Common localization of retention determinants in hepatitis B virus L protein from different strains

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Hepatitis B virus L protein is retained intracellularly, and trans-inhibits secretion of the related S and M proteins, as particulate HBsAg, at high L/S–M ratios. Comparison of equivalent A and D strain mutants suggested that the retention mechanism does not vary with genotype. Contrary to an earlier suggestion, the N-terminal extension specific for A–C strains was found to be inactive as a retention signal. Intact L was more completely retained than any mutated protein. Retained mutants had either a critical PreS stretch, or N-terminal myristate. Also, mutants of the latter class did not completely inhibit particulate budding, and could, in minor amounts, reach the Golgi. We conclude that (i) the principal retention determinant can be traced to the same PreS segment in distinct strains and (ii) myristic acid does reinforce retention in wild-type L, while acting in part as an HBsAg membrane anchor in mutants lacking the internal determinant.

Hepatitis B virus (HBV) Env proteins are unique in that they can leave the cell either with or without an encapsidated core particle (Ganem, 1991). They are inserted into endoplasmic reticulum (ER) membrane as multispan molecules (Fig. 1, top), and as such they are arrested at a pre-Golgi compartment. In the absence of interactions with other viral proteins they produce single-shelled lipoprotein particles (the hepatitis B s antigen, HBsAg), which are secreted into pre-Golgi cisternae (Huovila et al., 1992) and extracellularly. On assembly with capsid, however, they generate the bilayered envelope of infectious HBV virions, secreted along a similar route.

HBsAg production is sustained by the smallest, S, and the middle-sized, M, of the HBV Env proteins. By contrast the largest variant, L, trans-inhibits S–M secretion at high L/S–M ratios (Ganem, 1991). A possible function of L retention is to nucleate virion assembly at the retention site, by binding capsids and diverting the other Env variants from default secretion (Bruss & Ganem, 1991b; Bruss & Thomssen, 1994; Bruss & Vieluf, 1995; Dyson & Murray, 1995). L-induced Env storage in hepatocytes triggers liver damage, mediated by a cytotoxic response, and neoplastic progression in transgenic mice (Chisari et al., 1989).

Mutational studies on the retention mechanism have focused on the PreS domain specific to L (Fig. 1, top), leading to partially discrepant results. Kuroki et al. (1989), analysing L protein from an A (adw) isolate [genotype nomenclature of Norder et al. (1994)], concluded that the L N-terminal myristic acid (Persing et al., 1987), and, independently, the N-terminal stretch 6–13, act as retention signals. On the other hand, work on D (ayw/adyw) subtypes led to a model linking retention to the specialized topogenesis of L (Prange et al., 1991; Gallina et al., 1992, 1994, 1995; Nemeckova et al., 1994). Indeed, recent reports (Ostapchuk et al., 1994; Bruss et al., 1994; Bruss & Thomssen, 1995; Prange & Streeck, 1995; Bruss & Vieluf, 1995) suggest that the L PreS domain, unlike the shorter N-terminal extensions of S and M, fails to be co-translationally translocated by downstream signals: only in a fraction of L molecules does it undergo delayed translocation into secretory cisternae (Fig. 1, top). This would enable PreS to act in distinct L polypeptides as either exposed spike, or matrix domain, lining the interior of the virion envelope. According to an extension (Gallina et al., 1995) of the cited model, PreS cytosolic anchorage would impose a transmembrane folding incompatible with HBsAg assembly–secretion. An internal PreS stretch (D aa 70–99) would be critical for both domain anchorage and protein retention. In this retention model myristic acid, which does not influence PreS membrane topology, was regarded as an accessory or spurious retention determinant. Since the putative A N-terminal signal overlaps an 11 aa N-terminal extension absent in D genotypes (Valenzuela et al., 1979), while the 70–99 stretch shows a few mismatches in the aligned A and D genotypes (Galibert et al., 1979; Valenzuela et al., 1979; Fig. 1, bottom), the possibility remained that conflicting results reflected real subtype differ-

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Fig. 1. HBV Env proteins and secretion phenotype of Env mutants. Top, structure and transmembrane topology of Env proteins. S, M and L share a C-terminal (CT) domain, and differ in their N-terminal extensions (reviewed in Ganem, 1991). CT transmembrane regions are depicted as cylinders. S coincides with the CT domain. M bears a 55 aa extension (PreS2 region), which is cotranslationally translocated into the ER lumen. The L N-terminal domain (PreS) extends a further 108–119 aa (PreS1 region) beyond PreS2, and is N-terminally myristylated. The represented myristic acid insertion into the ER bilayer is purely hypothetical. The PreS domain undergoes a delayed (post-translational) transmembrane translocation only in a proportion of L molecules (Ostapchuk et al., 1994; Bruss et al., 1994; Prange & Strelecki, 1995); PreS is consequently not N-glycosylated at two potential sites, unlike M PreS2, which is modified at its unique N-linked glycosylation site. N, exploited glycosylation site; (N), facultative site; N, cryptic site; two cryptic sites within the CT domain were omitted. Bottom, A (adw2) and D (ayw) strain L PreS domains (Valenzuela et al., 1979; Galibert et al., 1979) expanded to highlight sequence...
Table 1. Transport phenotype of strain A and D Env variants expressed in 293 cells

Protein names reflect HBV genotype nomenclature after Norder et al. (1994). Plasmids inducing the synthesis of the proteins listed above have in part been described (Gallina et al., 1992, 1994, 1995); all D (ayw) variants; L-A and Δ27-132-A (adw2). Constructs expressing Δ2-26-A, Δ2-80-A and Δ2-110-A were obtained by introducing into the A (adw2) strain preS-S gene PCR-mediated deletions, as described for the corresponding D constructs (Gallina et al., 1995). In Δ2-99-D and Δ2-110-D, the extranumerary Ala residue is between Met1 and the first PreS residue downstream from the deleted segment (Pro100 D; Pro 111 A). Ala2/13-A and Ala2/13-A27-132-A coding genes were created by amplifying the preS region with a 5' mutagenic primer introducing Ala codon (GCA) substitutions at Gly codons 2 and 13. Gly13 is the myristylated N-terminal residue in the minority of L-A chains translated starting from ATG12. In immunoprecipitation (IP) experiments, secretion was checked by visual evaluation of fluorograms. For enzyme immunoassay (ELIA), the reported secretion ratios are the average of three replicates. Ratios are expressed as mean ± variation interval. Results obtained with HepG2 cells are in parentheses. ELIA secretion values are large overestimates for retained forms, as their intracellular breakdown and lowered immunoreactivity were not taken into account.

<table>
<thead>
<tr>
<th>Protein/genotype</th>
<th>Mutations within PreS</th>
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<th>EIA</th>
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<td>−</td>
<td>−</td>
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</table>

ences in retention mechanisms. This was tested here by comparing equivalent A and D strain L variants, bearing informative mutations within the PreS domain.

The proteins analysed are listed in Table 1. Taking L of either strain and M-D as wild-type retained or secreted controls, respectively, mutations were placed to evaluate the correlation between retention and the presence/absence of the following elements: (i) N-terminal myristic acid; (ii) the PreS N-terminal portion spanning the supposed A strain N-terminal retention signal; and (iii) the PreS internal stretch (D aa 70-99, corresponding to 81-110 in A) implicated in both cytosolic anchoring and L retention (see Fig. 1, bottom). All proteins were produced in 293 cells via transfection (calcium phosphate method) of the corresponding genes, cloned into pRPRSV vector.

To measure protein export, transfectants (~ 3 x 10^6 cells per 25 cm² flask) were processed for pulse/chase labelling and immunoprecipitation 36 h post-transfection, as described (Gallina et al., 1995). Methionine-starved cells were pulsed in Met-free medium with [35S]Met (150 μCi/ml; ~ 1000 Ci/mmol).
Fig. 2. (a) Pulse/chase analysis of cell line 293 transfectants. Cells expressing the indicated proteins and the corresponding culture supernatants were processed for immunoprecipitation as described in the legend to Table I. (b) Deleted, N-terminally myristylated L derivatives can reach the Golgi. Transfectants were pulsed/chased as above. Alternatively (c), they were lysed 3 days post-transfection without prior radioactive labelling, and subjected directly to immunoprecipitation. After SDS-PAGE, proteins were blotted onto nitrocellulose membrane and probed with anti-PreS2 MAb 4408 (Institute of Immunology, Tokyo). Alkaline phosphatase-conjugated anti-mouse serum was used as the secondary antibody, and nitro blue tetrazolium plus 5-bromo-4-chloro-3-indolyl phosphate as the substrates for visualization. In (a)–(c) mass and position of wild-type S and M (D strain) and L (A strain) polypeptides are indicated. Symbols indicate the glycosylation state of wild-type and mutant Env forms: •, nonglycosylated Env polypeptide; ○, Env glycosylated in the CT domain; *, Env glycosylated in the PreS domain. PreS glycosylation takes place at the PreS2 N terminus in M-D, and at the PreS1 site in mutants (see Fig. 1). The vertical bars at the right of some lanes indicate Golgi glycosylated Env forms. (d) S protein helper effect on secretion of L variants. 293 cells were transfected with a mixture of the plasmids encoding the indicated Env variants plus a plasmid inducing the synthesis of S (Gallina et al., 1992, 1995), at decreasing ratios. Secretion at 3 days was measured by EIA, as described in the legend to Table 1.

Cell lysates and culture media were subjected to immunoprecipitation with Sepharose-conjugated sheep anti-HBs IgGs. After SDS–PAGE, fixed gels were soaked for 30 min in Amplify (Amersham) solution, dried and exposed to X-ray film overnight at −70 °C. Results (summarized in Table 1; key A mutants in Fig. 2a) indicated a strict parallelism between transport phenotypes of corresponding A and D variants. Namely, retention was observed when either myristic acid, or the internal stretch crucial for PreS cytosolic anchoring, or both, were present. The PreS N-terminal portion of either subtype was ineffective by itself in blocking both domain translocation (signalled by a glycosylated PreS sequence; Table 1 and Fig. 2a–b) and whole polypeptide secretion.

To quantify secretion, intra- and extracellular HBsAg were
measured in transfectants with an enzyme immunoassay (EIA). Cells and media were collected 3 days post-transfection. Washed cells were lysed in 1 ml 10 mM-Tris–HCl, pH 7.5, 150 mM-NaCl, 0.5% Triton X-100 supplemented with complete protease inhibitors cocktail (Boehringer), and centrifuged for 5 min in a minifuge to pellet nuclei. Cleared lysates were diluted with 5 vols of DMEM–10% foetal bovine serum. Transfection media (5 ml) were cleared of cell debris and 0.2 vols of lysate from mock-transfected cells was added. Intracellular and secreted Env antigen was then assayed with the Auszyme EIA test (Abbott). Data (Table 1) confirmed the immunoprecipitation outcome, while suggesting some differentiation of retained mutants. First, L was shown to be the most strictly retained. The non-myristylated, full-length mutants were slightly less inhibitory of secretion, like A and D mutants with a central deletion spanning the PreS1 C-terminal tract but maintaining a myristylated N terminus (Δ22-123-D and Δ27-132-A). Directional shortening of PreS through D aa 70 (A 81) resulted in a moderate increase of secretion, while further removal of aa 70–99 (A 81–110) resulted in a sudden rise of secretion to M-like values. To further verify these results in a cell host supportive of HBV replication, the EIA experiment was repeated in HepG2 hepatoblastoma cells with a choice of plasmids. Results (Table 1) confirmed those with 293 cells.

It has been shown that in D strain L, as well as in mutants conserving the internal determinant, retention involves the inhibition of Env budding into pre-Golgi cisternae lumen as lipoprotein particles. On the contrary, HBsAg Budding is not completely inhibited in internally deleted mutants still bearing myristic acid (Prange et al., 1991; Gallina et al., 1995). This fact was confirmed for mutant Δ27-132-A. Transfectants were subjected to a cell disruption-fractionation procedure, which releases luminal HBsAg from intracellular cisternae, as described (Prange et al., 1991; Gallina et al., 1995). In agreement with previous results with the equivalent D variant (Δ22–123-D), the extractable Δ27–132-A proportion accounts for 35% of the total protein. Furthermore, a minor proportion of the internally deleted mutants can leak to the Golgi, as indicated by the presence of a smear of slow-migrating electrophoretic forms, suggestive of Golgi modification of N-linked sugars, revealed in some immunoprecipitation experiments (Fig. 2a, b). Smeared forms comigrate with the secreted fraction of the corresponding non-myristylated mutant (Fig. 2a, b). These forms were better visualized by Western blotting (Fig. 2c) and were found to be resistant to endoglycosidase H (data not shown). An interpretation of these findings is that myristic acid alone does not completely inhibit particulate budding, and is even permissive for subsequent HBsAg transport to the Golgi stack. Since HBsAg budding takes place in an intermediate compartment preceding cis-Golgi (Huovila et al., 1992), myristic acid is seemingly acting in mutants lacking the internal retention determinant as an HBsAg lipid anchor, appending the budded particles to the luminal face of the secretory cisternae bilayer, and allowing some further transport to distal Golgi compartments. We are presently investigating whether appended HBsAg can reach, and be exposed on, the cell surface. However, we surmise that, even in internally deleted mutants, the action of isolated myristic as a retention determinant must involve principally interference with HBsAg biogenesis/transport, as the majority of polypeptides in such mutants are retained in a pre-Golgi compartment.

Our results seem to disprove a role as linear retention signal of the N terminus of A strain L. This was proposed to contain a basic retention motif (KPRK) similar to that found at the C terminus of type I glycoprotein cytoplasmic domains (Prange et al., 1991). However, in the absence of a PreS cytosolic anchoring/retention determinant, the A strain N terminus undergoes early (cotranslational) translocation into the ER lumen (Gallina & Milanesi, 1993; Gallina et al., 1995; this work), and might no longer mediate the hypothetical retention. In addition, the work by Kuroki et al. (1989) exploited an SV40 vector/COS-7 cell system producing S (and to a lesser extent, M) in amounts exceeding the directionally deleted mutants under study. When coexpressed, secretable variants trans-suppress the retention induced by the internal PreS stretch (Gallina et al., 1995; Bruss & Vieluf, 1995), in a dose-dependent manner. Then, the possibility remained that the A strain N-terminal sequence requires a complete, cytosolically disposed PreS domain to act as a retention signal. An additional N-terminal signal would be masked by the internal determinant when L is the most abundant envelope form (as happens in our system), and unmasked when an excess of S suppresses the internal determinant. To check this point, secretion of wild-type L, of the non-myristylated, full-length variant and of the deletion variant lacking N terminus were compared at decreasing variant/S ratios, in both strain series, by cotransfecting cells with decreasing ratios of mutant-encoding/S-encoding plasmids. Plotting secretion values against variant/S ratios (Fig. 2d), curves of all the mutants were found to converge to moderate secretion levels at low ratios, regardless of the strain. In contrast, for both strains, the wild-type L curve significantly diverged, indicating greater inhibition of secretion at the lowest ratios. Thus, these results also fail to reveal a special role for the A strain L N-terminal stretch – either alone or as an accessory element – in retention, while supporting myristic acid as an element enhancing the internal determinant effect.

The present paper, extending earlier work, suggests that sequence differences between L protein of A and D subtypes are irrelevant with respect to L secretion inhibitory properties, which are caused by common retention determinants. These are the internal stretch which also blocks early PreS translocation, and the N-terminal myristic acid. Upstream PreS1 sequence strengthens the action of the internal determinant, probably contributing to a retention-proficient PreS conformation. The two principal determinants cooperate in wild-type L to produce a maximal retention effect.
The internal determinant, unlike myristic acid alone, is sufficient to impose both delayed PreS domain translocation and a strictly pre-Golgi transport arrest (Gallina et al., 1995; this work). Its action in molecular terms must now be defined. That it alters the conformation of the PreS2 region rendering PreS unsuitable for early translocation, that it represents a protein–protein interaction module mediating intra- or inter-L chain contacts, that it binds cellular chaperones, or that it is targeted by a cellular kinase (as phosphorylation of the homologous duck hepatitis B virus PreS domain might indicate; Grigacic & Anderson, 1994) are among the hypotheses to be explored.

Myristic acid has been known from case to case either to act as a constitutive/switchable membrane targeting signal, or to mediate intraprotein contacts (Johnson et al., 1994). Since the location of wild-type L myristic acid adduct is presently unknown—involved in hydrophobic interactions with the L polypeptide chain, or buried in a membrane bilayer?—it is equally possible that it contributes to retention by stabilizing the secretion-inhibitory L folding, or by independently interfering with HBsAg budding. In mutants with the internal determinant removed, the fatty acid seemingly has a mixed effect, including either interference with budding, or intracisternal anchoring of budded HBsAg. Whether myristic acid anchoring is indicative of any biological function in intact L remains to be ascertained. The myristic acid adduct is dispensable for virogenesis but essential for cell infection (Gripon et al., 1995, and references therein). One might thus speculate that the anchoring function has a role at early stages of cell penetration by HBV (e.g. in cell surface attachment or in virus–membrane fusion), when the viral envelope might undergo structural changes exposing L fatty acid. With this hypothesis, the anchoring action observed in the anterograde transport of particulate L mutants would be the converse of the essential action, relevant for infection.

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References


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