Characterization of Nuclear Targeting Signal of Hepatitis Delta Antigen: Nuclear Transport as a Protein Complex

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Hepatitis delta antigen (HDAg) is the only protein encoded by hepatitis delta virus (HDV). HDAg has been demonstrated in the nuclei of HDV-infected hepatocytes, and its nuclear transport may be important for the replication of HDV RNA. In this report, we investigated the mechanism of nuclear transport of HDAg. By expressing fusion proteins consisting of the different portions of HDAg and α -globin, we have identified a nuclear localization signal (NLS) within the N-terminal one-third of HDAg. It consists of two stretches of basic amino acid domains separated by a short run of nonbasic amino acids. Both of the basic domains are necessary for the efficient nuclear transport of HDAg. The nonbasic spacer amino acids could be removed without affecting the nuclear targeting of HDAg significantly. Thus, the HDAg NLS belongs to a newly identified class of NLS which consists of two discontinuous stretches of basic amino acids. This NLS is separated from a stretch of steroid receptor NLS-like sequence, which is also present but not functioning as an NLS, in HDAg. Furthermore, we have shown that subfragments of HDAg which do not contain the NLS can be passively transported into the nucleus by a *trans*-acting full-length HDAg, provided that these subfragments contain the region with a leucine zipper sequence. Thus, our results indicate that HDAg forms aggregates in the cytoplasm and that the HDAg oligomerization is probably mediated by the leucine zipper sequence. Therefore, HDAg is likely transported into the nucleus as a protein complex.

Human hepatitis delta virus (HDV) is an infectious agent whose transmission requires the helper function of hepatitis B virus (HBV) (27). HDV may cause fulminant or chronic hepatitis by coinfecting with HBV or by superinfecting chronic HBV carriers (1, 11, 14). HDV is a 36-nm particle consisting of (i) an envelope with HBV surface antigen and (ii) a nucleocapsid protein, hepatitis delta antigen (HDAg) (4, 13). The HDV genome is a single-stranded, circular RNA of 1.7 kb (16, 20, 34). HDAg is encoded by the antigenomic RNA of HDV and is the only protein encoded by HDV. HDAg has been detected in the nuclei of hepatocytes in hepatitis delta patients (28) and in hepatic and nonhepatic cell lines transfected with HDAg-encoding cDNA (5). It has been suggested that in the initial stage of HDV genomic replication, HDAg binds to HDV RNA and transports it into the nucleus, where RNA replication occurs (19). HDAg consists of two protein species of 27 and 24 kDa, termed the large and small HDAg, respectively, which are identical in sequence except for the C-terminal extension (2, 3, 26, 38). These two HDAg species are encoded separately by two different HDV RNA species (35). HDAg is a phosphoprotein with an RNA-binding activity (5). The RNA-binding site has been mapped to the middle one-third region of HDAg (18). The exact function of HDAg in the HDV life cycle is not yet clear. It has been suggested that the small HDAg is required for HDV RNA replication, while the large one may interfere with it (6).

To understand the structure and function of HDAg, we have undertaken systematic studies to analyze the properties of different functional domains of HDAg and the mechanism of its transport into the nucleus. In this study, we have demonstrated that the nuclear transport of HDAg is directed by a specific nuclear localization signal (NLS) which con-

tains two discrete basic amino acid domains separated by a short stretch of nonbasic amino acids. Both of the basic domains, but not the spacer sequence, are required for nuclear localization. Furthermore, we have shown that a subfragment of HDAg containing heptad repeats of leucine residues but lacking the NLS can be transported into the nucleus as a result of a complex formation with the full-length HDAg. Thus, this study has suggested that HDAg oligomerizes in the cytoplasm through a leucine zipper sequence and that HDAg can be transported into the nucleus as a protein complex.

MATERIALS AND METHODS

Cell line and DNA transfection. The monkey kidney cell line COS-7 (10) was used as the recipient cells for DNA transfection studies. The cell line was maintained in Dulbecco's modified essential medium containing 10% fetal calf serum. DNA plasmids were transfected by using the calcium phosphate precipitation method as described previously (12).

Construction of HDAg deletion mutants. The simian virus 40-derived expression vector PECE1 (17) was used throughout this study. For the construction of PECE(1-88)D and PECE(89-163)D, plasmid PECE- δ -BE (5), which contains the full-length large HDAg, was first digested with either *StuI* or *SmaI*. A second digestion with *EcoRI* was made in the cloning site of the PECE vector to remove the unwanted HDAg-coding region. The fragment containing the vector and the truncated HDAg open reading frame was isolated, blunt ended with Klenow DNA polymerase, and religated. PECE(89-163)D and PECE(164-211)D were constructed by digesting PECE- δ -BE with *StuI* and *SmaI* and with *SmaI* and *SalI*, respectively. Inserts were isolated, blunt ended, and inserted into the *KpnI* site on the expression vector PECE-ATG, which has been described previously (36).

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PECE- δ -SM, which expresses the full-length small HDAG, was constructed by replacing the *SalI*-*StuI* fragment of PECE- δ -BE with the fragment from plasmid HD473 (20).

Construction of an HDAG-globin chimera. An α -globin gene insert was prepared by digesting plasmid PECE-core- α -globin (36) with *Bam*HI and *Eco*RI. The fragment containing the globin cDNA was isolated from the gel, blunt ended, and cloned into appropriate sites downstream of the HDAG-specific sequence. For PECE(1-88)DG and PECE(1-163)DG, the globin-coding region was inserted into the *Xba*I site on PECE(1-88)D and PECE(1-163)D, whereas for PECE(89-163)DG and PECE(164-211)DG, the globin-coding region was inserted into the *Eco*RI site on PECE(89-163)D and PECE(164-211)D by blunt-end ligation.

Construction of HDAG N-terminal deletion mutants. Because of the lack of convenient restriction sites on the N-terminal one-third of HDAG, different strategies were used to construct various HDAG N-terminal deletion mutants. The nested-set deletion mutants were constructed by exonuclease III digestion at the *SalI* site of PECE(1-88)DG to remove different lengths of fragments from the carboxyl end of the HDAG-coding region. Modifications on the PECE(1-88)DG construct were first made to allow unidirectional deletions of the HDAG-coding sequence toward its amino terminus. A polylinker was prepared by digesting PECE1 vector (17) with *Bgl*II and *Xba*I. The 45-bp polylinker from the vector was purified from a 6% polyacrylamide gel, blunt ended, and cloned into the *Sac*I site, which was located between the coding regions of HDAG and α -globin of PECE(1-88)DG. The two in-frame stop codons present in the polylinker were removed by digestion with *Sac*I and *Bam*HI. The resulting construct was then linearized with *SalI* and *Kpn*I and treated with exonuclease III for the generation of nested-set deletion mutants. Several in-frame mutants were identified by DNA sequencing analysis. Additional in-frame mutants were generated by further digesting the plasmid with *Bam*HI, blunt ending, and religating. All of the constructs were confirmed by DNA sequencing analysis.

A second group of site-specific deletion mutants was constructed also by using plasmid PECE(1-88)DG. For making PECE(67-88)DGP, two primers, 314 (TCGAAGATGAGCGGGGCACCCCGGCGAAGAAGCTCCGGATGGAC, complementary to nucleotides 1607 to 1596, which contains the initiating codon of HDAG, and 1371 to 1404 of HDV RNA) and 301 (GGCTCCAGCTTAACGGTATT, corresponding to nucleotides 454 to 474 of the globin gene), were used to amplify an HDAG-coding region (residues 67 to 88) and the downstream globin sequence on PECE(1-88)DG by polymerase chain reaction (PCR). The PCR product was gel purified, phosphorylated, blunt ended, and inserted into the *Sma*I site on the PECE1 vector. PECE(58-88)DGP and PECE Δ (76-82)DGP were constructed in a similar way, but instead of using primer 314, several different primers were used. For PECE(58-88)DGP, primer 300 (TCGAAGATGAGCATCGGAAAGAAGGATAAGGAT, complementary to nucleotides 1607 to 1596, which contains the initiating codon of HDAG, and nucleotides 1430 to 1410 of HDV RNA) was used. For PECE Δ (76-82)DGP, primer 432 (AAGATGAGCCCCCGGCGAAGAAGCTCCGGGCGCGACCTAGGAAGAGG, complementary to nucleotides 1607 to 1599, which contains the initiation codon of HDAG, and nucleotides 1397 to 1377 and 1355 to 1338 of HDV RNA) was used. PECE(58-74)DGRP was constructed by digesting PECE(58-88)DGP with *Mro*I and *Eco*RI; the 2.9-kb fragment was then isolated from agarose gel, blunt ended, and ligated

to the α -globin gene, which was generated by PCR amplification with the primers named globin-ATG (ATGGTGCTGTCTCCTGCCGAC, corresponding to nucleotides 38 to 58 of globin) and 301. PECE(75-88)DGR was constructed by digesting PECE(1-88)DG with *Mro*I and *Xba*I; the insert was blunt ended and cloned into the *SalI* site on the PECE-ATG vector. The resulting plasmid was subjected to a second *SalI* digestion. Both the vector and the insert were blunt ended and religated.

PECE(32-48)DGP was also generated by PCR cloning. Two primers, 362 (TCGAAGATGAGCAGAGACCTCCGGAAGTTAAAG, complementary to nucleotides 1607 to 1596, which represent the sequence around the initiating codon of HDAG, and 1488 to 1508 of HDV RNA) and 301, were used to amplify the corresponding region on the precursor construct, PECE(1-48)DG.

PECE(67-77)DGO was constructed by inserting a double-stranded fragment,

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TCGAAGATGAGCGGGGCACCCCGGCGAAGAAGCTCCGGATGGAC
AGCTTCTACTCGCCCGTGGGGCCGCTTCTTCGAGGCCTACCTG
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corresponding to nucleotides 1607 to 1596, which represent the sequence around the initiation codon of HDAG, and 1404 to 1371 of HDV RNA into the *Sma*I site on PECE. The globin fragment was prepared by digesting PECE-core- α -globin (36) with *Nco*I and *Eco*RI; the insert was purified, blunt ended, and subsequently inserted at the *Eco*RI site on PECE. All of the constructs were confirmed by DNA sequencing analysis.

Immunofluorescence. Immunofluorescence was performed by a method modified from the published procedure (24). Rabbit anti-HDAG-TrpE antibody (18) and rabbit anti-human hemoglobin antibody (Dako Corp., Santa Barbara, Calif.) were the primary antibodies. Rhodamine-conjugated goat anti-rabbit antibody (Boehringer Mannheim) was used as the secondary antibody.

RESULTS

Nuclear localization of the large and small HDAG. We have previously shown that the HDAG of 27 kDa was localized exclusively in the nuclei of the transfected COS cells (5). The protein was distributed evenly throughout the entire nuclei in some cells but localized exclusively in the nucleoli in other cells (5). Since there are two different forms of HDAG, we compared the patterns of nuclear localization of both forms. The plasmids containing the open reading frame for either the large or small HDAG were transfected into COS cells, and the transfected cells were examined for the localization of HDAG by immunofluorescence staining using an antibody specific for a TrpE-HDAG fusion protein (18). The results showed that the both forms of HDAG were localized exclusively in the nuclei. There were several patterns of nuclear localization (Fig. 1). In the majority of cells, HDAG was either distributed evenly throughout the entire nuclei or present exclusively in the nucleoli (Fig. 1a and b); however, in a small number of cells, it was present only in the nucleoplasm, excluding nucleoli (Fig. 1c). No difference could be detected in the pattern of distribution between the large (data not shown) and small (Fig. 1) HDAG.

Identification of the NLS in HDAG. To determine whether there was a specific sequence in HDAG responsible for its nuclear transport and localization, we tested the ability of different portions of HDAG (Fig. 2) to be transported to the nuclei. The results showed that the N-terminal one-third portion (88 amino acids) of HDAG was localized exclusively

FIG. 1. Patterns of nuclear localization of small HDag. (a and b) HDag localized in the nucleoli only or evenly in the whole nuclei (c) HDag localized in the nucleoplasm, sparing nucleoli. Plasmids PECE- δ -SM (expressing the small HDag) were transfected into COS cells, and immunostaining was performed with rabbit anti-HDag antibody.

in the nuclei of the transfected COS cells (Fig. 3a). Furthermore, when this portion was fused with human α -globin, which is a cytoplasmic protein, the chimeric protein was also localized exclusively in the nuclei (Fig. 3b). In contrast, the chimeric proteins consisting of globin and the middle one-third (amino acids 89 to 163) or carboxy terminal one-third (amino acids 164 to 211) of HDag were localized exclusively in the cytoplasm (Fig. 3c and data not shown). As expected, the N-terminal two-thirds of HDag was also localized in the nuclei (data not shown). When the PECE vector alone was transfected, no immunofluorescence staining was detected (Fig. 3d). These results indicated that the N-terminal one-third of HDag contained an NLS. It is worth noting that the HDag-globin chimeric proteins were localized only in the nucleoplasm, sparing nucleoli. This was in contrast to the nuclear localization pattern of the HDag of comparable regions without globin (Fig. 3a and b).

To map precisely the NLS of HDag, various portions of the N-terminal one-third of HDag were fused with α -globin, and their subcellular localization in transfected COS cells was examined. The results suggested that the NLS of HDag was localized within residues 67 to 88 (Table 1). To rule out the possibility that the cytoplasmic localization of some of the proteins was due to the instability of fusion proteins,

FIG. 2. Schematic diagram of the structure and subcellular localization of plasmids used in this study. Dark box, HDag-specific sequence; shaded box, α -globin. The subcellular localization of these constructs is indicated. N, nucleus; C, cytoplasm.

FIG. 3. Immunofluorescence of COS cells expressing various truncated HDag. (a) PECE(1-88)D; (b) PECE(1-88)DG; (c) PECE(89-163)DG; (d) PECE vector. Immunostaining was performed with the antibody specific for HDag (a) or with the antibody specific for α -globin (b to d).

some of the HDag-globin chimeric proteins were also studied by immunofluorescence, using antibodies specific both for HDag and for globin, and the same results were obtained (data not shown). Furthermore, immunoprecipitation of the transfected cell lysates with these two antibodies showed that the chimeric proteins were intact (data not shown).

Residues 67 to 88 of HDag contain two clusters of basic amino acids with one or two proline residues, i.e., PPAK KLR (residues 69 to 75) and PRKR (residues 85 to 88). This feature bears resemblance to the NLSs described for other nuclear proteins (8). To examine whether both stretches of basic amino acid residues were necessary for nuclear trans-

port, we tested two chimeric proteins, each of which contained only one part of these two basic regions. PECE(67-77)DGO, which contained the upstream portion of the basic group, PPAKKLR, was distributed in both the cytoplasm and nuclei, whereas PECE(75-88)DGR, containing the downstream PRKR sequence, was localized only in the cytoplasm (Fig. 4b and c). This was in contrast to the protein containing residues 67 to 88, which was localized exclusively in the nuclei (Fig. 4a). Thus, neither of the basic amino acid-rich regions alone was sufficient for efficient nuclear transport. We also tested a chimeric protein which included the adjacent basic amino acids KKDK (nucleotides 60 to 63)

TABLE 1. Subcellular localization of constructs containing different domains of the NLS sequence of HDag^a

Construct	HDag sequence	Localization ^b
	30 48 58 67 74 77 88 LERDL <u>RLK</u> <u>KKKIKK</u> LEEDNPWLGNIGKIKDKDGEGAPPAKKLRMDQMEIDAGPRKR	
PECE(67-88)DGP	GAPPAKKLRMDQMEIDAGPRKR	N
PECE(75-88)DGR	RMDQMEIDAGPRKR	C
PECE(67-77)DGO	GAPPAKKLRMD	C/N
PECE(58-74)DGRP	IGKKDKDGEGAPPAKKL	C/N
PECEΔ(76-82)DGP	PPAKKLR AGPRKR	N
PECE(32-48)DGP	RD <u>RLK</u> <u>KKKIKK</u> LEEDN	C

^a All of the constructs were fused to globin, but only the HDag-derived sequences are indicated. The dotted line represents the nonbasic residues deleted. The basic amino acids in the NLS are underlined, and the putative steroid receptor NLS-like sequence is double underlined. The leucine zipper sequence is identified by dots.

^b N, nucleus; C, cytoplasm.

FIG. 4. Immunofluorescence of COS cells expressing HDag containing various portions of an NLS. Immunostaining was performed with the antibody specific for globin. (a) PECE(67-88)DGP; (b) PECE(75-88)DGR; (c) PECE(67-77)DGO; (d) PECE(58-74)DGRP.

and PPAKKLR. The subcellular distribution of this protein (residues 58 to 74) (Fig. 4d) was similar to that of the protein containing residues 67 to 77 only, suggesting that KKDK did not facilitate nuclear transport. Thus, both stretches of basic residues (residues 69 to 75 and 85 to 88) were necessary and sufficient for nuclear transport. Furthermore, to test the importance of the nonbasic residues between these two basic regions, we studied construct PECE Δ (76-82)DGP, in which seven of the nine nonbasic residues were deleted. The chimeric protein expressed was detected predominantly in the nuclei (data not shown), suggesting that the nonbasic spacer region was not necessary for HDag nuclear transport.

There was another stretch of basic amino acids (residues 35 to 44), which had been noted to resemble the NLS of steroid receptors (25). However, as shown with the protein containing residues 1 to 68, this sequence was probably not capable of transporting the α -globin into the nucleus (Fig. 2). To rule out the possibility that the activity of this NLS-like sequence was interfered with by the presence of other sequences, another chimeric protein containing residues 32 to 48 was examined. The results showed that the protein containing the steroid receptor NLS-like sequence was detected only in the cytoplasm (data not shown). Therefore, we concluded that PPAKKLR and PRKR constituted the minimum NLS required for the nuclear transport of HDag and that it was the only NLS active in HDag.

Nuclear transport of HDag subfragments without an NLS. Chang (11) has shown that the in vitro-translated full-length

HDag or its N-terminal one-third alone can dimerize and oligomerize in vitro. Thus, it is possible that HDag forms oligomers in the cells and is transported into the nuclei as a protein complex. To test this possibility, we cotransfected COS cells with a construct expressing a chimeric protein containing globin fused to an HDag subfragment which did not contain an NLS [PECE(1-68)DG] and the full-length HDag which was not fused with globin (PECE- δ -BE). The globin-specific antibody was then used for immunofluorescence staining to determine the subcellular distribution of the HDag-globin chimeras. As shown earlier, the full-length HDag was localized exclusively in the nuclei (Fig. 1), whereas the protein containing residues 1 to 68 was localized exclusively in the cytoplasm (Fig. 5a). However, when the two plasmids were cotransfected, the latter protein became localized in the nuclei of some cells (Fig. 5b). As the ratio of PECE- δ -BE to PECE(1-68)DG increased, more chimeric protein was found in the nuclei. Furthermore, a control experiment showed that there was no cross-reaction between HDag and the globin-specific antibody (data not shown). These results suggested that the HDag (residues 1 to 68)-globin chimera and the full-length HDag could form a complex, and as a result, the HDag-globin chimeric protein, which did not have an NLS, was transported to the nuclei. In contrast, another cytoplasmic protein, PECE-core- α -globin, which contained part of the HBV core protein sequence fused to globin (36), remained in the cytoplasm even when cotransfected with PECE- δ -BE (Fig. 5c). This result suggested that the facilitation of nuclear transport of the HDag-

FIG. 5. Immunofluorescence of COS cells cotransfected with the full-length HDag and various HDag-globin chimeras without an NLS. Immunostaining was performed with the antibody specific for α -globin. (a) PECE(1-68)DG only; (b) PECE- δ -BE and PECE(1-68)DG; (c) PECE- δ -BE and PECE-core- α -globin.

globin chimeric protein by the full-length HDag in *trans* was a result of interaction between the HDag-specific sequences. Thus, we concluded that HDag interacted with itself to form a protein complex in the cytoplasm and that HDag could be transported into the nucleus as an aggregated form.

Complex formation of HDag was likely mediated by a leucine zipper sequence. We have previously noted that HDag contains two leucine zipper motifs, one of which resides in the N-terminal portion (amino acid residues 30 to 51, LERDLRKLKKKIKKLEEEDNPL), although the last

helical turn is likely interrupted by a proline. Since the HDag subfragment consisting of residues 1 to 68, which could be transported by an NLS in *trans*, contained this leucine zipper sequence, it was possible that the in *trans* facilitation of nuclear transport was mediated through the interaction between the leucine zipper sequences. We thus studied whether HDag subfragments without the leucine zipper sequence could also be transported to the nuclei by the full-length HDag in *trans*. We cotransfected into COS cells a constant amount of PECE- δ -BE and various HDag-globin chimeras, which included different fractions of this leucine zipper motif but no NLS sequence. Two of the proteins, which contained residues 1 to 33 and 1 to 36, respectively, and included only one of the four leucine residues, were detected only in the cytoplasm even when cotransfected with PECE- δ -BE (Fig. 6a and b). However, the third protein (residues 1 to 48), which contained three of the four leucine residues, was present in significant amount in the nucleus when cotransfected with PECE- δ -BE (Fig. 6c). Furthermore, two proteins (residues 1 to 57 and 1 to 68) which contained the complete leucine zipper-like motif were detected almost exclusively in the nucleus when cotransfected with the full-length HDag (Fig. 5b and 6d). These results suggested strongly that the leucine zipper sequence was the sequence mediating HDag aggregation in vivo.

DISCUSSION

In this study, we have demonstrated that HDag contains an NLS which is composed of two stretches of sequence rich in basic amino acids, PPAKKLR and PRKR, within residues 67 to 88. Both of these two domains were required for efficient nuclear transport of HDag. The nonbasic spacer sequence between the two stretches of basic amino acids could be deleted without affecting nuclear localization significantly. Thus, HDag belongs to a newly identified class of nuclear proteins which require the presence of two discontinuous stretches of arginine-rich sequence for nuclear transport. This class of proteins include influenza virus polymerase basic protein 1 (23), *Xenopus* protein N1 (15), adenovirus DNA-binding protein (22), and nucleoplasmin (29). The activities of the various parts of this bipartite NLS in HDag are different. The N-terminal part, PPAKKLR, appears to play a major role in directing HDag nuclear transport, whereas the downstream part, PRKR, functions in conjunction with the upstream part to increase the efficiency of nuclear transport. These NLS sequences are mostly conserved in various HDV isolates. All of the divergence observed so far consisted of conservative substitutions (7). It is also interesting to note that this NLS is separated from the RNA-binding domain, which is localized in the middle one-third region of HDag (18).

HDag also contains a stretch of sequence (RKLKK KIKKL, residues 35 to 44) homologous to the glucocorticoid receptor nuclear localization signal NL1 (RKTKKKIKGI) (25). The homology is more than 70%, and the basic residues in NL1 are conserved in all of the HDV isolates studied so far (7). The NL1 sequence of the glucocorticoid receptor fused to galactosidase has been shown to be functional in targeting the fusion protein to the nucleus (25). Surprisingly, the fusion protein consisting of α -globin and the NL1-like sequence in HDag failed to be transported to the nuclei (Fig. 2b). This observation suggests that a slight variation of the NLS sequence can abolish its function and that HDag utilizes a completely different NLS for its nuclear transport.

Our studies also demonstrated that HDag molecules

FIG. 6. Immunofluorescence of COS cells cotransfected with the full-length HDag and various HDag-globin chimeras containing different portions of the leucine zipper sequence. Immunostaining was done with the antibody specific for α -globin. Cells were cotransfected with PECE- δ -BE and (a) PECE(1-33)DG, (b) PECE(1-36)DG, (c) PECE(1-48)DG, and (d) PECE(1-57)DG.

interacted with one another through a leucine zipper-like sequence. This interaction allowed the proteins which did not have an NLS sequence to be transported into the nuclei. This finding demonstrated that HDag could form an oligomer in the cytoplasm and suggested that the nuclear transport of HDag may normally be mediated through this complex formation. This observation provided another example of nuclear transport of proteins as protein complexes. This mechanism has been shown in the nuclear cotransport of adenovirus DNA polymerase and preterminal protein (37), p53 and simian virus 40 T antigen (32), and histones H2A and H2B (21). This cotransport of nuclear proteins may be a mechanism to regulate the functions of these proteins. On the other hand, protein complex formation between the cytoplasmic and nuclear proteins, such as those between the transcription factor NF- κ B and its inhibitor I κ B (9), glucocorticoid receptor and heat shock protein hsp90 (31), and *Drosophila* dorsal protein and cactus protein (30, 33), may inhibit the NLS activity of the nuclear proteins, resulting in the cytoplasmic accumulation of these nuclear proteins. The NLS activity of these proteins can be restored only after the protein complex is disrupted. These examples show that protein interactions can alter their subcellular distributions and functions. In addition, aggregation of HDag through a leucine zipper sequence raises the interesting possibility that HDag could potentially interact with cellular proteins containing leucine zipper sequences. This interaction may alter the subcellular localization and function of certain cellular

proteins and may be one of the mechanisms of HDV pathogenesis.

The significance of nuclear targeting of HDag is still not clear. HDag may carry HDV RNA into the nuclei, so that HDV RNA can be replicated by the nuclear machinery, including RNA polymerases (19). HDag may also participate in HDV RNA synthesis in a more direct way, since it binds to HDV RNA specifically (18). Thus, its nuclear targeting may serve an important role in the life cycle of HDV.

Another interesting observation was the nucleolar localization of the full-length large and small HDag. The functional importance of such a phenomenon is not clear. However, we noticed that proteins expressed from PECE(1-88)D and PECE(1-163)D were localized in either nucleoli or whole nuclei including nucleoli (Fig. 3a and data not shown). But none of the HDag subfragments fused to globin were detected in nucleoli (Fig. 3b and 4a and data not shown). Thus, the nucleolar targeting of HDag may require a stringent protein conformation; once fused to globin, the protein conformation may be altered and result in the inability of HDag to associate with nucleoli. Therefore, the nucleolar targeting of proteins may require a specific structural conformation rather than a primary signal sequence.

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