VIRAL HEPATITIS REVIEWS

1999, Vol. 5, No. 1, 13–26

Shutting the Door on Hepatitis Delta Virus (HDV): Sensitivity to Prenylation Inhibition Prompts New Therapeutic Strategy

JEFFREY S. GLENN

Division of Gastroenterology, Stanford University School of Medicine, and Veterans Administration Medical Center, Palo Alto, California 94305–5487, USA

(Received 29 July 1998 and accepted 14 October 1998)

Key Words: Hepatitis delta virus; prenylation; viral assembly; antiviral agents

Hepatitis delta virus (HDV) is an important cause of acute and chronic liver disease. To date, no medical therapy is effective at eradicating HDV infections. In recent years, much has been learnt about the molecular biology of the HDV life cycle which hopefully can be translated into new treatment strategies. For example, studies have begun to provide a molecular description of the virus assembly process. A key step in this virus particle formation involves the post-translational lipid modification of a specific HDV protein, namely prenylation of large delta antigen. Disruption of prenylation abolishes particle formation. Pharmacologic agents capable of inhibiting prenylation are being developed. Early results with such drugs show promise for their effective use as a new class of antiviral agents, with HDV as a first target. Here, the HDV life cycle is reviewed with an emphasis on the role of prenylation and implications for therapy.

Introduction

Hepatitis delta virus (HDV) causes acute and chronic liver disease with variable incidence throughout the world (1). Because HDV occurs exclusively in the setting of a concurrent hepatitis B virus (HBV) infection, the major reservoir of HDV resides within the over 300 million world-wide chronic carriers of HBV. It has been estimated that at least 15 million of the latter have been infected with HDV (2). Regions of highest prevalence include the Mediterranean and Amazon basins, the Middle East, Central and West Africa, and Central Asia. Surprisingly, to date, it is less common in East Asia which harbours large regions of HBV endemicity. In the United States, where the incidence is low, approximately 3% of an annual 250,000 acute HBV infections involved HDV, and the overall prevalence of chronic HDV carriers has been estimated at 70,000 (3).

Since its discovery approximately two decades ago (4), HDV has revealed itself to be an agent harbouring a rich array of fascinating biology (5, 6). A variety of models have been developed to study HDV. Animal models include the chimpanzee (7) and woodchuck (8), where experimental transmission recapitulates many aspects of human disease, and to a lesser extent the mouse which is capable of supporting only a single round of replication (9). Primary cultures of hepatocytes can be infected with HDV, although these systems also appear limited to a single cycle of infection (10, 11, 12). The availability of a cloned genome (13, 14, 15) has permitted the successful initiation of HDV infection upon direct inoculation of cloned HDV cDNA into the liver of a chimpanzee (16) or woodchuck (17, 18) already harboring HBV. Moreover,

Correspondence should be addressed to: jeffrey.glenn@stanford.edu.

1081–7050/99/010013+14 $12.00/0

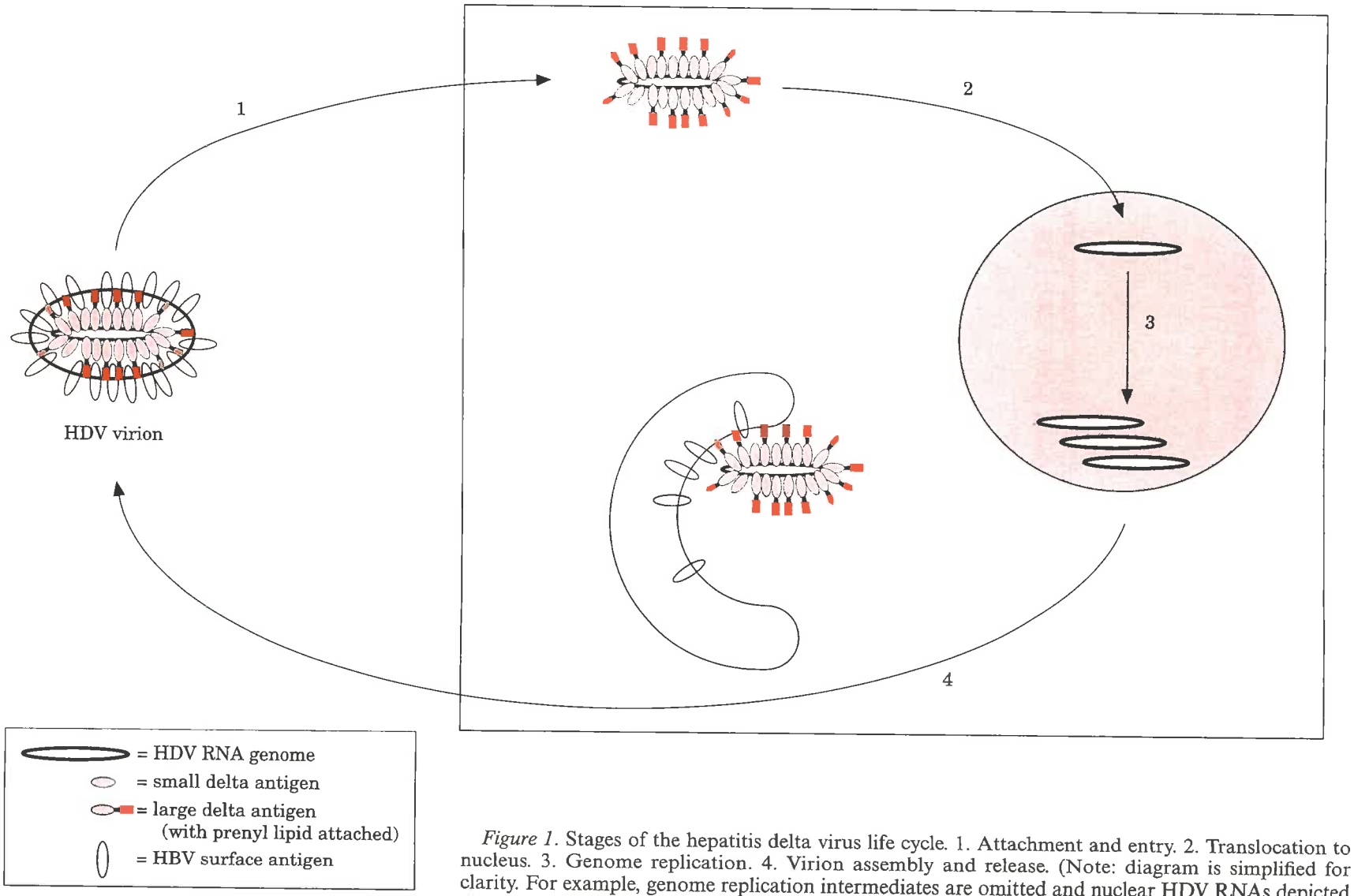

Figure 1. Stages of the hepatitis delta virus life cycle. 1. Attachment and entry. 2. Translocation to nucleus. 3. Genome replication. 4. Virion assembly and release. (Note: diagram is simplified for clarity. For example, genome replication intermediates are omitted and nuclear HDV RNAs depicted without associated protein. No specific virion assembly site is intended. See text for further details.)

transfection of in-vitro produced HDV RNA or cDNA into cultured cell lines can both initiate genome replication (19, 20, 21) and, if co-transfected with HBV DNA, produce infectious particles (22). Both the direct injection into liver and in-vitro transfection approaches permit studies with genetically defined inoculums and can take advantage of the power of defined in-vitro mutagenesis to answer detailed questions about the HDV life cycle. Finally, a variety of in-vitro systems have been established to study specific reactions crucial to the HDV life cycle such as auto catalytic RNA cleavage (23, 24, 25), RNA editing (26, 27), and RNA-dependent RNA replication (28, 29). Although each of the above experimental models has its own limitations, collectively, they have enabled us to better understand most stages of the HDV life cycle. Many important details and underlying mechanisms, however, remain to be explored. As is the case for the best questions in science, the resulting answers have often raised intriguing new questions of their own. After a brief survey of some of the salient features of the HDV life cycle, this Short Review will focus on the final events in the formation of HDV viral particles. In particular, the role of prenylation, a type of site-specific lipid modification of proteins, will be discussed—both as a key element of the viral assembly process, and as a novel target for specific antiviral therapy.

The HDV life cycle

The HDV particle is composed of a single-stranded circular 1.7 kb RNA genome, small and large delta antigen, and a lipid envelope containing hepatitis B virus (HBV) surface antigens (HBsAgs) (30, 13) (see Figure 1). The fully assembled particle diameter is about 36 nm (31). The HDV genome has several features reminiscent of viroids (32) including a high degree of potential intra-molecular base-pairing such that the circular genome is thought to assume a configuration where it is largely collapsed on itself, forming a linear unbranched rod-like structure (15). Although there are several conserved open reading frames (ORFs) in the HDV genome, only that encoding delta antigen is known to be expressed in all infections, and this ORF encompasses nearly the entire length of one side of the collapsed rod structure.

Reflecting the fact that HDV requires HBsAgs for envelopment, natural infections of HDV always occur in the presence of a co-existing HBV infection. HDV contains the same three surface antigen proteins—S, M and L—as HBV (33), although in different relative proportions. These envelope proteins appear to be the only essential factors provided by HBV. Indeed, once inside a target cell, HDV needs no HBV gene products until envelopes for new progeny delta virions are required.

At least four stages of the HDV life cycle can be defined (see 1 through 4, Figure 1). Even more so than for HBV (34), little is known about the details of virus attachment to, and mechanism of entry into, its target cell, the hepatocyte. Experiments with primary hepatocytes show that L surface antigen is required for infectivity (22). Interestingly, the M surface antigen appears to be dispensable in that system (35). How and where the incoming virion actually gains entry into the cell's interior is not yet known.

The second stage, translocation to the nucleus, has long been inferred by the observations that most viral nucleic acids and encoded proteins are found in the nucleus in liver biopsy specimens from infected patients and animals (4, 36, 37). Recently, elegant experimental systems have been developed (38, 39) which not only reproduce this step in the viral life cycle, but also provide an explanation for why experiments employing exogenously added viral RNA have needed the prior or simultaneous presence of delta antigen in order to efficiently initiate genome replication (20, 40, 41). It appears that the incoming viral genome gains access to the nucleus as a complex with delta antigen, forming a ribonucleoprotein particle (RNP). The nuclear localizing signal (NLS) domain contained in delta antigen (42, 43, 6, 44) has been shown to be exposed on the surface of HDV RNPs (45). The NLS would thus be available for interaction with nuclear import pathway

proteins (46). Indeed, direct binding of delta antigen with host cell karyopherin has been described (38), suggesting this nuclear import protein helps mediate shuttling of the HDV RNP through the nuclear pore complex, as described for an increasing number of other viruses (47).

The third stage, genome replication occurs in the nucleus (10, 36, 48). Based on analyses of the detected intermediates, it appears that HDV replicates via a rolling circle mechanism, as proposed for plant viroids (32). An incoming genome of negative or 'genomic' polarity serves as template for the production of multimers of opposite or 'antigenomic' polarity. The latter self-process via a site-specific ribozyme activity (23, 25) to linear monomers which each circularize to generate a template for production of linear genomic multimers. Another ribozyme activity and intramolecular ligation event are needed to generate the first new progeny genomes (49).

There are no DNA intermediates to this replication scheme. Rather, only RNA to RNA transcription is involved—an enzymatic activity not classically thought to reside in the hepatocyte. Since HDV encodes no known polymerase, however, such a host cell activity is implied. RNA polymerase II has been suggested to fulfill this role (28, 29, 50), perhaps with some still incompletely defined assistance from delta antigen (28). Which other host factors are involved is not known, but their eventual identification is likely to be quite interesting, especially as their function in RNA replication is probably not limited to ensuring HDV propagation. One approach to exploring these factors is to characterize proteins capable of interacting with delta antigen. Using a yeast two hybrid system, such a host cell protein has been identified (51), and may prove to be a useful probe of the cellular machinery employed by HDV.

During replication of the HDV genome, a critical RNA editing event occurs (52, 26, 27) which destroys the normal translation stop codon for the 195 amino acid long small delta antigen. As a result, translation of the edited RNA continues to the next downstream stop codon, adding an additional 19 amino acids to the small delta antigen reading frame. The result is the 214 amino acid large delta antigen. These two isoforms of delta antigen have dramatically different functions, the first of which is evident during this third stage of the viral life cycle. Small delta antigen promotes genome replication, while large delta antigen is a potent transdominant inhibitor (53, 54, 5). The mechanisms are incompletely defined. Small delta antigen could directly promote RNA transcription or it may act via effects on RNA processing or stabilization. Inhibition by large delta antigen may involve both intermolecular interactions between the antigen isoforms (55, 56, 57, 58) and their redistribution within subnuclear domains (59, 60, 61). The end-product of genome replication is a nascent ribonucleoprotein complex of genome and delta antigen. Such complexes are extractable from cell nuclei and virions (62). Interestingly, a supra molecular complex is maintained even after the RNA is experimentally released (45), suggesting the presence of a nucleocapsid-like protein structure composed of delta antigens, although a detailed capsid structure and geometry awaits definition.

The fourth stage of the viral life cycle is concerned with assembling the complex of newly replicated genome and associated delta antigens into an enveloped particle for release. To date, the precise intracellular location of assembly is not known (no specific membrane is implied in Figure. 1). Nevertheless, substantial progress has been made in defining the key determinants of HDV assembly. This has been greatly facilitated by experimental systems capable of producing particles after transfection of DNA clones encoding various portions of the HBV and HDV genomes into cultured cells (63). When full length clones of each virus are used, infectious virus particles are released into the media supernatants. As noted above, the middle or M HBV surface antigen appears to be dispensable for infectious particle production. Furthermore, while the L surface antigen is necessary for infectivity of the particles, the S surface antigen alone is sufficient to sustain particle production and release (64, 65).

On the delta antigen side, only the large form is capable of producing particles with HBsAg (66, 65). The small delta antigen cannot, although it can be co-packaged by large delta antigen into particles (67, 57). These interactions are also reflected in in-vitro binding studies (68, 69).

Thus a second key difference in function between the delta antigen isoforms is revealed. Moreover, the aforementioned RNA editing event which changes the form of delta antigen produced from small to large can be viewed as a molecular switch in the viral life cycle where genome replication is inhibited at the same time as packaging is favored (70).

Interestingly, in addition to small delta antigen, an intact RNA genome can also be experimentally omitted without affecting the ability of large delta antigen to be packaged and released with HBsAg (66). It is possible that such particles also exist in natural infections, although they have not yet been specifically sought for. Therefore, although it is to be expected that in an intact virion multiple interactions between the various components exist and can be detected in various binding assays (69, 71), it appears that the indispensable elements for particle formation are large delta antigen and HBV S surface antigen. Finally, mutation of large delta antigen's oligomerization domain does not impair its packaging with HBsAg, indicating the minimal requirement for envelopment lies within a large delta antigen monomer (67, 57, 72). Thus large delta antigen plays a key role in virion assembly, and an understanding of the underlying mechanism forms the basis of a new type of proposed antiviral therapy.

Prenylation and viral assembly

Large delta antigen is prenylated.

Within the 19 amino acids unique to large delta antigen lies a four amino acid sequence motif conserved in all isolates of HDV sequenced to date (73, 70, 74, 75, 76). This motif, composed of a cysteine situated exactly three amino acids from the carboxyl terminus, is termed a 'CXXX box' (where C=cysteine, and X=any amino acid) (See Figure 2A). CXXX boxes have been observed in a variety of proteins and are significant because these motifs represent substrates which can be recognized by a family of enzymes called prenyltransferases (77, 78, 79, 80). The latter catalyze the covalent attachment of hydrophobic prenyl lipid to the CXXX box cysteine via a reaction called prenylation. Farnesyltransferase adds a farnesyl group and geranylgeranyltransferase adds a geranylgeranyl group. These 15 (farnesyl) and 20 (geranylgeranyl) carbon prenyl lipids are both derived from mevalonic acid. Each prenyltransferase enzyme exhibits a generally non-overlapping substrate specificity determined by the amino acid composition of the CXXX box. Examples of farnesylated proteins include ras (81), lamin B (82), and yeast mating pheromone a-factor (83), while the γ-subunit of G proteins (84) and the Rab proteins involved in intracellular vesicular trafficking (85) undergo geranylgeranylation. One important consequence of prenylation is to render the modified protein more lipophilic. In the case of ras, prenylation is responsible for promoting the membrane association which is essential for its function. Of what relevance is all this to HDV?

As noted in the previous section, the fourth stage of the HDV life cycle depends on the proper targeting of newly replicated viral genomes and associated delta antigen to cellular membranes containing the HBsAg envelope proteins, in order to complete the assembly of new viral particles. Only the longer isoform of delta antigen was found to be essential for this assembly process. Prenylation of the CXXX box unique to large delta antigen, offered an attractive hypothesis to explain both the requisite role of large delta antigen in particle assembly, and the underlying molecular mechanism. In its simplest version, prenylation of large delta antigen would provide a lipophilic tag to help target large delta antigen, and its associated RNP, to the requisite membranes for envelopment.

The first evidence in support of this hypothesis required demonstrating that large delta antigen was indeed subject to prenylation. This was necessary not only to rule out other possibilities to explain the conserved CXXX box cysteine, but also because not all CXXX boxes undergo prenylation (86).

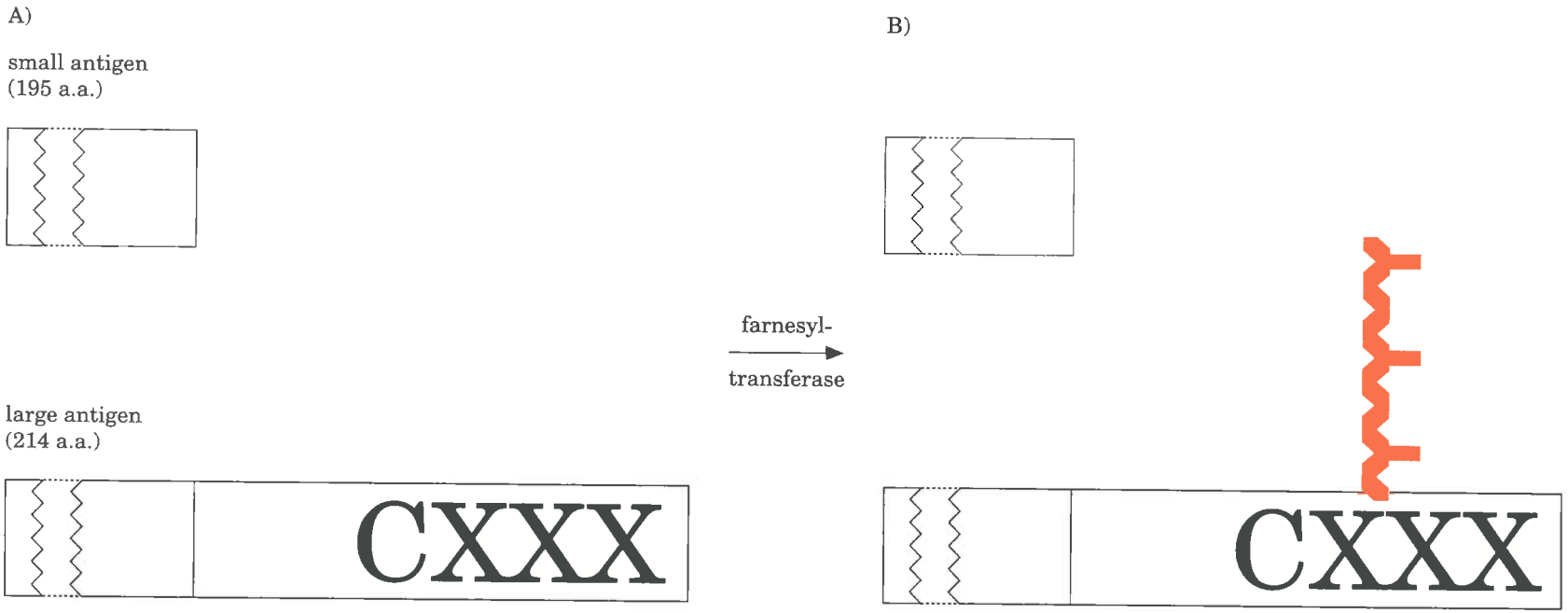

Figure 2. Small and large delta antigens. The 19 amino acids unique to large delta antigen harbors a CXXX box (A), which is subject to prenylation by farnesyltransferase (B). (Note: each half of the diagram depicts small and large delta antigens with their amino termini on the left, most of their central regions omitted and represented by dashed lines, and their carboxyl termini on the right. The one letter amino acid codes for the last four amino acids of large delta antigen are shown, with C = cysteine, and X = unspecified amino acid. The prenyl lipid farnesyl covalently attached to the CXXX box cysteine is shown in orange.)

Immunoprecipitates of large delta antigen from cells metabolically labelled with [^{3}H] mevalonic acid were found to specifically incorporate label, indicating that large delta antigen is indeed prenylated. A similar result was obtained upon expression of large delta antigen in rabbit reticulocyte lysate in-vitro translation reactions, which harbor all the enzymes needed to support prenylation. Small delta antigen, which has no CXXX box, could not be labelled in either assay (Figure 2B). In addition, genetic mutation of the CXXX box cysteine to serine abolished the observed prenylation of large delta antigen (70). Analogous results have since been obtained by several groups of investigators in a variety of cell types and expression systems (87, 88, 89, 90). HDV is thus the first virus shown to have a protein which undergoes prenylation, although there is reason to believe other viruses may also encode similarly modified proteins (91). The particular prenyl lipid which modifies large delta antigen has recently been shown to be farnesyl (90).

Prenylation is required for particle formation

The ability to produce HDV virus-like particles upon co-transfection of cultured cells with the genes encoding HBsAg and large delta antigen provided an opportunity to directly test the hypothesis that prenylation of large delta antigen was important for triggering assembly with HBsAg. Indeed, replacement of large delta antigen in such a co-transfection with either small delta antigen (which lacks a prenylation site) or large delta antigen with a genetically mutated prenylation site (replacement of the CXXX box cysteine with serine or phenylalanine) abolished particle formation (70, 89). That these results reflect the requirement for a prenylation-competent CXXX box rather than a specific amino acid sequence *per se* was further supported by additional detailed mutational analyses (89). Amino acid substitutions were shown to be tolerated in each of the X positions of the CXXX box. A strict correlation, however, was observed between a particular mutation's effect on prenylation and particle assembly. When prenylation was abolished, so was the ability to be packaged with HBsAg into particles released into the media.

One interpretation of prenylation's apparent key role in virion assembly is that, as suggested above, the hydrophobic nature of the attached prenyl lipid helps target the thus modified large delta antigen to the cellular membranes containing HBsAg. Alternatively, the attached farnesyl moiety could act as a specific ligand recognized by HBsAg, somewhat analogous to the interaction of geranylgeranylated Rab3 and GDI (85, 92).

Prenylation could also trigger additional changes in large delta antigen which themselves are important for interaction with HBsAg and particle assembly. These changes could be conformational in nature (88) or subsequent post-translational modifications. With respect to the latter, prenylation of most proteins triggers additional reactions including proteolytic removal of the three -XXX amino acids in the CXXX box and carboxylmethylation of the resulting new cysteine terminus. Each of these processing reactions further increases the hydrophobicity of the modified protein and its interaction with membranes (93, 94). An endoproteolytic activity has been found in liver microsomal membranes which is capable of specifically recognizing and cleaving off the carboxyl terminal 3 amino acids of a synthetic large delta antigen CXXX box tetrapeptide substrate (95). Similar proteolytic processing of full length large delta antigen in cells or viral particles, however, has yet to be demonstrated.

It appears that while prenylation is necessary for virion assembly, it is not sufficient. For example, transferring just the large delta antigen CXXX box onto the carboxyl terminus of small delta antigen does not result in packaging of the fusion protein with HBsAg (89). Rather, this experiment as well as combined deletion analysis data (67, 57) suggest that other amino acids immediately upstream of the prenylation site are important for productive packaging with HBsAg. If, however, in addition to the CXXX box, the immediately preceding 15 amino acids are also transferred to the carboxyl terminus of a protein not normally packaged by HBsAg, the

resulting fusion protein can now be co-secreted with HBsAg (96). Further deletion of 5 amino acid stretches within these 15 amino acids, abolishes the ability of large delta antigen to form particles (89). Interpretation of the results of such deletion experiments, however, is subject to the caveat that these mutations change not only the primary amino acid sequence but could also simply disturb a critical orientation, or the accessibility, of the prenyl lipid.

It is possible that either epitopes defined by the amino acids upstream of the prenylation site, or differences in the phosphorylation status of these residues could complement prenylation in triggering particle assembly. For example, the combination of phosphorylation and prenylation is required for maximal interaction of the ras-like protein smg p21B and its GDP/GTP exchange protein GDS (97). Phosphorylation of delta antigens has been reported by several investigators (87, 98) with serines being the predominant target of detected modification (99). It is particularly intriguing that one study described a difference between the phosphorylation status of large delta antigen found in cells versus that contained in secreted particles (100). Mutation of conserved serines unique to large delta antigen, (presumed candidate phosphorylation sites), had no significant effect, however, on the ability of large delta antigen to be either phosphorylated or packaged with HBsAg (98, 100).

In any case, the role played by the 15 amino acids immediately adjacent to the CXXX box is likely to be more complex than simply a reflection of their primary sequence, because almost none of these amino acids are strictly conserved among all sequenced isolates.

Since only large delta antigen appears to be able to productively interact with HBsAg to trigger particle assembly, how then is small delta antigen incorporated into viral particles? Although interaction with a common RNA genome could be one possibility, the data are most consistent with direct binding of small delta antigen to large delta antigen via their common oligomerization domains (101). The HDV genome itself appears to be incorporated via the previously described RNA binding activities of both delta antigens (102, 103, 104). Co-packaging of small delta antigen has been shown to increase the efficiency of genome incorporation (105).

Finally, although virion assembly requires prenylation, the exact intracellular site where such assembly occurs remains to be localized. The observation that a large delta antigen mutated to prevent nuclear localization can still be assembled into particles with HBsAg (101) would favour a cytoplasmic location. HBV is thought to bud into the ER (34) and a pre-Golgi compartment has been defined as an assembly site for HBsAg particles (106). Whether HDV assembly occurs in these same locations, the nuclear envelope, or yet other membrane compartments is unknown. This question remains an interesting challenge for future investigation.

The key role played by prenylation in the HDV life cycle is the prototype for a novel mechanism of virion morphogenesis. It also provides an attractive target for the design of potential new antiviral strategies.

Implications for therapy.

Therapy for HDV infections is sub-optimal to date. Efficacy of interferon in obtaining a biochemical and virologic response has been shown by randomized controlled trial (107, 108, 109). Higher doses than for HBV infection alone may be more effective. Unfortunately, even with prolonged treatment, most patients who respond initially have a relapse of hepatic inflammation and return of viral markers after cessation of therapy .

A more radical approach, liver transplantation of HDV patients, can yield good long term clinical outcome. Overall, HDV is associated with decreased rates of HBV re-infection of the donated liver (1). A further reduction in the rate of re-infection can be achieved by using immunoprophylaxis with anti-HBs. With such immunoprophylaxis, the detection rate of delta markers is as low as 5% after 2-years follow-up post-transplantation (110). Interestingly, Newer

therapies for HBV hold promise for ameliorating HDV infections, but if they fail to suppress HBsAg synthesis, as is often the case, their ability to eradicate HDV may be disappointing.

The key role played by prenylation in the HDV life cycle, however, offers a new target for specific anti-HDV therapy. While molecular genetic mutation of the prenylation site clearly abolishes HDV particle formation (70, 89), this would not be a practical clinical approach. Achieving the same result—namely prevention of prenylation—by *pharmacologic* means, however, is appealing. Furthermore, the requisite candidate drugs are already under development, albeit for a different purpose. Indeed, because of the role of ras in carcinogenesis, and the fact that its transforming activity is dependent on prenylation, a variety of prenylation inhibitors have been synthesized and are being evaluated for clinical use as potential anticancer agents (111, 112, 113). We therefore hypothesized that one of these prenylation inhibitors could be found to inhibit large delta antigen prenylation, and if so HDV particle formation might be sensitive to treatment with such an agent.

BZA-5B (produced by Genentech, South San Francisco, CA) is a farnesyltransferase inhibitor capable of preventing ras prenylation (114). Treatment of H-rasV^{12}-transformed rat-1 cells with BZA-5B resulted in reversion to a normal growth pattern without obvious toxicity (115). Because, like ras, large delta antigen is prenylated by farnesyl, the effect of BZA-5B on large delta antigen prenylation was tested. The results show that large delta antigen prenylation is significantly inhibited at BZA-5B concentrations as low as 5M and completely abolished by 50M (116), the concentration range which prevents ras prenylation. Furthermore, BZA-5B treatment of HDV-particle producing cells completely abolished virus-like particle production (116). Finally, no significant effect was observed on the secretion of a control non-prenylated protein over any of the BZA-5B concentrations tested, reflecting both a specificity for prenylation inhibition and a lack of gross toxicity. These results provide the first evidence for the feasibility of a novel antiviral strategy based on prenylation inhibition (91).

What is particularly exciting, is that farnesyltransferase inhibitors appear to be surprisingly well tolerated by host cells (117, 118). Perhaps this reflects some combination of a relative lack of essential host proteins which absolutely require farnesylation, a redundancy resulting from the presence of multiple prenyltransferase enzymes, or partial flexibility in prenyl group or CXXX box substrate requirements of particular prenyltransferases. In any case, HDV does not appear to be able to adequately benefit from these 'back-up' measures, and its assembly seems to be quite vulnerable to inhibition of large delta antigen prenylation. The availability of animal models of HDV (119) should further accelerate the evaluation of a prenylation inhibition-based antiviral therapy.

Future directions and conclusions

Both interesting biologic questions and additional potential antiviral targets remain to be evaluated. For example, does prenylation function as a trafficking signal targeting delta antigen to specific intracellular membranes, and if so how does that signal interact with other known targeting functions of delta antigen such as nuclear localization? Although prenylation inhibition now appears to be a promising antiviral therapy, if prenylation-induced further processing of large delta antigen occurs, as is the case for many other prenylated proteins, compounds which inhibit these specific proteolytic or carboxylmethylation reactions may also prove to be useful antiviral agents. Our laboratory is currently addressing these issues.

In summary, there are two isoforms of the major protein encoded by HDV, small and large delta antigens. Only the latter is essential for triggering assembly with the viral envelope proteins to make a virus particle. This requirement appears to reflect the fact that large delta antigen undergoes prenylation. Disruption of prenylation abolishes particle formation. This not only highlights a new mechanism of virus assembly, but also offers a promising new target for specific

antiviral therapy. The requisite agents are currently being developed and may soon find use as valuable new antiviral drugs. This was made possible as the result of many years of work by numerous investigators, and is an example of how basic molecular virology research can lead to promising new potential therapies.

Acknowledgements

This work was supported in part by a Howard Hughes Medical Institute Physician Postdoctoral Fellowship Award, and a Veterans Administration Merit Review Award. Additional support is provided by an Amgen/AASLD American Liver Foundation Award and a Burroughs Wellcome Fund Career Award.

References

1 Rizetto, M and A Ponzetto. 1995. Hepatitis delta virus infection: Medical aspects. In *The unique hepatitis delta virus*, G Dinter-Gottlieb ed. Austin: RC Landes Publishing Company, pp. 125–39.
2 Rizzetto, M, A Ponzetto and I Forzani. 1991. Epidemiology of hepatitis delta virus: Overview. In *The hepatitis delta virus*, J L Gerin, R H Purcell and M Rizetto ed. New York: Wiley-Liss, Inc., pp. 1–20.
3 Alter, M J and S C Hadler. 1993. Delta hepatitis and infection in North America. In *Hepatitis delta virus: Molecular biology, pathogenesis, and clinical aspects*, S J Hadziyannis, J M Taylor and F Bonino ed. New York: Wiley-Liss, Inc., pp. 243–50.
4 Rizzetto, M, M G Canese, S Arico, *et al.* 1977. Immunofluorescence detection of new antigen-antibody system (δ/anti-δ) associated to hepatitis B virus in liver and in serum of HBsAg carriers. *Gut* **18:** 997–1003.
5 Lazinski, D W and J M Taylor. 1994. Recent developments in hepatitis delta virus research. *Advances in Virus Research* **43:** 187–231.
6 Lai, M M C. 1995. The molecular biology of hepatitis delta virus. *Annu Rev Biochem* **64:** 259–86.
7 Rizzetto, M, M G Canese, J L Gerin, *et al.* 1980. Transmission of the hepatitis B virus-associated delta antigen to chimpanzees. *J Infect Dis* **141:** 590–602.
8 Ponzetto, A, P J Cote, H Popper, *et al.* 1984. Transmission of the hepatitis B virus-associated δ agent to the eastern woodchuck. *Proc Natl Acad Sci USA* **81:** 2208–12.
9 Netter, H J, K Kajino and J M Taylor. 1993. Experimental transmission of human hepatitis delta virus to the laboratory mouse. *J Virol* **67:** 3357–62.
10 Taylor, J, W Mason, J Summers, *et al.* 1987. Replication of human hepatitis delta virus in primary cultures of woodchuck hepatocytes. *J Virol* **61:** 2891–5.
11 Choi, S-S, R Rasshofer and M Roggendorf. 1988. Propagation of woodchuck hepatitis delta virus in primary woodchuck hepatocytes. *Virology* **167:** 451–7.
12 Sureau, C, J R Jacob, J W Eichberg, *et al.* 1991. Tissue culture system for infection with human hepatitis delta virus. *J Virol* **65**(7): 3443–50.
13 Wang, K-S, Q-L Choo, A J Weiner, *et al.* 1986. Structure, sequence and expression of the hepatitis delta viral genome. *Nature* **323:** 508–14.
14 Makino, S, M-F Chang, C-K Shieh, *et al.* 1987. Molecular cloning and sequencing of a human hepatitis delta virus RNA. *Nature* **329:** 343–6.
15 Kuo, M Y P, J Goldberg, L Coates, *et al.* 1988. Molecular cloning of hepatitis delta virus RNA from an infected woodchuck liver: Sequence, structure and applications. *J Virol* **62:** 1855–61.
16 Sureau, C, J Taylor, M Chao, *et al.* 1989. Cloned hepatitis delta virus cDNA is infectious in the chimpanzee. *J Virol* **63**(10): 4292–7.
17 Rapicetta, M, A R Ciccaglione, N D'Urso, *et al.* 1993. Chronic infection in woodchucks infected by a cloned hepatitis delta virus. In *Hepatitis delta virus: Molecular biology, pathogenesis, and clinical aspects*, S J Hadziyannis, J M Taylor and F Bonino ed. New York: Wiley-Liss, Inc., pp. 451.
18 Yang, A, P Karayiannis, D Cheng, *et al.* 1993. emergence of the long form of hepatitis delta virus antigen in transfected cells after intrahepatic transfection and during natural infection. In *Hepatitis delta virus: Molecular biology, pathogenesis, and clinical aspects*, S J Hadziyannis, J M Taylor and F Bonino ed. New York: Wiley-Liss, Inc., pp. 145–8.
19 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:** 1945–50.

20 Glenn, J S, J M Taylor and J M White. 1990. In vitro synthesized Hepatitis Delta virus RNA initiates genome replication in cultured cells. *J Virol* **64:** 3104–7.
21 Tai, F-P, P-J Chen, F-L Chang, *et al.* 1993. Hepatitis delta virus cDNA monomer can be used in transfection experiments to initiate viral RNA replication. *Virology* **197:** 137–42.
22 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**(1): 366–72.
23 Sharmeen, L, M Y-P Kuo, G Dinter-Gottlieb, *et al.* 1988. Antigenomic RNA of human hepatitis delta virus can undergo self-cleavage. *J Virol* **62:** 2674–9.
24 Belinsky, M and G Dinter-Gottlieb. 1993. Self-cleavage of internally deleted hepatitis delta RNAs. In *Hepatitis delta virus: Molecular biology, pathogenesis, and clinical aspects*, S J Hadziyannis, J M Taylor and F Bonino ed. New York: Wiley-Liss, Inc., pp. 89–97.
25 Tanner, N K. 1995. The catalytic RNAs from hepatitis delta virus: Structure, function and applications. In *The unique hepatitis delta virus*, G Dinter-Gottlieb ed. Austin: RC Landes Publishing Company, pp. 11–31.
26 Casey, J L and J L Gerin. 1995. Hepatitis delta virus: RNA editing and genotype variations. In *The unique hepatitis delta virus*, G Dinter-Gottlieb ed. Austin: RC Landes Publishing Company, pp. 111–24.
27 Polson, A G, B L Bass and J L Casey. 1996. RNA editing of hepatitis delta virus antigenome by dsRNA-adenosine deaminase. *Nature* **380:** 454–6.
28 MacNaughton, T B, E J Gowans, S P McNamara, *et al.* 1991. Hepatitis δ antigen is necessary for access of hepatitis δ virus RNA to the cell transcriptional machinery but is not part of the transcriptional complex. *Virology* **184:** 387–90.
29 Fu, T-B and J Taylor. 1993. The RNAs of hepatitis delta virus are copied by RNA polymerase II in nuclear homogenates. *J Virol* **67**(12): 6965–72.
30 Bonino, F, J Hoyer, W-K Shih, *et al.* 1984. Delta hepatitis agent: structural and antigenic properties of the delta-associated particle. *Infect Immun* **43:** 1000–5.
31 He, L-F, E Ford, R H Purcell, *et al.* 1989. The size of the hepatitis delta agent. *J Med Virol* **27:** 31–3.
32 Branch, A and H Robertson. 1984. A replication cycle for viroids and other small infectious RNAs. *Science* **223:** 450–5.
33 Bonino, F, K H Heermann, M Rizzetto, *et al.* 1986. Hepatitis delta virus: Protein compostion of delta antigen and its hepatitis B virus-derived envelope. *Journal of Virology* **58:** 945–50.
34 Ganem, D. 1996. Hepadnaviridae and their replication. In *Fields Virology*, B N Fields, D M Knipe, P M Howley and et al. ed. Philadelphia: Lippincott-Raven Publishers, pp. 2703–37.
35 Sureau, C, B Guerra and H Lee. 1994. The middle hepatitis B virus envelope protein is not necessary for infectivity of hepatitis delta virus. *J Virol* **68**(6): 4063–6.
36 Gowans, E J, B M Baroudy, F Negro, *et al.* 1988. Evidence for replication of hepatitis delta virus RNA in hepatocyte nuclei after *in vivo* infection. *Virology* **167:** 274–8.
37 Cullen, J M, C David, J-G Wang, *et al.* 1995. Subcellular distribution of large and small hepatitis delta antigen in hepatocytes of hepatitis delta virus superinfected woodchucks. *Hepatology* **22:** 1090–100.
38 Chou, H-C, T-Y Hsieh, G-T Sheu, *et al.* 1998. Hepatitis delta antigen mediates the nuclear import of hepatitis delta virus RNA. *J Virol* **72**(5): 3684–90.
39 Dingle, K, V Bichko, H Zuccola, *et al.* 1998. Initiation of hepatitis delta virus genome replication. *J Virol* **72**(6): 4783–8.
40 Bichko, V, H J Netter and J Taylor. 1994. Introduction of hepatitis delta virus into animal cell lines via cationic liposomes. *J Virol* **68**(8): 5247–52.
41 Hwang, S B, K-S Jeng and M M C Lai. 1995. Studies of functional roles of hepatitis delta antigen in delta virus RNA. In *The unique hepatitis delta virus*, G Dinter-Gottlieb ed. Austin: RC Landes Publishing Company, pp. 95–109.
42 Chang, M-F, S C Chang, C-I Chang, *et al.* 1992. Nuclear localization signals, but not putative leucine zipper motifs, are essential for nuclear transport of hepatitis delta antigen. *J Virol* **66**(10): 6019–27.
43 Xia, Y-P, C Yey, J-S Ou, *et al.* 1992. Characterization of nuclear targeting signal of hepatitis delta antigen: Nuclear transport as a protein complex. *J Virol* **66:** 914–21.
44 Chang, M-F, S C Baker, L H Soe, *et al.* 1988. Human hepatitis delta antigen is a nuclear phosphoprotein with RNA-binding activity. *J Virol* **62**(7): 2403–10.
45 Bichko, V V, S M Lemon, J-G Wang, *et al.* 1996. Epitopes exposed on hepatitis delta virus ribonucleoproteins. *J Virol* **70**(9): 5807–11.
46 Goerlich, D and I W Mattaj. 1996. Nucleocytoplasmic transport. *Science* **271:** 1513–8.

47 Whittaker, G R and A Helenius. 1998. Nuclear import and export of viruses and virus genomes. *Virology* **246:** 1–23.
48 MacNaughton, T B, E J Gowans, A R Jilbert, *et al.* 1990. Hepatitis delta virus RNA, protein synthesis and associated cytotoxicity in a stably transfected cell line. *Virology* **177:** 692–8.
49 Taylor, J M. 1990. Hepatitis delta virus: *cis* and *trans* functions required for replication. *Cell* **61:** 371–3.
50 Taylor, J M. 1995. HDV as a precedent for RNA-directed RNA synthesis by RNA polymerase II. In *The unique hepatitis delta virus*, G Dinter-Gottlieb ed. Austin: RC Landes Publishing Company, pp. 47–54.
51 Brazas, R and D Ganem. 1996. A cellular homolog of hepatitis delta antigen: implications for viral replication and evolution. *Science* **274:** 90–4.
52 Luo, G, M Chao, S-Y Hsieh, *et al.* 1990. A specific base transition occurs on replicating hepatitis delta virus RNA. *J Virol* **64:** 1021–7.
53 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:** 5066–9.
54 Glenn, J S and J M White. 1991. Trans-dominant inhibition of human hepatitis delta virus genome replication. *J Virol* **65**(5): 2357–61.
55 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**(11): 6641–8.
56 Chang, M-F, C-Y Sun, C-J Chen, *et al.* 1993. Functional motifs of delta antigen essential for RNA binding and replication of hepatitis delta virus. *J Virol* **67**(5): 2529–36.
57 Lazinski, D W and J M Taylor. 1993. Relating structure to function in the hepatitis delta virus antigen. *J Virol* **67**(5): 2672–80.
58 Wang, J-G and S M Lemon. 1993. Dimeric and multimeric forms of hepatitis delta virus antigen are present in infected woodchuck liver. In *Hepatitis delta virus: Molecular biology, pathogenesis, and clinical aspects*, S J Hadziyannis, J M Taylor and F Bonino ed. New York: Wiley-Liss, Inc., pp. 61–7.
59 Wu, J-C, C-L Chen, S-D Lee, *et al.* 1992. Expression and localization of the small and large delta antigens during the replication cycle of hepatitis D virus. *Hepatology* **16**(5): 1120–7.
60 Bichko, V V and J M Taylor. 1996. Redistribution of the delta antigens in cells replicating the genome of hepatitis delta virus. *J Virol* **70**(11): 8064–70.
61 Cunha, C, J Monjardino, D Chang, *et al.* 1998. Localization of hepatitis delta virus RNA in the nucleus of human cells. *RNA* **4:** 680–93.
62 Ryu, W-S, H J Netter, M Bayer, *et al.* 1993. Ribonucleoprotein complexes of hepatitis delta virus. *J Virol* **67**(6): 3281–7.
63 Wu, J-C, P-J Chen, M Y-P Kuo, *et al.* 1991. Production of hepatitis delta virus and suppression of helper hepatitis B virus in a human hepatoma cell line. *J Virol* **65:** 1099–104.
64 Wang, C J, P J Chen, J C Wu, *et al.* 1991. Small-form hepatitis B surface antigen is sufficient to help in the assembly of hepatits delta virus-like particles. *J Virol* **65**(12): 6630–6.
65 Ryu, W-S, M Bayer and J Taylor. 1992. Assembly of hepatitis delta virus particles. *J Virol* **66**(4): 2310–5.
66 Chang, F-L, P-J Chen, S-J. Tu, *et al.* 1991. The large form of hepatitis δ antigen is crucial for assembly of hepatitis δ virus. *Proc Natl Acad Sci U.S.A* **88:** 8490–4.
67 Chen, P-J, F-L Chang, C-J Wang, *et al.* 1992. Functional study of hepatitis delta virus large antigen in packaging and replication inhibition: Role of the amino-terminal leucine zipper. *J Virol* **66**(5): 2853–9.
68 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**(12): 7659–62.
69 de Bruin, W, W Leenders, T Kos, *et al.* 1994. In vitro binding properties of the hepatitis delta antigens to the hepatitis B virus envelope proteins: potential significance for the formation of delta particles. *Virus Res* **31:** 27–37.
70 Glenn, J S, J A Watson, C M Havel, *et al.* 1992. Identification of a prenylation site in delta virus large antigen. *Science* **256:** 1331–3.
71 Hourioux, C, C Sureau, F Poisson, *et al.* 1998. Interaction between hepatitis delta virus-encoded proteins and hepatitis B virus envelope protein domains. *J Gen Virol* **79:** 1115–9.
72 Sheu, S Y, K-L Chen, Y-H W Lee, *et al.* 1996. No intermolecular interaction between the large hepatitis delta antigens is required for the secretion with hepatitis B surface antigen: A model of empty HDV particle. *Virology* **218:** 275–8.

73 Chao, Y-C, M-F Chang, I Gust, *et al.* 1990. Sequence conservation and divergence of hepatitis D virus RNA. *Virology* **178:** 384–92.
74 Lee, C-M, F-Y Bih, Y-C Chao, *et al.* 1992. Evolution of hepatitis delta virus RNA during chronic infection. *Virology* **188:** 265–73.
75 Casey, J L, T L Brown, E J Colan, *et al.* 1993. A genotype of hepatitis D virus that occurs in northern South America. *Proc Natl Acad Sci* **90:** 9016–20.
76 Tang, J R, O Hantz, L Vitvitski, *et al.* 1993. Discovery of a novel point mutation changing the HDAg expression of a hepatitis delta virus isolate from Central African Republic. *J Gen Virol* **74:** 1827–35.
77 Maltese, W A. 1990. Posttranslational modification of proteins by isoprenoids in mammalian cells. *FASEB J* **4:** 3319–28.
78 Chow, M, C J Der and J E Buss. 1992. Structure and biological effects of lipid modifications on proteins. *Curr Opin Cell Biol* **4:** 629–36.
79 Schafer, W R and J Rine. 1992. Protein prenylation: Genes, enzymes, targets, and functions. *Annu Rev Genet* **30:** 209–37.
80 Zhang, F L and P J Casey. 1996. Protein prenylation: Molecular mechanisms and functional consequences. *Annu Rev Biochem* **65:** 241–69.
81 Hancock, J F, A I Magee, J E Childs, *et al.* 1989. All ras proteins are polyisoprenylated but only some are palmitoylated. *Cell* **57:** 1167–77.
82 Farnsworth, C C, S L Wolda, M H Gelb, *et al.* 1989. Human lamin B contains a farnesylated cysteine residue. *J Biol Chem* **264**(34): 20422–9.
83 Anderegg, R J, R Betz, S A Carr, *et al.* 1988. Structure of *Saccharomyces cerevisiae* mating hormone a-factor. *J Biol Chem* **263**(34): 18236–40.
84 Yamane, H K, C C Farnsworth, H Xie, *et al.* 1990. Brain G protein γ subunits contain an all-trans-geranylgeranyl-cysteine methyl ester at their carboxyl termini. *Proc Nat'l Acad Sci USA* **87:** 5868–72.
85 Novick, P and P Brennwald. 1993. Friends and family: The role of the Rab GTPases in vesicular traffic. *Cell* **75:** 597–601.
86 Sanford, J, J Codina and L Birnbaumer. 1991. γ-subunits of G proteins, but not their α- or β-subunits, are polyisoprenylated. *J Biol Chem* **266**(15): 9570–9.
87 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:** 413–22.
88 Hwang, S B and M M C Lai. 1994. Isoprenylation masks a conformational epitope and enhances trans-dominant inhibitory function of the large hepatitis delta antigen. *J Virol* **68**(5): 2958–64.
89 Lee, C-Z, P-J Chen, M M C Lai, *et al.* 1994. Isoprenylation of large hepatitis delta antigen is necessary but not sufficient for hepatitis delta virus assembly. *Virology* **199:** 169–75.
90 Otto, J C and P J Casey. 1996. The hepatitis delta virus large antigen is farnesylated both in vitro and in animal cells. *J Biol Chem* **271**(9): 4569–72.
91 Glenn, J S. 1995. Prenylation and virion morphogenesis. In *The unique hepatitis delta virus*, G Dinter-Gottlieb ed. Austin: RC Landes Publishing Company, pp. 83–93.
92 Casey, P J. 1995. Protein lipidation in cell signaling. *Science* **268:** 221–5.
93 Hancock, J F, K Cadwallader and C J Marshall. 1991. Methylation and proteolysis are essential for efficient membrane binding of prenylated $p21^{K\text{-}ras(B)}$. *EMBO J* **10**(3): 641–6.
94 Silvius, J R and F l'Heureux. 1994. Fluorimetric evaluation of the affinities of isoprenylated peptides for lipid bilayers. *Biochemistry* **33:** 3014–22.
95 Ma, Y-T and R R Rando. 1993. Endoproteolysis of non-CAAX-containing isoprenylated peptides. *FEBS Letters* **332**(1,2): 105–10.
96 Lee, C-Z, P-J Chen and D-S Chen. 1995. Large hepatitis delta antigen in packaging and replication inhibition: Role of the carboxyl-terminal 19 amino acids and amino-terminal sequences. *J Virol* **69**(9): 5332–6.
97 Shirataki, H, K Kaibuchi, M Hiroyoshi, *et al.* 1991. Inhibition of the action of the stimulatory GDP/GTP exchange protein for *smg* p21 by the geranylgeranylated synthetic peptides designed from its C-terminal region. *J Biol Chem* **266**(31): 20672–7.
98 Yeh, T-S, S J Lo, P-J Chen, *et al.* 1996. Casein kinase II and protein kinase C modulate hepatitis delta virus RNA replication but not empty viral particle assembly. *J Virol* **70**(9): 6190–8.
99 Chang, M F, S C Baker, L H Soe, *et al.* 1988. Human hepatitis delta antigen is a nuclear phosphoprotein with RNA-binding activity. *J Virol* **62**(7): 2403–10.

100 Bichko, V, S Barik and J Taylor. 1997. Phosphorylation of the hepatitis delta virus antigens. *J Virol* **71**(1): 512–8.
101 Chang, M-F, C-J Chen and S C Chang. 1994. Mutational analysis of delta antigen: effect on assembly and replication of hepatitis delta virus. *J Virol* **68**(2): 646–53.
102 Lin, J-H, M-F Chang, S C Baker, *et al.* 1990. Characterization of hepatitits delta antigen: specific binding to hepatitis delta virus RNA. *J Virol* **64**(9): 4051–8.
103 Lee, C Z, J-H Lin, M Chao, *et al.* 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**(4): 2221–7.
104 Chang, M-F, C-H Chen, S-L Lin, *et al.* 1995. Functional domains of delta antigens and viral RNA required for RNA packaging of hepatitis delta virus. *J Virol* **69**(4): 2508–14.
105 Wang, H-W, P-J Chen, C-Z Lee, *et al.* 1994. Packaging of hepatitis delta virus RNA via the RNA-binding domain of hepatitis delta antigens: Different roles for the small and large delta antigens. *J Virol* **68**(10): 6363–71.
106 Huovila, A-P J, A M Eder and S D Fuller. 1992. Hepatitis B surface antigen assembles in a post-ER, pre-golgi compartment. *J Cell Biol* **118**(6): 1305–20.
107 Rosina, F, C Pintus, C Meschievitz, *et al.* 1991. A randomized controlled trial of a 12–month course of recombinant human interferon-alpha in chronic Delta (type D) hepatitis: a multicenter Italian study. *Hepatology* **13**(6): 1052–6.
108 Farci, P, A Mandas, A Coiana, *et al.* 1994. Treatment of chronic hepatitis D with interferon alfa-2a. *NEJM* **330**(2): 88–94.
109 Rosina, F and R Cozzolongo. 1994. Interferon in HDV infection. *Antiviral Research* **24**(2–3): 165–74.
110 Samuel, D, A-L Zignego, M Reynes, *et al.* 1995. Long-term clinical and virological outcome after liver transplantation for cirrhosis caused by chronic delta hepatitis. *Hepatology* **21**(2): 333–9.
111 Koblan, K S, N E Kohl, C A Omer, *et al.* 1996. Farnesyltransferase inhibitors: a new class of cancer chemotherapeutics. *Biochem Soc Trans* **24:** 688–92.
112 Baringa, M. 1997. From bench top to bedside. *Science* **278:** 1036–9.
113 Sebti, S and A D Hamilton. 1997. Inhibitors of prenyl transferases. *Current Opinion in Oncology* **9:** 557–61.
114 Marsters, J C, Jr, R S McDowell, M E Reynolds, *et al.* 1994. Benzodiazepine peptidomimetic inhibitors of farnesyltransferase. *Bioorganic and Medicinal Chemistry* **2**(9): 949–57.
115 James, G L, J L Goldstein, M S Brown, *et al.* 1993. Benzodiazepine peptidomimetics: Potent inhibitors of ras farnesylation in animal cells. *Science* **260:** 1937–42.
116 Glenn, J S, J C Marsters Jr and H B Greenberg. 1998. Use of a prenylation inhibitor as a novel antiviral agent. *J Virol* **72**(11): 9303–6.
117 James, G L, M S Brown, M H Cobb, *et al.* 1994. Benzodiazepine peptidomimetic BZA-5B interrupts the MAP kinase activation pathway in H-Ras-transformed Rat-1 cells, but not in untransformed cells. *J Biol Chem* **269**(44): 27705–14.
118 Dalton, M B, K S Fantle, H A Bechtold, *et al.* 1995. The farnesyl protein transferase inhibitor BZA-5B blocks farnesylation of nuclear lamins and p21ras but does not affect their function or localization. *Cancer Research* **55**(15): 3295–304.
119 Negro, F. 1996. Animal models of hepatitis delta virus infection. *Viral Hepatitis Reviews* **2**(3): 175–85