Ala was synthesized and conjugated to IgG(r) (Fig. 5A). The resultant IgG(r)-NES*(HDAg-L) was co-injected with IgG(m) into the nuclei of Xenopus oocytes. Immunoblot analysis demonstrated a retention of IgG(r)-NES*(HDAg-L) in the nucleus (Fig. 5B, right panel). Because HDAg(P205A) was shown to be package-defective (Fig. 1B), these results indicated that Pro-205 is critical for both the nuclear export of HDAg-L and the assembly of HDV.

Leptomycin B Has Little Effect on the Assembly of HDV—HDAg-L harbors an amino acid substitution of Pro-205 to Ala. The resultant IgG(r)-NES*(HDAg-L) represents a conjugate in which the HDAg-L peptide spanning amino acid residues 198–210, whereas IgG(r)-NES(HDAg-L) represents a rabbit IgG conjugate of the HDAg-L peptide spanning amino acid residues 198–210, whereas IgG(r)-NES*(HDAg-L) represents a conjugate in which the HDAg-L peptide harbors an amino acid substitution of Pro-205 to Ala. The HDAg-L peptide spanning amino acid residues 198–210 possesses an NES activity. The rabbit IgG conjugates of HDAg-L peptides as indicated were coinjected with a control IgG(m) into the nuclei of Xenopus oocytes. At 1.5 h after injection, two oocytes from each of the injected groups were randomly selected and dissected manually into nuclear (N) and cytoplasmic (C) fractions. Immunoblot analysis was performed with goat anti-rabbit IgG and goat anti-mouse IgG as indicated.

CRM1-dependent pathway

Leucine-rich NES

Rev(73-83) LQPPLERLTL
PKI(37-46) LALKLGLDI

Serine & acidic amino acid-rich NES

hnRNP K(338-361) GFSAMENTWSADTWPSEWQMAY

Proline-rich NES

HDAG-L(198-210) ILFPADPPFSPQS

N NES(HDAg-L), ILFPADPPFSPQS*, is rich in proline residues. Previous studies have identified CRM1 as a receptor for the leucine-rich NES (30–32). In addition, CRM1-dependent nuclear export could be specifically blocked by leptomycin B (33, 34). To investigate whether CRM1 is involved in the nuclear export of HDAg-L and the assembly of HDV, a system in which leptomycin B blocked the nuclear export of the HIV Rev protein was first established. The nucleolus-localized Rev protein exported to the cytoplasm of transfected COS7 cells when RNA synthesis was inhibited by actinomycin D. Such a translocation was abolished when leptomycin B was added to the culture media at 24 h posttransfection (Fig. 7A). This system was used to study the effect of leptomycin B on HDV assembly.
In this study, we have demonstrated the nuclear trafficking of HDAG-L in heterokaryons and determined that amino acid residues 198–210 within the unique C-terminal domain of the HDAG-L can function as a nuclear export signal. In addition, Pro-205 is critical for the package activity and nuclear export of HDAG-L, but a mutation at the adjacent residue Pro-204 has little effect on the package and subcellular distribution. The cytoplasm targeting of HDAG-L is a prerequisite for the assembly of HDV.

Nuclear export signals have been identified in both viral and cellular proteins (Fig. 6) (35–37). The Rev protein of HIV-1 binds to Rev response element and escorts unspliced and partially spliced viral RNAs to the cytoplasm; the export is mediated by a leucine-rich NES (35–38). In addition, leptomycin B specifically abrogated the export of the Rev protein and viral RNA by binding to the cysteine residue in the middle domain of CRM1, a receptor for the leucine-rich NES (39). These results indicate that the nuclear export of the Rev protein plays an essential role in the life cycle of HIV-1. In the present study, we have identified an NES of HDAG-L spanning amino acid residues 198–210 (Fig. 5). The NES(HDAG-L) represents an atypical NES that is rich in proline (Fig. 6). In addition, the feature of insensitive to leptomycin B (Fig. 7) suggest that the NES(HDAG-L) drives nuclear export of the HDAG-L via a CRM1-independent pathway different from that of the Rev-like NES. Interestingly, a recent study also identified a CRM1-independent nuclear export of heterogeneous RNP K (40). However, the NES of heterogeneous RNP K is rich in serine and acidic residues (Fig. 6). These indicate that different mechanisms mediated by a variety of nuclear export signals are involved in the nuclear export pathway. The molecular mechanism of HDV assembly is not fully understood, and cellular receptors participate in the nuclear export remain to be elucidated. However, the present study has provided some information. Our current working hypothesis is that HDV genomic RNA forms RNP complexes with the HDAGs that translocate from nucleoplasm to the nuclear membrane via an interaction between NES(HDAG-L) and an unidentified NES receptor. The RNP complexes then move further to the endoplasmic reticulum for assembly with HBsAg. The isoprenylation motif that is known to be important for the package activity of HDAG-L (10, 14, 26, 27) and additional cellular factors, including nucleoporins, may also participate in the translocation process. To complete the life cycle of HDV, progeny viral particles transport to the plasma membrane and exocytosis occurs to release mature viral particles into the extracellular milieu.

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