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# Isoprenylation Mediates Direct Protein-Protein Interactions between Hepatitis Large Delta Antigen and Hepatitis B Virus Surface Antigen

SOON B. HWANG AND MICHAEL M. C. LAI*

*Howard Hughes Medical Institute and Department of Microbiology, University of Southern California School of Medicine, Los Angeles, California 90033-1054*

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**Hepatitis delta antigen (HDAg) consists of two protein species of 195 and 214 amino acids, respectively, which are identical in sequence except that the large HDAg has additional 19 amino acids at its C terminus and is prenylated. Previous studies have shown that the large HDAg and the surface antigen of hepatitis B virus (HBsAg) together can form empty hepatitis delta virus (HDV) particles. To understand the molecular mechanism of HDV virion morphogenesis, we investigated the possible direct protein-protein interaction between HDAg and HBsAg. We constructed recombinant baculoviruses expressing the major form of HBsAg and various mutant HDAgs and used these proteins for far-Western protein binding assays. We demonstrated that HBsAg interacted specifically with the large HDAg but not with the small HDAg. Using mutant HDAgs which have defective or aberrant prenylation, we showed that this interaction required isoprenylates on the cysteine residue of the C terminus of the large HDAg. Isoprenylation alone, without the remainder of the C-terminal amino acids of the large HDAg, was insufficient to mediate interaction with HBsAg. This study demonstrates a novel role of prenylates in HDV virion assembly.**

Hepatitis delta virus (HDV) is a human pathogen causing fulminant hepatitis and liver cirrhosis (9, 14). HDV is infectious only in the presence of a coinfecting hepatitis B virus (HBV), which provides hepatitis B surface antigen (HBsAg) for the HDV virion envelope. The requirement of HBsAg in HDV particle formation has been demonstrated in cell culture and in animals (3, 25, 26). HDV consists of a single-stranded circular RNA of approximately 1.7 kb (15, 20, 28) and an internal protein, hepatitis delta antigen (HDAg), surrounded by the HBsAg envelope. Two species of HDAg, a small HDAg (SHDAg) of 24 kDa (195 amino acids) and a large HDAg (LHDAg) of 27 kDa (214 amino acids), are present in the liver and sera of infected patients and animals (1, 2). The two proteins are identical in sequence except that the large HDAg contains an additional 19 amino acids at its C terminus (29). Both proteins have an RNA-binding activity (13) and are apparently complexed with HDV RNA (19). The LHDAg, but not the SHDAg, is isoprenylated (8, 13). Functionally, the SHDAg is required for HDV RNA replication (16), whereas the LHDAg has negative effects on genome replication (5) but is required for HDV assembly (3, 25, 26). It has been demonstrated that LHDAg without HDV RNA can be packaged by HBsAg and form an empty virus particle (3, 25), while SHDAg can be packaged only in the presence of LHDAg, probably through dimerization between SHDAg and LHDAg (6). Previous studies have implicated isoprenylate residues as essential for HDV particle formation (8). However, it is not clear whether HDAg interacts directly with HBsAg or through other components, e.g., lipid bilayer. This issue is particularly intriguing since HBsAg is mainly localized in the cytoplasm (22) while HDAg is localized in the nucleus (4). Thus, how these two proteins interact to form virus particles remains unknown.

**Direct protein-protein interaction between LHDAg and HBsAg.** To study the mechanism of HDV virion assembly, we first examined whether HDAg and HBsAg can interact directly with each other by a far-Western protein blotting procedure. For this purpose, several recombinant baculoviruses were constructed which express the wild type (13) and various mutant forms of HDAgs and the small form of HBsAg in Sf9 insect cells. The small form of HBsAg has been shown to be the major component of HDV particles (2) and is responsible for HDV assembly (27). While HDAg accumulated in the nuclei of the recombinant baculovirus-infected Sf9 cells (13), HBsAg remained in the cytoplasm and also was released into the culture medium. HBsAg particles purified from the culture medium contained mainly the nonglycosylated (p24) and glycosylated (gp27) forms of HBsAg (Fig. 1A), and were used in the far-Western assay (18). In this assay, both the small and large HDAgs from Sf9 cells were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Fig. 1B), transferred to nitrocellulose membrane, and renatured by sequential treatments with decreasing concentrations of guanidine HCl. The purified HBsAg particles were disrupted with Nonidet P-40 and then incubated with the membrane for 12 to 16 h at 4°C. The membrane was further incubated with a polyclonal antibody to HBsAg (anti-HBsAg), followed by incubation with $^{125}$I-protein A (12). The results showed that HBsAg bound to the large, but not the small, HDAg (Fig. 1C). Since the two proteins differ only by the presence of 19 extra amino acids at the C terminus of LHDAg, this result suggests that HBsAg interacts directly with LHDAg, most likely through the C-terminal 19 amino acids of the latter.

**Role of isoprenylation in LHDAg-HBsAg interaction.** The carboxyl terminus of LHDAg contains a CaXX box (where C is cysteine, a is an aliphatic amino acid, and X is any amino acid), in which the cysteine residue is isoprenylated (8, 13). Therefore, we examined whether isoprenylation was responsible for the LHDAg-HBsAg interaction detected in far-Western blots. Two site-specific mutants of HDAg-recombinant baculoviruses were made in which cysteine 211 of the CaXX box of LHDAg was changed to serine (mutant L-C211S), or

* Corresponding author.

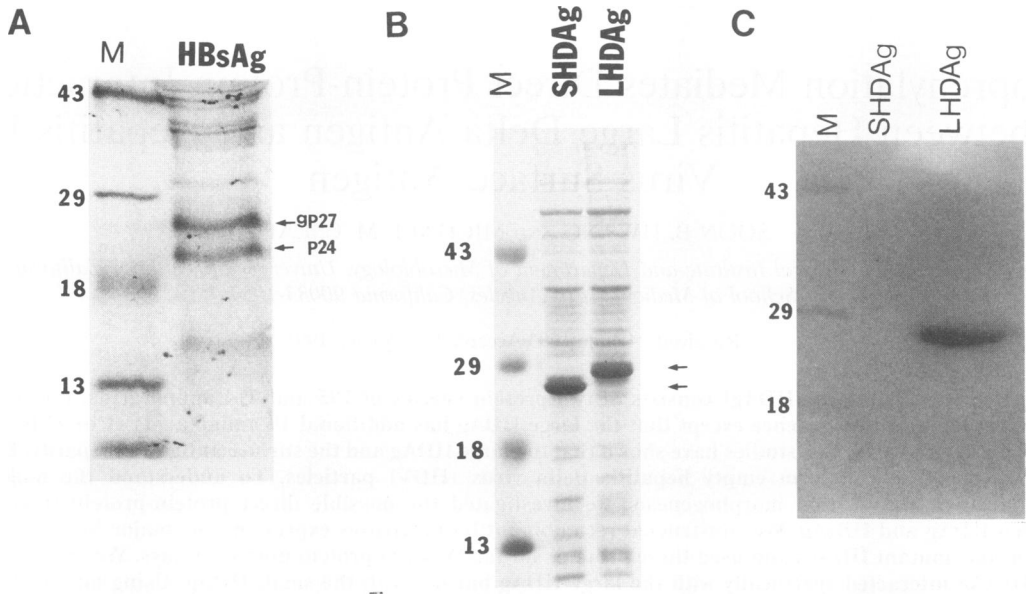


FIG. 1. Protein-protein binding assay between HBsAg and HDAg. (A) HBsAg expressed by recombinant baculovirus. Recombinant baculovirus expressing the small (major) HBsAg (*Autographa californica* nuclear polyhedrosis virus-HBsAg) was constructed according to the published method (13). Culture medium from virus-infected Sf9 cells was harvested at day 5 postinfection and clarified of cell debris, and HBsAg particles were pelleted by ultracentrifugation at 35,000 rpm at 4°C for 17 h in an SW41 rotor through a 20% sucrose cushion. The virus pellets were lysed by boiling in Laemmli sample buffer (17), separated by SDS-PAGE on a 12.5% polyacrylamide gel, and stained with Coomassie Brilliant Blue. (B and C) Protein-protein blotting assays. The Sf9 cells infected with recombinant baculoviruses expressing either SHDAg or LHDAg (13) were lysed by Laemmli sample buffer, separated by SDS-PAGE, and either stained with Coomassie Brilliant Blue (panel B) or electrotransferred to a nitrocellulose membrane (panel C). The membrane was washed with buffer A (10 mM HEPES-KOH [pH 7.5] [HEPES is *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid], 60 mM KCl, 1 mM EDTA, 1 mM 2-mercaptoethanol) and incubated with 6 M guanidine HCl for 15 min at 4°C and then sequentially with 3, 1.5, 0.75, 0.38, 0.19, and 0.09 M guanidine HCl for 5 min each to renature proteins. The membrane was subsequently blocked for 1 h at 4°C with 5% nonfat dry milk in buffer A containing 0.05% Nonidet P-40. The purified HBsAg particles were resuspended in buffer A and disrupted with 1% Nonidet P-40, mixed with uninfected Sf9 cell lysates, and incubated with the membrane in buffer A containing 3% nonfat dry milk overnight at 4°C. Unbound proteins were removed by washing with TNT buffer (10 mM Tris-HCl [pH 7.5], 150 mM NaCl, 0.05% [vol/vol] Tween 20), and the membrane was incubated with anti-HBsAg polyclonal antibody (Calbiochem) for 3 h and followed with $^{125}$I-protein A for 2 h. Membrane was then washed 3 times with TNT, and protein binding was detected by autoradiography (panel C). M, molecular size marker (in kilodaltons).

the last four amino acids of SHDAg were replaced with CRPQ (S-192Cp), such that this mutant SHDAg has the same isoprenylation motif as in the LHDAg (Fig. 2A). These mutant proteins were expressed in Sf9 cells to roughly the same extent as the wild-type proteins (Fig. 2B). The state of prenylation of the mutant proteins was examined by labeling the infected cells with [$^3$H]mevalonolactone (Fig. 2C). As shown previously, LHDAg was prenylated while SHDAg was not (8, 13). Interestingly, S-192Cp became prenylated whereas L-C211S was not (Fig. 2C). These results indicated that the CaXX box is sufficient and necessary for protein prenylation not only in mammalian cells but also in insect cells.

We then tested how isoprenylation affected protein interactions between HDAg and HBsAg (Fig. 3). As shown previously, HBsAg bound to the wild-type LHDAg but not to the wild-type SHDAg. The mutant LHDAg, L-C211S, which is isoprenylation defective, did not bind HBsAg. This result indicates that isoprenylation is necessary for the LHDAg-HBsAg interaction. Surprisingly, the mutant form of SHDAg (S-192Cp), which was isoprenylated, did not bind HBsAg. These results suggest that isoprenylate itself is not sufficient for the HBsAg-HDAg interaction. From these results, we conclude that both the isoprenylate and the C-terminal 19 amino acids of LHDAg are required for the specific HDAg-HBsAg interaction.

To determine the HBsAg domain which interacts with the HDAg, we performed an experiment in which the purified HBsAg particles without pretreatment with Nonidet P-40 were directly incubated with HDAgs. No binding was detected (data not shown). This finding suggested that the HDAg-HBsAg binding was through the internal domain of HBsAg, which was exposed after the detergent treatment. This is consistent with the idea that the direct interaction between HBsAg and LHDAg is the basis for HDV particle formation, since HDAg is located inside the HBsAg envelope of HDV particles (2, 24).

We also examined the possible interactions between HBsAg and HBV core antigen (HBcAg), since HBcAg is also enclosed inside the HBsAg envelope in HBV (23). Unlike the LHDAg, HBcAg did not bind to the HBsAg (data not shown), suggesting that the direct protein-protein interaction through isoprenylate is unique to HDV.

This study established a unique role of isoprenylae in HDV biology. Protein prenylation has been thought to increase protein hydrophobicity and thus allows protein localization on membranes (7). However, recent studies of *ras* protein suggested that prenylation alone is not sufficient for the protein to be membrane localized (10); rather, additional protein sequence is required, prompting a suggestion that prenylates mediate protein-protein interaction rather than protein-membrane interaction (21). Our studies here indicate that the presence of isoprenylate on the LHDAg allows a specific protein-protein interaction between LHDAg and HBsAg. This

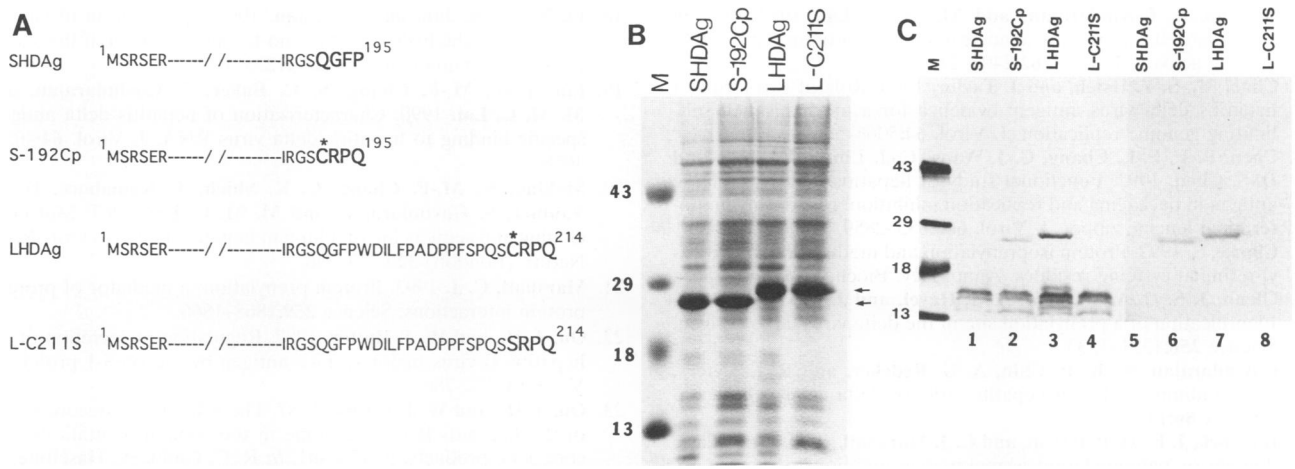


FIG. 2. Structure and properties of isoprenylation mutants of HDAg. (A) Partial protein sequences of wild-type and mutant HDAgs. The four C-terminal amino acids, including the isoprenylation motif (CaXX), are shown in boldface. The cysteine residue with an isoprenylate group is indicated with an asterisk. (B) The expression of HDAg mutants by recombinant baculoviruses. All of the recombinant baculoviruses expressing the mutant HDAg were constructed by standard site-specific mutagenesis procedures (13). Lysates from recombinant virus-infected Sf9 cells were analyzed by SDS-PAGE and stained with Coomassie Brilliant Blue. HDAgs are indicated (arrows). M, molecular size marker (in kilodaltons). (C) Isoprenylation of HDAgs. The recombinant baculovirus-infected Sf9 cells were incubated with media containing the *RS*-[5-$^3$H] mevalonolactone (27.5 Ci/mmol; NEN) at 24 h postinfection for 20 h. The radioactively labeled cells were washed twice in phosphate-buffered saline and then lysed in RIPA buffer (1% Nonidet P-40, 1% sodium deoxycholate, 150 mM NaCl, 50 mM Tris-HCl [pH 8.0], 0.1% SDS, 0.1 mM phenylmethylsulfonyl fluoride). The $^3$H-labeled cell extracts were immunoprecipitated with rabbit anti-HDAg serum, followed by SDS-PAGE and fluorography as previously described (13). M, molecular size marker (in kilodaltons); lanes 1 to 4, before immunoprecipitation; lanes 5 to 8, immunoprecipitates.

interaction requires not only prenylates but also other amino acid residues on the C terminus of the LHDAg. This study thus adds HDAg to the growing list of prenylated proteins, including p21*ras* and lamin A proteins (10, 11), in which prenylates mediate protein-protein interactions. In *ras* proteins, palmitoylation or a polybasic domain in combination with a CaaX motif is required for protein targeting into plasma membranes (10). Nuclear targeting of lamin A also requires a nuclear localization signal in addition to a CAAX motif (11). Similarly, our data suggest that HBsAg interacts not only with prenylates but also with neighboring amino acids of LHDAg. It is not clear whether isoprenylate is directly involved in protein-protein interaction or indirectly by altering protein conformation so that the C-terminal 19 amino acids could interact with HBsAg. Using a monoclonal antibody which recognizes a conformational epitope at the C terminus of SHDAg but not LHDAg (12), we have recently shown that prenylation alters the HDAg protein conformation around the C terminus of HDAg (unpublished observation). Conceivably, the level of phosphorylation also may affect protein-protein interactions, since LHDAg is more heavily phosphorylated than SHDAg (13). The precise residues, in addition to prenylate, involved in the HBsAg-HDAg interaction will require further studies.

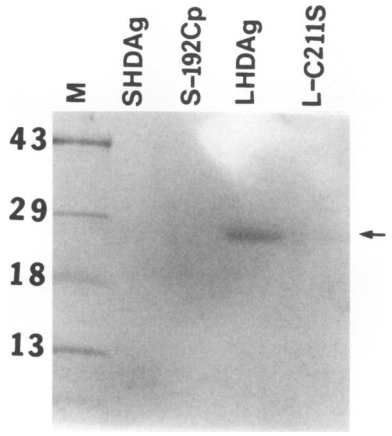


FIG. 3. Protein blotting assay of isoprenylation mutants of HDAg. Both wild-type and mutant forms of HDAgs were separated and transferred to nitrocellulose membrane as described in the legend to Fig. 1C. The membrane was incubated with purified HBsAg particles disrupted with 1% Nonidet P-40 and incubated sequentially with anti-HBsAg antibody and $^{125}$I-protein A. M, molecular size marker (in kilodaltons).

This direct protein-protein interaction between HBsAg and HDAg may provide the molecular basis for HDV virion assembly. However, HDAg is localized in the nucleoplasm and nucleoli (4) and HBsAg is localized in the cytoplasm of the infected cells (22). How these two proteins in different cellular compartments come into direct contact remains a puzzling issue.

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