Dual Topology of the Hepatitis B Virus Large Envelope Protein

DETERMINANTS INFLUENCING POST-TRANSLATIONAL PRE-S TRANSLOCATION*

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The large (L) envelope protein of the hepatitis B virus (HBV) has the peculiar capacity to form two transmembrane topologies via a nascent topology that is established at the endoplasmic reticulum (ER) membrane rather than at a post-ER compartment as originally supposed. Together our data provide evidence to suggest that the topological reorientation of L is facilitated by a host cell transmembrane transport machinery such as the ER translocon.

The transmembrane topology of most eukaryotic polytopic proteins is established cotranslationally at the endoplasmic reticulum (ER) membrane and is maintained during subsequent steps of folding and transport. The biogenesis of these proteins involves a series of coordinated translocation and membrane integration events that is directed by topogenic determinants within the nascent chains and that ultimately leads to a uniform topology for any given polypeptide (1, 2). In recent years, however, it has become evident that certain cellular polytopic proteins exhibit variations in biogenesis such that two or more distinct topological orientations are generated (3, 4). For example, the multidrug resistance P-glycoprotein (5), the transporter ductin (6), and the aquaporin-1 water channel protein (7) have been observed to be expressed in alternate topological forms with the diversity apparently generated at the time of translocation at the ER membrane. Because different orientations seem to serve different functions (6, 8), it is less surprising that viral proteins such as the hepadnavirus large L envelope protein exploit a similar strategy of topological heterogeneity (9–13), likely to optimize the generally limiting information potential of viral genomes.

The hepatitis B virus (HBV) L envelope protein with its pre-S domain plays vital roles in the viral life cycle by mediating receptor binding during host cell attachment, by performing a matrix-like function in nucleocapsid envelopment, and by exerting various regulatory functions (14–16). The multifunctional nature of L depends on its dual transmembrane topology that is established after the completion of polypeptide synthesis via an as yet uncharacterized mechanism (9, 11, 12). Upon biogenesis, L as well as two closely related homologues, the middle (M) and small (S) envelope proteins, are expressed from a single open reading frame of the viral genome by means of three different start codons that are spaced at intervals of 108 (or 119, depending on subtype) and 55 codons. Therefore, the 226-amino acid sequence of S is repeated at the C termini of M and L, which carry the additional pre-S2 domain or pre-S2 and pre-S1 domains, respectively (Fig. 1A) (17). All three proteins are cotranslationally integrated into the ER membrane, likely directed by the action of a signal (anchor) and a stop-transfer sequence encoded within the first and second transmembrane (TM) segments (TM1 and TM2) of their S domains (18, 19). Two further membrane-spanning segments are predicted in the C-terminal third of the S domain (Fig. 1B) (20, 21); it has not been established whether these putative TM3 and TM4 segments contain topogenic information. In contrast, the hydrophilic pre-S1 and pre-S2 domains have been shown to lack any signal sequence activity (11, 22). Hence, M and L are translocated into the ER membrane by the proximal signals of their S domains that also govern cotranslational translocation of the upstream pre-S2 region of M into the ER lumen (Fig. 1B) (11, 22). Conversely, the pre-S2 plus pre-S1 (pre-S) domain of L fails to be translocated and thus faces the cytosolic side of the ER membrane in nascent chains. During maturation, approximately half of the L molecules post-translationally translocate the pre-S region into the microsomal vesicle lumen, thereby generating a dual topology that is maintained in the secreted virion envelope (Fig. 1B) (9, 11, 12). By disposing the pre-S domain either at a cytosolic (i.e. inside the virus) or luminal (i.e. outside the virus) location, L seems to serve its topological...
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EXPERIMENTAL PROCEDURES

Plasmid Construction—The mammalian expression vector carrying the HBV L gene (pN12.L) under the transcriptional control of the human metallothionein IIA promoter has been described (12). For epitope tagging, an influenza virus hemagglutinin (HA) epitope (YPY-DVDPYDASL) was fused in frame to the C terminus of L by site-directed mutagenesis using a recombinant M13mp19.HBV bacteriophage and two antisense oligonucleotides, 5′-AGGTACCAA-

3′ and 5′-TACCCAAAG-3′ (restriction sites are underlined, and mismatches are in bold-itself). The polymerase chain reaction product obtained was then used to replace the XhoI (nt 127)-NdeI (nt 830) fragment of plasmid pN12.L, generating the construct pN12.L-MHV that encoded the first 155 aa of L, an 8-residue spacer (SRKSNPNI), and the N-terminal 82 aa of the MHV M protein, followed by the HA tag. To abolish concomitant expression of the HBV M and S envelope proteins from the L open reading frame, their translational frame shifts (Met109→Ala110 and Met164→Leu164 (Fig. 1A)) were changed to threonine residues (pN12.Lo) as described (25). Subsequently, most deletion mutants described above were introduced into this Lo background.

Cell Culture, Transfection, Trypsin Protection Assay, and Western Blotting—To produce viral envelope proteins, transient transfection of CHO cells by electroporation was used. Two days post-transfection, micromasses were prepared and subjected to the trypsin protection assay essentially as described (12). Briefly, cells were disrupted by dounce homogenization, and micromasses were recovered by ultracentrifugation prior to proteolysis with trypsin (25 μg/ml) in the presence or absence of 0.5% Nonidet P-40. After incubation on ice for 60 min, trypsin was inactivated by the addition of 30 μg/ml aprotinin. Each sample was then adjusted to 0.5% Nonidet P-40 and solubilized for 20 min on ice. After centrifugation for 5 min at 13,000 × g and 4 °C, proteins of the supernatants were precipitated with 10% trichloroacetic acid (TCA) and washed twice with 5% TCA and once with acetone. TCA precipitates were resolved by SDS-PAGE and Western blotted to nitrocellulose membranes. Immunoblots were incubated with a mouse monoclonal antibody against the HA epitope (Babco), diluted 1:1000 in blotting buffer (phosphate-buffered saline with 5% skim milk) and peroxidase-labeled goat anti-mouse secondary antibody (Dianova) (1:5000 dilution in blotting buffer), and developed with enhanced chemiluminescence detection reagents (Amersham Pharmacia Biotech).

Metabolic Labeling and Immunoprecipitation—For metabolic labeling, cells were washed 48 h after transfection and starved for 40 min in methionine-free minimal essential medium without fetal calf serum. Cells were pulsed for 10 min with 300 μCi of [35S]methionine/cysteine (PerkinElmer Life Sciences) (1000 Ci/mmol) and either harvested directly or harvested after a chase of 45 min performed by replacing the medium with Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum and 1.5 mg/ml unlabeled methionine and cysteine. Metabolically labeled cells were prepared and subjected to SDS-PAGE as described above except the proteins were immunoprecipitated with an L-specific (i.e. pre-S1-specific) polyclonal antisemur (23). Immunoprecipitates were separated by SDS-PAGE and analyzed by PhosphoImager scanning (Molecular Dynamics). For protein synthesis in the presence of dithiothreitol (DTT), the reducing agent was added 5 min before electroporation. Microsomes were prepared and subjected to trypsin digestion as described above except the phosphate-buffered saline washing buffer and the homogenization buffer were supplemented with 20 mM N-ethylmaleimide to block free sulhydryl groups.

Subcellular Fractionation—Transfected cells were washed twice in ice-cold homogenization buffer (10 mM triethanolamine, 10 mM acetic acid, 250 mM sucrose, 1 mM EDTA, and 1 mM DTT and suspended in the same buffer supplemented with 0.1% SDS, 0.5% Triton X-100, 0.1 mM each of leptomit, leupeptin, antipain, and pepstatin. The cells were homogenized by passing 12 times through a 25-gauge needle. The homogenate was centrifuged for 5 min at 1500 × g and 4 °C to obtain a postnuclear supernatant. The postnuclear supernatant was separated on nycodenz gradients (Nycodisc) and the distribution of L was analyzed with anti-HA. The postnuclear supernatant was separated on nycodenz (Nycodisc) step gradients that were formed by the method described by Hammond and Helenius (26). Briefly, a discontinuous gradient of 10, 14.66, 19.33, and 24% nycodenz in 0.75% NaCl, 10 mM Tris, pH 7.4, and 3 mM KCl, and 1 mM EDTA was formed manually and centrifuged for 4 h at 235,000 × g and 4 °C in an SW40 rotor (Beckman). The postnuclear supernatant was then loaded on top of the gradients and centrifuged for 1.5 h as described above. Fifteen fractions were collected from the bottom, and proteins from each fraction were precipitated with TCA immediately or subjected to immunoblot analysis. The assignments of ER, Golgi, and intermediate compartment (IC) fractions were based on the marker analysis using anti-ID3 (a generous gift from S. Fuller), anti-58K (Sigma), and anti-rab2 (Santa Cruz) for immunoblotting, respectively, whereas the distribution of L was analyzed with anti-HA.

RESULTS

Experimental Guide—Topological analyses have demonstrated previously that the initial topology of the hepatadnaviral L envelope protein at the ER membrane contains its entire pre-S domain oriented to the cytosolic side, whereas the mature protein disposes this domain in addition to the luminal side of intracellular membranes (Fig. 1B) (9–13). The topological re-
The four hydrophobic segments in the common to M and L, whereas L additionally contains the N-terminal 226-aa sequence of the S domain. The 55-aa long pre-S2 domain is 1, 109, and 164, respectively. Accordingly, all three proteins share the codons in the HBV envelope open reading frame located at aa positions 1–4. Partial N-glycosylation occurring at Asn309 within the S domain is indicated by the HBV envelope proteins.


determination of pre-S domain across membranes, can be scored by its protection from exogenously added trypsin in the absence but not presence of nondenaturing detergents. While the pre-S domain of newly synthesized L chains is almost fully sensitive to cleavage with trypsin, it becomes increasingly protected over time, yielding up to 50–80% resistant chains at steady state (9, 12). The post-translational mode of this translocation event is evidenced further by the absence of N-linked glycosylation of the pre-S domain that carries two modification-competent glycosylation sites (Asn4 and Asn123) (12, 23). Based on these observations, partial trypsin protection at steady state and the lack of pre-S-linked N-glycans are convenient markers for post-translational pre-S translocation and are thus used herein.

Porcine transgenic pre-S translocation Does Not Require the Helper Function of S and M Proteins—Because of the genetic organization of the HBV envelope open reading frame, L expression is accompanied by the synthesis of S and M, which together assemble the virion envelope (17). The complexity of envelope subunits has led to the proposal that post-translational pre-S translocation of L may occur through a channel created from lateral interactions between the amphipathic TM regions in the S domains of M and/or S chains (10, 20, 21, 24).

To examine this hypothesis, we probed whether S and M are required for pre-S translocation. To prevent concomitant M and S synthesis from the L gene, the start codons were inactivated by mutagenesis (designated L only (Lo)), and an HA-epitope tag was engineered into the C terminus of mutant and wild-type (wt) L to enable detection by Western blotting. The topology of the proteins synthesized in transiently transfected COS-7 cells was then determined by trypsin protection assays of microsomes, prepared 2 days post-transfection, and immunoblotting with an HA-specific monoclonal antibody. As shown in Fig. 2, L was obtained in nonglycosylated (p39) and single-glycosylated (gp42) forms as a consequence of partial modification at Asn309 in its S domain (lane 1) (17). In addition, nonglycosylated and glycosylated forms of both the S (p24 and gp27) and the M (gp33 and gp36) proteins appeared because of the internal initiation of translation (lane 1) (17). Importantly, the M and S forms were absent in cells transfected with mutant Lo (Fig. 2, lane 4), confirming the prevention of their expression. Consistent with previous works (9, 12), proteolysis with trypsin in the absence of detergent (Nonidet P-40) yielded two fractions of wt L: trypsin-resistant full-length molecules and trypsin-sensitive chains with cleavage occurring at least at a very distal site within pre-S, most likely at Arg136 (Fig. 2, lanes 3; see also Fig. 1A). Therefore, a 25-kDa nonglycosylated (T) and a 28-kDa glycosylated (gT) HA-reactive trypsic fragment were generated that migrated slightly slower than the 24- and 27-kDa forms of S. Upon disruption of the microsomal membranes with detergent, trypsin completely converted L to these two fragments (Fig. 2, lane 3), thus demonstrating that trypsin resistance is likely to be a consequence of pre-S translocation to the microsomal vesicle lumen rather than intrinsic protease resistance of the domain itself. Importantly, however, when L was synthesized in the absence of M and S chains, basically the same pattern of protection was obtained, because ~67% of Lo chains were found to be inaccessible to trypsin unless Nonidet P-40 was present, whereas the remaining chains were cleaved to the gT and T fragments (Fig. 2, lanes 4–6). The slightly more intense band obtained for the T fragment is likely to be caused by comigration of trypsin (molecular mass, ~33.5 kDa) and its unspecific staining in Western blotting. These results demonstrate that L formed its dual topology even when synthesized without S and M chains.

Porcine transgenic pre-S translocation Does Not Depend on Disulfide-linked Dimerization of L Chains—Alternatively, we reasoned that L on its own might create a putative pre-S-conducting channel that has been predicted to build up during dimerization and/or oligomerization of envelope chains concomi-
and Lo TM4 and constructed two mutants, L TM3/4, chased for the same period in the absence (with the Lo gene were metabolically pulse-labeled for 20 min and COS-7 cells transfected segments (Fig. 4 A). According to the channel model, we initiated with intermolecular covalent disulfide linkage formation (20, 21, 27). Therefore, we assessed whether the inhibition of disulfide bond formation in newly synthesized L chains had an effect on pre-S translocation. To this aim, Lo-transfected cells were treated with DTT during metabolic pulse-chase labeling with [35S]methionine/cysteine, followed by trypsin protection analysis and immunoprecipitation with a pre-S1-specific antiserum. As shown in Fig. 3, L molecules, synthesized and matured in the presence of DTT, translocated their pre-S domains to the microsomal lumen as efficiently as Lo polypeptides expressed in untreated cells (lanes 1–6). This is evident from the almost identical degree of trypsin protection in intact microsomes. Under both assay conditions, a significant fraction of Lo chains were resistant to trypsin unless the protecting membranes were destroyed (Fig. 3, compare lanes 2 and 5). Of note, the L-tryptic fragments were undetectable in this experiment, because a pre-S1-specific antiserum was used for immunoprecipitation. Amphipathic TM1, TM3, and TM4 Segments Are Dispensable for Post-translational pre-S Translocation—Although the above data raised first doubts on the channel hypothesis, it nonetheless seemed possible that noncovalent complexing of L chains, such as ionic interactions between the hydrophilic faces of the TM segments, may enable dimerization and pore formation. Because such interactions are difficult to interrupt in living cells, we employed a mutagenesis approach by constructing a series of mutant L proteins carrying deletions of TM segments (Fig. 4A). According to the channel model, we initially focused on the amphipathic a-helices TM1, TM3, and TM4 and constructed two mutants, L::TM1 and L::TM3/4, devoid of either the first or last two TM segments, respectively. In the latter mutant, flanking residues of the upstream luminal peptide loop including the glycan acceptor Asn309 were also deleted (Fig. 4A). Consistent with previous reports (18, 19), the deletion of TM1 did not affect cotranslational membrane integration as evidenced by the membrane association of mutant L::TM1 and its partial N-glycosylation at Asn309 (Fig. 4B, lane 4). Presumably, the stop-transfer segment TM2 substitutes for the loss of the signal (anchor) TM1 (18, 19). After trypsin digestion, protease-resistant chains of the 37-kDa nonglycosylated and 40-kDa single-glycosylated forms of mutant L::TM1 were identified in the absence but not the presence of detergents (Fig. 4B, lanes 5 and 6), indicating a partial luminal pre-S location and hence post-translational pre-S translocation. A similar degree of trypsin protection in intact microsomes was observed with mutant Lo::TM3/4 that appeared only in the nonglycosylated form of 32 kDa caused by the missing Asn309 residue (Fig. 4B, lanes 7–9). In combining these data, it seems that none of the three putative channel-forming TM segments of L is required for post-translational pre-S reorientation.

Hydrophobic TM2 Segment Is Essential and Sufficient for Post-translational pre-S Translocation—These results prompted us to investigate next which role the most hydrophobic TM2 helix played in this process. Therefore, we analyzed the mutants Lo::TM2 and Lo::TM2/3/4 that lacked this segment either alone or in combination with TM3 and TM4, respectively (Fig. 4A). The individual deletion of TM2 (L::TM2), however, yielded an unstable polypeptide that failed to associate with microsomal membranes and thus could be only detected in the crude cellular lysate (Fig. 4B, lane 10). Although this observation hints to an essential role of TM2 in the cotranslational membrane integration of L, it precludes definition of its precise role in post-translational pre-S reorientation. Evidence for an important contribution of TM2 to the latter event was obtained by the mutant Lo::TM2/3/4 that left only TM1 as a hydrophobic element (Fig. 4A). This mutant was stably expressed as a membrane-bound 29-kDa polypeptide in accordance with the molecular mass calculated for its nonglycosylated chain (Fig. 4B, lane 14). Its proper membrane integration was further confirmed by extraction with sodium carbonate (data not shown). Digestion with trypsin revealed that this mutant was fully sensitive to proteolytic attack even in intact microsomes, because no protected chains could be detected by either HA-specific (Fig. 4B, lanes 15 and 16) or pre-S1-specific immunoblotsing (data not shown). Because mutant Lo::TM2/3/4 no longer supported pre-S translocation and mutant Lo::TM3/4 did, TM2 seemed to be a critical determinant in pre-S reorientation. Addressing this point, we finally constructed a mutant missing all but the second TM segment (Lo::TM1/3/4) (Fig. 4A). This mutant, synthesized in membrane-nonglycosylated 28-kDa form, clearly acquired partial protease protection by the microsomal membrane (Fig. 4B, lanes 17–19). From these data we conclude that among the hydrophobic domains of L, only TM2 is needed for pre-S translocation.

To unequivocally demonstrate that pre-S translocation of mutant Lo::TM1/3/4 occurs post-translationally, the kinetics of its chain maturation was monitored using a pulse-chase labeling protocol that we had established previously for wt L (12). As shown in Fig. 5, after a 10-min pulse without chase, the majority of newly synthesized Lo::TM1/3/4 polypeptides were sensitive to trypsin, independent of whether membranes were disrupted (lanes 1–3). By contrast, after a chase for 45 min, about 30% of these polypeptides were found to resist protease digestion (lanes 4–6). The delayed pre-S translocation of mutant Lo::TM1/3/4 indicates that it reoriented its pre-S domain after polypeptide synthesis.

Foreign TM Segments Are Unable To Substitute for TM2 in Post-translational pre-S Translocation—To unravel the critical role of TM2 in pre-S transfer, we then evaluated whether it could be functionally replaced by heterogeneous sequences. Because of the structural homology between the S domain of L and the coronavirus MHV M envelope protein, a triple-spanning protein with its first and second TM segments likely serving as a signal anchor and a stop-transfer signal, respectively (28), these two segments were chosen to substitute for TM1 and TM2 of L (Fig. 6, A and B). The chimera L::MHV was engineered in the wt pre-S background to simultaneously monitor the topology of the M::MHV construct, which was derived from the internal translational initiation at the HBV M-specific start codon within pre-S. As shown in Fig. 6C, both L::MHV and M::MHV fusion proteins were efficiently expressed in transfected cells (lane 1). In intact microsomes, the M::MHV
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Fig. 4. Role for the TM segments in post-translational pre-S translocation. A, schematic representation of mutant L proteins carrying deletions in the S domains. As in Fig. 1A, the pre-S1, pre-S2, and S domain structures of wt L are shown in the most upper line with open ovals indicating its four TM segments (1–4). The positions of aa residues at the borders of these segments are shown, and the C-terminally fused HA-epitope tag is depicted by a hatched symbol. Partial N-glycosylation occurring at Asn309 is indicated by the crossed lines symbol in parentheses. The HA-tagged mutant L proteins carrying C-terminal truncations and/or internal deletions are aligned below. Deletions are indicated by dotted lines with the deleted aa residues shown beneath. B, COS-7 cells were transfected with the constructs as indicated above each panel. Two days post-transfection, microsomal vesicles were prepared and subjected to trypsin protection analysis and HA-specific Western blotting as described in the Fig. 2 legend. For mutant LΔTM2, the crude lysate of transfected cells was analyzed in addition (lane 10). Because samples were run on different gels, positions of molecular mass standards in kDa are shown on the left of each panel, and arrow tips mark L and its derivatives.

Fig. 5. TM2 is sufficient for post-translational pre-S translocation. Analysis of the topology of the mutant LΔTM1/3/4 using trypsin and pulse-chase labeling is shown. Transfected cells were pulse-labeled for 10 min and either lysed immediately (lanes 1–3) or chased for 45 min (lanes 4–6). Microsomes were mock-treated or digested with trypsin in the absence (−) or presence (+) of Nonidet P-40 (NP-40) as denoted above each lane. As a control for unspecific immunoprecipitation, nontransfected cells were pulse-chase labeled and processed in a similar manner (lanes 7–9).

construct was clearly protected from trypsin digestion (Fig. 6C, lane 2), which indicates that the foreign TM segments translocated the upstream pre-S2 domain into the vesicle lumen in a manner similar to the HBV-specific segments. It therefore came out as a surprise that the MHV-specific TMs failed to confer partial pre-S reorientation as evidenced by the full trypsin sensitivity of the L::MHV construct in intact membranes (Fig. 6C, lane 2). We took these data as further proof for the specific requirement of TM2 in L topogenesis.

Post-translational pre-S Translocation Occurs at the ER Membrane—Because our results altogether do not favor the need of a specialized HBV envelope structure for pre-S translocation, we finally determined where in the secretory pathway pre-S translocation takes place. Because of (i) the lack of pre-S-linked N-glycosylation, (ii) the inability of canine pancreas microsomes to confer pre-S reorientation, and (iii) the prevailing channel hypothesis, we and others have suggested previously that L maturation occurs at a post-ER intermediate compartment (9–13), where the HBV envelope assembly is thought to take place (27). Addressing this issue, we used fractionation of Lo-transfected cells to separate the ER, IC, and Golgi complex. As shown by the distribution of markers for the ER (protein disulfide isomerase), IC (Rab2), and the Golgi complex (58K), the organelles banded according to density, with the ER being the heaviest and the Golgi complex being the most buoyant (Fig. 7A). The majority of organelle-specific markers were found to peak in distinct fractions, indicating a sufficient resolution of the three compartments (Fig. 7A). Fractions were next assayed for the distribution of Lo that was predominantly present in the ER as expected (Fig. 7A). To our surprise, fractions of the IC, where pre-S translocation had been assumed to occur, were almost free of Lo, while it was also found in cis/medial Golgi fractions (Fig. 7A), an as yet unknown intracellular location of L. Next, the Lo polypeptides present in a peak ER fraction (no. 4) were digested with trypsin as described above. This analysis clearly showed the typical partial protease protection of about half of the Lo chains in the absence of detergent (Fig. 7B, lane 2). Virtually identical results were obtained when transport of L out of the ER was blocked with brefeldin A (data not shown). Together these findings demonstrate that the dual L topology is generated at the ER membrane.

DISCUSSION

The HBV L envelope protein is the first example of a viral protein exhibiting different transmembrane topologies for functional diversity (9, 11, 12, 14, 16) and hence is an addition to a restricted but growing number of membrane proteins that seem to have developed variations on cotranslational biogene-
sis pathways (3-7). In contrast, however, and in being novel, the topological heterogeneity of L is generated in a noncotranslational manner (9,11,12). Accordingly, a prevailing model predicts post-translational pre-S translocation of L to be enabled by an HBV-specific structure created during virion envelope assembly (10,20,21,24). By analyzing the parameters influencing L topogenesis, the results of this work raise doubts on the validity of such a channel model.

First evidence that pre-S refolding of L may be uncoupled from envelope assembly was obtained by the finding that neither the S nor the M envelope protein is required for this event. The dispensability of S is particularly surprising because this protein is the predominant constituent of the viral envelope and forms its scaffold (17), which is why it has been implicated as the likely candidate for providing the pre-S conducting channel (20). Indeed, recent studies on the pre-S translocation of the related L protein of duck HBV demonstrated an essential role for the avian S counterpart in this process (24). We do not yet know the reason for this discrepancy, but assume that the envelope proteins of the two virions may have evolved different folding pathways to acquire their final transmembrane topology, as has been also shown recently for the two closely related aquaporin-1 and aquaporin-4 proteins (29). In favor of this view, assuming different folds, is the inability of the duck HBV envelope proteins to substitute for the HBV homologues in virus envelope assembly (30).

As post-translational pre-S reorientation is an intrinsic feature of the HBV L protein, it nonetheless may proceed via a channel created by L itself. Analysis of L deletion mutants, however, revealed that the topological switch of L does not require any of its amphipathic TM helices but rather the most hydrophobic TM2 segment. By analogy to other viral single-spanning membrane proteins, such as the M2 protein from influenza A and the NB protein from influenza B, that form homotetramer bundles of transmembrane helices surrounding a central ion-permeable pore (31), mutant LΔTM1/3/4 might also still homo-oligomerize with the helical packaging of its single TM2 segment. Because of its extremely hydrophobic character, we consider TM2, however, unlikely to line a pore of sufficient hydrophilicity to enable transmembrane pre-S passage.

Although being in seeming conflict with a current view of post-translational pre-S translocation, our results do not rule out that aqueous pores may exist in the secreted virion envelope. This has been implicated by low pH treatment of hepadnaviral particles that triggers further exposure of pre-S domains of L that were originally hidden and even surface display of the TM1 segment, a putative fusion sequence involved in viral entry (9,32,33). Such repositioning events indeed may proceed through aqueous pores in the extracellular envelope, the formation of which is likely facilitated by the viral lipid structure that is devoid of a unit membrane, because hepadnaviral envelope assembly is accompanied by a substantial reorganization of membrane lipids (34). Therefore, the mechanisms responsible for the pre-S translocation of L across intracellular membranes and its rearrangement(s) in the extracellular envelope might not necessarily be coincident.
In combining these data, we consider other transmembrane transport mechanisms more feasible at generating the dual topology of L at a time when it is embedded in microsomal membranes. Oss and Hildt (35) have recently identified a cell-permeable motif within the pre-S2 region of M and L that enables pre-S, if separated from the S domain, to cross over the plasma cell membrane. Involvement of this motif in post-translational pre-S translocation, however, is largely excluded, because this sequence has been shown to be dispensable for morphogenesis and infectivity of HBV that both depend on the alternate L structure (14, 16, 36). Moreover, the failure of mutant LoATM2/3/4 to refold pre-S, as shown herein, argues against a role for the pre-S2-specific motif in this process. Alternatively, the structural change of L might be achieved by reversible flipping of pre-S across the lipid bilayer, as has been demonstrated for a subdomain of the colicin Ia channel protein (37). Although such a transmembrane slipping back and forth might well explain the partial mode of pre-S translocation, a further puzzling feature of L maturation, the phenotypes of distinct L derivatives, does not support this proposal. First, mutant L proteins, in which cotranslational pre-S translocation has been artificially enforced, maintained their uniform luminal pre-S orientation (14, 23). Second, mutant LoATM2/3/4 uniformly disposed its pre-S domain to the cytosol, thus contradicting a reversible pre-S flipping mechanism.

Rather, according to our fractionation data, it is reasonable to assume that pre-S reorientation of L is governed by the ER translocation machinery, with TM2 being the most crucial topogenic determinant. It seems possible that upon cotranslational translocation, the TM domains of nascent L chains might not be integrated into the bilayer immediately but instead are held within the Sec61p translocon even after their synthesis is completed. This would be consistent with the large internal diameter of the translocon pore (20–60 Å) as well as with recent studies suggesting that TM segments, even those of several chains of a single protein, may accumulate within or near the translocon before entering the membrane (38–40). Inconsistently, however, the detachment of the ribosome after chain termination (41) would keep the Sec61p transloconal channel, occupied with L chain(s), in an unphysiologically open state. Possibly, binding of the Hsc70 ribosome chaperone with cytosolically disposed pre-S domains (39) somehow might accomplish channel gating, similar to established chaperone functions in post-translational protein translocation in yeast and bacteria (42). The bound Hsc70 molecule might additionally serve as a regulatory device, preventing the premature exit of L from the translocon. Alternatively, it is also quite possible that L chains may be released into the lipid after translation but reenter the translocon channel for reorientation. Re-association of proteins with the Sec61p channel after they have been integrated into the lipid bilayer is suggested by experiments that implicate the Sec61p complex in retrograde transport of, for example, MHC class I molecules (43) or apoprotein B100 (44). Irrespective of the path L could use, the translocation channel would then provide the hydrophilic environment needed for transmembrane pre-S transport. In the simplest case, this would involve a 90° rotation of TM1, that had been originally left out or ignored by the translocation machinery, and a concurrent translocation of the flanking N-terminal pre-S domain to the luminal space (see Fig. 1B). A similar topological reorientation of internal TM segments and adjacent peptide loops has recently been observed to occur during the maturation of aquaporin-1 that, in contrast to L, is initiated during translation but enhanced after synthesis is almost completed (7). Although we cannot formally exclude that such a TM1 rotation may direct the alternate pre-S topology of wild-type L, the results of our mutagenesis analysis indicate that TM1 is not actually involved in pre-S repositioning, at least of mutant L proteins. Rather, our data hint to an essential and specific function for TM2 in this process that could be fulfilled by neither TM1 nor foreign TM segments. Considering the mandatory requirement of TM2, it is tempting to speculate that this domain confers a putative prolonged interaction of L with the ER translocon to promote pre-S refolding in a subset of L chains. To do so, TM2 must reorient from a membrane-spanning to a membrane-lining segment or to a helical hairpin structure. To account for the absence of pre-S-linked N-glycosylation in this scenario, initial folding of pre-S before its post-translational delivery to the luminal ER space may hamper recognition by the oligosaccharyl transferase. In regard to recent suggestions that certain polytopic proteins may require specialized components to achieve their proper topology in addition to the basic components needed for protein translocation (3, 45), it also seems conceivable that post-translational pre-S translocation may occur in a specialized ER subdomain where glycosylation enzymes might be missing. Our previous observation that canine pancreas microsomes are unable to reconstitute pre-S reorientation (12) would fit with such a proposal. Intriguingly, a similar result has been reported for aquaporin-1 (noted above) that likewise failed to reorient and thus to mature in canine rough microsomal membranes (7).

To conclude, the precise mechanism(s) by which the functionally important dual topology of L is generated is still elusive and remains to be defined. Our demonstration that the topological reorientation of L is seemingly not established by a virus-specific structure but is physically linked to the ER membrane renders the HBV L protein as a challenging substrate to unravel the complex and dynamic nature of events occurring during polytopic protein topogenesis.

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