Ser-123 of the large antigen of hepatitis delta virus modulates its cellular localization to the nucleolus, SC-35 speckles or the cytoplasm

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Hepatitis delta virus (HDV) is a defective virus and requires hepatitis B virus (HBV) to supply envelope proteins (HBsAg) for maturation and secretion. It is known that two proteins produced by HDV, the small (SDAg) and large (LDAg) antigens, are located in the nucleolus, speckles and the cytoplasm and are involved in genome replication and virion packaging. However, little is known about how they are targeted to the specific sites where they act. A green fluorescence protein fused to LDAg (GFP–LD) has been shown previously to translocate from the nucleolus to SC-35 speckles in the presence of the casein kinase II inhibitor dichlororibofuranosyl benzimidazole. In this study, we determined which amino acids of GFP–LD were responsible for the translocation from the nucleolus to SC-35 speckles and created three GFP–LD derivatives, GFP–LDS2A, GFP–LDS123A and GFP–LDS2/123A. Fluorescence microscopy studies showed that Ser-123 mutants had a high tendency to target SC-35 speckles in both transfected HeLa and HuH-7 cells and suggested that Ser-123, but not Ser-2, plays a role in modulating LDAg translocation to the nucleolus or to SC-35 speckles. This study also demonstrated that HBsAg plays a role in facilitating the transportation of LDAg from the nucleus to cytoplasm. Compared with GFP–LD and GFP–LDS2A, mutants of Ser-123 were less efficiently transported to the cytoplasm and resulted in a lower level of secretion. In contrast, little or no isoprenylation mutant was observed in the cytoplasm of HuH-7 cells expressing HbsAg, suggesting that the isoprenylation of LDAg plays a role in export from the nucleus. Thus, the current study demonstrated that both cis and trans elements modulate HDAg translocation to various subcellular sites.

INTRODUCTION

Hepatitis delta virus (HDV) is the smallest RNA virus found that causes human infection and results in acute and chronic liver disease when co- or superinfected with the helper virus, hepatitis B virus (HBV) (Rizzetto et al., 1980; Chen et al., 1997). The HDV genome is a 1.7 kb single-stranded, negative-sense circular RNA (Chen et al., 1986; Kos et al., 1986; Wang et al., 1986). Two HDV antigens (HDAs), the small and large forms (SDAg and LDAg, respectively), are encoded by the same open reading frame of the antigenomic RNA. The SDAg contains 195 aa while the LDAg contains an additional 19 aa at its C terminus (Weiner et al., 1988). SDAg trans-activates HDV RNA replication. In contrast, LDAg suppresses this process and interacts with the envelope proteins of HBV (HBsAg) to assemble a mature virion for secretion and infection (Kuo et al., 1989; Chao et al., 1990; Chang et al., 1991; Ryu et al., 1992; for a review, see Lai, 1995, and references therein).

Because both SDAg and LDAg lack any enzyme activity, HDV genome and antigenome replication require host RNA polymerases (Modahl et al., 2000; Chang & Taylor, 2002; MacNaughton et al., 2002). HDV genome and antigenome replication occur via a rolling-circle method, which produces multiple copies of HDV RNA in linear form (Lai, 1995; Taylor, 2003). Ribozymes in both the HDV genome and antigenome then self-cleave the linear RNA into single units, which are ligated into a circular form (Lai, 1995; Taylor, 2003). During the RNA replication cycle, host enzymes called ADARs (adenosine deaminases that act on double-stranded RNA) edit a portion of HDV RNA to convert the amber stop codon (UAG) of SDAg to a tryptophan codon (UGG), which results in the production of LDAg (Casey & Gerin, 1995; Sato et al., 2001; Jayan & Casey, 2002). Thereafter, LDAg inhibits HDV RNA replication and, together with SDAg and the HDV genome, assembles into a ribonucleoprotein complex, which is then transported to the cytoplasm to form a mature virion with HBsAgs. Although HDAs have been identified in many subcellular structures, such as the nucleolus, nuclear speckles and the Golgi apparatus (Xia et al., 1992; Wu et al.,...
Many reports have revealed that post-translational modifications of HDAGs are important for the execution of HDAg function; for example, isoprenylation of LDAG is crucial for its interaction with HBsAg for secretion (Glenn et al., 1992; Hwang & Lai, 1993; Sheu et al., 1996). Although both SDAG and LDAG are phosphoproteins, the SDAG can be phosphorylated at both serine and threonine, while the LDAG can be phosphorylated only at serine (Chang et al., 1988; Mu et al., 1999), which may account for their distinct biological roles. The serine residues at positions 2 and 177 of SDAG modulate HDV RNA replication but have no significant role in subviral particle formation (Yeh et al., 1996; Yeh & Lee, 1998; Mu et al., 1999, 2001). These results suggest that post-translational modifications of HDAGs may play a role in targeting HDAGs to a specific location.

Previously, we used a green fluorescent protein fused to LDAG (GFP–LD) to demonstrate that translocation of GFP–LD from the nucleolus to SC-35 speckles could be induced by treatment of the casein kinase II inhibitor dichlororobifuranosyl benzimidazole (DRB) (Shih & Lo, 2001). However, direct evidence showing that the dephosphorylated form of GFP–LD favours residence at the SC-35 speckles is lacking. In this study, we constructed GFP–LD derivatives containing a single mutation at Ser-2 or Ser-123, or a double mutation at both sites, and GFP–LD derivatives containing a single mutation at SC-35 speckles is lacking. In this study, we constructed

**METHODS**

**Site-directed mutagenesis of the plasmid pGFP-LD.** Four oligonucleotide primers used for the site-directed mutagenesis were synthesized by a local manufacturer (Mission Biotech). They were designated S2AF (5’-GATCTGAGGCGCCTTCGGAGAAG-3’), S2AR (5’-CTCTTCGAGGCGCCTTCGGAGAAG-3’), S123AF (5’-GGAAGGAGCTCGGACGGAGGAG-3’), and S123AR (5’-CTCTTCGAGGCGCCTTCGGAGAAG-3’). The pGFP-LD plasmid, expressing GFP fused to full-length LDAG (Shih & Lo, 2001), was used as a template and the paired primers (S2AF and S2AR; S123AF and S123AR) were used for PCR for 16 cycles resulting in the single-mutation plasmids pGFP-LDS2A and pGFP-LDS2/123A, respectively. The double-mutation plasmid pGFP-LDS2/123A was obtained by PCR using pGFP-LDS2A as the template and the primers S123AF and S123AR. The mutation sites of these three plasmids were confirmed using an automatic sequencer. The GFP fusion proteins encoded by these plasmids were designated GFP–LDS2A, GFP–LDS2/123A and GFP–LDS2/123A, respectively. Other plasmids used in this study were pSVL-d2g, pMTSD, pMTLD, pMTS and pGFP-LDM and have been described previously (Yeh et al., 1996; Hu et al., 1996; Sheu & Lo, 1994; Shih & Lo, 2001). The proteins or RNAs encoded by these plasmids were summarized as follows: (i) pSVL-d2g encodes a replication-competent HDV RNA and two HDAGs; (ii) pMTSD and pMTLD encode SDAG and LDAG, respectively; (iii) pMTS encodes the major surface antigen of HBV (HBsAg); and (iv) pGFP-LDM encodes GFP fused to a non-isoprenylated mutant of LDAG.

**Cell culture and transfection of HuH-7 and HeLa cells.** Two human cell lines were used in this study; one was a well-differentiated human hepatoma cell line, HuH-7, and the other was an epitheloid carcinoma cell line, HeLa. Both cell types were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, penicillin (100 IU ml$^{-1}$), streptomycin (100 µg ml$^{-1}$), Fungizone (50 µg ml$^{-1}$) and 2 mM l-glutamine and grown at 37°C under 5% CO$_2$. Plasmids in a supercoiled form were obtained using the Qiagen Plasmid Maxi kit and then used for transfection. Cells at 60% confluence in a 10 cm Petri dish were transfected with 10 µg of the indicated plasmids by the calcium phosphate/DNA precipitation method (Graham & van der Eb, 1973) or by adding lipofectamine (Invitrogen). For visualization of the various expression patterns of HDAG encoded by pSVL-d2g, HuH-7 cells at 24 h post-transfection were scrapped off and reseduced onto cover slips and cultured for an additional 3–10 days.

**Fluorescence microscopy.** To visualize HDAG expression patterns, pSVLd2g-, pMTLD- or pMTSD-transfected cells were fixed with 4% paraformaldehyde/PBS for 30 min at room temperature, then stained with anti-HDAG antibody followed by secondary goat anti-rabbit antibody conjugated to FITC. Cells expressing the various GFP fusion proteins (GFP–LD, GFP–LDS2A, GFP–LDS123A or GFP–LDS2/123A) were fixed and stained with anti-SC35 antibody followed by a secondary antibody conjugated to rhodamine. Cells co-transfected with pMTS and the various plasmids expressing GFP fusion proteins were fixed and stained with anti-HBs antibody followed by a secondary antibody conjugated to rhodamine. In parallel, the cells were stained with Hoechst 33258 to visualize the nucleus. Finally, cells were mounted on glass slides with mounting solution and examined using a fluorescence microscope (Olympus B-Max 60 or Leica DMRBE) or a confocal microscope (Leica DMRE) using an FITC or rhodamine filter.

**Western blotting.** To detect the secretion efficiency of various GFP fusion proteins with HBsAg, HuH-7 cells were co-transfected with pMTS and the various plasmids expressing the GFP fusion proteins GFP–LD, GFP–LDM, GFP–LDS2A and GFP–LDS123A. Growth media from 3 and 6 days post-transfection were collected and the secreted empty viral particles (EVPs) were concentrated by ultracentrifugation. Protein samples from secreted EVPs and the total lysates of cells cultured for 6 days post-transfection were separated by SDS-PAGE and electrotransferred onto PVDF membranes. The membrane containing total cell lysate was incubated with anti-HDAG antibody and anti-actin antibody (as a reference for the amount of protein loaded) in the presence of 5% non-fat milk, and the membrane containing EVPs was probed with anti-HDAG antibody and anti-HBsAg (as a reference for the amount of protein loaded). After incubation with the secondary antibody conjugated to horseradish peroxidase, the blots were developed by enhanced chemiluminescence using a commercial kit (Amersham). The intensity of protein bands was quantified by the program ImageQuant TL software (Amersham BioSciences). The secretion ratio of GFP–LD plays a role in its targeting to SC-35 speckles.

**RESULTS AND DISCUSSION**

**Different localization patterns of HDAGs appear in groups of cells transfected with plasmids containing a dimer of HDV cDNA**

Localization of HDAG to the nucleolus, nuclear speckles and Golgi apparatus has been reported previously (Xia et al., 1992; Chang et al., 1992; Wu et al., 1992; Bichko & Taylor, 1996; Bell et al., 2000) and it is possible that...
post-translational modifications play a role in this localization. To study this possibility, we transfected pSVL-d2g into HuH-7 cells. Twenty-four hours post-transfection, cells were detached from the plates, reseeded onto coverslips and cultured for an additional 3–10 days to examine HDAg distribution. The pSVL-d2g plasmid contained a tandem dimer of HDV cDNA, which was able to direct HDV RNA replication and synthesis of both SDAg and LDAg (Yeh et al., 1996). If newly divided cells are at the same stage of the cell cycle, modification of HDAg should be synchronized and appear as a similar uniform distribution pattern in the two daughter cells.

Results of immunofluorescence microscopy revealed that HDAg appeared in many distribution patterns but was uniform in paired cells that had been cultured for 3–5 days (Fig. 1A) and in clusters of cells that had been cultured for 5–10 days (Fig. 1B). HDAg appearing in the nucleolus and in various sizes of nuclear speckles (Fig. 1A, C and D) was commonly seen in both the short- and long-term cultured cells, similar to previous reports (Xia et al., 1992; Chang et al., 1992; Wu et al., 1992). In contrast, the presence of HDAg in the perinuclear region containing the Golgi apparatus was not seen in short-term cultured cells but was frequently observed in cells cultured for longer periods (Fig. 1B, indicated by arrows; Bichko & Taylor, 1996). This uniform staining pattern of HDAg did not occur in adjacent or clustered positively stained cells that had been transfected with plasmids expressing only LDAg or SDAg and cultured for more than 10 days (data not shown).

The observation of a uniform distribution of HDAg within a group of cells and LDAg localization to the Golgi apparatus in cells 18 days post-transfection has been reported previously (Bichko & Taylor, 1996). However, the interpretation that cells showing a uniform pattern of HDAg are probably the progeny of a single transfected cell (Bichko & Taylor, 1996) requires further explanation, since the uniform pattern for HDAg occurred in adjacent cells containing pSVL-d2g but not in adjacent cells transfected with pMTLD or pMTSD. Our explanation is that the former are probably generated by self-replicable HDV RNA and that the latter are produced by subgenomic HDV RNA. If cells contain self-replicable HDV RNA, HDV genome replication and HDAg expression will be synchronized in daughter cells, while in cells harbouring pMTSD or pMTLD, the transcription of HDV mRNA and HDAg protein synthesis would not be synchronized in daughter cells. This supposition is supported by the fact that HDV RNA replication requires RNA polymerase II and transcriptional factors (Fu & Taylor, 1993; Modahl et al., 2000; Chang & Taylor, 2002; MacNaughton et al., 2002) and that SDAg binds to RNA polymerase II to regulate elongation activity (Yamaguchi et al., 2001). The subunits of RNA polymerase II are substrates for casein kinase II (CKII) (Stetler & Rose, 1982) and the CKII activity is cell-cycle dependent, which is evident from phosphorylation of nucleolin and nucleophosmin (Meisner & Czech, 1991).

Previously, we demonstrated that inhibition of CKII activity by DRB moved GFP–LD from the nucleolus to SC-35 speckles (Shih & Lo, 2001), but this provided no direct evidence to show that localization of GFP–LD to the SC-35 speckles resulted from dephosphorylation of GFP–LD.

Ser-123 mutated proteins of GFP–LD are preferentially located in the SC-35 speckles

To confirm that CKII is regulating GFP–LD phosphorylation and that dephosphorylation of GFP–LD is responsible for its targeting to SC-35 speckles, we constructed three
GFP–LD-derived mutants, designated GFP–LDS2A, GFP–LDS123A and GFP–LDS2/123A, and analysed their localization in transfected cells. Serine residues at positions 2 and 123 of LDAg are known target sites of CKII; thus, if the three mutants were located in the SC-35 speckles in the absence of DRB, then dephosphorylation of GFP–LD must be required for its localization to the SC-35 speckles. On the other hand, if the three mutated proteins were distributed in the same way as the wild-type GFP–LD, then the effect of DRB must not be on GFP–LD dephosphorylation but by another mechanism that allows targeting to the SC-35 speckles.

Results obtained from fluorescence microscopy of cells expressing the four different GFP fusion proteins are shown in Fig. 2. When the distribution of GFP–LD, GFP–LDS2A, GFP–LDS123A and GFP–LDS2/123A was examined in cells 24, 48 and 72 h post-transfection, it was shown that the longer the post-transfection time of the cells, the higher the percentage of cells expressing GFP–LDS123A and GFP–LDS2/123A with a green fluorescent signal in the speckles (Fig. 2G–I and J–L). In contrast, a higher percentage of cells expressing GFP–LD and GFP–LDS2A had a signal in the nucleolus, although some cells had a signal only in the speckles (Fig. 2A–C and D–F). To

![Fig. 2. Distribution patterns of GFP–LD and its derived mutants. HeLa cells were transfected with plasmids expressing wild-type GFP–LD (A–C), the single-alanine-substitution mutants GFP–LDS2A (D–F) or GFP–LDS123A (G–I), or the double-substitution mutant GFP–LDS2/123A (J–L). The protein distribution patterns were visualized by fluorescence microscopy and are shown in the first column (A, D, G and J) for cells 24 h post-transfection, in the second column (B, E, H and K) for cells 48 h post-transfection and in the third column (C, F, I and L) for cells 72 h post-transfection. The distribution patterns were classified as type I, II or III, as indicated. A type I pattern indicated that proteins were present mainly in the nucleolus; type II indicated that there were some proteins present in the nucleolus but they were predominantly in the speckles; and type III indicated that proteins were solely in the speckles. All micrographs are at the same magnification, indicated by the scale bar in (L).](image-url)
quantify the percentage of cells showing these different distributions of GFP fusion proteins, we classified the patterns into three types: type I, with protein distribution mainly in the nucleolus; type II, with protein distributed in both the nucleolus and the speckles; and type III, with protein found only in the speckles (also see Fig. 2 legend). Three independent experiments were performed to transfect the four different plasmids into HeLa cells. Between 200 and 700 cells with green fluorescent signals were randomly selected, their patterns classified and the results summarized.

As shown in Table 1, GFP–LD cells showed an increase in the type III pattern from 1·8% at 24 h post-transfection to 10·1% at 72 h post-transfection, while the type I pattern decreased from 83·4% at 24 h post-transfection to 45% at 72 h post-transfection for these cells. GFP–LDS123A cells showed an increase in the type III pattern from 16·2% at 24 h post-transfection to 66·3% at 72 h post-transfection, while the type I pattern decreased from 53·2 to 11·9%. It was noted that the increasing trend of the type III pattern from 24 to 72 h post-transfection was also observed with GFP–LDS2A from 5·3 to 18·7%, which was similar to that of the wild-type, while for GFP–LDS2/123A, the increase from 17·2 to 70·6% was similar to that found for GFP–LDS123A. These results suggested that Ser-123 of GFP–LD, but not Ser-2, plays a role in regulating its localization to speckles. However, there are two different sizes of speckles, ranging from 0·1 to 3 μm, that are found in cells constantly expressing GFP–LD (Shih & Lo, 2001). The smaller ones (<1 μm) do not co-localize with SC-35, while the larger ones do. When GFP–LD-expressing cells are treated with DRB, most of the GFP–LD is targeted to SC-35 speckles (Shih & Lo, 2001). Therefore, whether the type III speckles of GFP–LDS123A and GFP–LDS2/123A also co-localized with SC-35 needed to be determined.

Forty-eight hours after transfection with the various plasmids expressing the GFP fusion proteins, HeLa cells were stained with anti-SC-35 antibody and examined using an immunofluorescence microscope. The double-fluorescent results showed that only the large-sized speckles co-localized with SC-35 in cells expressing GFP–LDS123A or GFP–LDS2/123S, while the small-sized speckles did not (Fig. 3). It appeared that cells showing the type I and II patterns had higher numbers of SC-35-negative speckles, in contrast with cells having the type III pattern, which showed more SC-35-positive speckles. Combining the results of Figs 2 and 3, we therefore concluded that Ser-123 mutants were preferentially located in SC-35 speckles at 72 h post-transfection.

Table 1. Distribution patterns of GFP–LD and its derivatives in HeLa cells

Each number represents the mean value (%) of three independent experiments. The three types of distribution pattern are as described in the legend for Fig. 2.

<table>
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<th>Time (h)</th>
<th>Expressed protein</th>
<th>Distribution pattern (%)</th>
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<tr>
<td></td>
<td>Type I</td>
<td>Type II</td>
</tr>
<tr>
<td>24</td>
<td>GFP–LD</td>
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</tr>
<tr>
<td></td>
<td>GFP–LDS2A</td>
<td>77·7</td>
</tr>
<tr>
<td></td>
<td>GFP–LDS123A</td>
<td>53·2</td>
</tr>
<tr>
<td></td>
<td>GFP–LDS2/123A</td>
<td>52·5</td>
</tr>
<tr>
<td>48</td>
<td>GFP–LD</td>
<td>55·8</td>
</tr>
<tr>
<td></td>
<td>GFP–LDS2A</td>
<td>57·3</td>
</tr>
<tr>
<td></td>
<td>GFP–LDS123A</td>
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</tr>
<tr>
<td></td>
<td>GFP–LDS2/123A</td>
<td>25·4</td>
</tr>
<tr>
<td>72</td>
<td>GFP–LD</td>
<td>45·0</td>
</tr>
<tr>
<td></td>
<td>GFP–LDS2A</td>
<td>39·5</td>
</tr>
<tr>
<td></td>
<td>GFP–LDS123A</td>
<td>11·9</td>
</tr>
<tr>
<td></td>
<td>GFP–LDS2/123A</td>
<td>11·3</td>
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Fig. 3. Co-localization of Ser-123 mutants of GFP–LD with SC-35. HeLa cells expressing GFP–LDS123A (A) and GFP–LDS2/123A (B) were stained with anti-SC-35 antibody and observed under a confocal microscope using the rhodamine channel. The yellow colour shows the co-localization of the GFP fusion protein with SC-35. The green colour indicates the nucleolus or small-sized speckles. Cells that failed to be transfected show their nucleus stained by anti-SC-35 in red.
As shown in Fig. 4, when cells expressing GFP–LDS2A, GFP–LDS123A and GFP–LDS2/123A were treated with DRB, all fusion proteins were located in SC-35 speckles similar to GFP–LD, as shown previously (Shih & Lo, 2001). These results supported the idea that other nuclear factors may be required, in addition to Ser-123 in its non-phosphorylated form, for targeting to SC-35 speckles. This supposition is further supported by the fact that GFP fusion proteins need to associate with other factors to form a complex with a density above 1.62 g ml\(^{-1}\), such as appears in the nucleolus (Huang et al., 2001; Shih et al., 2004). Modification of GFP–LD might change its conformation and this could result in its association with nuclear factors that have not yet been identified. Nevertheless, the current results showed that only the Ser-123 mutant played a role in modulating the distribution of the GFP fusion protein in the nucleolus or SC-35 speckles, although CKII catalyses phosphorylation of both Ser-2 and Ser-123. Since Ser-123 in a non-phosphorylated state appears in the nucleolus (Huang et al., 2001; Shih et al., 2004), modification of GFP–LD might change its conformation and this could result in its association with nuclear factors that have not yet been identified. Nevertheless, the current results showed that only the Ser-123 mutant played a role in modulating the distribution of the GFP fusion protein in the nucleolus or SC-35 speckles, although CKII catalyses phosphorylation of both Ser-2 and Ser-123. Since Ser-123 in a non-phosphorylated state favors GFP–LD localization to the SC-35 speckles, the question then arises as to which phosphatase is responsible for dephosphorylation of Ser-123. This is a topic that needs further exploration.

**Transportation of GFP–LD proteins from the nucleus to the cytoplasm is facilitated by HBsAg**

To determine whether Ser-123 mutants of GFP–LD were also preferentially located in SC-35 speckles in hepatoma cells, three independent experiments were performed to transfect each of the four described plasmids or pGFP-LDM, which encodes a non-isoprenylated GFP–LD, into HuH-7 cells. As shown in Table 2, qualitatively similar results were obtained, in that GFP–LD and GFP–LDS2A had a lower rate of increase for the type III pattern, while GFP–LDS123A and GFP–LDS2/123A had a higher rate. However, there was a two- to fourfold increase in the type III pattern in HuH-7 cells compared with HeLa cells expressing GFP–LD and GFP–LDS2A. This difference might be due to variation in cellular factors that maintain the GFP fusion proteins in the speckles between HuH-7 and HeLa cells. The non-isoprenylated mutant, GFP–LDM, had a lower rate of increase in the type III pattern, similar to GFP–LD and GFP–LDS2A.

It has already been demonstrated that GFP–LD can interact with HBsAg to form EVPs (Shih & Lo, 2001), but whether the presence of HBsAg can facilitate GFP–LD translocation from the nucleus to cytoplasm remains unknown. Three independent experiments were conducted to co-transfect pMTS plasmids with each of the five plasmids encoding the various GFP–LD fusion proteins into HuH-7 cells. Double-positive cells expressing both the GFP fusion protein and HBsAg were randomly selected and classified. A new pattern, type IV, with the GFP fusion protein in the cytoplasm, was observed (Fig. 5B–D). As shown in Table 2, these data showed a significant increase in the type IV pattern in the four GFP fusion proteins, with the exception of GFP–LDM, in cells at 48 and 72 h post-transfection when HBsAg was also expressed. Furthermore, cells expressing GFP–LD and GFP–LDS2A had a twofold higher percentage of type IV pattern than cells expressing GFP–LDS123A and GFP–LDS2/123A. Nevertheless, the latter two had more than a twofold higher percentage of...
Table 2. Distribution patterns of GFP–LD and its derivatives in HuH-7 cells

Each number represents the mean value (%) of three independent experiments. Distribution type III is as described in the legend for Fig. 2 and type IV is the presence of GFP fusion protein in the cytoplasm.

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<th>Time (h)</th>
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<th>Distribution pattern (%)*</th>
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<td></td>
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</tr>
<tr>
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</tr>
<tr>
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<td>GFP–LDS2A</td>
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<tr>
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<td>GFP–LDS2/123A</td>
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<tr>
<td></td>
<td>GFP–LDM</td>
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*−HBsAg and +HBsAg, absence or presence of HBV envelope proteins, respectively. ND, Not detectable.

When these results were compared with the less than 1% of cells having GFP fusion proteins in the cytoplasm when cells did not express HBsAg, it was concluded that HBsAg helps LDAg translocate to the cytoplasm (Table 2). Results showing a higher percentage of cells expressing GFP–LD and GFP–LDS2A in the cytoplasm than those expressing GFP–LDS123A and GFP–LDS2/123A (37-8 and 29-5% versus 15-6 and 16-9%) led to the conclusion that Ser-123 mutants are less efficiently transported to the cytoplasm.

To determine whether or not the lower percentage of cells with GFP–LDS123A accumulated in the cytoplasm affected its secretion with HBsAg, analysis of the amount of secretion of the various GFP–LD fusion proteins was performed. Results of Western blotting indicated that there were no obvious differences in the amount of secretion among the four different GFP–LD fusion proteins in the total cell lysate (data not shown). However, in the media of cells cultured for 3 days post-transfection, the amount of GFP–LDS123A in the secreted empty viral particles was 21-31% less than that of GFP–LD and GFP–LDS2A (Fig. 6, left panel). The amount of GFP–LDS123A found in the EVPs in media from cells cultured for 6 days post-transfection was about half that found for GFP–LD and GFP–LDS2A (Fig. 6, right panel). These data indicated that the slower transportation of LDAg to the cytoplasm resulted in a lower amount of secreted EVP. As indicated in Table 2, GFP–LDM was not detected in the cytoplasm of cells after culture for 24 and 48 h post-transfection, but a low level was detected after 72 h. No GFP–LDM was found in secreted EVPs at 3 days post-transfection, but a low level was detected after 6 days (Fig. 6).

Fig. 5. Representative images of HuH-7 cells co-expressing GFP–LD and HBsAg. The GFP–LD distribution was detected by FITC (green), while the expression pattern of HBsAg was detected by the rhodamine channel (red). The merged pictures are shown in the left column (A–D). The same field of cells, stained with the DNA dye Hoechst 33258, are shown in the right column (A’–D’). Cells in (A) were cultured for 48 h and showed no GFP–LD in the cytoplasm, while cells in (B) and (C), which were cultured for more than 48 h, appeared to have less GFP–LD inside the nucleus; instead GFP–LD co-localized with HBsAg in the ER region. The Golgi complex region is indicated by an arrow.
Although it has been shown that GFP–LDM cannot be secreted with HBsAg (Shih & Lo, 2001), the underlying mechanism is unknown. It has been suggested that this could be due to GFP–LDM having lost its ability to insert into the endoplasmic reticulum (ER) or Golgi membrane, which is the site of GFP–LD interaction with HBsAg, or, alternatively, that GFP–LDM cannot be exported out of the nucleus. The current study has shown for the first time using double-fluorescence microscopy that the majority of GFP–LDM cannot be exported out of the nucleus. The results shown in Table 2 and Fig. 5 clearly demonstrated that HBsAg can facilitate GFP–LD transportation from the nucleus to cytoplasm, although the underlying mechanism remains unknown. Apparently, HBsAg is not a crucial factor for LDAg export out of the nucleus, since the absence of HBsAg, wild-type HDAg could appear in the Golgi when transfected cells had been cultured for more than 5 days (Fig. 1B). Since LDAg alone, without SDAg and the HDV RNA genome, can be secreted with HBsAg (Sheu et al., 1996), it is suggested that LDAg requires modification to be exported out of the nucleus. The modification of LDAg includes isoprenylation at Cys-211 and phosphorylation or dephosphorylation at Ser-123. Enzymes for LDAg modification, such as CKII, may fluctuate during the cell cycle. The presence of HBsAg may transduce signals to the nucleus and alter enzyme activity to modify LDAg. This hypothesis is supported by the demonstration of cross-talk between HBsAg and nuclear factors (Xu et al., 1997). The reason that the majority of GFP–LDM could not be transported out of the nucleus is unknown. It is possible that isoprenylation changes LDAg so that it exposes its NES, allowing it to exit the nucleus. It is not clear whether HBsAg is necessary for LDAg export or whether HBsAg is involved in the translocation pathway of LDAg.

The translocation pathway of GFP–LD and its co-localization with HBsAg

Although this study did not include a dynamic study of GFP–LD movement from the nucleus to the cytoplasm, it should be possible to identify the sequential process of GFP–LD distribution based on the double-positive cells at seen at 24, 48 and 72 h post-transfection. Representative images were selected, as shown in Fig. 5. In the cells 24 h after transfection, most GFP–LD remained inside the nucleus, appearing either in a type II or type III pattern, while HBsAg was distributed in the ER (Fig. 5A). This image was selected to show that two adjacent cells had different distribution patterns of GFP–LD, in contrast to the uniform distribution of HDAg in two adjacent cells, as shown in Fig. 1(A) and (B). It might be that cells expressing only LDAg or SDAg have no mechanism to modify HDAg at the same time in the daughter cells as described above. In some of the cells 24 and 48 h post-transfection, green speckles of type III were found to align on the edge of the nuclear membrane (Fig. 5B). At this stage, co-localization between GFP–LD and HBsAg was occasionally observed near the nuclear membrane and cytoplasm (Fig. 5B). Meanwhile GFP–LD alone was detected in the region of the Golgi apparatus (Fig. 5B and C). In the cells 72 h post-transfection, co-localization of GFP–LD and HBsAg became more evident in the cytoplasm and the amount of GFP–LD gradually diminished inside the nucleus (Fig. 5C and D).

The results shown in Table 2 and Fig. 5 clearly demonstrated that HBsAg can facilitate GFP–LD transportation from the nucleus to cytoplasm, although the underlying mechanism remains unknown. Apparently, HBsAg is not a crucial factor for LDAg export out of the nucleus, since in the absence of HBsAg, wild-type HDAg could appear in the Golgi when transfected cells had been cultured for more than 5 days (Fig. 1B). Since LDAg alone, without SDAg and the HDV RNA genome, can be secreted with HBsAg (Sheu et al., 1996), it is suggested that LDAg requires modification to be exported out of the nucleus. The modification of LDAg includes isoprenylation at Cys-211 and phosphorylation or dephosphorylation at Ser-123. Enzymes for LDAg modification, such as CKII, may fluctuate during the cell cycle. The presence of HBsAg may transduce signals to the nucleus and alter enzyme activity to modify LDAg. This hypothesis is supported by the demonstration of cross-talk between HBsAg and nuclear factors (Xu et al., 1997). The reason that the majority of GFP–LDM could not be transported out of the nucleus is unknown. It is possible that isoprenylation changes LDAg so that it exposes its NES, allowing it to exit the nucleus. It is not clear whether HBsAg is necessary for LDAg export or whether HBsAg is involved in the translocation pathway of LDAg.

Based on the current study, we suggest a simple scheme regarding LDAg translocation and its interaction with HBsAg. The sequential pathway of LDAg could be depicted as follows. (i) LDAg is synthesized in the cytoplasm and transported into the nucleus by interaction of its NLS with importin and then accumulates in the nucleolus. (ii) Ser-123 of LDAg is dephosphorylated and interacts with unknown nuclear factors for targeting to the SC-35 speckles. (iii) Prenylation occurs at Cys-211 and phosphorylation or dephosphorylation at Ser-123. Enzymes for LDAg modification, such as CKII, may fluctuate during the cell cycle. The presence of HBsAg may transduce signals to the nucleus and alter enzyme activity to modify LDAg. This hypothesis is supported by the demonstration of cross-talk between HBsAg and nuclear factors (Xu et al., 1997). The reason that the majority of GFP–LDM could not be transported out of the nucleus is unknown. It is possible that isoprenylation changes LDAg so that it exposes its NES, allowing it to exit the nucleus. It is not clear whether HBsAg is necessary for LDAg export or whether HBsAg is involved in the translocation pathway of LDAg.
LDAG conformation and allows its export out of the nucleus. (iv) Using its prenylated tail, LDAG inserts into the Golgi membrane and LDAG-containing vesicles bud off from Golgi and are retro-transported to the ER, where LDAG and HBsAg form a subviral particle or mature virion.

Previously, we postulated that LDAG could follow two alternative pathways (Sheu et al., 1996). Here, we have demonstrated that only one pathway occurs, in which LDAG must enter the nucleus, and isoprenylation then allows it out of the nucleus. Thus LDAG is first located in the Golgi apparatus and then moves to the ER for subviral or viral particle formation. This differs from the central paradigm of the secretion pathway and may reflect the fact that HDV requires a tighter control for its secretion than other conventional secretion pathways (Nickel, 2003). To date, there is no evidence showing how HBV and HDV form a mature virion in the same cell. Generally, it is believed that HBV follows the conventional secretion pathway forming virions in the ER and then moving through the Golgi apparatus and secretory vesicles (Huovilla et al., 1992). HDV differs because it is present in the Golgi apparatus first and then moves to the ER, as indicated in this work by Figs 1(B) and 5(B) and (C). Previous results showing that non-glycosylation of HBsAg retards HDV secretion but not HBV also support this hypothesis that the maturation pathway of HDV and HBV is different (Wang et al., 1996; Sureau et al., 2003). Nevertheless, more work is needed to elucidate both the HDV and the HBV maturation pathways.

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