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Identification of a Prenylation Site in Delta Virus Large Antigen

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During replication, hepatitis delta virus (HDV) switches from production of small to large delta antigen. Both antigen isoforms have an HDV genome binding domain and are packaged into hepatitis B virus (HBV)–derived envelopes but differ at their carboxyl termini. The large antigen was shown to contain a terminal CXXX box and undergo prenylation. The large, but not the small, antigen formed secreted particles when expressed singly with HBV surface antigen. Mutation of Cys[211] in the CXXX box of the large antigen abolished both prenylation and particle formation, suggesting that this site is important for virion morphogenesis.

Hepatitis delta virus (HDV) infections cause both acute and chronic liver disease and can be fatal (*1, 2*). This RNA virus contains a 1.7-kb single-stranded circular genome and delta antigen, the only known HDV-encoded protein. These elements are encapsulated by a lipid envelope in which hepatitis B virus (HBV) surface antigens are embedded (*3*), which explains why HDV infections occur only in the presence of an accompanying HBV infection (*4, 5*). Two isoforms of delta antigen exist in infected livers and serum (*6, 7*). This heterogeneity arises from a unidirectional mutation at a single nucleotide in the termination codon for delta antigen (codon 196: UAG → UGG), which occurs during replication (*8*). Thus, although small delta antigen is 195 amino acids long, large delta antigen is identical in sequence except that it contains an additional 19 amino acids at its COOH-terminus. Although both forms of delta antigen contain the same RNA genome binding domain (*9*), they have dramatically different effects on genome replication. The small form is required for replication, whereas the large form is a potent trans-dominant inhibitor (*10, 11*).

The last four amino acids of large delta antigen are Cys-Arg-Pro-Gln-COOH. This COOH-terminal configuration, termed a CXXX box (where C is cysteine and X is any amino acid), has been implicated as a substrate for prenyltransferases that add to the cysteine 15 (farnesyl) or 20 (geranylgeranyl) carbon moieties derived from mevalonic acid (*12–14*). The resulting hydrophobic modification may aid in membrane association of the derivatized protein, as suggested for p21 Ras (*15, 16*) and lamin B (*12, 17*). We therefore examined whether large delta antigen was similarly modified.

To determine whether large delta antigen is a substrate for prenylation, we labeled three cell lines, SAG, LAG, and GP4F, with [^{3}H]mevalonic acid. GP4F cells are a derivative of NIH 3T3 cells (*18*). SAG (*19*) and LAG (*20*) cells are derivatives of GP4F cells that stably express the small and large delta antigens, respectively. Labeled cell lysates were analyzed on immunoblots (Fig. 1A) to detect steady-state amounts of small and large delta antigen. The lysates were also subjected to immunoprecipitation with an antibody to the delta antigens (anti-delta), SDS–polyacrylamide gel electrophoresis (SDS-PAGE), and fluorography (Fig. 1B). The large, but not the small, antigen was labeled with [^{3}H]mevalonic acid, suggesting that large delta antigen undergoes prenylation in cultured cells.

We obtained similar results using in vitro translation reactions (*13*) performed in the presence of [^{3}H]proline or [^{3}H]mevalonate (Fig. 2). Both the small and the large antigens were labeled with [^{3}H]proline (Fig. 2A), whereas only the large isoform was labeled with [^{3}H]mevalonate (Fig. 2B). To determine whether modification by [^{3}H]mevalonate was dependent on the presence of Cys[211] in the terminal CXXX box, we constructed a mutant that contains a serine at this position (*20*). Cys[211] is the only cysteine in large delta antigen. Mutating Cys[211] to Ser did not interfere with the synthesis of large delta antigen (Fig. 2A) but abolished its modification by [^{3}H]mevalonate (Fig. 2B). The specific type of mevalonate modification of large delta antigen appears to be geranylgeranyl rather than farnesyl (*21*). Although the first described CXXX boxes contained aliphatic residues at the first and second positions after Cys, other types of amino acids can be found in prenylation sites (*13, 14*). We do not yet know whether the COOH-terminal sequence Cys-Arg-Pro-Gln-COOH, which differs from that of previously described CXXX boxes, implies the existence of a novel prenylation enzyme or whether it reflects a broader substrate specificity of known prenyltransferases.

For HDV particle formation, delta antigen and associated genomes are presumably targeted to cell membranes that contain HBV envelope proteins. We hypothesized that prenylation of large delta antigen could be involved in this process. We therefore first examined whether large delta antigen was sufficient for HDV-like particle formation. HBV surface antigen (HBsAg) was expressed transiently in COS-7 cells together with small or large delta antigen. Virus-like particles consisting of delta anti-

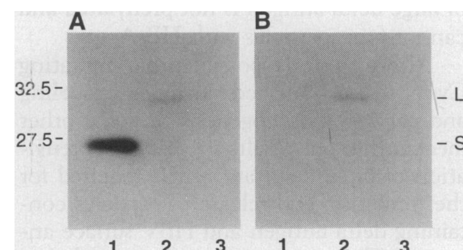

Fig. 1. Large delta antigen is prenylated in cultured cells. The cell lines SAG (*19*) (lane 1), LAG (*20*) (lane 2), and GP4F (*18*) (lane 3) were grown overnight in Lovastatin (25 μM) and (*R,S*)-[5-^{3}H]mevalonate (140 μM) (*30*), and lysed in RIPA buffer [50 mM tris (pH 7.5), 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS] (*20*). (**A**) Aliquots were subjected to immunoblot analysis (*11*). The blot was treated with serum from an HDV-infected patient that contained antibody to delta antigen (α-δAg) and horseradish peroxidase–conjugated rabbit antibody to human immunoglobulin G (Promega), followed by chemiluminescence (Amersham) development. (**B**) Immunoprecipitates (with α-δAg) from cell extracts were subjected to SDS-PAGE and fluorography. S, small delta antigen. L, large delta antigen. Molecular size markers are shown at the left (in kilodaltons).

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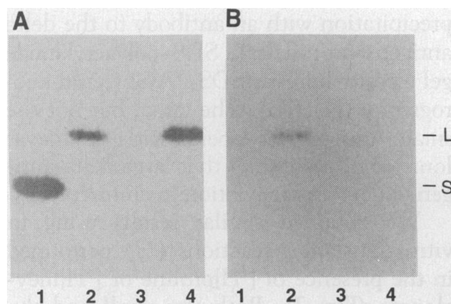

Fig. 2. Mutation of Cys^{211} of large delta antigen to Ser and loss of prenylation. In vitro translation reactions were performed with rabbit reticulocyte lysates (Promega) in the presence of either (**A**) *L*-[2,3,4,5-^{3}H]proline (19 μM) (94 Ci/mmol, Amersham) or (**B**) [^{3}H]mevalonate (200 μM) (*30*). For (A) and (B), translation reactions contained small delta antigen mRNA (lane 1); large delta antigen mRNA (lane 2); water (lane 3); or large delta antigen ($Cys^{211} \rightarrow$ Ser) (*20*) mRNA (lane 4). A portion (20 μl) of each reaction was added to 1 ml of RIPA buffer, immunoprecipitated with α-δAg, and analyzed as described (Fig. 1).

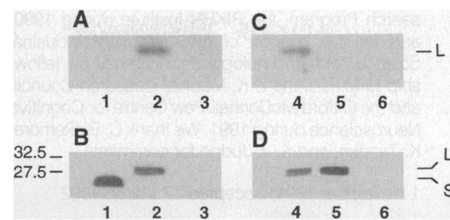

Fig. 3. Particle formation with large delta antigen and HBsAg. (**A** and **B**) COS-7 cells were transiently transfected with the following plasmids: SV24H, which expresses HBV surface antigen (*31*), and SVLAg, which expresses small delta antigen (*19*) (lane 1); SV24H and SVL-large, which expresses large delta antigen (*20*) (lane 2); and calcium phosphate precipitate without DNA (lane 3). (**C** and **D**) COS-7 cells were transfected with SV24H and SVL-large (lane 4); SV24H and SVL-large (Ser^{211}) (*20*) (lane 5); and calcium phosphate precipitate without DNA (lane 6). For (A) and (C), 48 hours after transfection, HBsAg-containing particles were immunoprecipitated from 2-ml aliquots of clarified media supernatants with anti-HBs (*31*) and subjected to immunoblot (with α-δAg) and chemiluminescence analyses as described (Fig. 1). For (B) and (D), the transfected cells were harvested in cell lysis buffer [50 mM tris (pH 8.8), 2% SDS] with protease inhibitors (*20*), and aliquots subjected to protein immunoblot and chemiluminescence analyses. Molecular size markers are shown at the left (in kilodaltons).

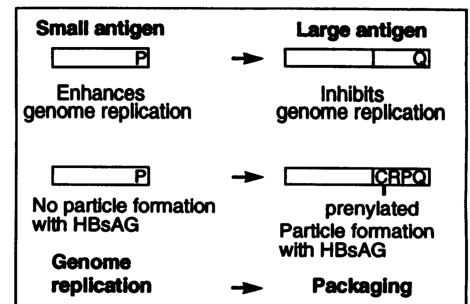

Fig. 4. Regulatory switch of S genomes to L genomes. During replication, S genomes encoding the small delta antigen mutate to L genomes, which encode the large delta antigen. This single base mutation has two effects on the COOH-terminus of delta antigen. The first is to change the nature of the COOH-terminal amino acid; Pro (P), which enhances genome replication (*20*), is replaced by Gln (Q), resulting in inhibition of genome replication. The second effect is the creation of a target prenylation site (CRPQ). C, cysteine; R, arginine; P, proline; Q, glutamine.

gen packaged into HBsAg-containing envelopes were analyzed by immunoprecipitation of clarified media supernatants with an antibody to HBsAg (anti-HBs). The presence of delta antigen in the immunoprecipitates was assayed by immunoblot analysis (Fig. 3A). Although both small and large antigens were synthesized in the transfected cells (Fig. 3B), only the large isoform was incorporated into secreted HBsAg-containing particles (Fig. 3A). Similar selective packaging has been observed (*22*).

We then examined the function of mevalonate modification in this particle formation. One explanation for the preferred packaging of large delta antigen is that the small antigen lacks the CXXX box and therefore cannot undergo modification. If so, the $Cys^{211} \rightarrow$ Ser mutant of large delta antigen should behave like small delta antigen and not be packaged. This was indeed found to be the case. Whereas both wild-type and Ser^{211} mutant large antigens were synthesized in transfected cells (Fig. 3D), only the wild-type form was packaged into particles (Fig. 3C). Thus, the mutated form of large delta antigen is not prenylated and cannot form particles with HBsAg.

Although it is possible that mutating Cys^{211} to Ser abolished mevalonate labeling and particle morphogenesis by some other mechanism, our results suggest that prenylation of large delta antigen is required for the formation and release of particles containing delta antigen and HBV surface antigens. The requirement of a prenylation site for productive viral infection is further suggested by other mutations of the CXXX box (*23*) and by the conservation of Cys^{211} and a CXXX box motif among all sequenced HDV isolates (*24*).

The ability of large, but not small, delta antigen to be prenylated and packaged into virus particles further highlights the significance of the mutation-induced heterogeneity at the termination codon of the small delta antigen. During HDV replication, S genomes (encoding the small antigen) mutate to L genomes (encoding the large antigen). At least two effects attributable to this mutation can be distinguished (see Fig. 4). The first is the conversion of an enhancer of genome replication (small delta antigen) into a potent trans-dominant inhibitor (large delta antigen) (*10, 11*). This dramatic difference in function appears to be determined solely by the nature of the COOH-terminal amino acid, with proline being sufficient to confer enhancer activity (*11, 25*). The second effect is the addition of a CXXX box to delta antigen, which allows the protein to be prenylated and presumably promotes its incorporation into HBsAg-containing particles. The combined effects of the switch from production of small to large delta antigen thus appear to have two roles: to suppress further genome replication and to promote the onset of packaging and virion morphogenesis.

Our results suggest prenylation as a new target for anti-HDV therapy. Several strategies designed to interfere with this stage of the HDV life cycle may be considered, including drugs that inhibit enzymes along the prenylation pathway, and CXXX box analogs. Both therapies have been considered for the inhibition of ras-mediated oncogenic transformation (*26*). Tetrapeptides that correspond to the CXXX box of p21 Ha-Ras inhibit prenylation of p21 Ha-Ras in vitro (*27*). Finally, the dual function of large delta antigen in the HDV life cycle suggests a further refinement of a proposed (*11*) defective interfering particle (DIP) (*28*)–like therapy aimed at cells infected with actively replicating S genomes. Because L genomes require a source of small delta antigen for replication (*19, 29*) but, once replicated, produce a potent trans-dominant inhibitor of further replication, a therapeutically administered L genome DIP could be specific for infected cells as well as possess an inherent shut-off mechanism (*11*). If the L genome also contained the Cys^{211} to Ser mutation, it could encode a delta antigen that not only inhibits replication but also affects packaging.

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Nondissociation of GAL4 and GAL80 in Vivo After Galactose Induction

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Transcription of galactose-inducible genes in yeast is regulated by interaction between the activator protein GAL4 and the negative regulatory protein GAL80. It has been suggested that GAL80 binds to and represses GAL4 under uninduced conditions and dissociates from GAL4 on induction. However, the possibility that GAL80 remains associated with GAL4 after induction has not been ruled out. Experiments to discriminate between these two models were performed and revealed that GAL80 stays bound after induction.

Transcriptional regulation in eukaryotes involves the interplay between positive and negative regulatory proteins. The yeast transcriptional activator GAL4 and its negative regulator GAL80 are said to be "poised" for activation. Under uninduced conditions (in the absence of galactose), GAL4 occupies its DNA binding site upstream of target genes, but its activation function is blocked by protein-protein interaction with GAL80. On induction, GAL4 becomes competent to activate the transcription of specific genes. The popular model (Fig. 1A) to explain this activation is that GAL80 dissociates from GAL4, allowing exposure of the GAL4 activation domain. Another possible model is that GAL80 remains bound after induction.

We devised a scheme (Fig. 1B) to discriminate between these two models in vivo. The basic components of the experiment were the following. First, a *GAL4* gene was used that encoded a protein that has reduced ability to activate transcription but still retains its normal affinity for the GAL80 protein (*1*). Second, a fusion was made between the GAL80 protein and the transcriptional activation domain of the VP16 protein of herpes simplex virus (*2*). The expectation was that the GAL80-VP16 fusion would be recruited to the enhancer region by GAL4 and activate transcription even in the uninduced state by virtue of the VP16 activation domain, because the acidic activation domain of VP16 is a strong activator in yeast (*3*) and a different activation domain had been fused to GAL80 to allow expression in the uninduced state (*4*).

We expected that the GAL4 proteins that were poor activators would produce high reporter gene expression in the uninduced state by binding the GAL80-VP16 protein. The question was what would happen on induction. If the GAL80-VP16 protein dissociates from GAL4, then the level of expression after induction should fall to the lower, GAL4 © mediated level. If, however, the GAL80-VP16 complex stays associated with GAL4 after induction, then the induced expression should be as high as or higher than that of the uninduced state. These two alternative outcomes are depicted in Fig. 1B.

As anticipated, the GAL80-VP16 hybrid stimulated high levels of transcription in noninducing media (Table 1). In inducing media, the level of expression was as high as or higher than uninduced for each of the GAL4 mutants tested, consistent with idea that GAL80 remains associated with the GAL4 after induction. We observed the same phenomenon for the wild-type GAL4. In this case, the level of expression is higher than that of the GAL80-VP16 plus GAL4 activation, suggesting that the GAL4 and VP16 activation domains are functioning synergistically under inducing conditions.

Several control experiments support the conclusion that GAL80 does not dissociate from GAL4 on induction. First, expression of the GAL80-VP16 hybrid or the VP16 domain itself in the absence of GAL4 gave no activation of the reporter gene (less than 1% of wild-type GAL4 activity), indicating that the VP16 stimulation of transcription is dependent on GAL4. To show that the VP16 stimulation is dependent on GAL4-GAL80 interaction, a $GAL4^c$ allele was tested. GAL4^c mutants do not interact with GAL80 but retain their ability to activate transcription. The protein encoded by the $GAL4^c\#18$ allele (*5*) did not interact with

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Table 1. A test of the dissociation versus transformation models. Yeast strain YJ0-Z that contained a deletion of *GAL4* and either a *GAL80* or *GAL80-VP16* integration was transformed with a CEN plasmid bearing the indicated *GAL4* allele. *LacZ* gene expression [β-galactosidase activity (β-Gal)] was measured in each strain under inducing (galactose) and noninducing (glycerol-lactate) conditions (*12*). The *GAL4-T858* (Thr^{858} → Leu; Tyr^{867} → Cys) and *GAL4-Y865* (Tyr^{865} → Ser; Tyr^{867} → Cys) alleles of *GAL4* have mutations in the COOH-terminal activation domain that decrease their activation but retain GAL80 interaction.

GAL4 derivative	GAL80 derivative	β-Gal activity (%)	
		Uninduced	Induced
GAL4 wild type	GAL80	<1	100
	GAL80-VP16	140	370
GAL4-T858	GAL80	<1	2
	GAL80-VP16	90	170
GAL4-Y865	GAL80	<1	70
	GAL80-VP16	190	220