Induction of hepatitis D virus large antigen translocation to the cytoplasm by hepatitis B virus surface antigens correlates with endoplasmic reticulum stress and NF-κB activation

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It is known that hepatitis D virus (HDV) requires hepatitis B virus (HBV) for supplying envelope proteins (HBsAgs) to produce mature virions, and the HDV large antigen (LDAg) is responsible for interacting with HBsAgs. However, the signal molecules involved in the cross-talk between HBsAgs and LDAg have never been reported. It has been previously demonstrated that the small form of HBsAg can facilitate the translocation of HDV large antigen green fluorescent protein (GFP) fusion protein (GFP–LD) from the nucleus to the cytoplasm. In this study, it was confirmed that the small form of HBsAg can facilitate both GFP–LD and authentic LDAg for nuclear export. It was also shown that the three forms of HBsAgs (large, middle and small) induced various rates (from 35–4 to 57–2 %) of GFP–LD nuclear export. Since HBsAgs are localized inside the endoplasmic reticulum (ER), this suggests that ER stress possibly initiates the signal for inducing LDAg translocation. This supposition is supported by results that show that around 9 % of cells appear with GFP–LD in the cytoplasm after treatment with the ER stress inducers, brefeldin A (BFA) and tunicamycin, in the absence of HBsAg. Western blot and immunofluorescence microscopy results further showed that the activation of NF-κB is linked to the ER stress that induces GFP–LD translocation. Combining this with results showing that tumour necrosis factor alpha (TNF-α) can also induce GFP–LD translocation, it was concluded that LDAg translocation correlates with ER stress and activation of NF-κB. Nevertheless, TNF-α-induced GFP–LD translocation was independent of new protein synthesis, suggesting that a post-translational event occurs to GFP–LD to allow translocation.

INTRODUCTION

Hepatitis D virus (HDV) is the smallest human RNA virus and has a genome of 1.7 kb in the form of a single-, negative-stranded circle (for a review see Lai, 1995). Although the genome structures of HDV and plant viroids share a common evolutionary origin, unlike viroids, which do not encode any proteins, HDV encodes two antigen isoforms, the small and large forms (SDAg and LDAg, respectively) during its replication cycle. The SDAg and LDAg share 195 aa at their N terminus, while the LDAg contains 19 extra aa at its C terminus (Weiner et al., 1988). SDAg is required for HDV RNA replication, whereas LDAg suppresses this process and interacts with hepatitis B virus (HBV) envelope proteins (HBsAgs) 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).

Replication of the HDV genome and antigenome is dependent on host cell RNA polymerases and occurs via a rolling-circle method, which produces multiple copies of HDV RNA in a linear form (Modahl et al., 2000; Macnaughton et al., 2002). Ribozymes in both the genome and antigenome then self-cleave the linear RNA into single units, which are then ligated into a circular form by cellular ligases (Lai, 1995; Reid & Lazinski, 2000; Taylor, 2003). During the HDV replication cycle, host enzymes called ADARs (adenosine deaminases that act on double-stranded RNA) edit a portion of the 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 then LDag together with SDAg and the HDV genome assemble into a ribonucleoprotein (RNP) complex (Ryu et al., 1993), which is then transported to the cytoplasm to form a mature virion with the HBsAgs.

In addition to the host RNA polymerases and ADARs, many other cellular enzymes involved in the post-translational modifications of HDAs are important for the execution of HDAg’s function (Mu et al., 2004; Li et al., 2004; Lai, 2005). For example, farneryl-transferase is required for LDag isoprenylation, which is crucial to the interaction with HBsAgs and mucomere assembly (Ryu et al., 1992; Hwang & Lai, 1993; Sheu et al., 1996). Different kinases are also required for LDag and LDag phosphorylation because the SDAg is phosphorylated at both serine and threonine, while the LDag is phosphorylated only at serine (Chang et al., 1988; Mu et al., 1999), which may account for their distinct functions. Furthermore, the serine residues at positions 2 and 177 of SDAg have been demonstrated to 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). Recently, methylation at arginine-13 and acetylation at lysine-72 of HDag have been reported to play an important role in virus replication (Mu et al., 2004; Li et al., 2004). These lines of evidence fully support the hypothesis that post-translational modifications of HDAs can drive HDAs to specific cellular compartments and functions (for a review see Lai, 2005). However, signals and mechanisms involved in HDAg post-translational modification have not yet been well studied.

Previously, we used a green fluorescent protein fused to LDag (GFP–LD) to demonstrate that translocation of GFP–LD from the nucleus to SC-35 speckles can be induced by treatment with the casein kinase II inhibitor, dichlororofuranosyl benzimidazole (Shih & Lo, 2001), in which serine-123 of LDag in the dephosphorylated state is responsible for preferentially targeting to the SC-35 speckles (Tan et al., 2004). How cellular kinases and phosphatases are regulated in order to modify LDag for translocation is still under question. We also demonstrated that the small form of HBsAg can facilitate the translocation of GFP–LD from the nucleus to the cytoplasm (Tan et al., 2004), but the mechanism remains unclear. In this study, we explore what signal molecules are generated by HBsAgs, which reside in the endoplasmic reticulum (ER) and can induce GFP–LD nuclear export.

**METHODS**

**Plasmids used in this study.** Based on the encoded proteins, plasmids used in this study can be grouped into four series: (i) pMTLD (Hu et al., 1996) and pN2LD encode authentic LDag under the metallothionein (MT) and cytomegalovirus (CMV) promoter, respectively; (ii) pMTS, pMTMMS[5] and pMTLS encode HBV small, middle and large (S, M and L) surface antigens, respectively (Sheu & Lo, 1994), and pN2LS encodes 1. HBsAg; (iii) those expressing different versions of HDag fused to a GFP, pGFP–LD, pGFP–LDM, pGFP–LD(31–214) and pSD–GFP (Shih & Lo, 2001; Shih et al., 2004); and (iv) pTKK, which encodes the wild-type 1xK kinase z subunit; pKA, which encodes a mutant form of IKKz; and pNF-xB-Luc, which contains the NF-xB-response element (TGGGACTTCCGC)3 fused to a luciferase gene (kindly provided by Dr Y. S. Chang, Chang Gung University, Republic of China). The characteristics of the GFP fusion proteins encoded by the series (iii) plasmids are summarized as follows: (a) pGFP–LD encodes GFP fused to wild-type LDag; (b) pGFP–LDM produces GFP fused to a non-isoprenylated mutant of LDag; (c) pGFP–LD(31–214) produces GFP fused to an N-terminal deletion (aa 1–30) mutant of LDag; and (d) pSD–GFP produces wild-type SDAg fused to GFP (Shih & Lo, 2001; Shih et al., 2004).

**Cell culture and plasmid transfection.** Two human cell lines were used in this study; one is a well-differentiated hepatoma cell line, HuH-7, while the other is a cervical carcinoma cell line, HeLa. Most of the experiments were carried out using HuH-7 cells and a few were done with HeLa cells. Both cell lines were cultured in Dulbecco’s modified Eagle’s medium (DMEM) 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% CO2. Plasmids in a supercoiled form were obtained using the Qiagen Plasmid Maxi kit and then used for transfection. Cells at 60–80% confluence in a 10 cm Petri dish or six-well plate were transfected with 10–20 μg of the indicated plasmids by the calcium phosphate-DNA precipitation method (Graham & van der Eb, 1973) or by adding lipofectamine (Invitrogen). At 1 or 2 days post-transfection, cells were treated with various drugs for different time intervals: (i) tunicamycin (TM; 2–5 μg ml–1) or breveldin A (BFA; 2–5 μg ml–1) for 2 h, and (ii) with or without pretreatment with cycloheximide (10 μg ml–1) for 30 min and then followed by treatment with tumour necrosis factor alpha (TNF-α; 10 ng ml–1) for 1 h.

**Fluorescence microscopy.** To visualize the co-expression of HBsAgs and various forms of GFP fusion proteins, transfected cells were seeded on 22 × 22 mm coverslips. After full attachment, cells were fixed and permeabilized using methanol for 30 min at −20°C and then stained with anti-HBs antibody followed by the secondary antibody conjugated with rhodamine. In parallel, cells were stained with Hoechst 33258 for 10 min to show the nucleus. Finally, cells were reseeded on 22-mm glass slides with mounting solution, and visualized using a fluorescence microscope (Olympus IX71) with a fluorescence isocytiosc (FITC) or rhodamine filter. For quantification of the GFP fusion protein, the distribution of the nucleus only (N), versus the nucleus–cytoplasm (N+C) pattern was observed, and between 100 and 500 GFP-positive cells were randomly selected and their patterns classified as described previously (Tan et al., 2004). For simplicity in this study, type I, II and III distribution patterns were redesignated ‘N’ pattern, nucleus only, while the type IV was redesignated ‘N+C’, nucleus and cytoplasm.

**Western blot analysis.** To detect the amount of GFP fusion proteins in the nucleus and cytoplasm, transfected cells were reseeded on 22 × 22 mm coverslips. After full attachment, cells were fixed and permeabilized using methanol for 30 min at −20°C and then stained with anti-HBs antibody followed by the secondary antibody conjugated with rhodamine. In parallel, cells were stained with Hoechst 33258 for 10 min to show the nucleus. Finally, cells were reseeded on 22-mm glass slides with mounting solution, and visualized using a fluorescence microscope (Olympus IX71) with a fluorescence isocytiosc (FITC) or rhodamine filter. For quantification of the GFP fusion protein, the distribution of the nucleus only (N), versus the nucleus–cytoplasm (N+C) pattern was observed, and between 100 and 500 GFP-positive cells were randomly selected and their patterns classified as described previously (Tan et al., 2004). For simplicity in this study, type I, II and III distribution patterns were redesignated ‘N’ pattern, nucleus only, while the type IV was redesignated ‘N+C’, nucleus and cytoplasm.

**Luciferase activity assay.** Measurement of NF-xB activity was carried out as described by Wu et al. (1998). Briefly, transfected cells
were lysed using a buffer supplied in a commercial luciferase assay kit (Promega) according to the manufacturer's instructions. The luciferase activity of each cell lysate was determined by using an Autolumat LB953 luminometer (Berthold). All experiments were carried out in duplicate and repeated three times.

RESULTS

The small form of HBsAg can induce nuclear export of both authentic and GFP fusion LDAg

Previously, we have demonstrated that the small form of HBsAg facilitated GFP–LD translocation from the nucleus to the cytoplasm (Tan et al., 2004). In this study, we first confirmed that HBsAg expression correlates with GFP–LD in the cytoplasm by co-transfection of pMTS and pGFP–LD into HuH-7 cells. A representative fluorescence microscopy image shows that three cells that have GFP appearing in an N + C pattern, while one cell has GFP located in the nucleus (N pattern) only [Fig. 1a(i) and a(ii)]. This particular N pattern cell had no HBsAg expression in contrast to the three N + C cells [Fig. 1a(iii)], indicating clearly that HBsAg can facilitate GFP–LD translocation to the cytoplasm. Consistently, the presence of authentic LDAg in the cytoplasm was highly correlated with the expression of HBsAg in HeLa cells [Fig. 1b(i), (ii) and (iii)], indicating that HBsAg can affect similarly on both GFP–LD and authentic LDAg. We then tested whether HBsAg can facilitate on other HDAg fusion proteins by performing single, double and triple plasmid transfection experiments, in which each of four plasmids [pGFP–LD, pGFP–LDM, pGFP–LD(31–214) and pSD–GFP] expressing a different version of HDAg fused to GFP was co-transfected with or without pMTS and pMTLD. As shown in Table 1, the percentage of N + C pattern cells for expressing GFP–LD (GFP fused to wild-type LDAg), GFP–LDM (GFP fused to a non-isoprenylated mutant of LDAg) and other GFP–HDAg fusion proteins were significantly higher than that for authentic LDAg. This result indicates that HBsAg can facilitate nuclear export of both authentic LDAg and various HDAg fusion proteins.

![Image](http://vir.sgmjournals.org) 1717

Fig. 1. The effect of HBsAg on GFP–LD [a(i)–(iii)] and authentic LDAg [b(i)–(iii)] distribution. [a(i)–(iii)] HuH-7 cells were co-transfected with pGFP–LD and pMTS. After 24 h post-transfection, cells were permeabilized and reacted with anti-HBsAg and followed by rhodamine-conjugated antibody. The same cells were photographed using different fluorescent filters and show: (i) cells stained with DNA dye, Hoechst 33258, to outline the nucleus, (ii) GFP–LD pattern inside of cells using an FITC filter, and (iii) HBsAg distribution using a rhodamine filter. [b(i)–(iii)] HeLa cells were co-transfected with pN2LD and pN2LS, which contain the CMV promoter to express L HBsAg and LDAg, respectively. After 24 h post-transfection, cells were fixed and stained with antibody conjugated with rhodamine to indicate the expression of LDAg and with FITC-conjugated antibody to indicate the expression of HBsAg. The same cells were photographed using different fluorescent filters and show: (i) cells stained with DNA dye, Hoechst 33258, to outline the nucleus, (ii) LDAg distribution using a rhodamine filter, and (iii) HBsAg distribution using an FITC filter. ‘N’ indicates the nucleus pattern and ‘N + C’ indicates the cytoplasm and nucleus pattern. Bars, 10 μm.
LDAg), GFP–LD(31–214) [GFP fused to an N-terminal deletion (aa 1–30) mutant of LDAg] or SD–GFP (wild-type SDAg fused to GFP) ranged from 1-1 to 5-1 % in single plasmid transfections. In the presence of HBsAg, the N+C pattern of GFP–LD cells increased to 26-5 %, while that of GFP–LDM, SD–GFP and GFP–LD(31–214) expressing cells increased ranging from 2-5 to 5-1 %, suggesting that HBsAg only facilitates the full-length of LDAg translocation to the cytoplasm but not the single point mutation, which cannot be isoprenylated (GFP–LDM), N-terminal deletion mutation [GFP–LD(31–214)] or SDAg. As compared with the result of double plasmid transfection, results of triple plasmid transfections suggested that LDAg can form a complex with GFP–LD(31–214) and GFP–LD, while 13-5 and 12-9 % increase in cells expressing GFP–LDM and SD–GFP, respectively (see the last column of Table 1). A higher increasing percentage of N+C pattern in GFP–LDM and SD–GFP expressing cells as compared with that of GFP–LD(31–214) cells in the presence of HBsAg and LDAg suggested that LDAg can form a complex with GFP–LDM and SD–GFP via the coiled-coil domain and this allows them export to the cytoplasm, in contrast, the coiled-coil domain of GFP–LD(31–214) is truncated (Chang et al., 1992; Shih & Lo, 2001). No significant increase of N+C pattern in GFP–LD cells between double and triple plasmid transfections suggested that GFP–LD behaves similarly to LDAg under the effect of HBsAg.

### The three forms of HBsAgs facilitate GFP–LD translocation to the cytoplasm to different degrees

It is known that there are three different forms (S, M and L) of HBsAg in various lengths of amino acids and degrees of glycosylation modification (Sheu & Lo, 1992). We were thus interested to see whether or not the three different forms of HBsAg exert different capability on the translocation of GFP–LD. Co-transfection of pGFp–LD with pMTS, pMTMMS["S"] or pMTLS, which express S, M and L HBsAg, respectively, into HuH-7 cells was performed. The N and N+C pattern of the GFP–LD cells were analysed at 24, 48 and 72 h post-transfection. As shown in Table 2, GFP–LD cells had an increased percentage of the N+C pattern when they were co-expressed with S, M and L HBsAg (10-4, 26-4 and 34-1 %, respectively) at 24 h post-transfection. A similar trend was also observed in the 48 h post-transfected cells (25-2, 36-3 and 47-0 %, respectively) as well as in the 72 h post-transfected cells (34-7, 47-8 and 56-5 %, respectively). Since the three forms of HBsAgs reside in the ER, it was speculated that the presence of HBsAg may induce ER stress and then in turn cause GFP–LD translocation. Western blot analyses were performed to test this supposition. Results clearly show that the GRP78 protein/BiP, an ER stress-induced marker, which functions as a protein chaperone at the ER cisternae (Schröder & Kaufman, 2005; Lee, 2005), was increased in the total lysate of cells co-expressing GFP–LD with S, M and L HBsAg (Fig. 2, lanes 3–5, bottom line) compared with cells that were mock transfected or co-expressing GFP–LD alone (Fig. 2, lanes 1 and 2, bottom line). For an unknown reason, the increasing folds of GRP78/BiP induced by different HBsAgs varied and were not reproduced consistently. In contrast, the amount of p65 subunit of NF-κB consistently increased in the nuclear fraction of cells co-expressing S, M and L HBsAg (Fig. 2, lanes 3–5, line 2). A decreased amount of GFP–LD in the nuclear fraction (Fig. 2, lanes 2–5, line 1) and, conversely, an increased amount of GFP–LD in the cytoplasmic fraction (Fig. 2, lanes 2–5, line 4) were found in cells expressing GFP–LD alone or co-expressing GFP–LD with S, M, or L HBsAg. These

<table>
<thead>
<tr>
<th>Expressed protein</th>
<th>Distribution pattern (%)</th>
<th>N+C pattern difference (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. GFP–LD</td>
<td>94-9</td>
<td>5-1</td>
</tr>
<tr>
<td>2. GFP–LD+S-HBsAg</td>
<td>68-4</td>
<td>31-6</td>
</tr>
<tr>
<td>3. GFP–LD+S-HBsAg+L-HDAg</td>
<td>70-9</td>
<td>29-1</td>
</tr>
<tr>
<td>1. GFP–LDM</td>
<td>98-7</td>
<td>1-3</td>
</tr>
<tr>
<td>2. GFP–LDM+S-HBsAg</td>
<td>96-2</td>
<td>3-8</td>
</tr>
<tr>
<td>3. GFP–LDM+S-HBsAg+L-HDAg</td>
<td>82-7</td>
<td>17-3</td>
</tr>
<tr>
<td>1. SD–GFP</td>
<td>97-7</td>
<td>2-3</td>
</tr>
<tr>
<td>2. SD–GFP+S-HBsAg</td>
<td>92-6</td>
<td>7-4</td>
</tr>
<tr>
<td>3. SD–GFP+S-HBsAg+L-HDAg</td>
<td>79-7</td>
<td>20-3</td>
</tr>
<tr>
<td>1. GFP–LD(31–214)</td>
<td>98-9</td>
<td>1-1</td>
</tr>
<tr>
<td>2. GFP–LD(31–214)+S-HBsAg</td>
<td>95-9</td>
<td>4-1</td>
</tr>
<tr>
<td>3. GFP–LD(31–214)+S-HBsAg+L-HDAg</td>
<td>95-6</td>
<td>4-4</td>
</tr>
</tbody>
</table>

**Table 1.** Effect of S-HBsAg on distribution pattern of various GFP fusion proteins in HuH-7 cells

These distribution patterns of various GFP fusion proteins were observed after 72 h post-transfection. Each number in the N and N+C column represents the mean value of three independent experiments. Numbers (1, 2 and 3) at the left represent for transfection conditions: 1, single plasmid transfection; 2, double plasmid transfection; and 3, triple plasmid transfection. ‘N’ and ‘N+C’ distribution pattern is described in the legend of Fig. 1.
**Western blot analyses of GFP–LD and NF-κB**

Fig. 2. Western blot analyses of GFP–LD and NF-κB distribution in the presence of three different forms of HBsAgs (L, M and S). HuH-7 cells were co-transfected by pGFP–LD with plasmids encoding L, M or S HBsAg. Cells were then fractionated into nuclear and cytoplasmic portions at 72 h post-transfection. Nuclear proteins (15 μg) were analysed with anti-GFP, anti-NF-κB p65 and anti-tubulin antibodies (the top three lines as indicated by nuclear fraction), while 15 μg cytoplasmic proteins were analysed with anti-GFP and anti-tubulin (as indicated by cytoplasmic fraction). Total cell lysate, in 20 μg protein, was analysed with GRP78/BiP and GFP–LD (the last two lines). Tubulin served as a positive control for the cytoplasmic fraction and as a negative control for the nuclear fraction.

**Table 2. Distribution pattern of GFP–LD in HuH-7 cells**

Each number in the N and N+C column represents the mean value of three independent experiments. ‘N’ and ‘N+C’ distribution pattern is described in the legend of Fig. 1.

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Expressed protein</th>
<th>Distribution pattern (%)</th>
<th>N+C pattern difference (%) in the presence of HBsAgs</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
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<td></td>
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</tr>
<tr>
<td>24</td>
<td>GFP–LD</td>
<td>99.6</td>
<td>0.4</td>
</tr>
<tr>
<td></td>
<td>GFP–LD + S-HBsAg</td>
<td>89.2</td>
<td>10.8</td>
</tr>
<tr>
<td></td>
<td>GFP–LD + M-HBsAg</td>
<td>73.2</td>
<td>26.8</td>
</tr>
<tr>
<td></td>
<td>GFP–LD + L-HBsAg</td>
<td>65.5</td>
<td>34.5</td>
</tr>
<tr>
<td>48</td>
<td>GFP–LD</td>
<td>99.4</td>
<td>0.6</td>
</tr>
<tr>
<td></td>
<td>GFP–LD + S-HBsAg</td>
<td>74.2</td>
<td>25.8</td>
</tr>
<tr>
<td></td>
<td>GFP–LD + M-HBsAg</td>
<td>63.1</td>
<td>36.9</td>
</tr>
<tr>
<td></td>
<td>GFP–LD + L-HBsAg</td>
<td>52.4</td>
<td>47.6</td>
</tr>
<tr>
<td>72</td>
<td>GFP–LD</td>
<td>99.3</td>
<td>0.7</td>
</tr>
<tr>
<td></td>
<td>GFP–LD + S-HBsAg</td>
<td>64.6</td>
<td>35.4</td>
</tr>
<tr>
<td></td>
<td>GFP–LD + M-HBsAg</td>
<td>51.5</td>
<td>48.5</td>
</tr>
<tr>
<td></td>
<td>GFP–LD + L-HBsAg</td>
<td>42.8</td>
<td>57.2</td>
</tr>
</tbody>
</table>

Western blot results are highly correlated with the results of the fluorescence microscopic observation (Table 2), indicating that all three forms of HBsAg have the capability to induce GFP–LD translocation, but to various degrees.

**Treatment with TM and BFA increases GFP–LD in the cytoplasm**

Although an increase of GRP78/BiP in the presence of HBsAgs is shown in Fig. 2, to further correlate with ER stress and GFP–LD distribution in the cytoplasm, we treated GFP–LD expression cells with the ER stress inducers, TM and BFA, for 2 h and then determined the percentage of N and N+C pattern cells by fluorescence microscopy. Around 8-8 to 9-3% N+C pattern increase was observed when they were treated with 5 μg TM and BFA ml⁻¹ (see Supplementary Table S1 available in JGV Online). Consistently, Western blot results showed a decreased amount of GFP–LD present in the nuclear fraction, while an increased amount of GFP–LD present in the cytoplasm when cells were treated with TM (Fig. 3a, lanes 2–5, lines 1 and 3) and BFA (Fig. 3b, lanes 2–5, lines 1 and 3). In addition, a slightly increased amount of GRP78/BiP was also observed in a dose-dependent manner when cells were treated with lower doses of TM and BFA (2–4 μg ml⁻¹) but not at higher doses (5 μg ml⁻¹) (Fig. 3a and b, line 6). The highest dose was unable to induce the highest amount of GRP78/BiP and GFP–LD in the cytoplasm (Fig. 3a and b lane 6, line 6), which could be due to a desensitization effect. Nevertheless, the data showing a good correlation between increasing GFP–LD in the cytoplasm and expression of GRP78/BiP indicate that even in the absence of HBsAg, inducing ER stress by an exogenous drug also causes GFP–LD translocation to the cytoplasm. Therefore, induction of ER stress is suggested to be an early event when HBsAg is expressed in cells to induce GFP–LD translocation.

**NF-κB plays a role in GFP–LD transportation from the nucleus to the cytoplasm**

Although an increasing amount of the p65 subunit of NF-κB in the nucleus has been demonstrated by Western blot in cells co-expressing GFP–LD and S, M or L HBsAg (Fig. 2), whether the nuclear form of NF-κB can actively transcribe its target genes remains to be determined. Similar experiments described in Table 2 were therefore conducted, in which pNF-κB-Luc containing the luciferase gene followed
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The NF-κB response element was co-transfected with pGFP–LD and plasmids encoding S, M or L HBsAg into HuH-7 cells. After 24, 48 and 72 h post-transfection, cells were lysed and luciferase activities were determined. Results clearly show that the luciferase activity (2-7 × 10^5 luminescence intensity unit, liu) was higher in cells co-expressing GFP–LD with S, M or L HBsAg as compared with those expressing GFP–LD without HBsAgs (1-3 × 10^5 liu) or in pNF-κB-Luc transfection cells (8-2 × 10^4 liu) at 24 h post-transfection (Fig. 4). A similar trend was also observed in 48 h post-transfection cells (3-1-4 × 10^5 liu versus 1-4-1-7 × 10^5 liu) and in 72 h post-transfection cells (4-3-7-3 × 10^5 liu versus 2-1 × 10^5 liu). Among the cells co-expressing various HBsAgs at 72 h post-transfection, the L HBsAg expression showed the highest luciferase activity (7-3 × 10^5 liu), while the S HBsAg expression cells showed the lowest activity (4-3 × 10^5 liu). These results are consistent with the Western blot results and indicate that the nuclear form of NF-κB is induced by three forms of HBsAgs (Fig. 2, line 2) and this varies to a range of different levels.

To obtain another line of evidence for the role of NF-κB in HBsAg-induced GFP–LD translocation, we co-transfected plasmid pKA, which expresses a dominant negative form of IKKa that results in inactivation of NF-κB, to examine whether the functioning of HBsAg will be blocked or not. Western blot results show that the GFP–LD remaining in the nucleus was increased proportionally to the amount of pKA transfected (Fig. 5a, line 1). Unlike the reciprocal change of GFP–LD between the nucleus and cytoplasm detected and shown in Fig. 2, a constant and similar amount of GFP–LD was present in the cytoplasm of cells with or without pKA transfection (Fig. 5a, line 3). However, the luciferase activity assay showed several fold decrease in cells expressing various amounts of mutant IKK (data not shown), suggesting that NF-κB was indeed inhibited in those cells and this resulted in a higher amount of GFP–LD being retained in the nucleus. When cells co-expressed GFP–LD and various amounts of active form of IkB kinase z subunit, an increasing amount of GFP–LD, proportional to the amount of pIKKa that was transfected was observed in the cytoplasmic fraction of cells (Fig. 5b, line 3). Conversely, a decreasing amount of GFP–LD was observed in the nuclear fraction of cells (Fig. 5b, line 1). Cells having the higher amount GFP–LD in the nucleus were highly correlated with the higher luciferase activity (data not shown). Taken together the evidence shown in Figs 4, 5(a) and (b) conclude that NF-κB indeed plays a significant role in HBsAg-induced GFP–LD translocation.

**TNF-α treatment also induces GFP–LD translocation to the cytoplasm in the absence of protein synthesis**

Since TNF-α induces cell responses largely through activation of NF-κB, to correlate further with activation of NF-κB and GFP–LD distribution, GFP–LD expressing cells were treated with TNF-α for 1 h and the percentage of N + C cells was determined by fluorescence microscopy. The results revealed that 1 h after TNF-α treatment induced more than...
50% of GFP–LD expressing HuH-7 or HeLa cells appeared to have the N+C pattern, while treatment with TM and BFA induced only 8-8 and 9-3% of the cells to have the N+C pattern (Supplementary Table S1 available in JGV Online). The robust effect of TNF-α allowed us to test whether the induction of GFP–LD translocation into the cytoplasm requires newly synthesized proteins or not. This kind of experiment could not be performed in cells expressing HBsAg because the protein translation inhibitor will also block HBsAg production. After pre-treatment with cycloheximide for 30 min, cells were treated with TNF-α for 1 h and the N+C pattern of GFP–LD was examined. Around 53-3% of cells appeared to have the N+C pattern (Supplementary Table S1), indicating that GFP–LD translocation into the cytoplasm does not require newly synthesized proteins. The inhibition effect by cycloheximide was also shown to occur by the luciferase activity assay, which showed that activity was greatly reduced in cells treated with both TNF-α and cycloheximide (data not shown). However, the Western blot result shows that the amount of NF-κB in the nucleus was similar after TNF-α treatment with or without cycloheximide but it was higher than that in TNF-α non-treated cells (Fig. 6, line 3). Western blot analysis also shows that the amount of GFP–LD in the nucleus and cytoplasm (Fig. 6, lines 1 and 4) correlated well with the fluorescence microscopic observations.

**DISCUSSION**

During the HDV replication cycle, the HDV RNP, which contains the HDV RNA genome and two isoforms of antigen (SDAg and LDAg), is known to be exported out of the nucleus in order to be enveloped by HBsAgs for production of mature viral particles. This process is very likely LDAg-mediated since there is a nuclear export signal (NES) localized at the C terminus of LDAg and a nuclear factor (NESI) that binds to the NES has been identified (Lee *et al.*, 2001; Wang *et al.*, 2005). However, signal molecules involved in HDV RNP export and the underlying mechanisms are largely unknown. In this study, we apply a previously established system, in which HBsAg can facilitate GFP–LD translocation into the cytoplasm, to investigate signals participating in the GFP–LD translocation. Several lines of evidence have demonstrated that ER stress and activation of NF-κB, which are induced by HBsAg, play a significant role (Figs 2, 4 and 5a). It is known that ER stress induced by many different viral proteins leads to various cellular effects (Su *et al.*, 2002; Tardif *et al.*, 2002; Dimcheff *et al.*, 2003), including full-length and truncated forms of the HBV L and M surface antigens (Wang *et al.*, 2003; Hsieh *et al.*, 2004; Hung *et al.*, 2004). The current study is the first

![Fig. 5. Western blot analyses of GFP–LD distribution under various degrees of NF-κB activation. (a) HuH-7 cells were co-transfected with pGFP–LD and pMTS (lanes 1–4) with 4, 6 or 8 μg plasmid pKA (lane 2, 3 and 4, respectively), which produces a dominant negative form of IKK. Post-transfected cells (72 h) were fractioned into nuclear and cytoplasmic portions as indicated in the legend of Fig. 2 and GFP–LD changes in the nuclear and cytoplasmic fractions were then analysed by anti-GFP. (b) HuH-7 cells were co-transfected with pGFP–LD with various amounts (4, 6 or 8 μg) of pIKK, which produces an active form of IκB kinase α subunit. Post-transfected cells (72 h) were fractionated into nuclear and cytoplasmic portions and GFP–LD changes in the nuclear and cytoplasmic fractions were analysed as indicated in (a).](http://vir.sgmjournals.org)

![Fig. 6. Western blot analyses to show the effect of TNF-α and cycloheximide on GFP–LD distribution. HuH-7 cells were transfected with pGFP–LD. After 72 h post-transfection, cells were untreated (lane 1), treated with 10 ng TNF-α ml⁻¹ for 1 h (lane 2) or pre-treated with cycloheximide (10 μg ml⁻¹) for 30 min followed by co-treatment of cycloheximide (10 μg ml⁻¹) and TNF-α (10 ng ml⁻¹) for 1 h (lane 3) and then fractionated into nuclear and cytoplasmic fractions as indicated in the legend of Fig. 2. GFP–LD and NF-κB (p65) changes in the nuclear and cytoplasmic fractions under various drug treatments were analysed as indicated in Fig. 2.](http://vir.sgmjournals.org)
report to show that induction of ER stress results in a nuclear protein translocation into the cytoplasm.

The current study also provides direct evidence that three different forms of HBsAgs induce various levels of NF-κB activities (Fig. 4) as well as 34-7–35-6 % cells having GFP–LD in the cytoplasm (Table 2). The various capabilities exhibited by different forms of HBsAgs could be due to their intrinsic properties, i.e. capability of ER retention (Bruss & Ganem, 1991; Sheu & Lo, 1994; Chau et al., 2005). The effects of HBsAg on NF-κB activity and GFP–LD translocation could be explained by either that they are two parallel events or that the GFP–LD translocation is the downstream event following the NF-κB activation. We prefer the second explanation because in the absence of HBsAg, GFP–LD cells treated with TNF-α, which induces a higher level of NF-κB, also appear to have a higher percentage of N+C pattern (Supplementary Table S1). At the present time, no direct evidence that HBsAg can trap the newly synthesized LDAg in the cytoplasm has been demonstrated. However, we favour the hypothesis that LDAg must enter into the nucleus and then translocate into the cytoplasm after post-translational modification, in which HBsAgs exert signals to facilitate the modification. This hypothesis is supported by results that cells co-expressing GFP–LD(31–214) and HBsAg with or without LDAg show a low N+C pattern from 1 to 3 days post-transfection (Table 1), while in 6 days post-transfected cells expressing GFP–LD(31–214) can be co-secreted with HBsAg (Shih & Lo, 2001). Therefore, the current study suggests that the sequence located between aa 1 and 30 may be important in helping LDAg translocation into the cytoplasm and post-translational modification of serine-2 and/or arginine-13 (Mu et al., 2004; Li et al., 2004) is likely to be involved in facilitating LDAg translocation into the cytoplasm.

In the absence of HBsAgs, GFP–LD translocation can also be facilitated by adding ER stress inducing drugs, TM and BFA (Fig. 3), but the effect is fivefold lower than that of L HBsAg on 72 h post-transfected cells (Table 2), suggesting that many signal pathways and molecules may participate in the HBsAg-induced GFP–LD translocation. This may explain why inactivation of NF-κB by co-transfection of dominant negative IKK does not significantly abolish the cytoplasmic distribution of GFP–LD (Fig. 5a) and the expression of GRP78/BiP and activity of NF-κB is not perfectly matched in some cases. Interestingly, no new proteins are required for TNF-α to induce GFP–LD translocation, indicating that a post-translational modification occurs to GFP–LD to allow this translocation (Fig. 6 and Supplementary Table S1). Results of low translocation of GFP–LD(31–214) and GFP–LDM in the presence of HBsAgs (Table 2) suggest that multimerization and farnesylation of GFP–LD are two important modifications for facilitating GFP–LD translocation. The present study cannot distinguish which step, farnesylation or multimerization, comes first when facilitating LDAg nuclear export but one report has shown that multimerization between SDAg and LDAg increases farnesylation of LDAg (O’Malley & Lazinski, 2005). Therefore, the farnesyl-transferase and other unidentified molecules that are directly or indirectly modified by the activated NF-κB are of interest for future exploration.

In conclusion, the current study presents the first report showing the presence of signals in the cross-talk between HDV and HBV using a system of GFP fusion proteins. However, to identify the target enzymes downstream to NF-κB and to understand how they are activated to modify LDAg are a great challenge for future studies. Moreover, whether the HDV RNP translocation into the cytoplasm can be facilitated by HBV and TNF-α remains to be tested.

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Signals for LDAg nuclear export


