Localization of isoprenylated antigen of hepatitis delta virus by anti-farnesyl antibodies

Ho-Pi Lin,1 Sheng-Chieh Hsu,1 Jaw-Ching Wu,2 I-Jane Sheen,2 Bo-Shiun Yan1 and Wan-Jr Syu1

1 Institute of Microbiology and Immunology, National Yang Ming University, Taipei 112, Taiwan
2 Department of Medicine, Veterans General Hospital, Taipei 112, Taiwan

Hepatitis delta virus (HDV) is a subviral pathogen that requires pre-existing or concurrent infection with hepatitis B virus (HBV). HDV expresses two forms of a single protein, the delta antigen (HDAg), which are identical except for an additional 19 residues at the C terminus of the large form. Within this C-terminal extension a cysteine residue is isoprenylated; this isoprenylation is critical for interaction with HBV envelope proteins to enable virus assembly and release into the medium. Therefore, large HDAG must be recruited to an extracellular compartment. However, immunostaining with HDAg-specific antibodies has localized the large antigen mainly to the nucleus and supports the notion that large HDAG suppresses virus replication in the nucleus. Since isoprenylation would increase the hydrophobicity of the protein and may favour transport towards specific membranes, the question remains whether the large HDAG detected in the nucleus carries an isoprenyl group. To address this issue, antibodies against the farnesyl modification were generated to allow direct visualization of the antigen by immunofluorescence microscopy. The anti-farnesyl antibodies specifically stained large HDAG expressed in Huh-7 cells, and the signal was largely restricted to the nucleus; the staining pattern could be superimposed on those of cells stained for large HDAG. The large HDAG translocated into the nucleus was therefore isoprenylated. In addition, antibodies specific for the farnesyl modification should be applicable to the study of other similarly isoprenylated proteins.

Introduction

Hepatitis delta virus (HDV) is a defective virus that needs hepatitis B virus (HBV) to supply envelope proteins in order to form infectious virions. Within the virion envelope are the HDV-specific delta antigen (HDAg) and the single-stranded 1.7 kb RNA genome (for review see Casey, 1996). Replication of the HDV RNA genome is believed to proceed via a rolling-circle model with an RNA-dependent RNA synthesis mechanism. Although several open reading frames are present in the genomic and anti-genomic RNA strands, only the largest open reading frame in the anti-genomic strand encodes a protein, HDAg, that is detected in the virions (Weiner et al., 1988).

HDAg is discernible in two forms when analysed by SDS–PAGE. These two forms are identical in their N-terminal sequence but large HDAG has a 19 amino acid extension at the C terminus (Weiner et al., 1988). The C-terminal four amino acids of large HDAG are Cys–Arg–Pro–Gln, which is the motif for isoprenylation. The cysteine residue in this motif is readily isoprenylated (Glenn et al., 1992; Hwang & Lai, 1994; Lee et al., 1994) and the isoprenyl group is farnesyl rather than geranylgeranyl (Otto & Casey, 1996).

Within the common N-terminal region of the small and large forms of HDAG the coiled-coil domain, the nuclear localization signal (NLS) and the RNA-binding motif are identical. However, the C-terminal 19 residue region of large HDAG shows structural differences to the small form. As a result, small HDAG stimulates RNA replication whereas the large form inhibits it (Chang et al., 1994). Furthermore, small HDAG can only be packaged with the HBV envelope proteins in the presence of large HDAG (Chang et al., 1991). Isoprenylation of the cysteine residue at the C terminus of large HDAG appears to play a key role in the interaction with HBV surface proteins and leads to virion release (Glenn et al., 1992; Hwang & Lai, 1993a; Lee et al., 1994).

Isoprenylation is a post-translational modification (for a review see Rando, 1996); the addition of a 15-carbon farnesyl
or a 20-carbon geranylgeranyl group to the C terminus of a protein results in a substantial increase in hydrophobicity. This property has been taken to explain the membrane affinity of many isoprenylated proteins such as the Ras-related GTP-binding proteins (Gelb, 1997). Although there is no evidence to indicate how and where within the cell large HDAG interacts with HBV envelope proteins, it seems reasonable to hypothesize that these events occur in the endoplasmic reticulum (ER) (Sheu et al., 1996). Isoprenylation would therefore facilitate secretion to this destination. However, large HDAG contains an NLS and is readily translocated into the nucleus. Due to the lack of a specific probe, it is not known whether the nucleus-translocated large HDAG is isoprenylated. In this report, antibodies specific to the isoprenyl modification were generated and used to address this question.

Methods

- **Preparation of antibodies.** To prepare the immunogen, N-acetyl-S-farnesyl-l-cysteine (Sigma) was conjugated to keyhole limpet haemocyanin (KLH) by 1-ethyl-3-(dimethylaminopropyl)-carbodiimide (Marchaloni & Warr, 1982). After conjugation, the mixture was dialysed against PBS. New Zealand white rabbits were intradermally immunized with the immunogen emulsified in complete Freund’s adjuvant. The next two immunizations, with a 1 month interval, were carried out similarly except that incomplete adjuvant was used. To assess the immune response, BSA was used as an alternative carrier protein for the conjugation. Additional haptons used for the conjugation were N-acetyl-S-geranylgeranyl-l-cysteine (ICN) and peptide T1C. Peptide T1C (KPWDLFPADDPPSPQSCRPQ) was synthesized by Genosys Biotechnologies and consisted of an N-terminal Lys followed by 20 residues of the C terminus of large HDAG. The T1C–KLH conjugate was used as the immunogen to generate anti-large HDAG. Procedures were similar to those described above except that the immunized animals were mice.

- **ELISA.** Proteins, diluted in 0.1 M Tris–HCl (pH 9.0) containing 0.15 M NaCl, were added to 96-well microtitre plates at 1 µg/ml. Following overnight incubation at 4 °C, plates were washed and blocked with 1% BSA in TBS (0.1 M Tris–HCl, pH 7.5, 0.5 M NaCl) as previously described (Sya et al., 1990). Rabbit sera were diluted in 1% BSA in TBS, added to each well of the plate and incubated for 1 h. The plates were washed five times with TBS. After the plates were washed, horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (heavy and light chain-specific; Sigma) was added to the plates at 1 µg/ml in diluent buffer. The plates were incubated as above and washed. Finally, the colorimetric reaction was developed with O-phenylenediamine (OPD) and absorbances were read at 490 nm with an ELISA reader (MR5000; Dynatech) (Yan et al., 1996).

- **Plasmid construction.** cDNA encoding HDAG was reverse-transcribed from viral RNA extracted from HDV-infected patients’ sera and was subsequently amplified by PCR (Vu et al., 1995). The DNA fragments obtained were ligated into a commercial TA cloning vector, pCRII (Invitrogen). The inserted fragments in the resulting plasmids were sequenced fully. Three such plasmids were used in the experiments described here. pCR-2577L and pCR16L encoded slightly different large HDAGs with sequences that were 88 and 96% identical to that of Taiwan type-I strain (Chao et al., 1991), respectively. pCR16S encoded a peptide constituting the N-terminal 195 residues of the large HDAG encoded by pCR16L.

Recombinant proteins expressed in *E. coli* are not isoprenylated. Therefore, the recombinant protein expressed by plasmid pMAL-DL2577 was included as a control. To construct this plasmid, the HDAG-coding sequence was amplified by PCR from pCR-2577L with primers D1 (5’ ATGACCGTTCGAGTCCA 3’) and D2 (5’ GCTCTAGAGCCATGCAGACCCGAAGAGC 3’). The amplified product was ligated to XmnI-restricted pMAL-c2 (New England Biolabs). As a result, large HDAG was fused to the C terminus of *E. coli* maltose-binding protein. For simplicity, this 67 kDa recombinant protein was named MBP–HDAG. MBP–HDAG was purified from bacterial lysates by affinity binding to amylose agarose (Hsu et al., 1997).

To express HDAG in Huh-7 cells (Nakabayashi et al., 1982), the HDAG-coding sequences in pCR16S and pCR16L were inserted separately into the Xbal site of plasmid pCMVBN (Clontech), in which the expression of HDAG was driven by a CMV promoter.

- **Western blotting.** Proteins were separated by SDS–PAGE and transferred to nitrocellulose membranes as previously described (Yan et al., 1996). In brief, rabbit antibodies were diluted with 5% skimmed milk in TBS and incubated with the nitrocellulose membranes. After incubation and washing, the blots were sequentially reacted with biotinylated goat anti-rabbit IgG (heavy and light chain-specific) and avidin–HRP conjugate, and finally developed with 4-chloro-1-naphthol and H2O2. When primary antibodies were derived from human or mouse, the immunochemical reactions were carried out in the same way except that the secondary antibodies used were biotinylated goat antibodies against immunoglobulins of the specified species (Sigma).

- **Cell transfection and immunofluorescence staining.** Huh-7 cells in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% foetal calf serum were maintained in 145 mm tissue culture dishes. Transient expression of HDAG was achieved by calcium chloride transfection of plasmids into Huh-7, according to a previously described procedure (Wu et al., 1991). Cells used for immunofluorescence staining were prepared similarly except that cells were cultured on cover slides. Cells were rinsed with PBS, fixed and permeated with methanol:acetone (1:1) 48 h after transfection. Non-specific binding was blocked with 1% BSA in PBS and the cells were reacted with primary antibodies diluted 1:100 in the same buffer. The secondary antibodies used were FITC-labelled goat anti-rabbit IgG (Sigma) for detecting the isoprenyl modification and Texas red-labelled goat anti-mouse IgG (Sigma) for detecting HDAG. These fluochrome-conjugated secondary antibodies were species-specific and did not cause cross-reaction. Both conjugates were diluted 1:250 in PBS containing 1% BSA. Other immunofluorescence staining procedures were carried out as previously described (Wu et al., 1992).

Results

The isoprenyl modification of large HDAG involves attachment of a farnesyl group to the sulphhydril group of a cysteine residue (Otto & Casey, 1996). To prepare the immunogen, we took advantage of the availability of N-acetyl-S-farnesyl-l-cysteine, which has been used as an inhibitor of S-farnesylcysteine methyltransferase (Volker et al., 1991), and conjugated it to KLH. ELISA was carried out to test the antibody production of immunized rabbits. Three different antigens were coated separately on plates and tested for antibody binding. The immunized rabbit serum reacted specifically with farnesyl-conjugated BSA, but not BSA alone.
Anti-farnesyl antibodies

Fig. 1. Specificity of anti-farnesyl antibodies demonstrated by ELISA. Different antigens were coated on polystyrene plates and assayed for binding of antibodies. Binding of rabbit anti-farnesyl antibodies (filled bars) to antigens was detected indirectly by HRP-conjugated goat anti-rabbit IgG. The control anti-HDAg (hatched bars) was HDV-infected human serum; binding of these antibodies was measured by HRP-conjugated goat anti-human IgG. The colour reaction was developed with OPD and H$_2$O$_2$, and was followed by measuring absorbance at 490 nm. Assays were carried out in triplicate and expressed as means.

Fig. 2. Western blotting analysis of antibody specificity. Proteins were run separately on different lanes of a 10% SDS–polyacrylamide gel and subsequently transferred to nitrocellulose membranes. Membranes were incubated with the indicated primary antibodies outlined in Fig. 1. Membrane-bound antibodies were detected by appropriate secondary antibodies conjugated with HRP. The colour reaction was developed with 4-chloro-1-naphthol and H$_2$O$_2$ (Yan et al., 1996). Bands below the marked antigens probably represent degradation products generated during antigen preparation.

Fig. 3. Analysis of HDAg expression in transfected Huh-7 hepatoma cells. Huh-7 cell lysates were prepared 2 days after DNA transfection and the lysate from a mock transfection was included as a control. Proteins in cell lysates were run on a 12% SDS–polyacrylamide gel. Subsequent Western blotting procedures were as described in Fig. 2; blots were incubated with anti-HDV (a) or anti-T1C (b) antibodies. L and S indicate the positions of large and small HDAg.

Fig. 4. Anti-HDAg antibodies demonstrated by Western blotting. BSA-farnesyl, BSA-geranylgeranyl, MBP-HDAg, and MBP–HDAg were used as antigens. The control anti-HDAg (hatched bars) was HDV-infected human serum; binding of these antibodies was measured by HRP-conjugated goat anti-human IgG. The colour reaction was developed with OPD and H$_2$O$_2$, and was followed by measuring absorbance at 490 nm. Assays were carried out in triplicate and expressed as means.

Examine whether these antibodies cross-reacted with HDAg, MBP–HDAg was used as the control. MBP–HDAg was produced in E. coli and contained large HDAg without isoprenyl modification. As shown in Fig. 1, while HDV-infected human serum (anti-HDV) reacted strongly with MBP–HDAg, negligible reaction with MBP–HDAg was observed with the anti-farnesyl antibodies.

Western blotting was used to demonstrate further the specificity of the antibodies towards the isoprenyl group. Two isoprenoid modifications have been observed in isoprenylated proteins: the farnesyl group has three isoprene repeats and the geranylgeranyl group has four. Anti-farnesyl antibodies would therefore be expected to cross-react with the geranylgeranyl modification. Fig. 2 shows that the anti-farnesyl antibodies indeed reacted with both farnesyl-conjugated and geranylgeranyl-conjugated BSA, but not with control BSA or the recombinant protein MBP–HDAg. The ELISA and Western blotting data therefore verified that the immunized rabbits produced antibodies that reacted specifically with isoprenyl groups.

Examination of the compartmentalization of HDAg was carried out in the Huh-7 transfection system (Wu et al., 1992). Western blotting was performed first to demonstrate that the small and large forms of HDAg were properly expressed. Huh-7 cell lysates were collected 2 days after transfection and the proteins in the lysates were analysed for HDAg by Western blotting with anti-HDV serum (Fig. 3a). Small HDAg had an apparent mass of about 24 kDa, whereas large HDAg had an apparent mass of about 29 kDa. Since large HDAg contains a unique peptide extension at the C terminus, it could be further differentiated from small HDAg by staining with anti-T1C antibodies (Fig. 3b).

The appearance of HDAg in transfected cell lysates but not...
Fig. 4. Immunofluorescence staining patterns of Huh-7 cells 2 days after transfection. (a)–(c) Cells expressing small HDag (HDag-S); (d)–(f) cells expressing large HDag (HDag-L). Each set of cells was triple-stained with anti-HDag antibody (a, d), anti-farnesyl antibody (b, e) and Hoechst 33258 (c, f); the anti-HDag serum used was anti-HDV (a) or anti-T1C (d). Anti-HDag was visualized with Texas red-labelled goat anti-human (c) or goat anti-mouse (d) IgG whereas anti-farnesyl was detected by FITC-labelled goat anti-rabbit IgG. Magnification ×1000.

in the control cell lysate (Fig. 3) indicated that HDag was appropriately expressed and the antibodies were specific. Therefore, the intracellular localization of HDag in these transfected cells was examined by immunofluorescence staining. Typical results are shown in Fig. 4. The cells shown in Fig. 4(a–c) expressed small HDag whereas those shown in Fig. 4(d–f) expressed large HDag. These cells were triple-stained with anti-HDag antibodies, anti-farnesyl antibodies and Hoechst dye 33258.

Cells expressing small HDag were stained by anti-HDV antibodies (Fig. 4a) and the antigen was visualized within the area of DNA staining (Fig. 4c). Our data localizing the small HDag to the nucleus are therefore consistent with previous reports (Bichko & Taylor, 1996; Macnaughton et al., 1990; Wu et al., 1992). When these cells were stained with anti-farnesyl antibodies, little signal was observed (Fig. 4b).

When cells expressing large HDag were stained, the anti-T1C serum also localized the antigen to the nucleus (Fig. 4d–f), as observed previously (Negro et al., 1991; Wu et al., 1992). In this experiment, we used the anti-T1C serum to avoid the possibility of staining N-terminal fragments of large HDag. Interestingly, the anti-farnesyl antibodies (Fig. 4e) stained the same cells that were stained by anti-T1C; the antigen was clearly in the nucleus and the staining obtained with anti-T1C and with anti-farnesyl antibodies could be superimposed (Fig. 4d, e). These results clearly indicate that under our culture conditions a significant amount of large HDag had moved to the nucleus and was isoprenylated.
Discussion

Both small and large forms of HDAG have the same NLS and both have been found in the nucleus during the process of virus replication (Bichko & Taylor, 1996; Macnaughton et al., 1990; Wu et al., 1992). Although the antigenicities of these two forms of HDAG are closely related, antibodies specific to certain epitopes allow them to be distinguished (Hwang & Lai, 1993b; Wang et al., 1992). Furthermore, HDAG is modified after protein synthesis. Phosphorylated HDAG can be differentiated from the non-phosphorylated form by two-dimensional gel electrophoresis (Bichko et al., 1997). In contrast, biochemical separation of isoprenylated large HDAG from the non-isoprenylated form has not been achieved. It is reasonable to believe that the post-translational modification and cellular location of HDAG will be correlated in some way with its different biological functions.

Two major functions of large HDAG have been reported (Wang et al., 1994). One of these functions is to suppress viral RNA replication (Chang et al., 1994; Wang et al., 1994), which is stimulated by small HDAG during the early replication stage. The site at which large HDAG acts on replication is presumably the nucleus. This rationale is consistent with the observations that both small and large forms of HDAG have been localized to the nucleus (this study; Macnaughton et al., 1990; Negro et al., 1991; Wu et al., 1992). Here, we have further demonstrated that large HDAG that is translocated to the nucleus carries the isoprenyl modification.

In eukaryotic cells, diverse proteins have been shown to be isoprenylated. These include the Ras proteins, many G proteins, the retinal cGMP phosphodiesterases, lamins and several fungal mating pheromones (Schafer & Rine, 1992). Isoprenylation enables the Ras-related family of proteins to be escorted to membranes where these signal transduction proteins exert their functions (Gelb, 1997). Farnesylation in conjunction with an NLS has been shown to be necessary to target newly synthesized lamins to the inner nuclear envelope membrane (Firmbach-Kraft & Stick, 1995; Holtz et al., 1989; Nigg et al., 1992), where filament formation is initiated. In HDV, our observations show that large HDAG was localized largely to the nucleus as speckled structures, although other patterns were occasionally observed. Further observation by confocal microscopy did not link large HDAG to a particular compartment of the nucleus (data not shown). Nevertheless, staining with anti-farnesyl antibodies could be superimposed on staining for large HDAG. Based on the notion that large HDAG suppresses virus replication in the nucleus, the functional large HDAG in the nucleus is therefore likely to be the isoprenylated form.

Large HDAG also plays a second role. In addition to the suppression of virus replication, it interacts with HBV surface proteins (Hwang & Lai, 1993a), presumably in the ER, to enable virus assembly and secretion (Sheu et al., 1996). However, immunofluorescence staining with anti-farnesyl antibodies gave signals within the nucleus. Staining for large HDAG and the farnesyl group was occasionally co-localized in both the cytoplasm and the nucleus. This suggests that large HDAG in the cytoplasm is also isoprenylated. An experiment was also carried out to determine the effect of co-expressed HBV surface antigen on the distribution of isoprenylated HDAG. No significant effect on the anti-farnesyl immunostaining pattern was observed (data not shown). We speculate that, in our transfection system, only a small portion of the over-expressed large HDAG was escorted to the particle assembly route, so the overall staining pattern for isoprenylated HDAG was not affected. Fine-tuning of the transfection conditions may enable the effect of the HBV surface antigen to be more clearly observed. To identify specifically the subcellular distribution of these isoprenylated HDAG molecules, high-resolution staining with immunoelectron microscopy would be required.

In conclusion, this is the first time to our knowledge that anti-farnesyl antibodies have been prepared and that large HDAG in the nucleus has been demonstrated to be isoprenylated. Although our current results do not provide direct evidence of how and where large HDAG exerts its functions, we strongly suggest that isoprenylated large HDAG is present in the nucleus. In addition, these data cast doubt on the model that non-isoprenylated large HDAG enters the nucleus and interacts with the other viral components to form ribonucleoprotein complexes that are then transported out of the nucleus for further modification and virus maturation. The anti-farnesyl antibodies we have produced should also be applicable to the study of other similarly modified proteins.

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