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Novel transmembrane topology of the hepatitis B virus envelope proteins

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The small (S), middle (M) and large (L) envelope proteins of the hepatitis B virus (HBV) are initially synthesized as multispanning membrane proteins of the endoplasmic reticulum membrane. We now demonstrate that all envelope proteins synthesized in transfected cells or in a cell-free system adopt more than one transmembrane orientation. The L protein disposes its N-terminal preS domain both to the cytoplasmic and the luminal side of the membrane. This unusual topology does not depend on interaction with the viral nucleocapsid, but is preserved in secreted empty envelope particles. Pulse-chase analysis suggests a novel process of post-translational translocation leading to the non-uniform topology. Analysis of L deletion mutants indicates that the block to co-translational translocation can be attributed to a specific sequence within preS, suggesting that translocation of L may be regulated. Additional topological heterogeneity is displayed in the S region of the envelope proteins and in the S protein itself, as assayed in a cell-free system. S proteins integrated into microsomal membranes exhibit both a luminal and a cytoplasmic orientation of the internal hydrophilic region carrying the major antigenic determinants. This may explain the unusual partial glycosylation of the HBV envelope proteins.

Key words: HBsAg/membrane proteins/preS domain/post-translational/protein translocation

Introduction

A large number of viruses contain a lipoprotein coat or an envelope that surrounds the internal nucleocapsid. The viral envelope proteins are transmembrane proteins that are synthesized on membrane-bound ribosomes in the endoplasmic reticulum (ER) and are transported to the site of virus assembly by the same process used by cellular membrane proteins. As a rule, the transmembrane orientation of envelope proteins acquired during biosynthesis is both absolute and maintained during virus assembly and release (reviewed in Perara and Lingappa, 1988; Stephens and Compans, 1988). In the present study we report on a remarkable exception to this rule occurring during biogenesis of the envelope of the hepatitis B virus (HBV).

The HBV virion is a double-shelled sphere, 42 nm in diameter, with an inner nucleocapsid enclosed by the viral envelope. The envelope carrying the major surface antigen (HBsAg) is composed of cellular lipids and three related viral proteins, the large (L), middle (M) and small (S) envelope proteins. They are encoded in a single open reading frame of the HBV genome and initiate at three separate in-phase AUG codons spaced at intervals of 108 (or 119, depending on serotype) and 55 codons. The segments downstream of the three initiation codons are called the preS1 and preS2 regions and the S gene (see Figure 1B). All three proteins are found in two forms, either glycosylated at Asn146 of the S sequence or unglycosylated at this site. The M protein is additionally glycosylated at Asn4 (reviewed in Heermann and Gerlich, 1991).

The S protein is the predominant constituent of the envelope. This protein is unique among viral envelope proteins in its capacity to self-assemble with host-derived lipids into secreted empty envelope particles. Formation of such particles is initiated by insertion of the S protein into the ER membrane. After aggregation of ~100 transmembrane S monomers, subviral particles are thought to mature by budding into the lumen of a pre-Golgi compartment and to leave the cell via the constitutive pathway of secretion (Simon *et al.*, 1988; Huovila *et al.*, 1992). Assembly of HBV virions is presumed to proceed along a similar pathway, including enveloping of cytoplasmic nucleocapsids by the membrane-embedded envelope proteins. The L protein has been shown to be absolutely necessary for virion formation, while the S protein is required, but not sufficient. The contribution of the M protein to this process is still disputed (Bruss and Ganem, 1991a; Ueda *et al.*, 1991).

The current models for the transmembrane structure of S assume a luminal disposition (i.e. external in the mature particle) of both the N- and C-termini (Guerrero *et al.*, 1988; Stirk *et al.*, 1992). Therefore, the protein must traverse the ER membrane at least twice. Of the four transmembrane α-helices predicted, only the two most N-terminal have been shown directly to determine membrane insertion of S (Eble *et al.*, 1987; Bruss and Ganem, 1991b; Prange *et al.*, 1992). These two domains, corresponding to amino acids 11–28 and 80–98 respectively, are spaced by a hydrophilic region exposed on the cytoplasmic side of the membrane (i.e. internal in the mature particle). The second hydrophilic loop (amino acids 99–168) is predicted to be on the luminal side, since it carries the major surface antigen and acquires carbohydrate modification (Eble *et al.*, 1987; Stirk *et al.*, 1992) (see Figure 8A). However, neither model accounts for the partial usage of the glycosylation site of S.

The M and L proteins are thought to adopt a similar topology, protruding their hydrophilic preS regions into the ER lumen (Eble *et al.*, 1990; Heermann and Gerlich, 1991). The accessibility of preS-specific epitopes of both

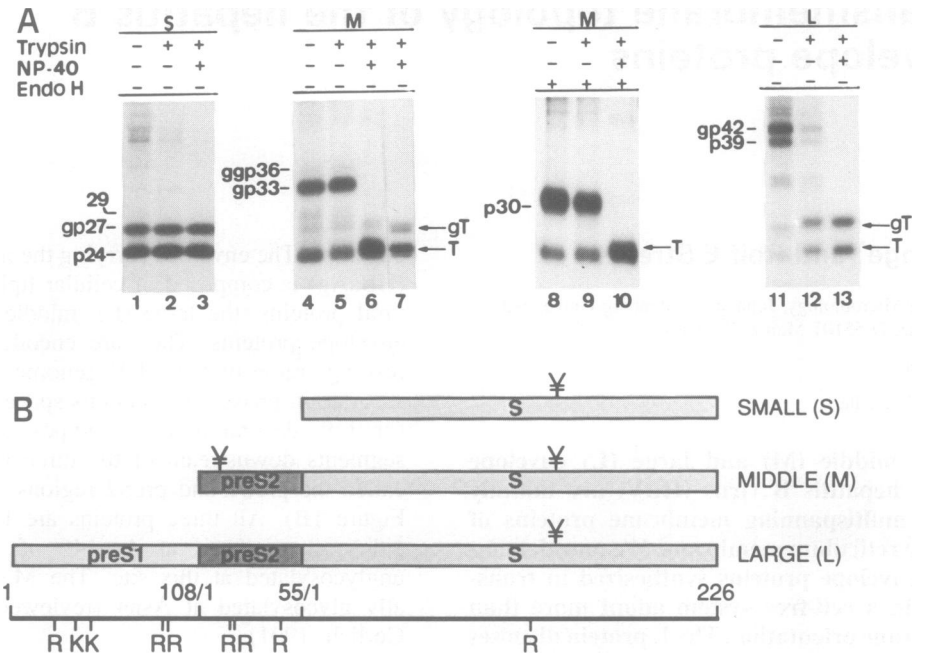

Fig. 1. (**A**) Analysis of the topology of the HBV env proteins using trypsin. COS7 cells transfected with the S (lanes 1–3), the M (lanes 4–10) or the L gene (lanes 11–13) were metabolically labeled for 2 h. Microsomal vesicles were prepared and either left untreated (lanes 1, 4, 8 and 11), or digested with trypsin in the absence (lanes 2, 5, 9 and 12) or presence of NP-40 (lanes 3, 6, 7, 10 and 13). Proteolysed microsomes of cells expressing the M protein were additionally treated with endo H (lanes 8–10). After immunoprecipitation, proteins were analyzed by SDS–PAGE. Non-glycosylated (p) and glycosylated (gp, ggp) forms of the proteins are indicated on the left of each panel; numbers refer to their molecular masses (in kDa) deduced from standards. The position of the 29 kDa mol. wt standard protein is shown. Non-glycosylated (T) and glycosylated (gT) tryptic fragments are indicated by arrows. (**B**) Schematic representation of the HBV env proteins. Sites of *N*-linked glycosylation are indicated by ¥. Below are depicted the positions of the potential trypsin cleavage sites (R, arginine, K, lysine). For simplification, only the known trypsin site of the S region (R at position 122) is denoted.

proteins on the surface of secreted particles supports this notion (Heermann *et al.*, 1984; Kuroki *et al.*, 1990). The glycosylation of the preS2 region of M is further evidence for its luminal disposition. Conversely, no glycan is linked to the preS2 region of L (Heermann *et al.*, 1984), suggesting an altered topology. Moreover, *in vitro* topogenesis studies have recently shown that the preS region of L is cytoplasmically disposed when synthesized in a cell-free system (Ostapchuk *et al.*, 1994). During HBV replication, the preS region of L plays a pivotal role by mediating attachment of HBV to liver cells (Neurath *et al.*, 1986, 1992) and by enveloping the nucleocapsid (Bruss and Ganem, 1991a; Ueda *et al.*, 1991). To accomplish this dual function, both a luminal and a cytoplasmic location of the preS region of L is required.

In this work we have approached this issue by analyzing the topology of wild-type and mutant L proteins synthesized in transfected cells. Our data demonstrate that a substantial alteration in the transmembrane conformation of L occcurs post-translationally, yielding two topologically different populations that are maintained upon secretion of empty envelope particles. In addition, we could map a sequence within the preS domain that may account for this post-translational event. Moreover, we provide evidence for a non-uniform transmembrane topology of part of the S region, which is common to all three envelope proteins of HBV, as assayed in a cell-free system.

Results

Topology of HBV envelope proteins in transfected cells: the L protein displays an unusual transmembrane configuration

To analyze the transmembrane topology of the HBV envelope (env) proteins, fragments of the HBV genome encoding the S, M or L proteins were separately transfected into COS7 cells. The topology of the proteins in the ER membrane was assayed using proteases to distinguish between domains accessible to proteolysis or protected from digestion by the lipid bilayer. Therefore, microsomal vesicles were prepared from the transfected cells and were subjected to trypsin treatment either in the absence or presence of detergent (NP-40). The env proteins were then immunoprecipitated with polyclonal HBsAg-specific antiserum recognizing antigenic determinants common to all three proteins and were subjected to SDS–PAGE.

When cells were metabolically labeled for 2 h, both the 24 kDa non-glycosylated and the 27 kDa glycosylated form of the small S protein were virtually resistant to digestion with trypsin (Figure 1A, lane 2), as shown by comparison with the mock-treated control (Figure 1A, lane 1). Trypsin is known to cleave S exclusively at Arg122 (Figure 1B) (Guerrero *et al.*, 1988), located in the second hydrophilic region, which is predicted to be luminally disposed. Therefore, protection of S was not surprising. However, when microsomes were disrupted with detergent, the trypsin cleavage site of S remained

inaccessible (Figure 1A, lane 3), indicating that protection from digestion was due to intrinsic protease resistance, rather than to membrane sequestration. We will return to this observation later.

Using the same proteolytic conditions, the middle M protein was cleaved by trypsin. The M protein was synthesized in two forms, glycosylated at Asn4 of the preS2 region (gp33) and, to a lesser extent, additionally partially glycosylated at Asn146 in the S region (ggp36) (Figure 1A, lane 4). In addition, the 24 and 27 kDa forms of the S protein were obtained due to internal translation initiation (Figure 1A, lane 4). Since *N*-linked glycosylation generally indicates a luminal location of at least the glycan attachment site (Doms *et al.*, 1993), the preS2 region of M was reasoned to be protected from proteolysis. As expected, neither form of M was degraded by trypsin (Figure 1A, lane 5) unless microsomal membranes were disrupted with NP-40. Under these conditions, trypsin converted the M protein into two fragments of ~25 and 28 kDa (Figure 1A, lane 6) that migrated slightly slower in polyacrylamide gels than the 24 and 27 kDa forms of S. The co-migration of each of these doublets is more clearly shown in lane 7 of Figure 1A. Keeping in mind that the S domain is trypsin-resistant, trypsin must have cleaved the M protein at a very distal site within preS2, most likely at Arg48 (Figure 1B). Thus the 25 and 28 kDa tryptic fragments represent N-terminally extended, non-glycosylated and glycosylated versions of the S molecule. They will be termed herein T and gT respectively. *N*-linked glycosylation was confirmed by treatment of the proteolysed samples with endoglycosidase H (endo H), which converted the M protein to the non-glycosylated form (p30) and gT to the T tryptic fragment (Figure 1A, lanes 8–10).

Proteolysis of the large L protein yielded quite a different pattern of protection. As shown in Figure 1A, the L protein was synthesized as the non-glycosylated (p39) and the glycosylated (gp42) polypeptide (lane 11), due to partial modification at Asn146 in the S domain. Unlike M, no glycan is attached to the preS domain of L, neither to Asn4 of preS2 nor to the potential glycosylation site Asn4 of preS1 (Heermann *et al.*, 1984), suggesting a cytoplasmic disposition. The preS region of L contains several potential trypsin cleavage sites, of which Arg156 (corresponding to Arg48 of preS2) is the most C-terminal (Figure 1B). Incubation with trypsin in the presence of NP-40 completely converted L to the same T and gT fragments (Figure 1A, lane 13) that were generated by tryptic digestion of M. Using either preS1- or preS2-specific antibodies for immunoprecipitation, we were unable to detect any protected product (data not shown), indicating that the preS region of L was entirely degraded except for the last seven residues attached to S. Rather unexpectedly, proteolysis with trypsin in the absence of NP-40 reproducibly yielded two fractions of L: trypsin-resistant, full-length molecules and trypsin-sensitive polypeptides with cleavage occurring at Arg156 (Figure 1A, lane 12). As protection of M (Figure 1A, lane 5) has proven the polarity and integrity of the microsomal membranes, the partial protection of L reflects the co-existence of both a cytoplasmic and a luminal disposition of its preS region.

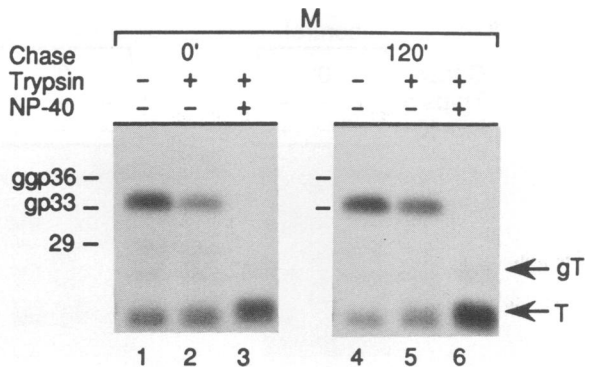

Fig. 2. Analysis of the topology of the M protein using trypsin and pulse-chase labeling. Transfected cells were pulse labeled for 10 min and were either lysed immediately (lanes 1–3) or chased for 120 min (lanes 4–6). Microsomes were mock-treated or digested with trypsin in the absence or presence of NP-40 as denoted above each lane. Numbers to the left show positions of mol. wt standards in kDa and deduced molecular masses of the glycosylated forms (gp, ggp) of the M protein. Arrows to the right indicate the tryptic fragments.

Topology of HBV env proteins in transfected cells: the transmembrane configuration of L is altered post-translationally

The surprising transmembrane topology of L observed during steady-state prompted us to study the time course of the protease sensitivity of the env proteins using pulse-chase labeling. The M protein was studied initially. After a 10 min pulse without chase, most of the newly synthesized M polypeptides were resistant to trypsin unless NP-40 was present (Figure 2, lanes 1–3). The small amount of M molecules cleaved in the absence of NP-40 is ascribed to degradation of immature translocation intermediates (Figure 2, lane 2). Since the protection pattern did not change when pulse-labeled cells were chased for 2 h (Figure 2, lanes 4–6), the preS2 region of M is most likely co-translationally translocated into the ER lumen.

In contrast, the majority of newly synthesized L polypeptides labeled during a 10 min pulse were sensitive to digestion with trypsin, independent of whether or not membranes were disrupted (Figure 3, lanes 4–6). To ensure detection of the L protein, the cell number was increased in this experiment; several tryptic polypeptides ranging from 27 to 33 kDa now appeared in the proteolysed samples that co-migrated with the T and gT tryptic fragments of L (Figure 3, lanes 5 and 6). These polypeptides were attributed to non-specific immunoprecipitation, as non-transfected cells labeled for 10 min displayed virtually the same pattern of bands (Figure 3, lanes 2 and 3). Importantly, when pulse-labeled cells were chased for various periods, the L protein became increasingly protected from proteolytic attack (Figure 3, lanes 4–15). Analysis by PhosphorImager scanning revealed that 17% of L proteins synthesized during the 10 min pulse were protected (Figure 3, lanes 4 and 5), while the amount of protected L proteins increased to 22 or 46% after a chase of 1 h (Figure 3, lanes 7 and 8) or 2 h (Figure 3, lanes 10 and 11) respectively. Under the same labeling conditions the internally initiated M protein (gp33) was completely resistant to trypsin (Figure 3, lanes 4 and 5, 7 and 8 and 10 and 11). In the presence of detergent both the non-uniformly oriented L and the uniformly oriented M proteins

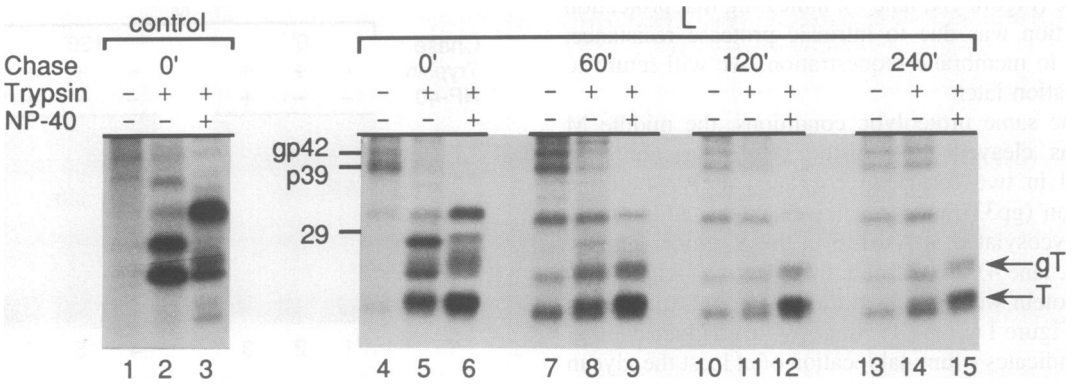

Fig. 3. Analysis of the topology of the L protein using trypsin and pulse-chase labeling. Transfected cells were pulse labeled for 10 min and were either lysed immediately (lanes 4–6) or chased for 60 min (lanes 7–9), 120 min (lanes 10–12) or 240 min (lanes 13–15). As a control, non-transfected cells were pulse labeled for 10 min without chase (lanes 1–3). Microsomes were mock-treated or digested with trypsin in the absence or presence of NP-40 as denoted above each lane. Numbers to the left of lane 4 show positions of mol. wt standards in kDa and deduced molecular masses of the non-glycosylated (g) and glycosylated (gp) L protein. Arrows to the right indicate the tryptic fragments. The corresponding polyacrylamide gel was scanned using a PhosphorImager Scan v5.60 (Molecular Dynamics).

were cleaved to the T and gT tryptic fragments, leading to an increase in these fragments (Figure 3, lanes 6, 9 and 12) as compared with the corresponding samples proteolysed without NP-40 (Figure 3, lanes 5, 8 and 11). A uniform topology of L (i.e. 100% protection) could be achieved after extending the chase for up to 4 h (Figure 3, lanes 13–15). Taken together, these results indicate that the preS region of L traverses the lipid layer post-translationally, as the same region is predominantly disposed on the cytoplasmic side during or soon after biosynthesis. It should be noted, however, that this post-translational transfer did not lead to glycosylation of preS.

Unlike the S and M proteins, the L protein is not secreted as empty envelope particles (McLachlan *et al.*, 1987; Ou and Rutter, 1987). Therefore, the unusual topology observed for L may be due to intracellular retention mediated through myristylation and/or N-terminal amino acid sequences. We and others have shown previously (Kuroki *et al.*, 1989; Prange *et al.*, 1991) that mutation of these sequences leads to partial secretion of mutant L proteins. We were therefore interested to analyze the topology of the secretion-competent mutant LΔ2–19, which lacks residues 2–19, including the myristylation signal (Prange *et al.*, 1991). Proteolysis of microsomes prepared from pulse or pulse-chase labeled cells transfected with the mutant gene basically revealed the same pattern of protection as obtained for the wild-type L protein. As shown in Figure 4, incubation with trypsin in the presence of NP-40 likewise converted the non-glycosylated 37 kDa and the glycosylated 40 kDa polypeptides of mutant LΔ2–19 to the T and gT tryptic fragments respectively (lanes 1–3). Like wild-type L, minor amounts of mutant LΔ2–19 were protected when synthesized during a 10 min pulse (Figure 4, lane 2), but this significantly increased during chase (Figure 4, lane 5). Therefore, intracellular retention of L does not account for the post-translational translocation of its preS region.

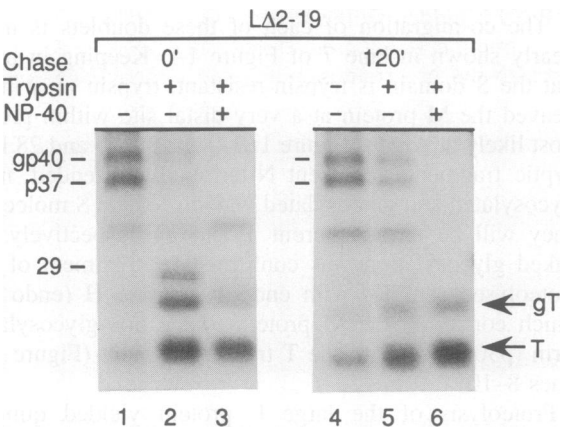

Fig. 4. Topology of the secretion-competent mutant LΔ2–19 protein. COS7 cells expressing the non-glycosylated (p37) and glycosylated (gp40) forms of mutant LΔ2–19 were pulse labeled for 10 min and either lysed immediately (lanes 1–3) or chased for 120 min (lanes 4–6). As denoted above each lane, microsomes were left untreated or digested with trypsin in the absence or presence of NP-40. Arrows to the right mark the tryptic fragments.

The non-uniform topology of L is maintained upon secretion of empty envelopes

Given the unexpected topology of the L protein, we were interested in analyzing whether the non-uniform disposition of its preS region was maintained during export out of the cell. To approach this issue, we took advantage of mutant LΔ2–19, which is secreted from transfected cells along with the internally initiated M and S proteins (Prange *et al.*, 1991). Empty envelope particles were harvested from the cellular supernatants, treated with trypsin and analyzed by immunoassays.

Native particles were strongly reactive in a preS1-specific ELISA (Table I) operating with the preS1-specific MAb 18/7 in the solid phase and HBsAg-specific MAbs in the detection phase. This indicates that the epitope of MAb 18/7, which has been mapped to the N-terminus of L (Heermann and Gerlich, 1991), was exposed on the surface of the particles and should therefore be susceptible to trypsin. As expected, trypsin treatment removed the epitopes of MAb 18/7 from the particles, even in the absence of NP-40 (Table I). Most importantly, preS1-reactivity was restored when the proteolysed particles were disrupted with NP-40 after inactivation of trypsin

Table I. Analysis of the topology of mutant LΔ2–19 in secreted empty envelope particles using trypsin and detergent combined with immunoassays

Treatment of empty envelope particles	E_{492} nm in ELISA	
	preS1 ELISA[a]	HBsAg ELISA[b]
Untreated	1.022	0.569
NP-40 − trypsin	1.857	0.552
Trypsin − NP-40	0.044	0.523
Trypsin + NP-40	0.063	0.476
Trypsin − NP-40 + post-proteolytic addition of NP-40	0.713	0.511

[a]Cut-off: 0.050.
[b]Cut-off: 0.068.

(Table I). We could exclude by control experiments (e.g. analysis of the same samples in an HBsAg-specific ELISA) that the antibodies used were affected by detergent (0.5% NP-40) (Table I; data not shown). Therefore, these data demonstrate that the preS region of a fraction of mutant LΔ2–19 is hidden within the envelope particles, while the other fraction is exposed on the surface. As MAb 18/7 binds to the N-terminus of L, the entire preS region of the trypsin-resistant fraction should be buried within the particles. This notion received further support from the observation that detergent treatment of native particles led to an increased exposure of preS1 epitopes (Table I).

Mapping a sequence that is involved in post-translational translocation of the preS region

To examine the structural requirements for the unusual topology of L, various mutant L proteins were analyzed after expression in transfected cells. Surprisingly, deletion of the 38 residues from the C-terminus of the preS1 region (LΔ70–107) dramatically altered the transmembrane orientation of preS, although it did not allow secretion. In addition to the expected non- and single-glycosylated forms, double- and triple-glycosylated polypeptides were found (Figure 5A, lane 1). *N*-linked glycosylation was confirmed by treatment with endo H (Figure 5A, lane 2). When microsomes prepared from cells transfected with mutant LΔ70–107 were digested with trypsin, the non-glycosylated T and the single-glycosylated gT fragments were again generated (Figure 5B, lane 3), demonstrating that the additional glycosides were linked to the preS region cleaved off by trypsin, most likely to Asn4 of preS1 and Asn4 of preS2. Since *N*-linked glycans are added co-translationally to polypeptide chains as the consensus sequence emerges on the luminal side of the ER membrane (Doms *et al.*, 1993), the glycosylation pattern of mutant LΔ70–107 indicates a co-translational translocation of its preS region. Consistent with this notion, most of the newly synthesized polypeptides of mutant LΔ70–107 were protected from trypsin cleavage unless NP-40 was present (Figure 5B, lanes 1–3). Thus we conclude that amino acids 70–107 of L interfere with the co-translational translocation of the preS domain.

Topology of HBV env proteins synthesized in vitro: nascent env polypeptides adopt two different transmembrane configurations

In parallel to the *in vivo* studies, we analyzed the topology of env proteins synthesized in a cell-free system. The env

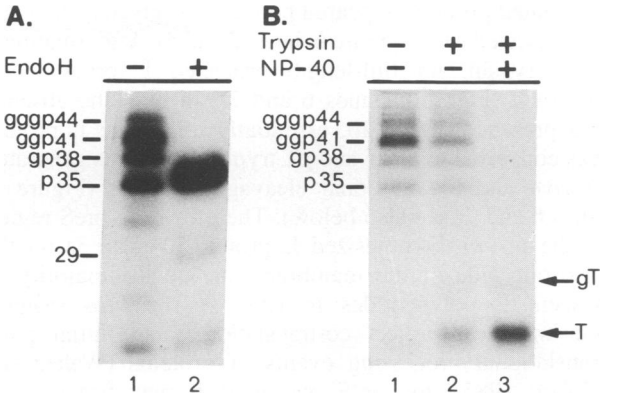

Fig. 5. Topology of the secretion-deficient mutant LΔ70–107 protein. (**A**) Transfected cells were labeled for 2 h and subjected to immunoprecipitation. Samples were divided in half and either mock-treated (lane 1) or digested with endo H (lane 2). Numbers to the left indicate positions of mol. wt standards in kDa and deduced molecular masses of the non-glycosylated (p) and glycosylated (gp, ggp, gggp) forms of mutant LΔ70–107. (**B**) Trypsin protection assay of mutant LΔ70–107. Microsomes were prepared from cells that were labeled for 10 min without chase and were subjected to proteolysis with trypsin as denoted above each lane. Arrows to the right mark the tryptic fragments.

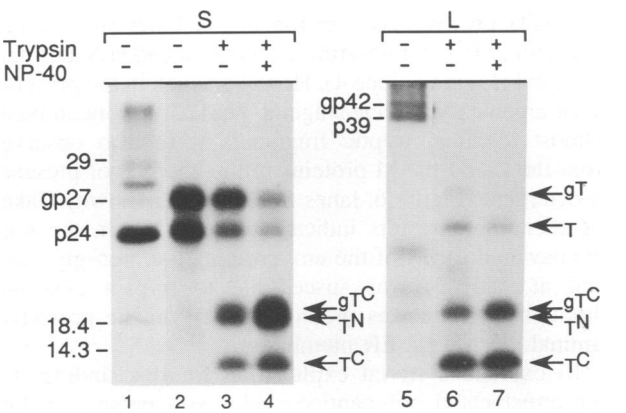

Fig. 6. Topology of the HBV env proteins synthesized in a cell-free system. The S (lanes 1–4) and the L proteins (lanes 5–7) were synthesized in a coupled transcription/translation/translocation system using a microsomal concentration of either 0.6 or 0.3 equivalents/25 μl translation mix respectively. Following synthesis, microsomes were sedimented through sucrose to separate them from non-translocated polypeptide chains remaining in the supernatant, as shown for the S protein (p24) in lane 1. Microsomes were subjected to proteolysis with trypsin as denoted above each lane. Samples were analyzed by SDS−PAGE without prior immunoprecipitation. Numbers to the left of lanes 1 and 5 indicate positions of mol. wt standards in kDa and deduced molecular masses of the non-glycosylated and glycosylated forms of the env proteins. Arrows to the right of lanes 4 and 7 mark the tryptic fragments described in the text.

genes were expressed in a one-step, coupled transcription/translation/translocation system using rabbit reticulocyte lysates and canine pancreatic microsomes. Non-translocated polypeptide chains were removed by sedimentation of microsomes through sucrose (see Figure 6, compare lanes 1 and 2) prior to proteolysis with trypsin and SDS−PAGE.

Synthesis of the env proteins in the *in vitro* system closely reflected the *in vivo* situation; the microsome-

associated proteins appeared both in non-glycosylated and glycosylated form (Figure 6, lanes 2 and 5). After treatment with trypsin, no full-length protected L protein was observed (Figure 6, lanes 6 and 7). In both the absence and presence of NP-40, essentially all of the L protein was converted to the T and gT tryptic fragments generated *in vivo* and to additional cleavage products (Figure 6, lanes 6 and 7; see also below). Therefore, the preS region of the *in vitro* synthesized L protein likewise is on the cytosolic side of the membrane, as are the majority of nascent L polypeptides *in vivo*. As *in vitro* systems are known to reflect co-translational and initial post-translational processing events of proteins (Walter and Blobel, 1983), the preS region of L may traverse the membrane in a post-translational process which does not seem to occur *in vitro*.

The appearance of the additional tryptic fragments of L was quite surprising. We therefore analyzed the transmembrane topology of the S protein using trypsin digestion and again obtained a rather unexpected pattern of protection: while the glycosylated form of S was protected in the absence of NP-40, the non-glycosylated form was partially cleaved yielding two fragments of ~16.6 kDa (T^N) and 13.2 kDa (T^C), corresponding to cleavage at Arg122 (Figure 6, lane 3). In the presence of NP-40, cleavage of the glycosylated S protein yielded the 16.2 kDa glycosylated version of the C-terminal tryptic fragment (gT^C) co-migrating with the 16.6 kDa N-terminal fragment (Figure 6, lane 4). However, even in the presence of detergent, trypsin cleavage at Arg122 was incomplete. Almost identical tryptic fragments were also observed from the L and the M proteins in the absence or presence of detergent (Figure 6, lanes 6–7; data not shown). Taken together, these results indicate that Arg122 in the non-glycosylated forms of the env proteins (i.e. non-glycosylated at Asn146) was susceptible to trypsin cleavage, although this site was predicted to be on the protective luminal side of the ER membrane.

To exclude a trivial explanation for this finding, i.e. non-translocated polypeptide chains still present leading to the observed partial trypsin sensitivity, two control experiments were performed. First, we analyzed the topology of a modified S protein (S^{polio}) carrying a poliovirus epitope inserted at residue 113 (Delpeyroux *et al.*, 1986). Unlike Arg122, the lysine residues present in the 11 amino acid insertion are readily accessible to trypsin (Delpeyroux *et al.*, 1986). In the presence of NP-40, both the non-glycosylated and the glycosylated form of S^{polio} were almost completely cleaved by trypsin to the expected limit products (Figure 7A, compare lanes 1 and 3). Most importantly, in the absence of detergent the glycosylated form was entirely protected, while the non-glycosylated form was entirely cleaved (Figure 7A, lane 2). Protection in the absence of detergent of a simple secretory protein, prepro-α-factor, which we included as a control, confirmed the integrity of the microsomes used (Figure 7A, lanes 4–6). These results demonstrate that the partial trypsin cleavage at Arg122 of the non-glycosylated wild-type S protein (Figure 6, lane 3) was due to steric hindrance, rather than to membrane sequestration. In a second control experiment carried out to verify that the trypsin-sensitive, non-glycosylated form of S^{polio} was indeed integrated into microsomal membranes, re-isolated microsomes were

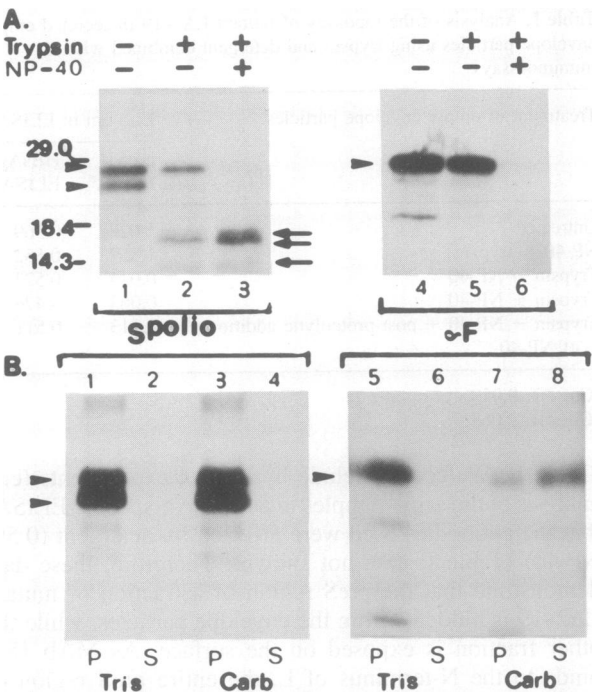

Fig. 7. Topology of a modified S^{polio} protein synthesized in the cell-free system. (**A**) The S^{polio} protein (lanes 1–3) or prepro-α-factor (α-F, lanes 4–6) were synthesized in a coupled transcription/translation/translocation assay. Re-isolated microsomes were subjected to proteolysis with trypsin as denoted above each lane. Numbers to the left indicate the positions of mol. wt standards in kDa. Arrowheads mark the positions of the 25.5 kDa non-glycosylated and the 28.5 kDa glycosylated form of S^{polio}, while the arrowhead to the left of lane 4 marks the 30 kDa prepro-α-factor. Arrows indicate the positions of the tryptic fragments. (**B**) Carbonate extraction of the S^{polio} protein (lanes 1–4) and prepro-α-factor (lanes 5–8). After *in vitro* synthesis, re-isolated microsome fractions were divided in half and treated with Tris-buffered sucrose (Tris) (lanes 1, 2, 5 and 6) or alkaline carbonate (Carb) (lanes 3, 4, 7 and 8). Following ultracentrifugation, the supernatants (S) and pellets (P) were separated and analyzed by SDS–PAGE. Arrowheads mark the positions of the S^{polio} protein and prepro-α-factor respectively.

subjected to high pH treatment. As shown in Figure 7B (lanes 3 and 4), alkaline carbonate treatment could release neither the non-glycosylated nor the glycosylated form of S^{polio} into the supernatant, indicating that both forms were firmly associated with the membrane. Under the same conditions, the secretory prepro-α-factor was largely extracted from the pelleted membranes (Figure 7B, lanes 7 and 8), but was retained in intact microsomes on treatment with Tris-buffered sucrose (Figure 7B, lanes 5 and 6).

In summary, these data demonstrate that nascent chains of the env proteins adopt more than one transmembrane orientation with reference to Arg122 located in the second hydrophilic loop of S. The strict correlation found between trypsin resistance (Arg122) and glycosylation (Asn146) and vice versa suggests that the entire second hydrophilic loop of S is on the cytoplasmic side of the ER membrane in non-glycosylated chains and on the luminal side in glycosylated chains. This heterogeneity explains well the rather uncommon, partial glycosylation of the S protein occurring *in vitro* and *in vivo*.

Discussion

In this work we have analyzed the topology of the envelope proteins of HBV. These proteins are initially synthesized as transmembrane proteins of the ER membrane (Eble *et al.*, 1986). Based on epitope mapping studies (Heermann *et al.*, 1984; Kuroki *et al.*, 1990), the current view of the transmembrane structure of the large L protein assumes that the entire preS region of L is on the luminal side of the ER membrane and is external in the virion (Heermann and Gerlich, 1991; Stirk *et al.*, 1992). We demonstrate in the present study that the L protein adopts more than one transmembrane orientation.

During or soon after biosynthesis of L in transfected cells at least the very C-terminus of the preS domain is not translocated across the ER membrane, according to its trypsin sensitivity, while the S region appears to be conventionally integrated into the ER membrane, as evidenced by glycosylation, intrinsic trypsin resistance and association with microsomes. Since the preS region lacks signal sequences that initiate co-translational translocation across the ER membrane (Eble *et al.*, 1990; Ostapchuk *et al.*, 1994) and stretches of hydrophobic sequences sufficient to span or to be embedded in the lipid layer, the entire preS region is most likely on the cytosolic side. Recent mutational analysis of tryptic sites of the preS region of L combined with *in vitro* topogenesis studies supports this notion (Ostapchuk *et al.*, 1994). The failure of the preS region of L to be co-translationally transferred may be due to a translocation-incompetent conformation. As L is targeted to the ER by the topogenic elements of its S region (Ostapchuk *et al.*, 1994), a polypeptide of ~200 amino acids must be synthesized until the signal sequence responsible emerges from the ribosome. Premature folding of preS may thus hinder its co-translational movement across the membrane. Similarly, one might expect co-translational myristylation of L in the cytoplasm to impair the translocation conducive structure of preS. However, the phenotypes of the myristylation-deficient mutant LΔ2–19 and the myristylation-competent mutant LΔ70–107 strongly argue against a role for myristylation in translocation of preS. Alternatively, the initial cytoplasmic disposition of preS could be governed by interaction with cellular regulatory molecules, such as chaperones. These proteins transiently associate with proteins as they are synthesized, thereby preserving (post-translational) translocation competence (Beckmann *et al.*, 1990).

Interaction of cytoplasmic cellular components with specific sequences of the preS1 domain was suggested by the finding that co-translational translocation concomitant with *N*-linked glycosylation of the preS region could be achieved by deletion of 38 residues from the C-terminus of the preS1 region. Larger lesions in the N-terminus of the preS1 region (e.g. Δ2–67) did not lead to glycosylation (Bruss and Thomssen, 1994; Hilfrich and Streeck, unpublished data), strongly indicating that the deleted sequence rather than its size accounts for the co-translational translocation of the preS domain of mutant LΔ70–107. Strikingly, this sequence (amino acids 70–107) overlaps with a region important for HBV virion assembly. Recent analysis of truncated L proteins (subtype adw) revealed that the N-terminal 5/6 of the preS1 region is dispensable for virion formation, whereas its last 17 residues are indispensable (Bruss and Thomssen, 1994). Most interestingly, only the assembly-incompetent L mutants were glycosylated in their truncated preS regions (Bruss and Thomssen, 1994) and thus presumably co-translationally translocated, like the deletion mutant LΔ70–107. Combining these data, the very C-terminus of preS1 seems to determine the mode of translocation of the preS domain. The initial cytoplasmic disposition of preS might thus be regulated to offer docking sites for envelopment of the viral nucleocapsid.

However, the entire preS domain of L gained access to the lumen of the microsomes even in the absence of nucleocapsids. The results of our pulse-chase analysis strongly suggest a post-translational transfer of preS which does not lead to glycosylation of preS. Therefore, this transfer seems to occur in a compartmental environment distal to the rough ER. The mechanism by which the preS domain traverses the lipid layer, however, remains enigmatic. As we have shown, this process neither depends on the envelopment of the viral nucleocapsid nor on secretion of empty envelopes from the transfected cells. Hence, it may be due to the intracellular assembly of empty envelopes. Although intracellularly retained in a transmembrane configuration (our unpublished results), the L protein still oligomerizes with S and M chains, thereby preventing the secretion of empty envelopes (McLachlan *et al.*, 1987; Ou and Rutter, 1987). Formation of S oligomers and their budding into the lumen of a pre-Golgi compartment (Huovila *et al.*, 1992) involve a substantial reorganization of membrane lipids (Gavilanes *et al.*, 1982; Satoh *et al.*, 1990). Therefore, oligomerization of L may induce local changes in the lipid structure that would allow passage of preS.

Apart from its mechanistic feature, the post-translational transfer of preS is unusual in being non-quantitative, thereby imposing a non-uniform topology upon the L protein. Only after extended chase periods could the intracellularly retained wild-type L protein be ultimately shifted into a uniformly oriented population. Importantly, however, the non-uniform disposition of preS is maintained upon secretion of empty envelopes and thus is preserved in the mature viral envelope. Bruss and co-workers (V.Bruss, personal communication) have recently demonstrated a similar dual topology of the L protein in secreted HBV virions isolated from the blood of virus carriers (Bruss *et al.*, 1994, published after completion of this manuscript). Therefore, our topogenesis study of L expressed in the absence of other viral components closely reflects the *in vivo* situation. Moreover, it proves that the partial cytosolic (i.e. internal) disposition of preS is not enforced upon entrapping the viral nucleocapsid. All the information required to achieve the unusual topology resides solely within L, again implying that translocation and thus topology of L may be subject to regulation rather than proceeding by default.

Rather without causality, we found that even the S region of the L and M proteins, as well as the S protein itself, are not uniformly oriented in the membrane of microsomes, as assayed in a cell-free system. The cytoplasmic disposition of the preS domain of nascent L polypeptide chains was confirmed by these experiments, in agreement with a recent *in vitro* topogenesis study

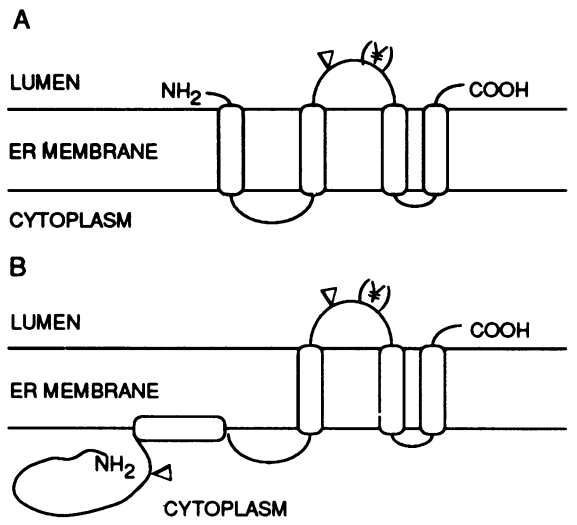

Fig 8. Models for the topology of the HBV env proteins in the ER membrane. The luminal and cytoplasmic sides of the membrane are indicated. (**A**) Transmembrane structure of the S protein proposed by others and discussed in the text. The predicted four membrane spanning segments (Stirk *et al.*, 1992) project the N- and C-termini of S into the ER lumen. The partial glycosylation at Asn146 is indicated by (¥). The triangle (Δ) marks the trypsin cleavage site, Arg122. (**B**) Model of the initial transmembrane topology of the L protein. The cytoplasmic disposition of the preS region of L enforces an altered structure on the first hydrophobic domain of the S region. Both the non-glycosylated and the glycosylated forms of L are susceptible to cleavage with trypsin at Arg156 of the preS region, as indicated by triangle (Δ). During maturation, the topology of L changes, as its entire preS region partially gains access to the ER lumen.

(Ostapchuk *et al.*, 1994). However, inconsistent with this study and previous work (Eble *et al.*, 1986, 1987, 1990), our data do not support the uniform topology of the S protein illustrated in Figure 8A. As we have shown, the trypsin cleavage site (Arg122) of the non-glycosylated form of S is on the cytosolic side of the membrane, while the same site is luminally disposed in the glycosylated form. We have excluded the possibility that trypsin cleavage of the non-glycosylated protein can be accounted for by non-translocated polypeptide chains, as previously postulated (Eble *et al.*, 1986, 1987, 1990; Ostapchuk *et al.*, 1994). The *in vitro* system employed here therefore faithfully reflects the partial glycosylation at Asn146 of the membrane-integrated forms of S observed *in vivo*. Thus the S protein seems to be unique in adopting two different conformational states on co-translational translocation. Multiple orientations of proteins in the ER membrane have until now only been reported for chimeric proteins (Parks *et al.*, 1989). To account for the unusual topology of S, co-translational intermolecular interactions of S polypeptide chains may be considered as dictating the two opposing orientations and yielding heterodimeric structures.

A heterodimeric structure of S consisting of two oppositely oriented molecules has previously been postulated by Tiollais and Wain-Hobson (1984) to explain the partial glycosylation of the S protein. However, inconsistent with their proposal, experimental data support a uniform disposition of the first hydrophilic loop and a transmembrane passage of the first hydrophobic domain (Eble *et al.*, 1986, 1990; Simon *et al.*, 1988) (see Figure 8A). To account for both a cytoplasmic and a luminal disposition of Arg122, located in the second hydrophilic loop of S, the flanking hydrophobic domains may not span the membrane as predicted. The size of the preceding domain (amino acids 80–98) does not favor an additional membrane passage. Therefore, we propose that this segment might be embedded in the plane of the bilayer, rather than spanning the membrane, to generate the topological heterogeneity of the second hydrophilic loop. The arrangement of the succeeding apolar domain(s) might be similarly altered, although their structure is poorly defined at present. In addition, the initial cytosolic location of the preS region of the L protein constrains an altered structure of even the first hydrophobic domain of the S region (Figure 8B).

In vivo, the non-uniform topology of S is less apparent, due to the intrinsic trypsin resistance of both of its forms. This resistance is likely to be conferred by the rapid intra- and intermolecular disulfide cross-linking of S monomers in the ER (Huovila *et al.*, 1992), which does not proceed with the same efficiency *in vitro* (our unpublished results). In support of this notion, trypsin fails to cleave the S protein present in (sub)viral envelope particles unless disulfide bonds are reduced (Guerrero *et al.*, 1988). Albeit hampered by the protease-resistant structure, further analysis should elucidate whether the dual topology of S is maintained during assembly and secretion of the viral envelope. Possibly, the dual topology may provide a mechanism through which the S protein can (re)organize membranes and assemble them into the viral envelope. Such a mechanism has been proposed for the assembly of liver-derived lipoprotein particles (Davies *et al.*, 1990).

Although the various steps of translocation determining the unique topologies of the HBV env proteins may reflect a specialized strategy used by the virus, our results emphasize that basic principles of the cellular translocation machinery may not be absolute. This raises the prospect that other membrane proteins may deviate from the generally accepted views of membrane topology.

Materials and methods

Plasmid constructions

For expression of the HBV env genes (subtype ayw), plasmid pNI2 carrying the human metallothionein IIA promoter was used. As previously described (Prange *et al.*, 1991), plasmid pNI2.L carrying the preS-S coding region and its polyadenylation signal has been obtained by inserting a 2.3 kb *Bgl*II–*Bgl*II fragment [nucleotides (nt) 2839–1986] of the HBV genome into the unique *Bam*HI site of pNI2. For construction of plasmids pNI2.M and pNI2.S, either a flush-ended *Bal*I–*Bgl*II fragment (nt 2972–1986) or a flush-ended *Xho*I–*Bgl*II fragment (nt 129–1986) of the HBV genome respectively, was cloned into the filled-in *Bam*HI site of pNI2. Deletion mutant LΔ70–107 was generated by digestion of pNI2.L with *Sau*I cutting at nt 3052 and 3166 (with reference to the HBV genome). After removal of the 114 bp *Sau*I fragment, plasmid DNA was religated, thereby maintaining the env reading frame.

For cell-free transcription and translation, the S gene was cloned into plasmid pSP64(polyA) (Promega) by insertion of a flush-ended *Xho*I–*Dra*I fragment (nt 129–835) into the filled-in *Hin*dIII site of the polylinker of pSP64(polyA). Using this strategy, a translational stop codon was regenerated at the authentic 3′ end of the S gene. To construct plasmids pSP64.M and pSP64.L, plasmid pSP64.S was digested with *Hin*dIII (nt 129) and *Spe*I (nt 681). The *Hin*dIII–*Spe*I fragment was replaced by either a corresponding *Sau*I–*Spe*I fragment (nt 3166–681) or a *Bgl*II–*Spe*I fragment (nt 2839–681) encoding in addition the preS2 or the preS1–preS2 regions respectively. For introduction of additional tryptic cleavage sites, plasmid pPAP (Delpeyroux *et al.*, 1986) was employed, which contains a modified S gene carrying a 39 bp insertion at nt 490 encoding a poliovirus type I VP1 neutralization epitope. The

wild-type S gene of plasmid pSP64.S was replaced by the modified S gene of pPAP.

Cell culture, transfection and metabolic labeling

Transient transfection of COS7 cells was done as described (Prange *et al.*, 1992) using the calcium phosphate precipitation technique. For metabolic labeling, 2×10^6 cells were washed 48 h after transfection and starved for 40 min in 0.6 ml of methionine-free minimal essential medium without fetal calf serum (FCS) (≤10 min pulse) or supplemented with 1% FCS (>10 min pulse). After addition of 250 μCi [^{35}S]-methionine/cysteine (1000 Ci/mmol; Dupont de Nemours), cells were pulse labeled for the indicated periods. Cells were either harvested directly or after a chase performed by replacing the medium with 2 ml of Dulbecco's modified Eagle's medium supplemented with 10% FCS and 1.5 mg/ml unlabeled methionine and cysteine. For metabolic pulse-chase labeling of the wild-type L and mutant LΔ2–19 protein, the number of cells was increased by a factor of three.

Preparation of microsomes, trypsin protection assay and immunoprecipitation

Labeled cells were washed twice with Tris-buffered saline (TBS, 50 mM Tris−HCl, pH 7.5, 150 mM NaCl) and were incubated with 0.9 ml 0.1× TBS/6 cm dish for 10 min on ice. Swollen cells were disrupted by Dounce homogenization (20 strokes) and adjusted to 1× TBS using 10× TBS. Unbroken cells and nuclei were removed by centrifugation for 20 min at 2500 r.p.m. and 4°C in a microfuge. The supernatant was layered on 2.7 ml of 250 mM sucrose/TBS and centrifuged for 30 min at 37 000 r.p.m. and 4°C in a SW60 rotor (Beckman). After a brief wash, the microsomal pellet was carefully suspended in 1 ml of TBS supplemented with 2 mM tetracaine to stabilize the microsomal membranes. For proteolysis with trypsin, the microsomes were divided into three equal aliquots and were adjusted to 2 mM $CaCl_2$. Trypsin (Boehringer) was either omitted or added to a final concentration of 25 μg/ml in the absence or in the presence of 0.5% NP-40. After incubation for 1 h on ice, proteolysis was terminated by the addition of 30 μg/ml aprotinin (Boehringer) followed by an incubation for 20 min on ice. Each sample was then adjusted to 0.5% NP-40 and solubilized for 20 min on ice. After centrifugation for 5 min at 10 000 r.p.m. and 4°C in a microfuge, the supernatants were subjected to immunoprecipitation as previously described (Prange *et al.*, 1992), except that polyclonal antiserum was used, which was raised in rabbits by immunization of animals with yeast-derived HBsAg particles (Engerix-B; Smith Kline Beecham). Alternatively, labeled cells were lysed in the presence of detergent and subjected to immunoprecipitation with the rabbit antiserum and, if indicated, treated with endo H as previously described (Prange *et al.*, 1992).

Isolation of subviral particles and immunoassays

Empty envelope particles were isolated from the supernatants of transfected COS7 cells. Supernatants were collected, clarified and concentrated by ultracentrifugation for 4 h at 35 000 r.p.m. and 4°C (SW40 rotor; Beckman). The pellets were suspended in TBS supplemented with 2 mM tetracaine and were digested with trypsin (25 μg/ml) in the presence or absence of 0.5% NP-40 as outlined above. Proteolysed samples were then subjected to immunoassays. HBsAg reactivity was determined by ELISA (Auszyme II; Abbott Laboratories). The preS1-specific ELISA was performed as described (Marquardt *et al.*, 1987) using the preS1-specific MAb 18/7 (Heermann *et al.*, 1984) in the solid phase and peroxidase-labeled HBsAg-specific MAbs (Auszyme II) in the detection phase.

Cell-free transcription, translation and translocation

In vitro transcription and translation were carried out using a coupled transcription/translation system (TNT™ Coupled Reticulocyte Lysate System; Promega) according to the instructions of the manufacturer. In a typical reaction mixture of 25 μl, 0.5 μg of circular plasmid DNA was transcribed with SP6 polymerase and translated using rabbit reticulocyte lysate in the presence of 20 μCi [^{35}S]methionine (1000 Ci/mmol; Amersham) and dog pancreas microsomes (Promega). As recommended, the optimal microsome concentration for different constructs was experimentally determined. Following incubation, microsomes were sedimented by layering the reaction on 1 ml of 250 mM sucrose/TBS and centrifuged for 30 min at 14 000 r.p.m. and 4°C in a microfuge. The microsomal pellet was proteolysed with trypsin as outlined above, except that trypsin was used at a final concentration of 80 μg/ml and the volume of each reaction sample was scaled down to 12.5 μl, since immunoprecipitation was omitted. For extraction with sodium carbonate, sedimented microsome fractions were divided in half and diluted 300-fold with either 100 mM sodium carbonate, pH 11.5 (Fujiki *et al.*, 1982), or 250 mM sucrose/20 mM Tris−HCl, pH 7.5. Following incubation on ice for 30 min, samples were centrifuged for 30 min at 37 000 r.p.m. and 4°C (SW60 rotor; Beckman). Proteins in the supernatants were precipitated with 10% trichloroacetic acid (TCA) and washed twice with 5% TCA and once with acetone. The precipitates and pelleted membranes were adjusted to the same volume using sample buffer and analyzed by SDS−PAGE.

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