JOURNAL OF VIROLOGY, May 1994, p. 2958–2964
0022-538X/94/$04.00+0

Vol. 68, No. 5

Isoprenylation Masks a Conformational Epitope and Enhances *trans*-Dominant Inhibitory Function of the Large Hepatitis Delta 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

Received 10 November 1993/Accepted 27 January 1994

Hepatitis delta antigen (HDAg) consists of two species, large (LHDAg) and small (SHDAg), which are identical in sequence except that the large form contains 19 extra amino acids at the C terminus. The large form is prenylated on the Cxxx motif. The small form can *trans* activate HDV RNA replication, while the large form inhibits it. To determine the molecular basis for their differential functions, we examined the effects of prenylation on the conformation and function of HDAg. We show that the presence of prenylates masks a conformational epitope which is present in SHDAg but hidden in wild-type LHDAg; this epitope becomes exposed in all of the nonprenylated mutant LHDAgs. Prenylation also plays a major role in conferring the *trans*-dominant negative inhibitory activity of LHDAg, since the loss of prenylation in LHDAg reduced its inhibitory activity. The primary amino acids of the C-terminal sequence also contributed to the maintenance of the HDAg protein conformation; a prenylated LHDAg mutant with a five-amino-acid deletion had an exposed C-terminal epitope. By examining LHDAg mutants which have deletions of various extents of C-terminal sequence, with or without the prenylation motif, we have further shown that all of the prenylated mutants have much higher levels of *trans*-dominant suppressor activities than do the corresponding nonprenylated mutants. Surprisingly, a few nonprenylated LHDAg mutants were able to *trans* activate HDV RNA replication, while all of the prenylated ones lost this function. These results suggest that isoprenylates cause the masking of a conformational epitope of HDAg and that conformational differences between the large and small HDAgs account for the differences in their *trans*-activating and *trans*-dominant inhibitory biological activities.

Hepatitis delta virus (HDV) is a defective RNA virus which contains a 1.7-kb single-stranded circular genome (16, 25, 35). HDV RNA is known to replicate by RNA-dependent RNA synthesis via a rolling-circle mechanism (24) and has autocatalytic cleavage and ligation activities (32, 33, 37, 38). The viral RNA encodes only a single protein, hepatitis delta antigen (HDAg), from the antigenomic strand. During RNA replication, an RNA editing event occurs at the amber termination codon for HDAg, resulting in the synthesis of a larger isoform of HDAg (21). These two species of HDAg, small HDAg (SHDAg) of 24 kDa (195 amino acids) and large HDAg (LHDAg) of 27 kDa (214 amino acids), are identical in sequence except that LHDAg contains 19 additional amino acids at its C terminus (36). However, the two HDAg forms have entirely different functions: SHDAg *trans* activates HDV RNA replication (17), whereas LHDAg inhibits HDV RNA replication (3) and is required for HDV assembly (1, 30, 34). Both forms are phosphoproteins (2, 15), but the large form is more heavily phosphorylated than the small form (15). LHDAg also contains a Cxxx box (where x is any amino acid) at its C terminus, in which the cysteine residue is isoprenylated (8, 15). Recently, it has been demonstrated that LHDAg interacts with hepatitis B virus major surface antigen (HBsAg) through the prenylate residues (14) and that these prenylates are necessary, but not sufficient, for virion assembly (19). Since both forms of HDAg are, for the most part, alike in sequence, the question of how these two proteins effect different functions arises. Our previous studies have demonstrated that both forms can form homo- and heterodimers (39) and that protein-protein interaction is required for the *trans*-activating function of SHDAg and the *trans*-dominant negative function of LHDAg (39). Thus, a model has been proposed in which SHDAg complex is required for HDV RNA replication and the LHDAg-SHDAg complex is not functional (39). This model is based on the assumption that the two forms have different protein conformations. Indeed, we have obtained a monoclonal antibody (MAb) which recognizes an epitope at the C terminus of SHDAg that is not present in LHDAg (13). Therefore, the C-terminal 19 amino acids of LHDAg likely alter the protein conformation of the HDAg, such that this epitope is not exposed. Since the C terminus of LHDAg is prenylated, which is known to increase the hydrophobicity of protein (6, 26), the prenylate may play a central role in maintaining the protein conformation and biological activity of LHDAg.

Prenylation has been implicated in various protein functions. It occurs posttranslationally in many eukaryotic proteins and is usually followed by proteolysis and methylation at the C terminus of proteins (6, 29). Prenylation promotes protein-protein interactions to facilitate membrane localization of proteins (11, 12, 26) and function in signaling pathways (27, 28).

To study the molecular basis of the different functions of the two HDAgs, we examined the effects of prenylation of LHDAg. We demonstrate that prenylates mask a conformational epitope at the C terminus of the protein either directly or as a result of the alteration of protein conformation. As a consequence, the prenylated LHDAg exhibits *trans*-dominant inhibitory activity. This study confirms the importance of protein

* Corresponding author. Mailing address: Department of Microbiology, University of Southern California School of Medicine, 2011 Zonal Ave., Los Angeles, CA 90033-1054. Phone: (213) 342-1748. Fax: (213) 342-9555.

conformation as the basis of HDAg functions and provides an insight into the effect of protein prenylation.

MATERIALS AND METHODS

Cells and cell culture. The monkey kidney cell lines Cos 7 (10) and H1δ9, which is a Cos cell line with an integrated HDV trimer cDNA (22) and constitutively expresses HDV RNA and HDAg, were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 5% fetal calf serum. Cos 7 cells were used as the recipient for DNA transfection.

Construction of mutant HDAg expression plasmids. All of the mutant constructs were made by using plasmid pECE-δ-BE (2), which contains the open reading frame for LHDAg behind a simian virus 40 T-antigen promoter. Mutations were made on this plasmid to generate desired deletions within the C-terminal 19 amino acids of LHDAg, containing either an intact (CRPQ) or a defective (SRPQ) isoprenylation motif. Mutations were performed by PCR by using a 5′ primer, which consists of a *Bam*HI endonuclease restriction site plus 12 nucleotides homologous to the HDAg initiation site, and a 3′ primer, which consists of a *Hin*dIII restriction site followed by sequences coding for either CRPQ or SRPQ in the desired region within the C terminus of LHDAg. Amplified DNA fragments were cloned into the *Bgl*II and *Hin*dIII sites of pECE vector (7). All mutations were confirmed by dideoxynucleotide-chain termination sequencing (31).

DNA transfection. All plasmid DNAs were purified by CsCl gradient centrifugation. For immunoprecipitation studies, each HDAg-encoding plasmid DNA (5 μg) was transfected into subconfluent Cos 7 cells in 60-mm-diameter dishes. For Northern (RNA) blot analysis, 5 μg of pECE plasmid containing a wild-type HDV dimer or replication-defective HDV dimer DNA (20, 39) was cotransfected with pECE plasmids (5 μg of each) encoding either the wild-type or mutant form of HDAg. The medium was removed 24 h later, and cells were refed with fresh DMEM containing 5% fetal calf serum. All transfections were performed with *N*-[1-(2,3-dioleoyloxy) propyl]-*N*,*N*,*N*-trimethylammonium methylsulfate (DOTAP; Boehringer Mannheim) as instructed by the manufacturer.

Cell labeling and immunoprecipitation. At 48 h posttransfection, Cos 7 cells were incubated in methionine-free DMEM for 1 h and labeled with Trans [^{35}S] label (0.1 mCi/ml; 1,162 Ci/mmol; ICN). After a 4-h incubation period, the labeling medium was removed, and the cells were washed with phosphate-buffered saline (PBS) and incubated in 500 μl of lysis buffer (150 mM NaCl, 0.5% Nonidet P-40, 50 mM Tris HCl [pH 8.0], 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride) on ice for 15 min. Lysed cells were transferred to 1.5-ml Eppendorf tubes by scraping with rubber policemen and further incubated for 15 min on ice with occasional vortexing. Cell extracts were further disrupted by passage through a 25-gauge syringe needle 7 to 10 times, and cell debris was cleared by centrifugation in a microcentrifuge for 15 min. Aliquots of cell lysates were incubated with either a rabbit anti-HDAg polyclonal antibody or an SHDAg-specific MAb (13) at 4°C. After incubation with constant rocking for 2 h, protein A-Sepharose (Pharmacia) was added, and the preparations were rocked for an additional hour. The Sepharose beads were washed three times with radioimmunoprecipitation assay buffer (150 mM NaCl, 1% Nonidet P-40, 1% sodium deoxycholate, 50 mM Tris HCl [pH 8.0], 0.1% sodium dodecyl sulfate [SDS], 0.1 mM phenylmethylsulfonyl fluoride), boiled in Laemmli sample buffer (18) for 5 min, and analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) on a 12.5% polyacrylamide gel. The dried gel was exposed for autoradiography.

In vitro transcription. ^{32}P-labeled HDV antigenomic RNA was synthesized by using 1 μg of *Hin*dIII-linearized plasmid S18, which contains a monomeric HDV cDNA behind the T7 promoter (39). Transcription was performed with T7 RNA polymerase and [α-^{32}P]UTP (3,000 Ci/mmol; ICN) as described previously (22). The transcribed RNAs were purified by passage through a G-25 column (5 Prime→3 Prime, Inc.) and heat denatured before use.

Northern blot analysis. Total cellular RNA was extracted from the transfected Cos 7 cells at day 5 posttransfection, using the guanidinium isothiocyanate method (5). The RNA samples were electrophoresed in formaldehyde-containing agarose gels and transferred onto a nitrocellulose membrane (Hybond C; Amersham) overnight. The membrane was baked for 2 h at 80°C, prehybridized for 6 h, and hybridized with ^{32}P-labeled HDV antigenomic RNA overnight at 55°C. The blots were washed briefly twice at room temperature and four times for 20 min each time at 80°C as previously reported (23). HDV RNA extracted from the cell line H1δ9 (22), in which HDV RNA constitutively replicates, was used as a positive control.

RESULTS

Effects of isoprenylation on HDAg protein conformation. To determine the possible effects of isoprenylation on the protein conformation of HDAg, we took advantage of the fact that MAb 9E4 specifically recognizes SHDAg but not LHDAg (13). The epitope for this MAb has been mapped within the C terminus of SHDAg (13). The corresponding sequence in LHDAg is not recognized by this MAb, most likely as a result of the conformational change induced by the C-terminal 19 amino acids. To determine whether this conformational difference between SHDAg and LHDAg is due to isoprenylation at the C terminus of LHDAg, we constructed several HDAg mutants with altered isoprenylation patterns. In mutant 211S, cysteine 211 of LHDAg was changed to serine; this mutant LHDAg has been demonstrated to be unprenylated (8, 14). Another mutant, 192C, contains the isoprenylation motif CRPQ, which replaces the last four amino acids (QGFP) of SHDAg; this mutant SHDAg has been shown to be prenylated (14). These mutant plasmids were transfected into Cos cells, and the radiolabeled proteins were immunoprecipitated with either rabbit polyclonal anti-HDAg serum or MAb 9E4. As shown in Fig. 1, both mutant HDAgs and wild-type HDAgs were precipitated by the polyclonal antibody. However, MAb 9E4 precipitated primarily wild-type SHDAg and very little LHDAg. A very faint background of LHDAg was detected; as previously demonstrated (13), this residual reactivity was probably due to denaturation of some of this LHDAg preparation. In contrast, MAb 9E4 did not precipitate 192C, suggesting that the epitope recognized by 9E4 is localized at the very end of the C terminus of SHDAg, which has been mutated in 192C. Interestingly, 9E4 precipitated the LHDAg mutant 211S, in contrast to wild-type LHDAg, suggesting that the removal of prenylates in the C terminus of LHDAg exposed the epitope, which is otherwise detected only in SHDAg (13). Therefore, we conclude that detection of the 9E4 epitope is dependent on protein conformation, which is masked in wild-type LHDAg as a result of prenylation. Thus, prenylates either directly mask this conformational epitope or induce conformational changes in the C terminus of HDAg.

Effects of isoprenylation on the *trans*-dominant suppressor function of LHDAg. LHDAg has been shown to exert inhibitory effects on HDV RNA replication (3), and this inhibitory

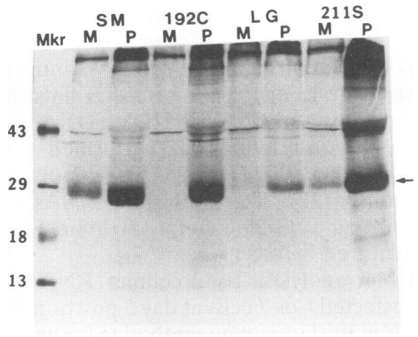

FIG. 1. Immunoprecipitation of wild-type and mutant HDAgs. Cos 7 cells transfected with various plasmids were labeled metabolically with Trans [^{35}S], and cell lysates were immunoprecipitated with MAb 9E4 (M) or a polyclonal antibody (P) made against HDAg. The immunoprecipitates were separated by SDS-PAGE on a 12.5% polyacrylamide gel. Mkr, molecular mass marker in kilodaltons. The arrow indicates HDAgs.

activity has been postulated to be due to conformational differences between SHDAg and LHDAg (39). To corroborate this possibility, we examined whether isoprenylation is responsible for the *trans*-dominant suppression function of LHDAg on HDV RNA replication. The HDAg isoprenylation mutants were cotransfected with a replication-competent HDV dimer cDNA into Cos 7 cells, and the effects of the mutant HDAgs on HDV RNA replication were examined. As demonstrated in Fig. 2, the replication of HDV RNA was completely inhibited by wild-type LHDAg, consistent with the previous finding (39). Interestingly, mutant 211S, which is isoprenylation defective (8, 14) but is otherwise identical to wild-type LHDAg, allowed some HDV RNA replication, indicating that 211S has a lower *trans*-dominant inhibitory activity than wild-type LHDAg. Furthermore, mutant 192C, which is similar to SHDAg but contains an isoprenylation signal (14), had a *trans*-dominant suppression activity comparable to that of wild-type LHDAg.

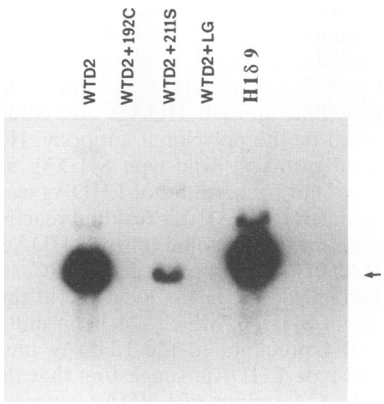

FIG. 2. *trans*-dominant suppressor activities of HDAg mutants. Cos 7 cells were cotransfected with replication-competent HDV dimer DNA (WTD2) (39) and equal amounts of different plasmids expressing either wild-type (LG) or a mutant form (192C or 211S) of HDAg. Total RNAs were extracted at 5 days posttransfection, analyzed on 1.1% agarose gels containing formaldehyde, transferred to nitrocellulose membrane, and hybridized with a ^{32}P-labeled HDV antigenome-sense probe. H1δ9 RNA (22) was used as a marker for HDV monomeric RNA. The arrow indicates monomer-length HDV RNA of 1.7 kb.

These results strongly suggest that the *trans*-dominant inhibitory activity of wild-type LHDAg and HDAg mutants is not caused by the primary inhibitory amino acid sequence but is due to conformational differences between SHDAg and LHDAg and that isoprenylation plays a major role in this conformational difference.

Effects of amino acid residues at the C terminus of LHDAg on protein conformation. Since prenylates are localized at the C terminus of the 19 amino acids unique to LHDAg, we examined whether the shortening of the amino acid spacer sequence could affect the influence of prenylates on HDAg protein conformation. We therefore constructed several LHDAg mutants with various degrees of deletion within the 19 amino acids. A pair of mutants was generated for each deletion, one with a functional isoprenylation motif (CRPQ) and the other with a mutated motif (SRPQ) (Fig. 3). Previous studies indicated that all mutants bearing a C-terminal CRPQ, regardless of the context of the upstream sequence, were prenylated, but those with SRPQ were not (14, 19). These mutants were transfected into Cos cells and precipitated with polyclonal or monoclonal antibodies. As shown in Fig. 4A, all constructs expressed similar levels of proteins, and all of them were precipitated by the polyclonal antibody against HDAg. The size differences among these mutant proteins are not evident in this gel; they are probably obscured by protein modifications and conformational differences. When these proteins were immunoprecipitated with MAb 9E4 (Fig. 4B), all of the LHDAg mutants with a C-terminal SRPQ were precipitated, regardless of the length of amino acid deletions. In contrast, 9E4 did not precipitate any CRPQ-containing LHDAg mutants except 206C. This result further confirmed the importance of isoprenylation in masking the SHDAg-specific conformational epitope near the C terminus of HDAg. Without isoprenylation, this epitope is exposed. It is not clear why MAb 9E4 was able to precipitate the mutant LHDAg 206C. It is possible that the remaining 10 amino acids present a steric hindrance and prevent the prenylates from coming into contact with the 9E4-specific epitope. As expected, neither 192C nor 192S was precipitated by MAb 9E4, because of the alteration of the primary sequence of SHDAg.

Effects of amino acid sequence deletion on the *trans*-dominant suppressor activity of LHDAg. We next examined whether C-terminal amino acid sequences of the LHDAg mutants and the state of isoprenylation are correlated with their abilities to inhibit HDV RNA replication. As demonstrated in Fig. 5, most of the mutant HDAgs which have an isoprenylation motif almost completely suppressed HDV RNA replication. The only exception was 196C, which had a slightly lower inhibitory activity. Even 192C, which contains an isoprenylation motif replacing the C-terminal amino acids of SHDAg, without additional amino acids, had a significant inhibitory function. In contrast, all of the nonprenylated mutants except 201S allowed significant levels of HDV RNA replication, indicating lower suppressor activities. Overall, all of the prenylated HDAg mutants suppressed HDV RNA replication to a much higher extent than the corresponding nonprenylated mutants. However, there is no obvious correlation between inhibitory activity and length of amino acid deletion. These results are consistent with the previous interpretation (39) that the extra 19-amino-acid sequence in LHDAg does not contain inhibitory information and further suggest that isoprenylation has a pivotal role in the inhibitory function. Since RNA expression levels in all transfected cells showed similar transfection efficiencies (data not shown), we conclude that isoprenylation enhances the *trans*-dominant inhibitory function of LHDAg. It is not clear how the isoprenylation-

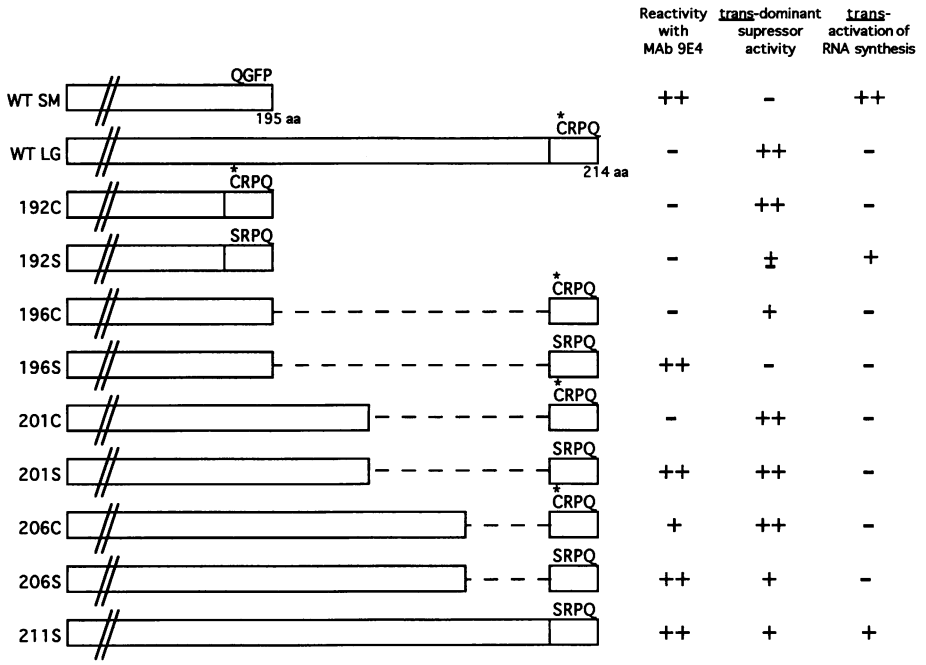

FIG. 3. Schematic diagram of the structure and summary of the biological properties of wild-type and C-terminal mutant HDAgs. 192C and 192S contain the authentic isoprenylation motif (CRPQ) and a defective motif (SRPQ), respectively, replacing the last four amino acids of SHDAg. The remaining mutants have successive deletions (indicated by dashed lines) of five amino acids, followed by either an intact or defective isoprenylation motif, of LHDAg. The cysteine residue with an isoprenylate group is indicated with an asterisk. The biological activities of the proteins are summarized from the data in Fig. 4 to 6. ++, strong activity; +, weak activity; −, no activity.

defective 201S mutant can inhibit HDV RNA replication completely. This mutant LHDAg likely has a unique conformation which results in the *trans*-dominant suppressor activity.

Some isoprenylation-defective mutant LHDAgs have *trans*-activating activities. SHDAg, but not LHDAg, has been demonstrated to be a *trans* activator of HDV RNA replication (17). Since the 9E4-specific epitope, a structural feature unique to SHDAg, is affected by the state of prenylation, we determined whether the removal of prenylation signals would allow LHDAg to serve as a *trans* activator. To address this possibility, Cos 7 cells were cotransfected with either wild-type or mutant HDAgs and Smd2, a replication-defective HDV dimer (20, 39) containing a frameshift mutation in the HDAg open reading frame, and the ability of any mutant HDAg to complement the defect of HDV RNA replication was examined. As shown in Fig. 6, Smd2 was unable to replicate by itself. However, in the presence of wild-type SHDAg, this mutant dimer could replicate. Interestingly, 192S, which is an SHDAg mutant containing a defective isoprenylation motif, still retained a partial *trans*-activating activity. In contrast, a comparable mutant, 192C, which is prenylated (14, 20), lost all *trans*-activating activity, suggesting that isoprenylation is a major factor affecting the *trans*-activating activity. Although most of the LHDAg mutants could not *trans* activate, a prenylation-defective mutant, 211S, which retained all of the 19 amino acids (except cysteine) unique to wild-type LHDAg, had the ability to complement the replication function partially. From these observations, we conclude that isoprenylation and other amino acid residues are responsible for altering the protein conformation of HDAg such that LHDAg and other HDAg mutants lost the *trans*-activating activity.

The biological properties of all the HDAg mutants are summarized in Fig. 3.

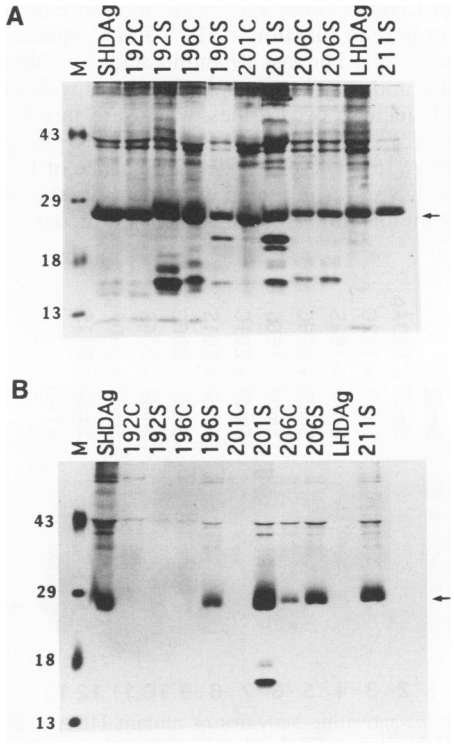

FIG. 4. Immunoprecipitation of deletion and prenylation mutants of HDAg. Cos 7 cells were labeled with Trans [^{35}S] at 48 h posttransfection and immunoprecipitated as described in the legend to Fig. 1. (A) Polyclonal antibody; (B) MAb 9E4. M, molecular mass marker in kilodaltons. HDAgs are indicated by arrows.

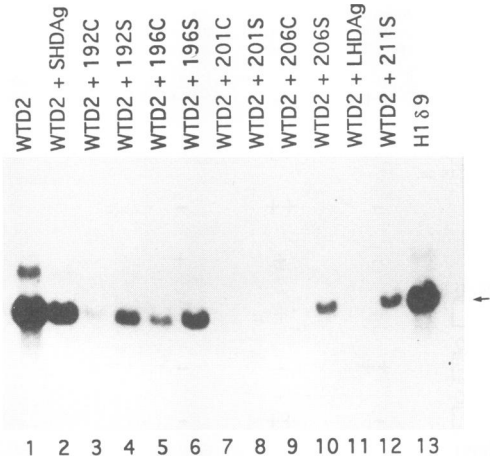

FIG. 5. *trans*-dominant suppression activities of deletion and prenylation mutants. Cos 7 cells were cotransfected with replication-competent wild-type HDV dimer (WTD2) (39) and either wild-type or mutant HDAg cDNA constructs. Total cellular RNA was analyzed by Northern blotting to detect genomic HDV RNA as described in the legend to Fig. 2. Lanes 2 to 12 represent cotransfection with wild-type HDV dimer and the indicated plasmids. The arrow at the right indicates monomer-length HDV RNA.

DISCUSSION

Both SHDAg and LHDAg share many structural characteristics and functional domains, including the coiled-coil sequence (39), nuclear localization signal (40), and RNA-binding motifs (20). However, the extra 19 amino acids at the C terminus of LHDAg cause a dramatic difference between these two HDAgs in their function in HDV RNA replication as well as HDV assembly. The last 19 amino acids contain an isoprenylation site and may be phosphorylated as well, since these amino acids include two serines, which are targets for phosphorylation (2), and LHDAg is more heavily phosphorylated than SHDAg (15). The biological significance of HDAg phosphorylation has so far not been examined. On the other hand, SHDAg contains an epitope which is recognized by an SHDAg-specific MAb (13), although the entire SHDAg sequence is contained within LHDAg. The isolation of this SHDAg-specific MAb suggests that SHDAg contains a unique conformation, which may account for the biological activity of SHDAg. However, the possibility that this MAb recognizes the primary amino acid sequence of the extreme C terminus of SHDAg, which is extended for 19 additional amino acids in LHDAg, had, prior to this study, not been ruled out. The data presented in this report provided unequivocal evidence that the 9E4 epitope indeed represents a unique SHDAg-specific conformational epitope, since the epitope can be detected in LHDAg if the prenylation signal is removed. The presence of an SHDAg-specific conformation may explain why LHDAg does not have *trans*-activating activity despite the fact that it contains all of the primary sequence of SHDAg.

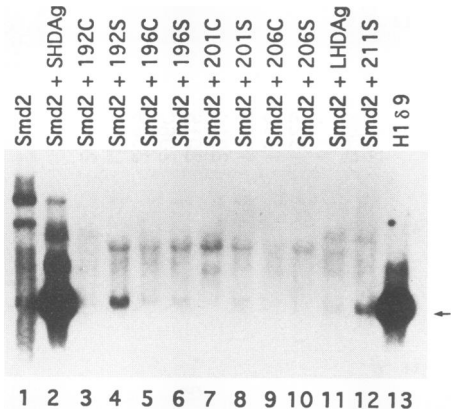

FIG. 6. *trans*-activating activities of mutant HDAgs. Replication-defective HDV dimer cDNA (Smd2) (20) and plasmids expressing either wild-type or mutant HDAgs were cotransfected into Cos 7 cells. Total cellular RNAs were subjected to Northern blot analysis to detect HDV genomic RNA. Lanes 2 to 12 are RNA samples from cells cotransfected with Smd2 and the indicated plasmids. Monomer-length HDV RNA is indicated by an arrow.

LHDAg has two unique biological activities. First, it can interact with HBsAg (14) and is involved in HDV virion assembly (1, 30, 34). This protein-protein interaction is mediated through the prenylate residues within the C-terminal 19 amino acids (14) and appears to be the basis of HDV virion assembly, since the prenylation-defective mutant LHDAgs cannot interact with HBsAg (14) and cannot be packaged (19). There are two possible mechanisms to explain the role of prenylation in HDAg-HBsAg interactions: the protein-protein interaction may be mediated directly through prenylates, or prenylation may alter HDAg conformation to allow other parts of HDAg to interact with HBsAg. These two possibilities are not mutually exclusive. Indeed, our previous studies indicate that both the prenylate and the 19 amino acids are required for HDAg-HBsAg interaction (14) and HDV virion packaging (19). Our current study further revealed that prenylates mask a unique conformational epitope of the C-terminal region of HDAg. The masking of this epitope can be mediated either directly by the prenylates or indirectly through protein conformational changes induced by prenylation. We cannot distinguish between these two possibilities. Nevertheless, we can conclude that prenylation not only increases protein hydrophobicity to target proteins to the membrane as previously thought (6, 26) but also changes overall conformation of the protein so as to facilitate its interaction with other proteins.

The second unique biological activity of LHDAg is its *trans*-dominant inhibiting activity on HDV RNA replication. Intuitively, the C-terminal 19 amino acids of LHDAg may be considered an inhibitory sequence. However, our previous results with use of mutant HDAgs which fail to dimerize suggest that the inhibitory activity is more likely due to the conformational differences between the LHDAg and SHDAg (39). Furthermore, the inhibitory activity of LHDAg has been shown to be quite variable and unpredictable with respect to the length and sequence of the C-terminal amino acids (9), making the possibility of inhibitory sequence less likely. Our results here clearly demonstrate that the prenylate plays a major role in the *trans*-dominant inhibitory activity of LHDAg. This conclusion was supported by the finding that the prenylated mutant in every pair of HDAg deletion mutants invariably had a much stronger inhibitory activity than its nonprenylated counterpart. Furthermore, an SHDAg mutant in which the last four amino acids were replaced with CRPQ while maintaining the same length as in wild-type SHDAg inhibited HDV RNA replication as efficiently as LHDAg, suggesting that isoprenylation alone can exert inhibitory activity. Nevertheless, some combinations of C-terminal amino acid sequences may also alter protein conformation such that some LHDAg mutants without an isoprenylation signal may confer partial inhibition.

SHDAg contains a unique activity, i.e., *trans* activation of

HDV RNA replication. In contrast, LHDAg does not have this activity, even though LHDAg contains the complete sequence of SHDAg. Our data suggest that part of the reason may be the conformational differences conferred or induced by prenylates. The surprising finding that at least one LHDAg mutant, which is not isoprenylated, contains a partial *trans*-activating activity supports this possibility. Since LHDAg and SHDAg share almost all of the functional domains, and all of the MAbs (except 9E4) recognize both HDAgs (13), it stands to reason that the region unique to SHDAg, i.e., the region containing the 9E4 epitope, is critical for the *trans*-activating activity of SHDAg. Our study indicates that the conformation of this domain is affected by the presence of nearby isoprenylates. How the C terminus of SHDAg participates in HDV RNA replication is currently unknown. Potentially, this domain may interact with the essential transcription factors. If this domain is masked or altered by prenylates, it will not be able to interact with other proteins. Indeed, recent studies in our laboratory have shown that HDAg interacts with some cellular transcription factors (4). The precise mechanism of *trans* activation by SHDAg remains to be studied.

ACKNOWLEDGMENTS

We thank Daphne Shimoda for the preparation of figures and editorial assistance.

This work was partially supported by grant AI 26741 from the National Institutes of Health. M. M. C. Lai is an Investigator of the Howard Hughes Medical Institute.

REFERENCES

1. **Chang, F.-L., P.-J. Chen, S.-J. Tu, C.-J. Wang, and D.-S. Chen.** 1991. The large form of hepatitis δ antigen is crucial for assembly of hepatitis δ virus. Proc. Natl. Acad. Sci. USA **88**:8490–8494.
2. **Chang, M.-F., S. C. Baker, L. H. Soe, T. Kamahora, J. G. Keck, S. Makino, S. Govindarajan, and M. M. C. Lai.** 1988. Human hepatitis delta antigen is a nuclear phosphoprotein with RNA-binding activity. J. Virol. **62**:2403–2410.
3. **Chao, M., S.-Y. Hsieh, and J. Taylor.** 1990. Role of two forms of hepatitis delta virus antigen: evidence for a mechanism of self-limiting genome replication. J. Virol. **64**:5066–5069.
4. **Chao, M., and M. M. C. Lai.** Unpublished observation.
5. **Chomczynski, P., and N. Sacchi.** 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal. Biochem. **162**:156–159.
6. **Clarke, S.** 1992. Protein isoprenylation and methylation at carboxyl-terminal cysteine residues. Annu. Rev. Biochem. **61**:355–386.
7. **Ellis, L., E. Clauser, D. O. Morgan, M. Edery, R. A. Roth, and W. J. Rutter.** 1986. Replacement of insulin receptor tyrosine residues 1162 and 1163 compromises insulin-stimulated kinase activity and uptake of 2-deoxyglucose. Cell **45**:721–732.
8. **Glenn, J. S., J. A. Watson, C. M. Havel, and J. M. White.** 1992. Identification of a prenylation site in the delta virus large antigen. Science **256**:1331–1333.
9. **Glenn, J. S., and J. M. White.** 1991. *trans*-dominant inhibition of human hepatitis delta virus genome replication. J. Virol. **65**:2357–2361.
10. **Gluzman, Y.** 1981. SV40-transformed simian cells support the replication of early SV40 mutants. Cell **23**:175–182.
11. **Hancock, J. F., H. Paterson, and C. J. Marshall.** 1990. A polybasic domain or palmitoylation is required in addition to the CAAX motif to localize $p21^{ras}$ to the plasma membrane. Cell **63**:133–139.
12. **Holtz, D., R. A. Tanaka, J. Hartwig, and F. McKeon.** 1989. The CaaX motif of lamin A functions in conjunction with the nuclear localization signal to target assembly to the nuclear envelope. Cell **59**:969–977.
13. **Hwang, S. B., and M. M. C. Lai.** 1993. A unique conformation at the carboxyl terminus of the small hepatitis delta antigen revealed by a specific monoclonal antibody. Virology **193**:924–931.
14. **Hwang, S. B., and M. M. C. Lai.** 1993. Isoprenylation mediates direct protein-protein interactions between hepatitis large delta antigen and hepatitis B virus surface antigen. J. Virol. **67**:7659–7662.
15. **Hwang, S. B., C.-Z. Lee, and M. M. C. Lai.** 1992. Hepatitis delta antigen expressed by recombinant baculoviruses: comparison of biochemical properties and post-translational modifications between the large and small forms. Virology **190**:413–422.
16. **Kos, A., R. Dijkema, A. C. Arnberg, P. H. van der Merde, and H. Schelekens.** 1986. The HDV possesses a circular RNA. Nature (London) **323**:558–560.
17. **Kuo, M. Y.-P., M. Chao, and J. Taylor.** 1989. Initiation of replication of the human hepatitis delta virus genome from cloned DNA: role of delta antigen. J. Virol. **63**:1945–1950.
18. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
19. **Lee, C.-Z., P.-J. Chen, M. M. C. Lai, and D. S. Chen.** 1994. Isoprenylation of large hepatitis delta antigen is necessary but not sufficient for hepatitis delta virus assembly. Virology **199**:169–175.
20. **Lee, C.-Z., J.-H. Lin, M. Chao, K. McKnight, and M. M. C. Lai.** 1993. RNA-binding activity of hepatitis delta antigen involves two arginine-rich motifs and is required for hepatitis delta virus RNA replication. J. Virol. **67**:2221–2227.
21. **Luo, G., M. Chao, S.-Y. Hsieh, C. Sureau, K. Nishikura, and J. Taylor.** 1990. A specific base transition occurs on replicating hepatitis delta virus RNA. J. Virol. **64**:1021–1027.
22. **Macnaughton, T. B., E. J. Gowans, A. R. Jilbert, and C. J. Burrell.** 1990. Hepatitis delta virus RNA, protein synthesis and associated cytotoxicity in a stably transfected cell line. Virology **177**:692–698.
23. **Macnaughton, T. B., E. J. Gowans, S. P. NcNamara, and C. J. Burrell.** 1991. Hepatitis δ antigen is necessary for access of hepatitis δ RNA to the cell transcriptional machinery but is not part of the transcriptional complex. Virology **184**:387–390.
24. **Macnaughton, T. B., Y.-J. Wang, and M. M. C. Lai.** 1993. Replication of hepatitis delta virus RNA: effect of mutations of the autocatalytic cleavage sites. J. Virol. **67**:2228–2234.
25. **Makino, S., M.-F. Chang, C. K. Shieh, T. Kamahora, D. M. Vannier, S. Govindarajan, and M. M. C. Lai.** 1987. Molecular cloning and sequencing of a human hepatitis delta (δ) virus RNA. Nature (London) **329**:343–346.
26. **Marshall, C. J.** 1993. Protein prenylation: a mediator of protein-protein interactions. Science **259**:1865–1866.
27. **Mizuno, T., K. Kaibuchi, T. Yamamoto, M. Kawamura, T. Sakoda, H. Fujioka, Y. Matsuura, and Y. Takai.** 1991. A stimulatory GDP/GTP exchange protein for smg p21 is active on the post-translationally processed form of c-Ki-ras p21 and rhoA p21. Proc. Natl. Acad. Sci. USA **88**:6442–6446.
28. **Ong, O. C., I. M. Ota, S. Clarke, and B. K.-K. Fung.** 1989. The membrane binding domain of rod cGMP phosphodiesterase is posttranslationally modified by methyl esterification at a C-terminal cysteine. Proc. Natl. Acad. Sci. USA **86**:9238–9242.
29. **Philips, M. R., M. H. Pillinger, R. Staud, C. Volker, M. G. Rosenfeld, G. Weissmann, and J. B. Stock.** 1993. Carboxyl methylation of Ras-related proteins during signal transduction in neutrophils. Science **259**:977–980.
30. **Ryu, W.-S., M. Bayer, and J. Taylor.** 1992. Assembly of hepatitis delta virus particles. J. Virol. **66**:2310–2315.
31. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74**:5463–5467.
32. **Sharmeen, L., M. Y.-P. Kuo, G. Dinter-Gottlieb, and J. Taylor.** 1988. Antigenomic RNA of human hepatitis delta virus can undergo self-cleavage. J. Virol. **62**:2674–2679.
33. **Sharmeen, L., M. Y.-P. Kuo, and J. Taylor.** 1989. Self-ligating RNA sequences on the antigenome of human hepatitis delta virus. J. Virol. **63**:1428–1430.
34. **Sureau, C., B. Guerra, and R. E. Lanford.** 1993. Role of the large hepatitis B virus envelope protein in infectivity of the hepatitis delta virion. J. Virol. **67**:366–372.
35. **Wang, K.-S., Q.-L. Choo, A. J. Weiner, J.-H. Ou, R. C. Najarian, R. M. Thayer, G. T. Mullenbach, K. J. Denniston, J. L. Gerin, and M. Houghton.** 1986. Structure, sequence and expression of the hepatitis delta (δ) viral genome. Nature (London) **323**:508–514.

36. **Weiner, A. J., Q.-L. Choo, K.-S. Wang, S. Govindarajan, A. G. Redeker, J. L. Gerin, and M. Houghton.** 1988. A single antigenomic open reading frame of the hepatitis delta virus encodes the epitope(s) of both hepatitis delta antigen polypeptides $p24^{\delta}$ and $p27^{\delta}$. J. Virol. **62:**594–599.
37. **Wu, H.-N., and M. M. C. Lai.** 1989. Reversible cleavage and ligation of hepatitis delta virus RNA. Science **243:**652–654.
38. **Wu, H.-N., Y.-J. Lin, F.-P. Lin, S. Makino, M.-F. Chang, and M. M. C. Lai.** 1989. Human hepatitis δ virus RNA subfragments contain an autocleavage activity. Proc. Natl. Acad. Sci. USA **86:**1831–1835.
39. **Xia, Y.-P., and M. M. C. Lai.** 1992. Oligomerization of hepatitis delta antigen is required for both the *trans*-activating and *trans*-dominant inhibitory activities of the delta antigen. J. Virol. **66:**6641–6648.
40. **Xia, Y.-P., C.-T. Yeh, J.-H. Ou, and M. M. C. Lai.** 1992. Characterization of nuclear targeting signal of hepatitis delta antigen: nuclear transport as a protein complex. J. Virol. **66:**914–921.