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Post-translational alterations in transmembrane topology of the hepatitis B virus large envelope protein

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The preS domain at the N-terminus of the large envelope protein (LHBs) of the hepatitis B virus is involved in (i) envelopment of viral nucleocapsids and (ii) binding to the host cell. While the first function suggests a cytosolic location of the preS domain during virion assembly, the function as an attachment site requires its translocation across the lipid bilayer and final exposure on the virion surface. We compared the transmembrane topology of newly synthesized LHBs in the endoplasmic reticulum (ER) membrane with its topology in the envelope of secreted virions. Protease sensitivity and the absence of glycosylation suggest that the entire preS domain of newly synthesized LHBs remains at the cytosolic side of ER vesicles. However, virions secreted from transfected cell cultures or isolated from the blood of persistent virus carriers expose antibody binding sites and proteolytic cleavage sites of the preS domain at their surface in approximately half of the LHBs molecules. Thus, preS domains appear to be transported across the viral lipid barrier by a novel post-translational translocation mechanism to fulfil a dual function in virion assembly and attachment to the host cell.

Key words: HBsAg/preS domain/protein translocation/viral envelope protein/virus morphology

Introduction

The viral envelope is responsible for the transport of the nucleocapsid from one cell to another and consists of host-derived lipids and virally encoded proteins. These envelope proteins are synthesized at the rough endoplasmic reticulum (ER) where they gain a defined transmembrane topology, fold and oligomerize. They are subsequently transported via vesicles to a cellular target membrane for virus particle formation (Copeland *et al.*, 1988). The virions are generated by binding of the nucleocapsid to one side of the target membrane containing the envelope proteins, budding across this membrane and release of the enveloped nucleocapsid at the opposite side of the lipid bilayer (Stephens and Compans, 1988). It is believed that the integrity of the lipid membrane is not disturbed by the budding process and that the membrane topology of the envelope proteins in the virion reflects their topology in the ER: cytosolic domains become internal in the virus particle while luminal domains become exposed to the outside. In this paper, we present data showing that the morphogenesis of the human hepatitis B virus (HBV) does not follow this paradigm.

HBV is the prototype of a family of hepatotropic, enveloped DNA viruses (hepadnaviridae) that cause acute and chronic infections of the liver [for a review, see Ganem and Varmus (1987)]. The HBV particle is a sphere, 42 nm in diameter and consists of a 27 nm icosahedral nucleocapsid surrounded by a lipid envelope, containing three closely related transmembrane proteins (Heermann *et al.*, 1984) known as hepatitis B surface (HBs) proteins. Unlike many other enveloped viruses, hepadnaviruses do not encode a matrix protein which links the nucleocapsid and envelope. The site of virion formation is probably the ER membrane, since the envelope proteins have not been found in the membranes of other cellular compartments. Rather, hepadnaviral envelope proteins, which are not incorporated into virions, form lipid-containing spherical or tubular particles of 20 nm diameter in the ER lumen and are secreted from infected cells in large quantities as hepatitis B virus surface antigen (HBsAg) particles (Simon *et al.*, 1988).

HBV encodes all three envelope proteins with one single open reading frame by using three different in-frame translation start codons and a common stop codon. As a consequence, the sequences of middle [MHBs, 281 amino acids (aa)] and small (SHBs, 226 aa) envelope proteins are identical with the sequence of C-terminal portions of the large protein (LHBs, 400 aa for HBsAg subtype ad; Figure 1) (Heermann *et al.*, 1984). The transmembrane folding of the SHBs and MHBs proteins has been determined in considerable detail: SHBs traverses the membrane at least twice, as determined by two topogenic signals (I and II) and forms a cytosolic loop of ~55 aa between the two transmembrane regions of signals I and II, and a luminal partially glycosylated loop that contains the major epitopes of the protein downstream of signal II (Eble *et al.*, 1987) (Figure 5B). The topology of MHBs is very similiar to that of SHBs: the additional N-terminal 55 aa of MHBs, referred to as the preS2 domain, are translocated into the ER lumen by signal I downstream in its S domain (Eble *et al.*, 1990). Consequently, the preS2 domain of MHBs and the major epitope domains of MHBs and SHBs are exposed on virions, whereas the loop between signals I and II is supposed to be internal (Heermann and Gerlich, 1991). The transmembrane topology of the LHBs protein in the ER membrane was less clear. It was known that epitopes of the additional 119 N-terminal aa (the preS1 domain) appeared on the virion surface and that the preS2 domain of LHBs is accessible to proteases in the virus particle (Heermann *et al.*, 1984). Therefore, most models assumed that the preS1 and preS2 domains were located in the ER lumen after synthesis of the LHBs protein, and that the topology of LHBs is very similar to that of MHBs (Heermann and Gerlich, 1991). It is believed that the preS domain plays an important role for the attachment of the virus to and penetration into the host cell (Pontisso

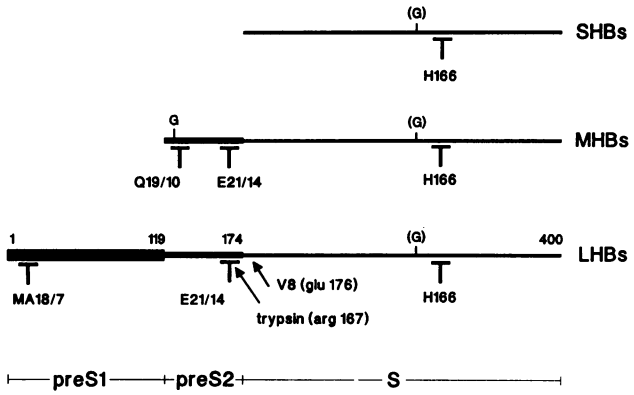

Fig. 1. Domain structure of the hepatitis B virus envelope proteins. All three HBV envelope proteins SHBs, MHBs and LHBs are encoded by one open reading frame with three different in-frame start codons. The LHBs protein consists of the 119 aa preS1 (for subtype ad), the 55 aa long preS2 and the 226 aa long S domains which are indicated by the different widths of the lines. The S domains of all three proteins are partially glycosylated, as indicated by a G in parenthesis. The preS2 domain of the MHBs, but not of the LHBs protein, is glycosylated in naturally occurring HBV envelope protein (as shown by the G without parenthesis). Trypsin efficiently cleaves the MHBs and SHBs proteins at Arg167 and probably at additional sites upstream. V8 protease cleaves LHBs and MHBs proteins only once at Glu2 in their S domains. Arg167 is part of the linear epitope of mAb E21/14 that recognizes aa 161–174 in LHBs and MHBs. The mAb MA18/7 binds to an N-terminal epitope in preS1, Q19/10 binds to a glycan-dependent epitope at the N-terminus of preS2 in the MHBs protein, but not in the LHBs protein, and the epitope of H166 is present in the S domain of all three proteins.

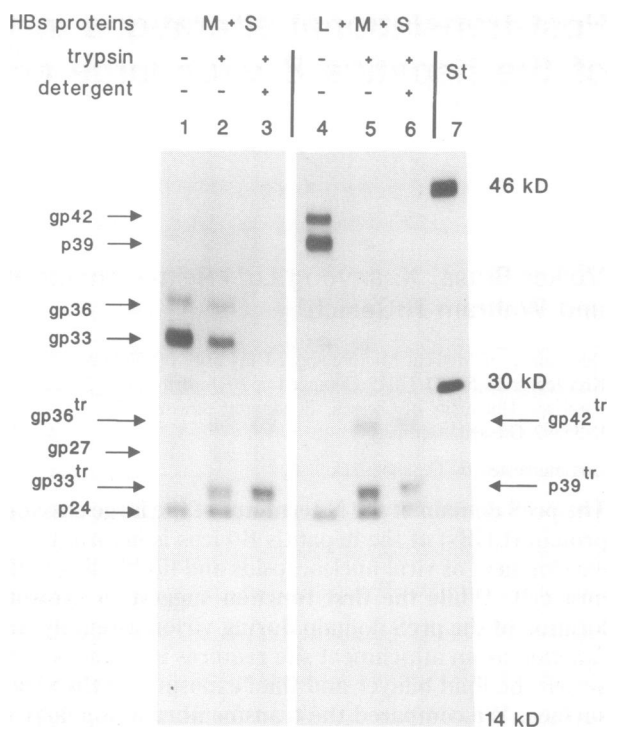

Fig. 2. Protease protection experiment to determine the transmembrane topology of the LHBs preS2 domain in ER vesicles. Microsomes were prepared from COS cells that express the MHBs and SHBs (lanes 1–3) or the LHBs and small amounts of MHBs and SHBs proteins (lanes 4–6) which were pulse-labelled with [^{35}S]methionine for 10 min without chase. The microsomes were either mock-treated (lanes 1 and 4) or incubated with trypsin in the absence (lanes 2 and 5) or presence (lanes 3 and 6) of detergent. After inhibition of the protease, envelope proteins were immunoprecipitated with polyclonal anti-HBs and depicted by PAGE and autoradiography. The LHBs protein was cleaved completely, generating 25 kDa (p39^{tr}) and 28 kDa (gp42^{tr}) C-terminal fragments, as indicated by arrows, even in the absence of detergent (lane 5). Only a minor fraction of the microsome-associated MHBs protein was cleaved by trypsin generating gp33^{tr} and gp36^{tr} (lane 2), but cleavage was complete when the microsomes were disrupted by detergent (lane 3). This shows that Arg167 was on the cytosolic side of the ER in the case of the LHBs protein and on the luminal side for the MHBs protein. Lane 7 contains a molecular mass standard.

et al., 1989; Neurath *et al.*, 1992; Budkowska *et al.*, 1993; X.Lu and W.H.Gerlich, unpublished data), and these functions were in agreement with the assumed translocation of the preS domain into the ER lumen.

The formation of HBV particles requires the participation of the LHBs and SHBs, but not of the MHBs protein (Bruss and Ganem, 1991a). This finding was surprising since the sequence of the SHBs protein is fully present at the C-terminus of LHBs and both proteins were supposed to expose identical peptide sequences at the cytosolic side of the ER membrane. Our experiments, however, show that the topology of newly synthesized LHBs protein in the ER membrane is fundamentally different from the folding of SHBs or MHBs. More interestingly, the topology of approximately half of the LHBs proteins changed dramatically relative to the lipid barrier during virion morphogenesis.

Results

Topology of newly synthesized transmembrane LHBs protein in ER vesicles

The preS domains of the LHBs (Heermann *et al.*, 1987) and MHBs (Stibbe and Gerlich, 1983) proteins of certain HBV subtypes contain several trypsin-sensitive sites, Arg167 being the most C-terminal (Figure 1). We tried to determine the location of this site relative to the ER membrane in newly synthesized LHBs proteins by protease protection experiments. The LHBs protein was expressed in COS7 cells together with MHBs and SHBs from an SV40 early promoter-driven expression vector. SHBs, and in particular MHBs, were only weakly expressed by this vector, due to the relatively weak activity of the MHBs/SHBs promoter that is internal in the preS1 sequence, relative to the SV40 early promoter driving LHBs expression. After labelling with [^{35}S]methionine for 10 min without chase, microsomal vesicles were prepared by douncing and were incubated with trypsin in the absence or presence of the mild detergent Nonidet P40 (NP-40). After inactivation of the protease, the HBV envelope proteins were immunoprecipitated with an antiserum against epitopes in the S domain present in all three proteins (anti-HBs) and depicted by PAGE and autoradiography.

Figure 2 (lane 4) shows the untreated envelope proteins. One characteristic of all three HBV envelope proteins is that they are only partially glycosylated at Asn146 of their S domain (Heermann *et al.*, 1984), resulting in the formation of doublets. Consequently, the LHBs protein was expressed as unglycosylated p39 and as once-glycosylated gp42. The glycosylation site at Asn4 in the preS2 domain of this protein (Heermann *et al.*, 1984) is not used, which provides a first hint for a cytosolic location of this site in the LHBs protein. The MHBs protein was glycosylated at Asn4 in its preS2 domain (gp33) and, in addition, was partially glycosylated

at Asn146 in its S domain (gp36). The unglycosylated SHBs protein p24 and its glycosylated version gp27 form the third doublet. COS7 cells do not glycosylate the S domain as efficiently as the infected liver. Thus, gp27 was only weakly visible.

Incubation with trypsin in the absence of detergent reproducibly converted almost all of the LHBs proteins into fragments of ~25 and 28 kDa (lane 5, $p39^{tr}$ and $gp423^{tr}$, marked by arrows). The fragments represent the expected C-terminal parts of the LHBs protein (Heermann *et al.*, 1987) (Figure 1), since (i) the 3 kDa difference in relative mass agrees with the 28 kDa fragment being the glycosylated version of the 25 kDa fragment, (ii) cleavage of the MHBs protein generated the same two fragments (lane 3), arguing for the fragments to be generated from the preS2/S domain, and (iii) the S domain is resistant to cleavage with trypsin in our experiment (see, for example, Figure 2, lanes 5 and 6). Therefore, these digestion products were generated by trypsin cleavage shortly upstream of the S domain in the LHBs protein at Arg167 (Figure 1). The weakly detectable MHBs protein was not cleaved in the absence of detergent (Figure 2, lane 5). This is in agreement with the known luminal location of the preS2 domain of this protein in the ER (Eble *et al.*, 1990) and served as an internal control of the polarity and integrity of the microsomes. This behaviour of MHBs is demonstrated more clearly in a control experiment by using microsomes prepared from cells expressing only MHBs and SHBs under the control of the SV40 early promoter (lanes 1–3). Without detergent added, more than half of MHBs was protected against trypsin (lane 2). In the presence of detergent, both the MHBs ($gp33^{tr}$ and $gp36^{tr}$, lane 6 or 3) and the LHBs protein (lane 6) were completely digested. These experiments show that in virtually all of the newly synthesized LHBs protein molecules Arg167 was at the cytosolic side of the ER membrane.

Topology of the LHBs protein in the envelope of virions secreted from cultured cells

As a second probe for accessibility of the preS domain, we used the monoclonal antibody (mAb) E21/14 which recognizes aa 161–174 of the preS sequence as a linear epitope (H.Meisel *et al.*, in preparation). This sequence also contains the trypsin cleavage site in the C-terminal region of preS2, as mentioned above (Figure 1). To determine whether this site is exposed on the surface of virions as a component of the LHBs protein, we used the antibody for immunoprecipitation of virions under non-denaturing conditions. The epitope of E21/14 is also present on MHBs (see Figure 1), but the MHBs protein is dispensable for virion formation (Bruss and Ganem, 1991a). Therefore, we employed a point mutant (M^-) of HBV secreted from transiently transfected cell cultures which lacks the MHBs protein due to an ATG to ACG mutation of its start codon. The immunoprecipitated virions were detected by the activity of the endogenous polymerase of the virus which incorporates radioactive dNTPs into a gap of the viral genome under appropriate conditions. The labelled viral genomes were isolated, separated on an agarose gel and visualized by autoradiography (Figure 3). In lane 1, wild-type (WT) virions from transiently transfected HepG2 cells were treated with NP-40 to open the viral envelope and to release the nucleocapsids which could be immunoprecipitated

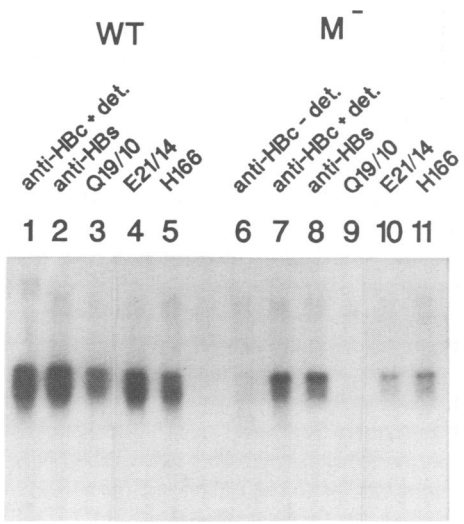

Fig. 3. Immunoprecipitation of wild-type and M^- virions. Wild-type (WT) virions and a point mutant (M^-) lacking the MHBs protein were expressed in HepG2 cells, immunoprecipitated with different antibodies and detected by the activity of the endogenous DNA polymerase incorporating radioactively labelled dNTPs into the viral genome. The genomes were isolated, separated on an agarose gel and visualized by autoradiography. The WT virus could be immunoprecipitated with a polyclonal antibody (anti-HBs, lane 2) or a mAb (H166, lane 5) against the S domain, with the monoclonal anti-preS2 antibodies Q19/10 and E21/14 (lanes 3 and 4, respectively), and with antibodies against the nucleocapsid (anti-HBc) after solubilization of the viral envelope with detergent (lane 1). The M^- virus was also immunoprecipitable with polyclonal (lane 8) or monoclonal (lane 11) anti-HBs or with anti-HBc in the presence of detergent (lane 7). In the absence of detergent, only a very minor fraction of nucleocapsids is bound to anti-HBc (lane 6), showing that the majority of the M^- virions possessed a complete envelope. Q19/10 is known to depend on the glycan linked to Asn4 in the preS2 domain of the MHBs, but not of the LHBs protein (see Figure 1). Consequently, the M^- virions did not bind to Q19/10 (lane 9). However, the monoclonal anti-preS2 antibody E21/14 immunoprecipitated the M^- virions (lane 10) almost as efficiently as the monoclonal anti-S antibody H166 (lane 11). This demonstrates that the epitope of E21/14, containing the trypsin cleavage site, was exposed on the surface of virions by LHBs protein.

with an antiserum against the nucleocapsid antigen (anti-HBc). The polyclonal anti-HBs antiserum immunoprecipitated the untreated WT virions with the same efficiency (lane 2). The monoclonal anti-HBs antibody H166 (lane 5), as well as the anti-preS2 antibodies Q19/10 (lane 3) and E21/14 (lane 4), also bound to WT virions. The differences in the amount of precipitated genomes probably reflect differences in the affinity of the antibodies to the virion and/or to the immobilized protein A.

The total amount of nucleocapsids of the M^- mutant in the culture medium was determined by immunoprecipitation with anti-HBc after solubilization of the viral envelope with NP-40 (lane 7). The same amount of virions could be found with polyclonal anti-HBs in the absence of detergent (lane 8). To show that the majority of M^- virus particles had a complete envelope, an equivalent aliquot of the culture medium was used for immunoprecipitations with anti-HBc in the absence of detergent. Only a very small amount of nucleocapsids was found (lane 6), demonstrating again that the absence of MHBs protein is compatible with the formation of a sealed envelope (Bruss and Ganem, 1991a). The monoclonal anti-preS2 antibodies gave different results: antibody Q19/10 is known to depend on the glycan bound

to Asn4 of the preS2 domain in the MHBs protein (Heermann *et al.*, 1988). Consequently, Q19/10 did not bind to the M^- virions (lane 9), since this site is not glycosylated in the LHBs protein. E21/14 binding to the C-terminal part of preS2 gave a signal (lane 10) almost as strong as that found by using the monoclonal anti-S antibody H166 (lane 11). These results show that the MHBs protein was indeed absent in the M^- variant and that the C-terminal portion of the preS2 domain of the LHBs protein was accessible on the virion surface.

Topology of LHBs in the envelope of carrier-derived virions

The findings described so far do not conclusively show that polypeptide sequences around Arg167 in the LHBs protein are transported from the cytosolic side of the ER membrane across a lipid barrier to the outside of the virion envelope. It could be argued, for example, that only a small fraction of the LHBs proteins gained a transmembrane topology with luminal preS2 domains, which was missed in the protease protection experiment, but which was sufficient to allow successful immunoprecipitation of the virions. In order to investigate this point, we tried to quantify the fraction of LHBs proteins with externally exposed preS2 domains by a combination of enzyme immune assay (EIA) and Western blotting before and after treatment of native virions with V8 protease. V8 protease cleaves LHBs and MHBs at a unique site, i.e. Glu2 of the S domain (Heermann *et al.*, 1984), which is very close to the junction with preS2 (Figure 1). The amount of virus secreted by transfected HepG2 cells appeared to be insufficient to perform Western blots or EIAs. Thus, virions from virus carrier plasma were partially purified by sucrose gradient centrifugation and used for these experiments. Furthermore, it appeared important to also study the topology of LHBs in natural virions secreted by the infected human liver.

V8 protease was able to remove all MHBs preS2 epitopes detectable by mAb Q19/10 in a solid-phase EIA and thus cleaved MHBs very efficiently (Table I). Also, the preS1 domain of LHBs, strongly detectable by mAb MA18/7 in untreated virions, was completely removed from the surface of native virions by V8 protease. This suggested that Glu2 in the S domain of LHBs was accessible. The kinetics of MHBs and LHBs digestions were very similar (data not shown). If the virion envelope contains LHBs protein with internal preS sequences, these LHBs proteins should be protected from V8 cleavage and should appear as full-length molecules on Western blots after detection with the same mAbs used for EIA. Figure 4 compares the amount of MHBs and LHBs before and after treatment with V8 protease. MHBs disappeared completely after V8 protease digestion (lane 5), whereas a weaker but still prominent LHBs band was present in the digested virions (compare lanes 1 and 2). Similarly to V8 protease, trypsin cleaved MHBs in virions completely at Arg167 of preS, which is the closest to the C-terminus (lane 6), while a fraction of LHBs was left uncleaved (lane 3). Analysis by scanning of the p39 and gp42 bands on the Western blot membrane revealed that 52 and 37% of the LHBs proteins were cleaved by trypsin, respectively.

Since cleavage near the junction of preS2 with the S domain removed the reactivity of preS1 with mAb MA18/7, which binds to an N-terminal epitope in preS1 (Figure 1), it is inferred that a cleavage-resistant fraction of LHBs had a preS1 domain which was completely hidden within the virions. The existence of hidden preS domains could also be demonstrated by the incubation of purified carrier-derived virions at pH 5.5 and 37°C overnight (Table II). The signal

Table I. Removal of preS domains from carrier-derived virions by V8 protease

mAb for binding of virions	Specificity	OD in EIA	
		Undigested	Digested
C20/02	S domain, conformational	>2.20	>2.20
Q19/10	preS2-glycan; MHBs	1.34	0.00
MA18/7	preS1 (31–34); LHBs	>2.20	0.03

Cutoff 0.05.

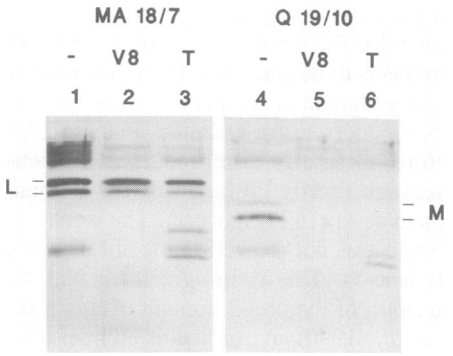

Fig. 4. Western blot of envelope proteins from mock- or protease-treated virions. Virions prepared from the blood of chronic carriers were treated under non-denaturing conditions with V8 protease, cutting at Glu176 (lanes 2 and 5), or with trypsin, cutting at Arg167 (lanes 3 and 6), in the preS2 domain of LHBs and MHBs (see Figure 1). The proteins of equivalent amounts of untreated (lanes 1 and 4) and treated virions were separated by SDS–PAGE, blotted on a membrane and detected by mAb MA18/7 binding to an N-terminal epitope in the preS1 domain of LHBs (lanes 1–3) or by mAb Q19/10 binding to an MHBs-specific, glycan-dependent N-terminal epitope in the preS2 domain (lanes 4–6). Whereas MHBs was completely sensitive to both proteases, indicating that all preS2 domains of this protein were exposed on the viral surface, approximately half of the LHBs proteins were resistant to protease cleavage, suggesting an internal location of these preS domains of LHBs.

Table II. Increased surface exposure of preS1 antigen on carrier-derived virions after incubation at pH 5.5 and 37°C overnight

Experiment No.	OD_{492} in EIA		
	Before	After pH 5.5	% Increase
1	0.762	1.033	36
	0.613	0.910	48
2	0.308	0.408	32
3	0.266	0.441	66
	0.281	0.488	74
4a	1.279	1.701	33
	1.402	1.788	28
4b (4°C)	1.650	1.544	−6
	1.422	1.382	−3
4c[a]	1.744	1.826	5
	1.768	1.847	4

[a]EIA for HBsAg after incubation at 37°C.

of the EIA for preS1 epitope MA18/7 increased after such a treatment by 28–74% in four independent experiments. A typical control experiment (No. 4b) at 4°C did not lead to additional exposure of preS1 epitopes. The reactivity of SHBs epitopes C20/02 (No. 4c) increased by only 4–5% during overnight incubation at pH 5.5 and 37°C. It remains open whether the pH-induced exposure of the previously hidden preS1 epitopes resembles the translocation of preS during virus maturation. The unaltered reactivity of SHBs epitopes suggests that the virions were not degraded by incubation at pH 5.5.

Discussion

Several independent observations made in the past led to the conclusion that the preS1 domain of the LHBs protein is exposed on the surface of HBV particles. Epitopes of the preS1 domain are detectable by EIA in HBV that has been purified from virus carrier blood (Heermann *et al.*, 1984) or more recently by immune electron microscopy (Possehl, 1993). PreS1 peptide sequence 21–47 mediates the attachment of natural HBV to HepG2 cells (Neurath *et al.*, 1986, 1992) or to human liver plasma membranes (Pontisso *et al.*, 1989; Budkowska *et al.*, 1993) and antibodies against that sequence neutralize the infectivity of HBV in chimpanzees (Neurath *et al.*, 1989). Furthermore, subtype-specific sensitivity of LHBs against trypsin suggests that the preS sequence at about position 100 is also surface-exposed (Heermann *et al.*, 1987). Since no hydrophobic stretches of sufficient length for crossing of a membrane are present in the preS1 or preS2 domain, location of the entire preS domain at the exterior side of the virion envelope appeared likely (Gerlich and Bruss, 1993). Our data on the accessibility of the C-terminal preS2 epitope for mAb E21/14 (Figure 3) are consistent with this topological model of LHBs. Generation of this topology of LHBs appeared plausible, because translocation of the preS2 domain in MHBs under the influence of signal I in the S domain has been shown experimentally (Eble *et al.*, 1990) and was confirmed *in vivo* by the *N*-glycosylation of the preS2 domain in natural MHBs (Stibbe and Gerlich, 1983). Based on this observation, it was expected that the preS domain of LHBs is co-translationally translocated into the ER lumen.

Many experimental facts on LHBs were, however, in conflict with this seemingly obvious model and rather support a cytosolic location of the LHBs preS sequence. LHBs carries the same glycosylation signal as MHBs in its preS2 domain, but is not glycosylated at this site, in contrast to MHBs (Heermann *et al.*, 1984). However, Asn4 in the preS2 domain and Asn15 in the preS1 domain of LHBs are efficient substrates for N-linked glycosylation when the preS domain is co-translationally translocated into the ER lumen by fusing a translocation signal to the N-terminus of LHBs (K.Vieluf and V.Bruss, in preparation). A complete translocation of the preS domain of LHBs to the ER lumen would generate the same transmembrane topology as with MHBs. However, MHBs is secreted as a spherical particle almost as efficently as SHBs (Sheu and Lo, 1992), but LHBs is not secreted and probably does not form spherical particles (Persing *et al.*, 1986). The mandatory requirement of LHBs, but not MHBs, for the assembly and secretion of virions (Bruss and Ganem, 1991a) is a strong hint that the topology of LHBs at ER membranes is different from that of MHBs. SHBs or the S domain of MHBs may not form suitable docking sites at the ER membrane for HBV nucleocapsids, although these proteins are, at least in principle, capable of enveloping a viral nucleocapsid such as that of hepatitis delta virus (Sureau *et al.*, 1993). A cytosolic location of the preS domain as a docking site for nucleocapsids is consistent with its essential role in virus assembly. Recent mutation studies showed that N-terminally truncated LHBs proteins were unglycosylated in preS and could still support HBV assembly and secretion unless truncations went beyond position 102 of the 119 aa long preS1 domain. Beyond this point, LHBs became glycosylated in preS2, suggesting co-translational translocation of the truncated preS domain to the ER lumen, and was no longer capable of supporting virion formation (Bruss and Thomssen, 1994). The data presented in this study prove that the C-terminal region of preS2 on LHBs is indeed not translocated to the ER lumen during or soon after its biosynthesis. Since there does not appear to exist an upstream translocation signal, this implies that the entire preS domain initially remains at the cytosolic side. Ostapchuk *et al.* (1994) have recently shown that the entire preS domain remains cytosolic after processing at microsomal membranes in a cell-free *in vitro* system.

Based on these observations and on our finding that approximately half of the LHBs proteins in the virion envelope expose preS sequences to the outside (Figure 4), we propose that the preS domain of LHBs traverses a lipid barrier post-translationally. Our data could alternatively be explained without postulating such a switch in transmembrane topology by a selection mechanism assuming that a small fraction of LHBs proteins gain co-translationally translocated preS sequences and that these protein chains become selectively enriched in viral envelopes. However, co-translational translocation of preS, as achieved by fusing a signal sequence to the N-terminus of LHBs, results in efficient N-linked glycosylation of this domain (K.Vieluf and V.Bruss, in preparation). Therefore, the glycosylation of preS is a suitable marker for co-translational translocation of the domain. The fact that all LHBs proteins in virions are not glycosylated in their preS sequence (Heermann *et al.*, 1984) argues strongly against the selection mechanism.

The preS sequence of the large envelope protein (LDHBs) of the duck hepatitis B virus (DHBV) can also be removed from the S domain by treatment with V8 protease. Digestion of DHBV particles with V8 under non-denaturing conditions removed the preS domain of only approximately half of the LDHBs proteins very efficiently, whereas the other half remained V8 resistant (I.Swamey and H.Schaller, personal communication). We take this as a hint that the phenomenon described here for the LHBs protein of HBV may be common to all hepadnaviruses.

The cytosolic location of preS possibly affects the topology of the S domain in LHBs as well. Signal I is believed to translocate its amino end in a luminal position if present in SHBs or MHBs (Eble *et al.*, 1990). On LHBs, the non-translocated preS domain may enforce another topology of signal I, as suggested in Figure 5A. This postulated alteration of the S topology could potentially explain the inability of LHBs to form secretable particles since an SHBs-like topology seems to be requisite for HBsAg particle secretion (Bruss and Ganem, 1991b). This model would also imply that the sequence between aa 103 in the C-terminal region of preS1 and aa 254 upstream of signal II in the S domain

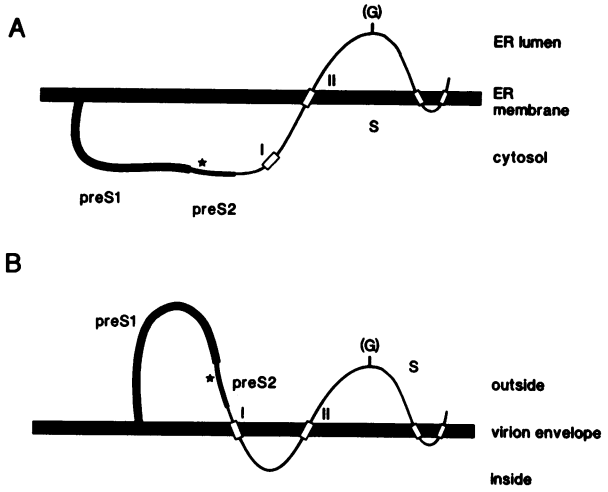

Fig. 5. Model of the LHBs protein topology in the ER membrane (A) and in the virion envelope (B). (A) LHBs protein has a cytosolic preS2 sequence shortly after synthesis at the ER membrane, as supported by the protease protection experiment (Figure 2) and the missing glycan at Asn4 in this preS2 domain indicated by an asterisk. The sequences downstream of signal II in the S domain shown as an open box (II) are translocated as in the SHBs and MHBs protein, as suggested by the partial glycosylation of this domain. The location of signal I in the S domain is unknown, but it is proposed to be on the cytosolic side. The N-terminus is assumed to be fixed to the membrane by the myristate group covalently linked to Gly2 of the preS1 domain (Persing *et al.*, 1987). (B) In the virion envelope, approximately half of the LHBs proteins show a different topology: the C-terminal portion of the preS2 domain containing the E21/14 epitope (see Figures 1 and 3, lane 10) and Glu2 of the S domain (Figure 4, lane 2) is exposed to the outside. Also, more N-terminal regions of preS1 are accessible from the outside, as shown by the binding of monoclonal preS1 antibodies (see Table I). Two α-helices at the C-terminus of the S domain are believed to traverse the membrane and to contribute to assembly of the HBs particles or the HBV envelope.

forms a docking site for nucleocapsids since the N-terminal 102 aa of preS1 are dispensable for virion formation (Bruss and Thomssen, 1994).

The current model of SHBs or the S domain in mature MHBs assumes four transmembrane α-helices, one of which (signal II) is completely hydrophobic, whereas the other three helices are amphipathic. It has been speculated that the six amphipathic helices of an SHBs dimer (Huovila *et al.*, 1992) would form a hydrophilic channel (Stirk *et al.*, 1992). Computer modelling shows that such a hypothetical channel could reach a diameter of 1.5 nm and would, thus, allow easy passage of a polypeptide chain through the viral lipid envelope (A.Berting and W.H.Gerlich, in preparation) once the HBs proteins have assembled to HBsAg or HBV particles. This model leaves open whether the S domain of matured LHBs or SHBs helper molecules would provide that channel. Another possible mechanism for the post-translational translocation of the preS domain may be analogous to the *sec*-independent membrane insertion of a mildly hydrophobic segment of the inner membrane protein leader peptidase (Lep) in *Escherichia coli* (Nilsson *et al.*, 1993).

The observation that a significant portion of the LHBs molecules in natural virions have an internal preS domain suggests that at least a fraction of the preS domains are also located in the cytosol during the virion assembly in infected human liver. On the basis of our *in vitro* data, it is likely that the great majority of LHBs initially has a cytosolic preS domain in infected human liver as well. The hypothesis that the occurrence of preS domains of LHBs at the surface is due to post-translational transmembrane transport is supported by the finding that incubation of natural virions at pH 5.5 may trigger further exposure of preS domains which were originally hidden. Our findings reconcile the two seemingly contradictory observations that the preS domain of LHBs may act both as a viral matrix protein and as a viral attachment protein for a receptor.

Materials and methods

Plasmids

Plasmid pSV45H contains the preS1, preS2 and S open reading frame and the HBV polyadenylation signal of a subtype adw HBV genome (Valenzuela *et al.*, 1980) under the control of an SV40 early promoter (Persing *et al.*, 1986). Transfection with this plasmid leads to the expression of the LHBs, MHBs and SHBs proteins. Plasmid pSV33H contains the preS2 and S open reading frame of the same HBV genome under the transcriptional control of an SV40 early promoter and directs the synthesis of MHBs and SHBs proteins. It was constructed by cutting plasmid pSV45H with *Oxa*NI 4 bp upstream of the preS2 start codon, filling up the single-stranded region with the Klenow enzyme, and generating a 690 bp fragment by cutting with *Spe*I in the S sequence. This fragment was inserted into *Hin*dIII/Klenow enzyme–*Spe*I-treated plasmid pSV24H (Persing *et al.*, 1986). Plasmid pRVHBV1.5 contains one and a half copies of the HBV genomic DNA in a tandem head-to-tail configuration (Bruss and Ganem, 1991a). Plasmid pRVM$^-$ is a derivative of pRVHBV1.5 with a single point mutation of the preS2 translation start codon from ATG to ACG (Bruss and Ganem, 1991a).

Cell culture, immunoprecipitation and endogenous polymerase reaction

Transient transfections of HepG2 cells, immunoprecipitations of virions and detection of virions by a radioactive endogenous polymerase reaction were carried out as described elsewhere (Bruss and Ganem, 1991a). Transient transfections of COS7 cells, metabolic labelling with [^{35}S]methionine and immunoprecipitations of HBV envelope proteins were carried out as described previously (Bruss and Thomssen, 1994), except that cells were seeded in 8.5 cm dishes and were placed on ice after labelling for 10 min without a following chase.

Preparation of microsomes and treatment with trypsin

After [^{35}S]methionine labelling for 10 min, the cells were washed with cold TBS [0.05 M Tris–HCl (pH 7.5), 0.15 M NaCl] and incubated with 1.2 ml cold 0.1 × TBS for 10 min on ice. Swollen cells were scraped off the dish and homogenized by douncing (20 strokes) on ice. After the addition of 0.13 ml 10 × TBS, the cell debris was sedimented by centrifugation for 15 min at 2500 r.p.m. and 4°C in a microfuge. The supernatant was layered on 2.7 ml of 10% (w/w) sucrose–TBS and spun at 37 000 r.p.m. and 4°C for 30 min in an SW60 rotor (Beckman). The pellet was washed once with cold TBS and resuspended in 1 ml TBS by passing 10 times through a 23G needle. The microsome preparation was divided into three 0.33 ml samples. To sample A nothing was added, to sample B 6.6 μl trypsin (5 mg/ml in TBS, Sigma) were added, and to sample C 6.6 μl trypsin and 18 μl 10% (v/v) NP-40 (Sigma) were added. All three samples were incubated for 30 min at 37°C and a further 30 min on ice. The protease was inhibited with 40 μl 25 mM phenylmethylsulphonyl fluoride and 40 μl aprotinin (24 000 IU/ml, Sigma), and the samples were incubated for an additional 10 min on ice. To samples A and B were added 18 μl 10% NP-40 and to all three samples 0.3 ml phosphate-buffered saline (PBS)–0.5% NP-40 and the incubation was continued for 20 min on ice. After centrifugation for 5 min in a microfuge, the supernatant was transferred to a fresh tube and 20 μl of a slurry of protein A–Sepharose CL-4B gel (Sigma) pre-incubated with 2 μl of polyclonal goat anti-HBs (DAKO) in PBS were added for immunoprecipitation.

Virion preparation from plasma

Virions from a highly viraemic virus carrier, with 3 × 10^9 HBV DNA molecules/ml plasma (genotype D), were purified by sucrose density centrifugation. Seventeen millilitres of plasma were layered on a step gradient consisting of 2 ml 65%, 4 ml 55%, 4 ml 45%, 5 ml 35%, 5 ml 25% sucrose (w/w) in 0.01 M Tris–HCl (pH 7.4), 0.13 M NaCl, 1 mM Na-EDTA (TNE) and centrifuged for 16 h at 10°C and 25 000 r.p.m. in a SW28

swing-out bucket rotor from Beckman. Fractions of 1.2 ml were collected from the bottoms of the tubes. Fractions 4–6 contained 48–41% sucrose and virions, as detected by DNA dot blot hybridization and electron microscopy, but only few HBs filaments or HBs spheres, as reported previously (Pontisso *et al.*, 1989). Serum proteins were also removed by more than a factor of 1000, as shown by the low UV absorbency of these fractions. Aliquots of the virion preparation were dialysed against the buffer suitable for the subsequent experiment.

Immunoassays and monoclonal antibodies

EIA of preS1, preS2 and S epitopes was carried out in microplates (Nunc polysorb) coated with 0.4 μg mAb/well. Post-coating and dilutions of samples and conjugates were performed with 2% bovine serum albumin in PBS. Virion samples at suitable dilutions (usually between 1:100 and 1:100 000) were incubated for 2 h at 37°C. After washing, a polyvalent anti-HBs–peroxidase reagent from the HBsAg micro ELISA (Behringwerke Marburg/Lahn) was added for 1 h and samples were processed further as with the standard HBsAg EIA.

mAb MA18/7 (Heermann *et al.*, 1984) is directed against the sequence preS1(31–34) (Sominskaya *et al.*, 1992), Q19/10 binds to glycosylated preS2 and is thus MHBs specific (Heermann *et al.*, 1988), E21/14 binds to the C-terminal portion of preS2 (H.Meisel *et al.*, in preparation), C20/02 reacts with a group-specific conformational epitope of SHBs (W.H.Gerlich, unpublished) and H166 reacts with a sequential epitope of the S domain (Peterson *et al.*, 1984).

Western blot and protease cleavage

Virus was digested with 1.2 mg/ml V8 protease (Boehringer Mannheim, Tutzing) at pH 7.4 and 37°C overnight in 0.05 M potassium phosphate buffer, or with 0.1 mg/ml trypsin in 0.05 M Tris–HCl (pH 7.4) for 2 h at 37°C. The proteases were removed by pelleting the virions through a 20% sucrose–TNE cushion for 8 h at 35 000 r.p.m. and 10°C in a SW41 rotor (Beckman). Protease-digested virus was resuspended in TNE. For Western blotting, ~1 μg virus protein in 10 μl was run through a 13.5% SDS–polyacrylamide gel in a Hoefer Mighty Small Tall electrophoresis chamber. The proteins were transferred to Immobilon membranes (Millipore) and developed by standard techniques using mAbs MA18/7 and Q19/10 (Gültekin and Heermann, 1988).

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