The hepatitis delta virus large antigen (lHDAg) is a virally encoded protein that contains a prenylation signal sequence at its carboxyl terminus consisting of the tetrapeptide Cys-Arg-Pro-Gln. Although the presence of the Gln as the COOH-terminal residue generally specifies addition of the 15-carbon farnesyl isoprenoid, earlier reports had suggested that the protein is modified by the 20-carbon geranylgeranyl. The prenylation of IHDAG was examined in vitro using a fusion protein between glutathione S-transferase and the COOH-terminal 117 amino acids of IHDAG (GST-IHDAG). When recombinant GST-IHDAG was incubated with bovine brain cytosol in the presence of either farnesyl diphosphate or geranylgeranyl diphosphate, GST-IHDAG was preferentially farnesylated. Geranylgeranylation of the fusion protein was also observed, although at a rate considerably less than that of farnesylation. Using purified recombinant protein prenyltransferases, GST-IHDAG was found to be an excellent substrate (apparent Km = 0.8 μM) for protein farnesyltransferase (FTase), while modification by protein geranylgeranyltransferase I (GGTase I) was not detected. FTase was also able to catalyze geranylgeranylation of GST-IHDAG at a very low rate, suggesting that the low level of geranylgeranylation of GST-IHDAG observed in cytosolic preparations was mediated by FTase. Consistent with our observations on the in vitro prenylation of the GST-IHDAG fusion protein, isoprenoid analysis of authentic IHDAG expressed in COS cells demonstrated that the protein was farnesylated. Geranylgeranylation of IHDAG expressed in COS cells was not observed. As prenylation of IHDAG is required for the assembly of the hepatitis delta viral particle, these results suggest that inhibitors of FTase may be useful therapeutic agents for treatment of delta virus infection.

The hepatitis delta virus (HDV) is a satellite virus of hepatitis B virus that can cause an increase in the incidence and severity of liver disease in individuals infected with both viruses (1, 2). HDV consists of the HDV RNA genome and two HDV-encoded proteins, designated as the small (sHDAg) and large (lHDAg) HDV antigens, encapsulated in an envelope composed of hepatitis B surface antigens (2). sHDAg and lHDAg contain identical deduced amino acid sequences for their first 195 amino acids, with lHDAg containing an additional 19-amino acid COOH-terminal tail (2). Despite their sequence identity, sHDAg and IHDAG have very different functions. Whereas sHDAg is essential for HDV replication (3), IHDAG is a dominant inhibitor of HDV replication (4) and is required for assembly of the HDV particle (5, 6). As IHDAG can package into pseudo-viral particles with hepatitis B surface antigens in the absence of both HDV RNA and sHDAg (6, 7), the current model of HDV assembly indicates that IHDAG functions by directly interacting with hepatitis B surface antigens that form the envelope of the viral particle (2).

Interestingly, IHDAG is modified by an isoprenoid lipid on a cysteine located near its COOH terminus (8), and this modification is necessary for IHDAG to facilitate HDV assembly (8, 9). The prenylation motif contained in IHDAG is the COOH-terminal tetrapeptide Cys-Arg-Pro-Gln (CRPQ) (8). This sequence is similar to the conventional prenylation motif which consists of the COOH-terminal tetrapeptide CAAX, where the cysteine residue is the prenylation site, “A” are generally aliphatic residues, and X can be one of several amino acids. Two distinct protein prenyltransferases modify proteins which contain a CAAX motif, farnesyltransferase (FTase), which modifies proteins with the 15-carbon farnesyl isoprenoid, and geranylgeranyltransferase I (GGTase I), which modifies proteins with the 20-carbon geranylgeranyl group (10, 11). The COOH-terminal amino acid (i.e., X) in general determines which of the two isoprenoid lipids modify a CAAX motif. If X is Met, Ser, or Gln, the sequence is a substrate for FTase (12), while Leu at this position directs modification by GGTase I (12–14). A third protein prenyltransferase, GGTase II, recognizes a different class of COOH-terminal motifs present in GTP-binding proteins of the Rab family (15). Following the prenylation of the CAAX motif, two additional processing events occur: proteolytic cleavage of the -AAX tripeptide and carboxymethylation of the new COOH-terminal prenylcysteine (10, 11).

As prenylation of IHDAG is required for HDV assembly, inhibition of the enzyme responsible for its prenylation is a potential treatment of HDV infection. Due to the presence of Gln at its COOH terminus, IHDAG would be predicted to be a FTase substrate (11, 12). However, two reports have appeared indicating that IHDAG is modified with the geranylgeranyl isoprenoid (8, 9). Thus, it is not clear which protein prenyltransferase is responsible for the prenylation of IHDAG. In this report, we have examined both the nature of the isoprenoid group attached to IHDAG and the identity of the enzyme responsible for its prenylation. Our results indicate that the predominant modification of IHDAG is in fact farnesylation, and the protein is exclusively a substrate for FTase.

Hepatitis delta virus large antigen fusion protein; MEV, mevalonate transport protein; PBS, phosphate-buffered saline; DMEM, Dulbecco’s modified Eagle’s medium; DTT, dithiothreitol.
EXPERIMENTAL PROCEDURES

Materials—Farnesyl diphosphate (FPP), geranylgeranyl diphosphate (GGPP), [3H]PP (15 Ci/mmmol), [3H]GGPP (15 Ci/mmmol), and [5-3H]mevalonolactone (35 Ci/mmmol) were obtained from American Radiolabeled Chemicals, Inc. (St. Louis, MO). Glutathione-Phenosephore 4B and protein A-Sepharose 4B were obtained from Pharmacia Biotech Inc. Lovastatin was a generous gift of Al Alberts (Merck Sharp and Dohme Research Laboratories, West Point, PA). JSV-LDAg, a mammalian cell expression vector containing the cDNA for the G protein (16), was obtained from American Type Culture Collection (Rockville, MD). Rabbit polyclonal antisera directed against a peptide in IHDAG (17) was a gift from Stanley Lemon (University of North Carolina, Chapel Hill, NC).

Protein Prenylation transfers and Protein Substrates—Bovine brain cytosol was prepared by homogenizing bovine brain in 50 mM HEPES, pH 7.4, containing a protease inhibitor mix, and subjected to homogenate centrifugation at 100,000 × g for 1 h. Recombinant human Ha-Ras and Ha-Ras-CVLL were expressed in Escherichia coli and purified as described (13). Recombinant rat FTase and rat GGTaseI were expressed in Sf9 cells and purified as described (18–20).

Expression and Purification of GST-IHDAD—The GST-IHDAD fusion protein was expressed in E. coli and purified essentially as described by Lee and Oh (5). A 500-mg flask of LB medium was inoculated with 1 ml of an overnight culture of M109 cells that had been transformed with the vector pGEX-ZT-IHDAD. After incubation for 4 h at 37 °C, isopropyl-1-thio-β-D-galactopyranoside was added to 1 mM, and the cells were grown for an additional 4 h. Cells were harvested by centrifugation at 3000 × g for 15 min, resuspended in lysis buffer (PBS containing 10 mM EDTA, 10 mM DTT, 1% Triton X-100, 10% streptomycin sulfate and protease inhibitor mix), and lysed by three passes through a French press. Cell debris was removed by centrifugation at 45,000 × g for 1 h, and the supernatant was incubated with glutathione-Sepharose 4B for 1 h at room temperature. The mixture was transferred to a column, and the column was washed with 20 volumes of PBS containing 50 mM NaCl, 10 mM EDTA, 10 mM DTT, 1% Triton X-100, 0.1% deoxycholate, 5 mM glutathione, and concentrated to 40 mg/ml using an Amicon Centricon-30.

In Vitro Prenylation Reactions—GST-IHDAD (20 µg), Ha-Ras (2 µg), or Ha-Ras-CVV (2 µg) were utilized in prenylation reactions that contained 50 mM Tris-HCl, pH 7.7, 5 mM MgCl₂, 5 mM ZnCl₂, 2 mM DTT, and 100 µM [3H]PP (8 Ci/mmol) or [3H]GGPP (8 Ci/mmol) (19, 20). The reactions were initiated by the addition of either bovine brain cytosol (500 µg protein) or purified recombinant FTase (75 ng) or GGTaseI (75 ng), incubated for 1 h at 37 °C, and stopped by addition of SDS-PAGE sample buffer and heating at 65 °C for 5 min. Samples were resolved on 12% SDS-PAGE gels. Proteins were visualized by Coomasie Blue staining, and the gels were prepared for fluorography by rinsing in water and soaking for 45 min in 1% salicylic acid. Prenylated proteins were visualized by exposing the gels to Fuji RX film.

For kinetic determinations, the concentrations of prenylation competent GST-IHDAD, Ha-Ras, and Ha-Ras-CVV were determined by driving the prenylation reactions to completion in the presence of excess FTase or GGTase I and the respective prenylphosphatase, and determining the concentration of protein prenylated as described below. Final concentrations of substrates ranging from 0.1 to 20 µM were utilized in kinetic assays. Assay conditions were identical to those described above, with the exception that the specific activities of [3H]PP and [3H]GGPP were 3 Ci/mmol. Reactions were initiated by the addition of 75 ng of FTase or GGTaseI and incubated for 15 min at 37 °C. The reactions were stopped by addition of 4% SDS, proteins precipitated by trichloroacetic acid, and prenylated protein was recovered from the cells and the cells were washed with PBS and harvested by scraping. Cells were pelleted by centrifugation, resuspended in 50 mM Hepes, pH 7.4, containing 1 mM EDTA, 1 mM DTT, and protease inhibitor mix, flash frozen in liquid nitrogen, thawed, and lysed by passing several times through a 27-gauge needle.

Immunoprecipitation of IHDAD from COS Cell Extracts—Cellular proteins were solubilized from the lysed cell extracts by addition of Triton X-100 and NaCl to final concentrations of 1% and 250 mM, respectively. Samples were incubated on ice for 30 min, passed through a 27-gauge syringe several times, and insoluble material precipitated by centrifugation at 100,000 × g for 1 h at 4 °C. SDS was added to the solubilized protein to a final concentration of 0.5% and the sample was heated at 65 °C for 5 min to denature proteins. The sample was then adjusted to 0.25% SDS, 125 mM NaCl, 1% Triton X-100, and 1% sodium cholate. IHDAD was immunoprecipitated by incubation of the final extract obtained with rabbit anti-IHDAD (17) for 15 min, followed by incubation with protein A-Sepharose 4B for 1 h. The protein A-Sepharose 4B beads were pelleted by centrifugation and were washed extensively with 0.1 mM Tris, pH 7.7, containing 0.1% Triton X-100. Immunoprecipitated proteins were eluted from the beads either by addition of SDS-PAGE sample buffer (for SDS-PAGE) or by two washes with 0.2 mM acetic acid (for isoprenoid analysis). Immunoprecipitation of IHDAD was monitored by fluorography of SDS-PAGE gels.

Isoprenoid Analysis—Isoprenoid analysis was conducted essentially as described by Farnsworth et al. (23). Solubilized COS cell proteins or immunoprecipitated IHDAD were precipitated in 15% trichloroacetic acid and the resulting protein pellets washed extensively in acetone at −20 °C. Precipitated proteins were subjected to trypsin digestion, and isoprenoids cleaved from the tryptic peptides by treatment with methyl iodide and 20% formic acid. Following deacylation, methyl iodide was removed under mild vacuum and the solution neutralized by addition of sodium carbonate. Isoprenoids were extracted into a solution of chloroform:methanol (9:1), and the extracted isoprenoids dried under nitrogen and resuspended in 50% acetonitrile containing 0.1% phosphoric acid. Farnesol and geranylgeraniol (15 nmol of each) were added to the samples as standards, and isoprenoids resolved by HPLC using a 50–100% gradient of acetonitrile in 0.1% phosphoric acid. Fractions containing H-labeled isoprenoids were identified by liquid scintillation spectroscopy.

RESULTS AND DISCUSSION

In Vitro Prenylation of GST-IHDAD in Bovine Brain Cytosol—It has been reported that IHDAD is modified by the geranylgeranyl isoprenoid (8, 9). In order to identify the prenyltransferase responsible for the modification of IHDAD, we first examined whether a fusion protein between glutathione S-transferase and the COOH-terminal 117 amino acids of IHDAD (GST-IHDAD) (9) was a substrate for these enzymes present in bovine brain cytosol. Surprisingly, our initial experiments indicated that GST-IHDAD was exclusively farnesylated, with no evident modification with the geranylgeranyl isoprenoid (Fig. 1, panel A, lanes 4 and 8). Prolonged exposure of the gel did, however, reveal that a low level of geranylgeranylation of GST-IHDAD did occur (Fig. 1, panel B, lane 8). The level of geranylgeranylation of GST-IHDAD was approximately 1% of the level of farnesylation. Two recombinant Ras proteins, Ha-Ras (containing the COOH terminus-CVLS, and thus a substrate for FTase) and Ha-Ras-CVV (containing the COOH terminus-CVLS, and thus a substrate for GGTase I) were used as control substrates for the two enzymes and clearly demonstrated the presence of each enzyme in bovine brain cytosol (Fig. 1, panel A). These results suggested that IHDAD is a substrate for FTase.

In Vitro Prenylation of GST-IHDAD Using Purified Recombinant Protein Prenyltransferases—Further to assess the ability of GST-IHDAD to serve as a substrate for FTase, we examined
was unable to modify GST-lHDAg using either FPP or GGPP as viral antigen (Fig. 4, readily immunoprecipitated from solubilized COS cell extracts lHDAg, could be faintly observed. This 27-kDa protein was additional labeled protein of 27 kDa, the predicted size of apparent protein was found to be an excellent substrate for FTase with an confirmed by a more detailed kinetic analysis of the interaction level of farnesylation (Fig. 2, level of geranylgeranylation, again approximately 1% of the GST-lHDAg. FTase efficiently transferred the farnesyl group from FPP to GST-lHDAg, and the enzyme also supported a low abilities of recombinant FTase and GGTase I to prenylate proteins in the COS cells, the cells were co-transfected with a mevalonate transport protein (MEV) and also treated with lovastatin. Expression of MEV second plasmid coding for a mevalonate transport protein (24). In order to increase the labeling of prenylated proteins (26). Isoprenoid analysis of the immunoprecipitated lHDAg, however, did not utilize GST-lHDAg as a substrate over the range concentrations tested (Fig. 3, panel B).

Isoprenoid Analysis of lHDAg Expressed in Animal Cells—Our in vitro studies indicated that lHDAg should be farnesylated in cells. In order to examine this directly, isoprenoid analysis was performed on lHDAg expressed in COS cells. COS cells transfected with a vector expressing lHDAg were subjected to the same procedure (Fig. 4, compare identical to that observed when COS cells not expressing lHDAg were subjected to the same procedure (Fig. 4, compare lanes 4 and 8, GST-lHDAg. Data shown are from a single experiment, which is representative of several such experiments.}

COS cells, and on immunoprecipitated lHDAg. For the total pool of prenylated proteins, approximately 20% of the protein-associated isoprenoid was farnesyl and 80% was geranylgeranyl (Fig. 4, panel B), a ratio consistent with previous studies (26). Isoprenoid analysis of the immunoprecipitated lHDAg, however, revealed that the protein was exclusively modified by the farnesyl isoprenoid (Fig. 4, panel C); the amount of geranylgeranyl lipid in immunoprecipitated lHDAg was essentially identical to that observed when COS cells not expressing lHDAg were subjected to the same procedure (Fig. 4, compare panels C and D). These results are completely consistent with the in vitro data that indicated that lHDAg was a FTase substrate, and we conclude that lHDAg is farnesylated in cells by
cancers that contain oncogenic Ras proteins (32–34). Recent reports have indicated that many of these inhibitors are effective against some tumors containing activated Ras proteins and are relatively non-toxic in animals (35, 36). Taken together, these findings indicate that FTase inhibitors may be an effective route to block the pathological consequences of HDV infections.

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FTase. We also predict that the geranylgeranylation of IHDAG observed by others in vitro (9) was mediated by FTase.

Mutational analysis of FTase has revealed that while the prenylation motif at the COOH terminus of the protein is not necessary for FTase to bind sHDAg or the RNA genome, it is essential for IHDAG to interact with hepatitis B surface antigen in vitro (27). Mutation or deletion of the prenylation motif also results in IHDAG that is unable to form pseudo-viral particles with hepatitis B surface antigens in transfected cells (8, 9, 28), providing strong evidence that prenylation of IHDAG is required for HDV particle formation. Although prenylation of IHDAG is necessary for interaction of the protein with hepatitis B surface antigen and for the formation of pseudo-viral particles, it is not sufficient in this regard in that a 15-amino acid cassette immediately upstream of the CAAX motif is also required (9, 27–29). This finding parallels those from studies of Ras proteins, in which it was determined that the proteins require either a polybasic domain or palmitoylated cysteine residues immediately upstream of a farnesylation motif for efficient association with membranes (30, 31). Thus, both in IHDAG and Ras proteins, a second “signal” is required for a function that is also dependent upon prenylation.

The requirement of the prenylation motif for HDV particle assembly, coupled with our finding that IHDAG is exclusively a substrate for FTase, indicates that inhibitors of FTase should prevent viral particle formation. A number of laboratories have developed potent FTase inhibitors for use in the treatment of HDV infections.
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