Identification of structural determinants of the first transmembrane domain of the small envelope protein of duck hepatitis B virus essential for particle morphogenesis

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The envelope of duck hepatitis B virus (DHBV) consists of the small (S) and large (L) envelope proteins, which share a common C-terminal mult spanning transmembrane region but differ by the long N-terminal pre-S domain of L, which is essential for interactions with both the receptor and nucleocapsid. To achieve these dual functions, L acquires mixed topologies through S-dependent post-translational translocation of its pre-S domain. This study has examined the role of S in this unusual mechanism of translocation by analysis of the α-helical transmembrane domains and their potential to engage in lateral interactions for envelope assembly. Through mutagenesis in constructs expressing the S and L envelope proteins independently, transmembrane domain 1 was identified as an essential structural determinant in S. Two polar residues in this helix were identified as contributing to L protein translocation through the assembly of S into particles, implying that the topological switch of L is part of the assembly and maturation process. The same domain in L was shown to be dispensable for L translocation and assembly, suggesting that transmembrane domain 1 of L and S have different functional roles and structural arrangements on the assembled particle. The conservation in all hepadnavirus envelope proteins of two polar residues at positions 24 and 27 of transmembrane domain 1, the former positively charged, points to this being a common determinant in particle morphogenesis for all hepadnaviruses.

Introduction

The envelope of duck hepatitis B virus (DHBV) consists of two surface proteins, the small (S) and the large (L) envelope proteins, which are produced by differential translation initiation from a single pre-S/S open reading frame (ORF) such that the L protein consists of a pre-S domain of 161 amino acids and the C-terminal S domain of 176 amino acids, which comprises the 17 kDa S protein (reviewed by Nassal, 1996). The S protein, and consequently all surface proteins, contains four hydrophobic regions, which are predicted to form α-helices that traverse the membrane bilayer. The location of these four α-helices is conserved in the Hepadnaviridae, except for DHBV, which appears to be missing the third conserved transmembrane (TM) region as indicated by incomplete alignment with the mammalian sequence (Stirk et al., 1992). Thus, the third and most C-terminal transmembrane domain of the DHBV envelope proteins is referred to as TM3.

The assembly of the envelope proteins and their involvement in entry of the virus are closely linked to a unique protein transport process adopted by the hepadnaviruses. This ability of the virus to translocate a large N-terminal portion of the fully translated L protein, and to limit this process to achieve mixed membrane orientations or topologies, are underlying features of hepadnavirus assembly and regulation. The L protein displays a more complex functional role than that expected of a viral structural protein and this is partly achieved by its mixed topology. The orientation of viral envelope proteins spanning the endoplasmic reticulum (ER) membrane is mirrored in the mature particle, so that those domains located in the ER lumen are found on the ectodomain of the particle and cytosolic domains remain internally disposed. Thus, the translocated form of L makes pre-S sequences available on the external surface of the mature virion for receptor binding (Klingmuller & Schaller, 1993; Le Seyec et al., 1999), while maintenance of an internal pre-S domain enables the L protein to take on the suggested role of a matrix protein for interaction with the nucleocapsid (Bruss et al., 1994) (Fig. 1A). The L protein also controls the size of the pool of
Fig. 1. S is required for changes to L protein topology. (A) Models of the internal form of L, present immediately after synthesis, with pre-S (black line) and TM1 being cytosolically disposed, the external form of L with a translocated N-terminal pre-S domain and the S protein, which has the same C-terminal transmembrane region as L. The arrows indicate the major sites susceptible to trypsin cleavage in pre-S. (B) Schematic representation of the L expression construct lacking a start site for S (L\(^{+}\)S\(^{-}\)) and the S expression construct created by deletion of the pre-S domain (L\(^{-}\)S\(^{+}\)) (Gazina et al., 1998). The TM domains are indicated by the numbered black boxes and the amino acid numbers in italics indicate the boundaries of TM1 and TM3. (C) Protease protection analysis of microsomes prepared from LMH cells co-transfected with L\(^{+}\)S\(^{-}\) and L\(^{-}\)S\(^{+}\) (L/S) or transfected with L\(^{+}\)S\(^{-}\) alone (L). Microsome samples were subjected to digestion with trypsin in the absence or presence of NP-40, or left untreated, as denoted above each lane. Protease protection of L was analysed by Western blotting with a monoclonal anti-S antibody, which detects both L and S proteins.

the replicative template, covalently closed circular DNA (cccDNA), at least partly by determining the assembly of core particles into virions and perhaps also by a more direct regulatory role (Summers et al., 1991).

A unique feature of the hepadnaviruses in their status as enveloped viruses is that little or no (hepatocyte) membrane proteins are present and the structural organization of the lipid bilayer is not maintained in the mature particle (Gavilanes et al., 1982; Satoh et al., 2000), suggesting that the translocation, assembly along the ER membrane and budding of the particle involve a more compact interaction between the envelope proteins. In this regard, it has been proposed that the post-translational translocation of L occurs through a channel created from lateral interactions between the amphipathic TM regions of the S domains, excluding TM2, which is the membrane anchor region and entirely hydrophobic (Guo & Pugh, 1997; Stirk et al., 1992). For such a complex to be functionally significant, the presence of S is expected to be an essential requirement for pre-S translocation. We have previously made an initial investigation of this hypothesis and have shown that S is indeed required for protease protection of L, i.e. the translocation of newly synthesized L protein, which in the absence of S remains cytosolically disposed and sensitive to protease digestion (Grgacic et al., 2000). We have also observed augmentation of L protein phosphorylation with S co-expression, providing a further indication that S causes a conformational change in the pre-S domain of L (Grgacic, 1996). Moreover, the ability of acid or reducing agents (Grgacic & Schaller, 2000; Guo & Pugh, 1997; Lu et al., 1996) to trigger the complete translocation of the pre-S domains held in an intermediate stage of translocation, as shown in mature DHBV particles, suggests that the translocation mechanism is associated with the assembly and maturation of the viral envelope.

Increasingly, TM domains are being identified as important structural elements in membrane protein assembly and for
many enveloped viruses, budding is dependent on the formation of an envelope lattice through lateral interactions of the TM domains (Garoff et al., 1998). Lateral interactions may occur through hydrogen bonding of the side groups of polar residues, such as asparagine, arginine and glutamic acid, or by helix packing through the regular meshing of side chains, as exemplified by heptad repeats or leucine zipper motifs (Gurezka et al., 1999; Ubarretxena-Belandia & Engelman, 2001). The latter have been implicated in the folding and/or oligomerization of a variety of cellular and viral membrane proteins (Gurezka et al., 1999), including gp41 of human immunodeficiency virus type 1 (Center et al., 1997). Assembly of the HBV envelope involves the accumulation of S monomers along the ER, where initial contacts may involve such lateral TM interactions before they bud into the lumen and are stabilized by disulphide bonds into dimers, eventually forming higher-order oligomers. However, of the 14 cysteine residues in HBV, only the three conserved cysteines of the first hydrophilic loop are essential for particle secretion and these do not form intermolecular disulphide bonds (Mangold & Streeck, 1993). That the DHBV envelope contains only these three cysteines further points to TM helices playing a role in the initial stages of hepadnavirus assembly.

The aim of this study was to investigate whether the transmembrane helices of the S protein play a role in translocation and morphogenesis. Through mutagenesis using constructs expressing the S and L envelope proteins independently, we have identified TM1 as an essential structural determinant in S, with charged, polar residues contributing to L protein translocation and particle assembly.

**Methods**

**Plasmids.** Constructs encoding wild-type DHBV L (L’S) and DHBV S (L’S), described previously (Gazina et al., 1998), were used for co-transfections and mutagenesis studies. Deletion mutants ΔTM1 and ΔTM3 were created by substitution of a KpnI–BstEII fragment from the plasmids pMDL ΔTM1 (A nt 1294–1358 of DHBV DNA (Mandart et al., 1984)) and pMDL Δlam–BstEII (A nt 1656–1847) (a gift from H. Schaller, ZMBH, Germany) with the corresponding fragment from L’S or L’S’. Amino acid substitutions were introduced into the S ORF of L’S by overlap extension PCR, as described previously (Grgacic et al., 1998). The outside non-mutating primers were sense strand 5’-ATGGTCGGGCAAATTTCC 3’ (DHBV nt 905–922) and antisense 5’-CCAAATGTATGCTGCTATTACT 3’ (DHBV nt 2050–2030). L’S was used as the template and the overlap extension PCR product containing the desired mutation was digested with KpnI (nt 1294) and BstEII (nt 1847) and introduced into L’S’ by subcloning into the same restriction sites via three-way ligation. Plasmids for multiple mutants were constructed sequentially with the same overlapping PCR method using the appropriate mutant DNA as a template.

**Cells and transfections.** Chicken hepatocyte LMH cells (Leghorn male hepatoma) were maintained in Dulbecco’s modified Eagle’s medium (DMEM-F12) supplemented with 10% foetal bovine serum. Transfections were carried out by the dextran sulphate method, as previously described (Grgacic et al., 1998) using 5 μg of DNA per well in six-well multiplates (Greiner).

**Protease protection analysis.** Microsomes were prepared according to the method of Prange & Streeck (1995) with modifications. Transfected LMH cells (two 30 mm diameter wells) were washed in cold Tris-buffered saline (TBS; 50 mM Tris–HCl, pH 7·5, 150 mM NaCl). The monolayers in each well were incubated on ice with 400 μl 0·1× TBS for 10 min and then harvested by scraping, pooled and dispersed by drawing five times through a 26G needle. The homogenate was adjusted to 1× TBS with 5× TBS and centrifuged for 20 min at 2500 r.p.m. at 4°C to remove unbroken cells and nuclei. The supernatant was removed and set aside while the pellet was again dispersed in 300 μl TBS and centrifuged as before. Supernatants were pooled and layered on to 2·7 ml 250 mM sucrose in TBS and centrifuged for 30 min at 38000 r.p.m. at 4°C in an SW60 rotor (Beckman). The microsomal pellets were washed once with TBS and resuspended in 65 μl TBS. For trypsin protection analysis, the microsomal preparation was divided into three 20 μl aliquots. One sample was left untreated while the remaining two were treated with 25 μg/ml of trypsin (TPCK treated; Worthington Biochemical Corporation) with or without 0·5% NP-40 for 1 h on ice. Proteolysis was halted by the addition of 30 μg/ml aprotinin (Boehringer) and further incubated on ice for 20 min. Five μl of 5× Laemmli buffer was then added to each sample and boiled for 5 min prior to separation by 13% SDS–PAGE followed by Western blotting to detect the L protein.

**Western blot analysis.** Proteins were separated by 13% SDS–PAGE and transferred to nitrocellulose membrane (Schleicher and Schüll) using a Trans-Blot SD semi-dry transfer cell (Bio-Rad). Membranes were blocked for 1 h with 3% skim milk in PBS plus 0·3% Tween 20 (PBST). Membranes were probed with monoclonal anti-S (7C.12) and anti-pre-S (H1·1) (Pugh et al., 1995) for 1 h in 1% skim milk in PBST, then washed with PBST and probed with goat anti-mouse Ig–horseradish peroxidase (Amersham) in 1% skim milk in PBST. After a final wash in PBST (3 × 10 min), protein bands were visualized by enhanced chemiluminescence (ECL) (Amersham).

**Pulse–chase analysis and carbonate extraction.** Transfected LMH cells were starved in methionine/cysteine-free media (ICN) for 30 min before pulse-labelling with 150 μCi/ml TRAN35S-label (ICN) for 30 min. Cells were harvested immediately after labelling and after a 4 h and 24 h chase with unlabelled media. A crude microsome preparation was prepared as described above except that the ultracentrifugation through sucrose was omitted. Microsomes were extracted with 0·1 M Na2CO3 according to the method of Bruss & Ganem (1991b) with modifications. Briefly, 200 μl microsome fractions were treated with 4 ml 0·1 M Na2CO3, pH 11·5, on ice for 30 min. The sample was then ultracentrifuged for 30 min at 100 000 g using an SW60 rotor. The top 3·5 ml of supernatant was removed, the next 0·5 ml discarded and the remaining 200 μl was regarded as the pellet. The supernatant and the pellet were neutralized with acetic acid and adjusted to 1× RIPA buffer with 10× RIPA buffer (100 mM Tris, 2·5 M NaCl, 10 mM EDTA, 10% NP-40, 5% sodium deoxycholate, 1% SDS), and labelled envelope proteins were immunoprecipitated with monoclonal anti-S (7C.12) (Pugh et al., 1995) at 4°C for 16 h. Immune complexes were pelleted following incubation with 60 μl of a 10% slurry of Protein A–Sepharose for 1 h at 4°C, washed twice with 1× RIPA buffer and boiled in Laemmli buffer for SDS–PAGE and autoradiography.

**Isolation of intracellular and extracellular particles.** For extracellular particles, media from transfected LMH cells was harvested 3 days post-transfection and clarified of non-adherent cells by centrifugation for 5 min at 2000 r.p.m.

For intracellular particles, cell monolayers were washed twice with PBS and harvested by scraping cells into 1 ml PBS. Harvested cells were
freeze/thawed three times with vigorous vortexing upon thawing. The cytosol fraction (supernatant) was obtained by centrifugation for 1 min at 10000 r.p.m. in an Eppendorf centrifuge. This procedure has been used in this laboratory to release DHBV particles capable of infecting primary duck hepatocytes from transfected cells. Particles in the clarified media or cytosol fraction were diluted to 6 ml with PBS and pelleted for 3 h at 38000 r.p.m. in an SW40 rotor through 3 ml of 20% sucrose on to a 2 ml 70% sucrose cushion. The fraction at the 20–70% interface was collected from the bottom and methanol-precipitated for 16 h at −20 °C, followed by separation by 13% SDS–PAGE and analysis by Western blotting.

For the cell membrane fraction, following removal of the cytosol fraction the pellet was resuspended in 100 µl PBS with 1% NP-40 by vigorous vortexing and centrifuged for 1 min at 10000 r.p.m. in an Eppendorf centrifuge. The supernatant or membrane fraction was removed and 20 µl was mixed with 5 µl 5 × Laemmli buffer for SDS–PAGE.

Results

TM3 of S and TM1 of L are dispensable for L protein translocation

In this study we have assessed the resistance to protease of microsomal membrane-associated L chains that were synthesized from an expression plasmid encoding full-length L protein but lacking the start site for S (L’S−) (Fig. 1B).

L chains expressed in the presence of S in microsomal vesicles were protected from protease digestion and deemed to have been translocated across the ER membrane to the lumen (Fig. 1C, L/S), while L chains expressed without S were susceptible to digestion (Fig. 1C, L).

To assess the role of the transmembrane domains of S in L translocation, L’S− was co-transfected with a construct only able to synthesize S (L’S+) in which various deletions of the amphipathic TM domains were made (Fig. 2A). Microsomes were prepared from co-transfected LMH cells, treated with trypsin, and protection of L was assessed by Western blotting. TM1 appeared to be a critical structural element of the S protein, since deletion of this domain resulted in little or no detectable S expression, most likely due to aberrant protein folding and degradation. As a consequence, L protein was not protected from trypsin digestion in the absence of detergent (Fig. 2B, ∆TM1). In contrast, S protein with a deletion in the region encompassing TM3 was tolerated and still able to protect L protein from protease attack (Fig. 2B, ∆TM3).

![Fig. 2. TM1 is essential for proper S folding. (A) Schematic representation of S TM deletion mutants. The transmembrane domains are indicated by the numbered black boxes and the amino acid numbers in italics indicate the boundaries of the deleted regions. (B) Microsomes were prepared from LMH cells co-transfected with L’S+ and the S expression construct L’S−, either with a deletion in TM1 (∆TM1), a deletion in the region encompassing TM3 (∆TM3) or wild-type for S expression (w.t.). Microsomes were subjected to protease protection analysis as for Fig. 1. The trypsin digestion product of approximately 17 kDa, derived from L through cleavage at the very C-terminus of pre-S is denoted by the asterisk.](image)
Role of TM1 in DHBV morphogenesis

Fig. 3. TM1 of L is dispensable for L protein translocation. (A) Schematic representation of L+S− with a deletion in the TM1 domain. The amino acid numbers in italics indicate the boundaries of the deleted region in TM1. (B) Microsomes were prepared from LMH cells co-transfected with either the L+S− construct with a deletion in TM1 of L and the S expression construct L−S+ (∆TM1) or the expression constructs for the wild-type proteins, L+S− and L−S+ (w.t.). Microsomes were subjected to protease protection analysis as for Fig. 1.

Because TM1 was a critical determinant of S protein stability, we therefore wished to examine whether this domain was also essential in the L protein. Co-transfections were conducted with L−S+ and L+S− but with the TM1 deletion in L (Fig. 3A). Deletion of TM1 in L had no adverse effect on L folding/expression or protection (Fig. 3B, ∆TM1), indicating that this region in L is dispensable for the normal protein folding and translocation process. This was surprising given the importance of the same domain for folding of the S protein and may reflect a stabilizing effect of the N-terminal pre-S extension. This result indicates that interactions between TM1 of L and S are not required for L translocation.

Multiple amino acid substitutions in the leucine zipper motif of TM1 of S affect L translocation

Possible motifs in TM1, which may impact on S subunit interaction and thus L translocation, were examined. Analysis of the amino acid sequence of this domain when displayed on an α-helical wheel diagram revealed that one face of the helix is rich in the hydrophobic residues leucine and isoleucine, and appears to constitute a leucine zipper-like motif (Fig. 4A, B). The heptad repeat consists of isoleucine and leucine at each seventh position but with an omission or stutter of three residues from the heptad pattern for leucine 26, as commonly found (Lupas, 1996; Stirk et al., 1992). To assess whether the...
leucine zipper-like motif in S plays a role in L translocation, each position of the repeat sequence (I8, L15, L22 and L26) was substituted with alanine. Single amino acid substitutions in S had no affect on the L protein in the protease protection assay (Fig. 5). Given the nature of the interaction of leucine zippers, where close side chain packing occurs, multiple hydrophobic contacts would be a likely requirement for adequate association. Further double, triple and quadruple substitutions with alanine were made sequentially from the N-terminal position in TM1 and assessed for protease protection of L (Fig. 6A, C). Two or more amino acid substitutions resulted in a loss of L protection, indicative of this structural motif in S affecting L translocation (Fig. 6A, ST1.2, ST1.3 and ST1.4). These multiple mutations in S had no affect on the membrane insertion and consequently the expression of S. However, two or more mutations appeared to have a global effect on S as can
be seen from the sequential increased mobility of these mutant S proteins in SDS–PAGE. Furthermore, a variable amount of a protein of approximately 20 kDa, co-migrating with the L protein trypsin digestion product, was seen in lanes without trypsin with all S mutants (Fig. 6A, lane marked with asterisk). Treatment of S mutant proteins under more stringent conditions of denaturation and/or reduction had no effect on the presence of this 20 kDa S-related protein (data not shown). However, treatment of ST1.4 with endoglycosidase H resulted in the removal of the higher molecular mass species (Fig. 6B, lane 2), indicating that a fraction of the mutant S proteins are glycosylated. DHBV envelope proteins contain a potential glycosylation site downstream of TM2, at asparagine 99 of S, which is not utilized in contrast with HBV (which exhibits partial glycosylation of all its envelope proteins). We concluded that the mutations in TM1 resulted in more global changes in S folding, which resulted in a small proportion of S glycosylation. However, the mutations did not affect S stability.

Amino acid substitution of the two polar charged residues in TM1 of S affects L translocation

In addition to the leucine zipper-like motif, analysis of the amino acid sequence of TM1 revealed two charged residues, lysine 24 and glutamic acid 27, on the opposite face of the α-helix (Fig. 4B). Since charged or polar residues in TM domains have been shown to be involved in inter-helical hydrogen bonding and the dimerization or stabilizing of oligomeric transmembrane proteins (Therien et al., 2001; Ubarretxena-Belandia & Engelman, 2001; Zhou et al., 2000), the effect on L translocation of alanine substitution of these two residues in S was also examined. LMH cells were again co-transfected with wild-type L and either wild-type L or L with a double alanine substitution at K24 and E27 (L w.t./S K24/E27). Microsomes were subjected to protease protection analysis as for Fig. 1.

Charged residues in TM1 are required for particle formation

While the above mutations in S were shown to affect L translocation, it was not clear that the effect was a direct result of a loss in S subunit interactions. To assess the role of the leucine zipper-like motif and the charged residues of TM1 on envelope assembly, LMH cells co-transfected with mutant S and wild-type L were examined for the presence of intracellular and extracellular particles. Media and the cytosolic cellular fractions, obtained from three cycles of freeze/thawing and centrifugation, were layered on a 20% sucrose cushion and any sedimented particles at the 70–20% interface were observed by Western blotting following methanol precipitation. Co-transfection with wild-type L and L resulted in both intracellular (I) and extracellular (E) particle formation as expected (Fig. 8A, panel 1). The K24A/E27A mutant was not able to assemble into particles, as neither intra- nor extracellular particles were detected (Fig. 8A, panel 2). Surprisingly, the ST1.4 quadruple mutant was able to assemble, as intracellular particles were clearly detected. However, few or none of these assembled particles were exported, indicative of aberrant particle formation (Fig. 8A, panel 3). Similar levels of L and S protein expression for all three co-transfections were confirmed by assessing the membrane fraction of the cells by Western blot (Fig. 8A, lanes 4–6).

During hepadnavirus budding, the envelope proteins assemble along the ER and are then extruded to the lumen thereby entering the normal vesicular, secretory pathway, at which point they are deemed to be no longer integral membrane proteins and are extractable with carbonate at pH 11.5. To further assess the budding and secretion process of the two S mutants and to confirm the above results obtained by ECL Western blotting, [35]methionine pulse–chase labelling of wild-type and mutant S proteins was performed, followed by carbonate extraction. Wild-type L and S budding were found to be extremely slow, with only partial conversion from the ER membrane pellet fraction (P) to the carbonate-extractable or supernatant fraction (S) after 24 h (Fig. 8B, panel 24 h, lane 2). The K24A/E27A S mutation resulted in greatly reduced L protein translocation as determined by the protease protection assay (Fig. 7).

Fig. 7. Polar residues in TM1 of S are required for L translocation. Microsomes were prepared from LMH cells co-transfected with wild-type L and either wild-type L or L with a double alanine substitution at K24 and E27 (L w.t./S K24/E27A). Microsomes were subjected to protease protection analysis as for Fig. 1.
Discussion

The S protein is the major structural component of hepadnaviruses. Able to form the envelope lattice independent of the viral capsid (or L protein), it also drives the budding and secretion of subviral and viral particles. The implication of this sequential mode of assembly is that specific, lateral interactions occur between the S subunits, with the transmembrane \( \alpha \)-helices being the candidate structures involved. In the context of the post-translational translocation of L, where S is a requirement in DHBV (Grgacic et al., 2000), we have assessed the candidate structural elements in S that may impinge on this vital modification of the receptor-binding L protein.

Using constructs that express L and S independently, we have demonstrated that envelope protein functional diversity is not limited to the pre-S domain and the mixed topologies it achieves, but that TM1, a common domain of L and S, appears to have different functional roles in L and S. This study has indicated that TM1 in S is essential for proper S folding and stability. Similar studies on HBV S protein have shown that although TM1 was not essential for membrane insertion, it played a role in assembly either directly or indirectly (Bruss & Ganem, 1991a; Prange et al., 1992). While this deletion in DHBV S had a more drastic effect than previously observed in HBs, together these results and the apparent non-essential role of TM3 strongly pointed to TM1 as the region engaged in envelope interaction. In contrast, deletion of TM1 in L was well tolerated, allowing L to be membrane-inserted, translocated to the ER lumen (Fig. 3B) and secreted with S in particles (data not shown). Thus, TM1 of L appears to be dispensable for the translocation/assembly process. Previously, we have shown that low pH treatment of particles released a hidden, potentially fusogenic, hydrophobic domain, identified as TM1 (Grgacic & Schaller, 2000). These results and studies with synthetic peptides of TM1 implicate this domain of L in the viral fusion process (Rodriguez-Crespo et al., 1999). We conclude that TM1 of L has a different functional role to that of S and, importantly, that the way L is incorporated into the particles must differ from S.

TM1 of S was shown to have two important structural determinants for particle assembly: (i) a leucine zipper-like motif on one face of the \( \alpha \)-helix, and (ii) two charged, polar...
residues, lysine 24 and glutamic acid 27, on the opposing face. Both these features were shown to play a role in the ability of S to facilitate L translocation as determined by the protease protection assay. Multiple mutations in the leucine zipper-like motif resulted in defects in L translocation and particle export, suggestive of aberrant particle formation. It is not clear whether L translocation is a prerequisite for particle secretion or whether a more global change had occurred in the envelope, which inhibited export. The partial glycosylation of these leucine zipper mutants implied such a change in S occurred, since the glycosylation site is located adjacent to TM2 well downstream of TM1. However, these changes were not sufficient to inhibit particle assembly.

The lack of particle assembly of the K24A/E27A S mutant suggests there is a relationship between assembly of S into particles and its ability to translocate L. Polar residues are unusual in transmembrane domains: the energetic cost of their insertion into a hydrophobic environment usually heralds some specific functional role (Bonifacino et al., 1991; Cocquerel et al., 2000; Ubarretxena-Belandia & Engelman, 2001). If exposed, they are predicted to be involved in inter-helical hydrogen bonding or, if charged, to form salt bridges with similar anti-parallel domains. Previously it has been predicted that the TM helices had the potential to form a proteinaceous similar anti-parallel domains. Previously it has been predicted hydrogen bonding or, if charged, to form salt bridges with some specific functional role (Bonifacino et al., 1991; Ubarretxena-Belandia & Engelman, 2001). If

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\[ \text{Fig. 1A} \]

which also contains several charged residues and may form the hydrophilic lining of the channel. One could further speculate that the same residues in L will necessarily be sequestered to stop unwanted helix interactions, again suggestive of a different conformational arrangement from S and possibly also accounting for the binding of Hsc70 chaperone to L (Hildt, 1997; Prange et al., 1999).

Although this study identified a link between particle assembly and L translocation, no direct interaction (dimerization) between the envelope proteins could be demonstrated in the ER membrane, either by chemical cross-linking with a membrane permeable cross-linker or by velocity sedimentation of envelope containing cell lysates (data not shown). This may be due to the technical difficulty in capturing non-covalent envelope interactions.

None the less, the results from this study provide further supportive evidence that L translocation in DHBV involves correct assembly of S into particles. These data, together with the demonstration of an intermediate L topology in DHBV, i.e. one where the pre-S domain is not fully translocated to the ER lumen but traverses the viral envelope in the mature particle, support the model that the envelope proteins assemble to form their own translocation channel. An envelope-formed channel would allow the long hydrophilic pre-S domain to not only traverse the viral envelope but also be retained in that topology in the mature particle for subsequent release by such conditions as low pH, as previously described (Gracic & Schaller, 2000; Guo & Pugh, 1997). Whether an envelope complex of this nature would still need, or be physically able, to engage with the host cell translocation machinery is not clear.

The mechanism of DHBV L translocation appears to be in contrast with recent studies in HBV (Lambert & Prange, 2001), which indicate that S is not required for L translocation. This difference in the translocation mechanism from DHBV may reflect use of a different folding pathway, indicated by the dominant retention of HBV L in the ER in the presence of excess S whereby L is largely excluded from subviral particles (SVPs) and cytosolic L is retained for virion formation (Gazina et al., 1998). In contrast, DHBV L is not actively excluded from SVPs, which thus contain the same ratio of L:S as virions (Schlicht et al., 1987). This difference in envelope folding between the human and avian hepadnaviruses is further supported by the inability of the avian envelope proteins to combine with mammalian HBs to form particles, while woodchuck and human HBs are readily interchangeable (Gerhardt & Bruss, 1995). An important question still remains with both the S-dependent (DHBV) and S-independent (HBV) modes of L translocation and that is how the translocation is regulated to 50% of L chains to meet the essential, dual pre-S domains.

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While this essential translocation mechanism appears to differ between the avian and mammalian viruses, the strong conservation of the N-terminal third of the S domain and two polar residues at positions 24 and 27 of TM1 for all hepadnavirus envelope proteins points to this being a common determinant in particle morphogenesis (Fig. 4C). Moreover, the results of this study indicate that TM1 has different functional roles in L and S, which suggest that the assembly and incorporation of L into the viral envelope lattice may differ markedly from S, despite their common membrane-spanning domains.

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