Interaction between hepatitis delta virus-encoded proteins and hepatitis B virus envelope protein domains

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Hepatitis delta virus (HDV) packaging requires prenylation of the HDV large protein (p27), as well as a direct protein–protein interaction between HDV proteins and hepatitis B virus (HBV) envelope protein domains. To investigate this interaction, we have analysed the binding capacity of baculovirus-expressed delta p24 and p27 proteins to synthetic peptides specific for the HBV envelope. Although a higher degree of binding was observed with p27, both p24 and p27 could bind HBV envelope peptides. One such peptide corresponded to residues 56–80 located in the cytosolic loop of the small HBV envelope protein, and another corresponded to 23 carboxy-terminal residues of the pre-S1 specific to the large HBV envelope protein. This indicates that in addition to p27, p24 may contribute to packaging of HDV through a protein–protein interaction with HBV envelope domains, and that an interaction between the pre-S1 polypeptide and delta proteins may play a role in infectivity.

Hepatitis delta virus (HDV), a satellite of hepatitis B virus (HBV), can cause fulminant hepatitis and liver cirrhosis in chronically infected patients (Rizzetto, 1983). The HDV virion is coated with an outer envelope composed of host cell-derived lipids and the envelope proteins of HBV. It surrounds the inner ribonucleoprotein (RNP) complex comprising a circular RNA genome and two proteins bearing the hepatitis delta antigen (HD Ag): the small HD Ag (p24, 195 residues) and the large HD Ag (p27, 214 residues) (Wang et al., 1986; Weiner et al., 1988). They are encoded by the HDV genome and their sequences are identical except for 19 additional residues at the carboxy terminus of the large protein (Weiner et al., 1988). The small protein, p24, is required for HDV RNA replication (Kuo et al., 1989) whereas the large protein, p27, is necessary for virion assembly (Chang et al., 1991; Ryu et al., 1992) and has an inhibitory effect on genome replication (Chao et al., 1990; Glenn & White, 1991). The 19 carboxy-terminal polypeptide residue of p27 contains a CXXX motif that directs prenylation of the protein. This modification to p27 results in its targeting to the host cell membranes (Glenn et al., 1992), but is not sufficient for virion assembly. Specific binding of delta proteins to HBV envelope proteins seems to be necessary for the formation of enveloped HDV (Lee et al., 1995). In vitro studies done with deletion mutants have suggested that this protein–protein interaction occurs through the 19 carboxy-terminal residues of p27 (Hwang & Lai, 1993; Lazinski & Taylor, 1993; Lee et al., 1994, 1995). However, this sequence is poorly conserved among various HDV isolates and its role in protein–protein interaction during HDV morphogenesis remains uncertain (Lai, 1995). The envelope of HDV particles is identical to that of HBV. It is composed of three HBV-encoded proteins designated large (L), middle (M) and small (S) (Tiollais et al., 1985). From amino to carboxy terminus, the L protein contains the pre-S1 (119 residues), pre-S2 (55 residues) and S (226 residues) domains. The M protein contains both pre-S2 and S domains, whereas the S protein contains only the S region. The S protein is unique among viral envelope proteins in its capacity to self-assemble with host-derived lipids and to form empty envelope particles (Simon et al., 1988). The amino terminus of S contains a type I transmembrane signal and an internal type II signal which result in the protein traversing the ER membrane at least twice to form a cytosolic loop and a lumenal domain (Heerman & Gerlich, 1991). The L protein has a more complex topology. The pre-S domain is exposed at either the cytosolic or the lumenal side of the ER membrane (Bruss et al., 1994; Ostapchuck et al., 1994; Prange et al., 1992). Although the three HBV envelope proteins, S, M and L, are found in the HDV virion in vivo (Bonino et al., 1986), it is clear that the S protein is sufficient for virion maturation (Sureau et al., 1993; Wang et al., 1991) and that the L protein is important for HDV infectivity (Sureau et al., 1992, 1993, 1994).
Fig. 1. Amino acid sequence of the HBV envelope (ayw subtype) and control peptides. The small HBV surface protein (S, 226 residues) spans the membrane at its carboxy-terminal type II signal (open boxes), and probably twice at its hydrophobic carboxy-terminal region. S exposes a loop of 50 residues at the cytosolic side of ER, covered by peptides S1 and S2. The middle HBV surface protein (M) contains the S domain plus the 55 residues of the pre-S2 domain, covered by peptide L5/M. The large HBV surface protein (L) contains the S plus pre-S2 domains and the 108 residues of the pre-S1 domain, covered by peptides L1, L2, L3 and L4, and two overlapping peptides, L4a and L4b. The four control peptides are from HIV GP 120 (HIV-P2 and HIV-P4) and HCV NS4 (HCV-9 and HCV 24).

The protein–protein interactions involved in the maturation process of HBV have previously been investigated by determining the binding capacity of purified HBV core particles to a panel of synthetic peptides mapping the HBV envelope proteins (Poisson et al., 1997). The 13 carboxy-terminal residues of pre-S1 and residues 56–80 in the cytosolic loop of S were found to bind efficiently to HBV core particles. In the present study, the binding capacity of the p24 and p27 delta proteins to the same panel of HBV envelope-specific peptides was analysed.

The synthesis of the HBV peptides has been described previously (Poisson et al., 1997). Peptides corresponding to pre-S1, pre-S2 and the cytosolic loop of S are shown in Fig. 1. Four control peptides were also included in this study: human immunodeficiency virus (HIV)-P2 and HIV-P4 (residues 242–264 and 411–436, respectively, of HIV gp120); and hepatitis C virus (HCV)-9 and HCV-24 (residues 1710–1728 and 1691–1708, respectively, of HCV genotype II NS4A).

For construction of the p27 and p24 delta protein vectors, a DNA sequence encoding p24 was cloned into the BsmI and XhoI restriction sites of the pVL1393 transfer vector (invitrogen) after DNA amplification by PCR, using a 5' primer containing a BsmI restriction site sequence and a 3' primer containing an XhoI restriction site sequence. The appropriate sequence was PCR-amplified from plasmid pSVLD3, which contains three copies of the HDV genome encoding the p24 form of the delta protein (Kuo et al., 1989). For cloning of the p27 DNA coding sequence, in vitro mutagenesis was performed.
by the PCR overlap extension method (Higuchi et al., 1988) to mutate the p24 TAG stop codon to TGG. A PCR-generated fragment encoding p27 was cloned into the BamHI and XbaI restriction sites of pVL1393. Two viruses, designated HD24A and HD27A, were isolated and characterized for their ability to express the p24 and p27 delta proteins.

For protein expression, SF9 insect cells were cultured in Grace's culture medium (Gibco BRL) supplemented with 10% foetal calf bovine serum. Cells (10^6) were infected with HD24A, HD27A or wild-type (WT) baculovirus at an m.o.i. of 10. HD27A-infected cells were harvested 2 days post-infection, whereas HD24A- and WT-infected cells were harvested 3 days post-infection. Expression of delta proteins p24 and p27 was detected in infected cells by immunocytochemistry, as previously described (Pol et al., 1989). The intracellular localization was predominantly nuclear. For purification, the cells were resuspended in a hypotonic lysis buffer (1 mM Tris pH 7.5, 0.1 mM MgCl2, 0.1% Triton), incubated at 4°C for 30 min and then lysed with a Dounce homogenizer. Membranes were removed by centrifugation at 1000 g for 10 min at 4°C. Pellets containing the nuclear fraction were resuspended in 1 ml lysis buffer and then sonicated at 4°C to disrupt nuclear membranes. Each step was monitored by light microscopy after toluidine blue staining. Proteins in each lysate were analysed by 11.5% polyacrylamide–SDS gel electrophoresis (PAGE) followed either by silver staining or transfer to nitrocellulose for Western blot analysis, using purified human anti-delta IgG antibodies and peroxidase-conjugated goat anti-human F(ab')2 (data not shown). (Fig. 2).

Staining of 24 and 27 kDa proteins was observed in the HD24A and HD27A baculovirus-infected cell lysates, respectively. An additional band at approximately 20 kDa was detected in both lysates, probably corresponding to a degradation product (Wang et al., 1992). Delta proteins in each lysate were quantified with a commercial HD Ag ELISA (Turnout, Organon Teknika) (Roinier et al., 1992). HD24A lysate was found to contain four times more delta proteins than HD27A lysate, while WT lysate was negative.

For the HD Ag–HBV envelope peptide interaction assay, all peptides were conjugated to BSA with carbodiimide as previously described (Poisson et al., 1997). Microtitre plate wells (Maxisorp, Nunc) were coated at 20 µg per well with BSA-coupled peptides in PBS for 12 h at 4°C. The coating efficiency was verified for the different peptides in preliminary assays using antibodies against pre-S1, pre-S2 and S epitopes described by Sureau et al. (1992). After coating, the wells were washed three times with PBS–0.5% Tween 20 (PBS-TW) and blocked by addition of 300 µl 1 × PBS containing 2% newborn calf bovine serum (NBCS) and incubation for 45 min at 37°C. After three washes in PBS-TW, HD24A and HD27A lysates were added at a dilution of 1:100, 1:25 and 1:100, respectively, in 0.1 × PBS, and the plates incubated for 3 h at 37°C. In some experiments, 10% WT lysate was added to the HD24A and HD27A lysate dilutions. After three washes in PBS-TW, delta antigen binding was evaluated with the same reagents as used in the Western blot described above: 100 µl of a 1:10000 dilution of human anti-HD purified IgG antibodies in PBS, 5% NBCS, 5% BSA, 0.5% Tween 20 (PBS-NBT) was added and the plates incubated for 30 min at 37°C. After three more washes, 100 µl peroxidase-conjugated goat anti-human F(ab')2 (Biosource International) diluted 1:10000 in PBS-NBT was added, and plates were incubated for 30 min at 37°C. Plates were then washed three times in PBS-TW, and 100 µl of a mixture containing hydrogen peroxide–o-phenylenediamine was added to each well and left in the dark for 15 min at room temperature. Colour development was stopped with 1 M H2SO4 and the absorbance was read at 492 nm. The binding capacity of each peptide for HD24A, HD27A and WT lysates was expressed as a multiple of the cut-off value (2.5 times the average value obtained with the four control peptides). All results are summarized in Table 1. Although we cannot exclude the possibility that the anti-HD IgG could have induced a dissociation of the delta antigens from coated peptides, peptides L1, L2, L3, L5/M and S1 did not bind to delta proteins in this assay. However, peptide S2 (residues 56–60 in the cytosolic loop of S) and peptide L4 (corresponding to the 23 carboxy-terminal residues of pre-S1) showed binding activity to HD Ags. Peptide L4a bound very efficiently to both HD24A and HD27A lysates, whereas peptide L4b had a lower binding capacity (Table 1). With the exception of peptide L4b, all peptides bound preferentially to HD27A lysate. This interaction was specific since addition of 10% WT lysate in HD24A or HD27A lysate dilutions did not decrease the signal (data not shown).

When binding to HBV envelope proteins of p24 versus p27 was determined the highest degree of binding was observed for p27, although both p27 and p24 bound to peptides representing different HBV envelope domains (Table 1). These
HBV protein domains involved in HDV maturation. The binding assay represents a useful alternative for exploring S, especially when deletion mutants are examined. Our results further suggest that specific binding of the delta proteins to HBV envelope proteins may occur in domains other than the 19 carboxy-terminal residues of p27. Whether these interactions have a function in morphogenesis or infectivity of HDV particles remains to be elucidated. Early studies suggested a possible function of the Pro/Gly-rich region (residues 146–214) of p27 in a possible interaction with the HBV envelope proteins (Lazinski & Taylor, 1993), but subsequent experiments have demonstrated the crucial role of the carboxy terminus of p27 (Hwang & Lai, 1993; Chang et al., 1994; Lee et al., 1994, 1995). It should be noted that the 19 carboxy-terminal residues of p27 are poorly conserved among the different HDV genotypes, and that their role in the interaction between HDV RNP and HBV envelope proteins may lie in their contribution to generating a maturation-competent conformation of p27, rather than an ability to bind S directly (Lai, 1995). Nevertheless, the present data show that both p27 and p24 are able to bind to HBV envelope proteins. Whether binding of p24 to HBV envelope proteins is of biological significance for HDV packaging into the HBV envelope remains to be determined. In vitro binding assays represent a convenient alternative to in vitro expression of deletion mutants for exploring a protein–protein interaction required for HDV maturation. Experiments done in tissue culture indicate the need for p24 and p27 for efficient HDV maturation. The p27 protein alone was able to mediate only a low level of HDV packaging, while the restoration of p24 enhanced HDV packaging 3- to 4-fold (Wang et al., 1994).

Table 1. Binding of HBV envelope peptides to HD24A, HD27A and WT baculovirus-infected cell nucleus lysates

<table>
<thead>
<tr>
<th>Peptide</th>
<th>L1</th>
<th>L2</th>
<th>L3</th>
<th>L4</th>
<th>L4a</th>
<th>L4b</th>
<th>L5/M</th>
<th>S1</th>
<th>S2</th>
</tr>
</thead>
<tbody>
<tr>
<td>HD24A</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>1·2±0·2</td>
<td>6·7±1·1</td>
<td>5·1±1·0</td>
<td>–</td>
<td>5·1±1·0</td>
<td>–</td>
</tr>
<tr>
<td>HD27A</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>3·5±0·7</td>
<td>10·6±1·5</td>
<td>4·3±1·5</td>
<td>–</td>
<td>6·0±0·6</td>
<td>–</td>
</tr>
<tr>
<td>WT</td>
<td>–</td>
<td>–</td>
<td>–</td>
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<td>–</td>
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</tr>
</tbody>
</table>

Results are given as multiples of the cut-off value (cut-off value = 2·5 times the average value obtained with the four HIV and HCV control peptides for a given lysate). Values presented represent the mean±standard deviations of four independent experiments performed with all peptides. –, below cut-off value.

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References


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