Evidence for structural differences in the S domain of L in comparison with S protein of hepatitis B virus

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The structures of the large (L), middle (M) and small (S) versions of the envelope proteins of hepatitis B virus remain poorly characterized due to the complex nature of their conformations. Several groups have proposed transmembrane topological models depicting the lumenally and cytosolically disposed regions of these proteins. Recently, post-translational topological changes in L have been described. However, no overall differences in the topology of the S domains of the L or M, to the S protein are predicted. In this report, we investigated a previously uncharacterized anti-S monoclonal antibody (MAb), 6B1, which recognizes a conformation-sensitive epitope in S. Unlike other anti-S MAb tested, this MAb did not recognize its epitope in the S domain of L protein. Interestingly, however, the M protein was efficiently recognized. This unique characteristic of MAb 6B1 has allowed us to study the intracellular distribution of L and S proteins. In cells expressing both L and S, L re-localized from the endoplasmic reticulum–Golgi intermediate compartment (ERGIC) to the membrane-associated distribution of S protein indicating that L and S interact with each other. This was confirmed by immunoprecipitation assays, which also showed that the interaction between L and S results in the secretion of L protein from cells. Overall, the ability of MAb 6B1 to selectively recognize S and M, but not L, strongly points to the existence of significant topological differences in the S domain of L. The availability of this important reagent should help further our understanding of the structure of HBV surface antigens.

Introduction

Hepatitis B virus (HBV), the prototypic member of the family Hepadnaviridae, is an enveloped hepatotropic virus which causes a chronic inflammatory liver disease in humans, often leading to the development of hepatocellular carcinoma. The envelope of HBV consists of cellular lipids and three related viral glycoproteins, the large (L), middle (M) and small (S) surface proteins. These proteins, which are co-terminal at their carboxy end, are expressed from a single viral open reading frame (env) carrying three in-frame ATG translational initiation codons. The translation of the env open reading frame (ORF) from the first ATG yields L [comprising 389 or 400 amino acids (aa) depending on subtype], whereas those from the ATG at aa positions 109 (or 120) and 164 (or 175) generate M and S, respectively. The aa domains at the N termini of L and M are called the preS1 and preS2 regions, respectively. All three proteins are transmembrane species which are co-translationally translocated across the endoplasmic reticulum (ER). These surface proteins interact with viral nucleocapsids to form virus particles which are thought to mature by budding into the lumen of a pre-Golgi compartment followed by secretion via the constitutive secretory pathway of the host cell (Bruss & Ganem, 1991; Heermann & Gerlich, 1991; Huovila et al., 1992; Ueda et al., 1991). The M and S proteins, when expressed alone, can be secreted as subviral particles (Huovila et al., 1992; Molnar-Kimber et al., 1988). In contrast, L cannot be secreted when expressed on its own: instead, it is retained within the ER/pre-Golgi regions in the form of intraluminal particles (Cheng et al., 1986; Chisari et al., 1987; Molnar-Kimber et al., 1988; Nemeckova et al., 1994; Ou & Rutter, 1987; Persing et al., 1986; Xu et al., 1997). However, co-expression of L with other surface proteins can lead to heteromultimerization and low levels of secretion where the efficiency of secretion is dependent on the relative amounts of the various surface proteins (Wunderlich & Bruss, 1996).

All three surface proteins undergo post-translational modification. Each carries a partially glycosylated site at Asn-146 of the S domain and glycosylation also occurs in the preS2 region. A potential N-linked glycosylation site (aa 4 or 15) in the preS1 domain of L is not used (Bruss et al., 1996a; Heermann et al., 2000).

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distribution of the HBV surface proteins and their interaction. The topology models for HBV surface antigens described above depict no overall differences in the structure of the S domain in HBV M and L. However, L is myristylated at Gly-2 in the preS1 domain, and this fatty acid moiety has a profound effect on the in vitro infectivity of the virion (Bruss et al., 1996b). Furthermore, the myristylation at Gly-2 is essential for the maintenance of infectivity of HBV in vivo, although Gly-2 to Ala substitution did not have any apparent effect on the presentation of the preS1 domain on the virion surface, or the particle morphology (Bruss et al., 1996b).

The anchorage and translocation of the surface proteins into the ER are achieved by the signal sequences in the S domain, which have multispansing transmembrane topologies (Berting et al., 1995; Eble et al., 1986, 1987, 1990; Gerlich et al., 1993, Guerrero et al., 1988, Sheu & Lo, 1994). Current models predict that S traverses the ER membrane at least twice so that both the termini as well as the region between the transmembrane domains II and III are exposed in the lumen (and thus externally on the mature virion) and the other regions of S are disposed to the cytosolic side (Guerrero et al., 1988; Löffler-Mary et al., 2000; Prange & Streeck, 1995; Stirk et al., 1992). The topology of the S domain in HBV M and L is predicted to be similar to that of the S protein (Bruss et al., 1996a; Gerlich et al., 1993; Prange & Streeck, 1995; Stirk et al., 1992). In addition, the preS2 domain of M is thought to be located in the ER lumen (Eble et al., 1990). Interestingly, L differs from M and S in that in approximately half of the L molecules the preS1 and preS2 (preS) regions remain cytosolic and therefore located internally in the virion providing a scaffolding function by interaction with the nucleocapsid. In the other half of the L molecules the preS region is lumenerously disposed and consequently exposed at the surface of the virion (Bruss et al., 1994, 1996a; Gerlich et al., 1993; O斯塔fchuk et al., 1994; Prange & Streeck, 1995). This dual topology of L would differ from M and S in that in approximately half of the L molecules the preS1 and preS2 (preS) regions remain cytosolic and therefore located internally in the virion providing a scaffolding function by interaction with the nucleocapsid. In the other half of the L molecules the preS region is lumenerously disposed and consequently exposed at the surface of the virion (Bruss et al., 1994, 1996a; Gerlich et al., 1993; O斯塔fchuk et al., 1994; Prange & Streeck, 1995). This dual topology of L would be consistent with its presumed multifunctional role, in cell receptor binding by the surface exposed region, and in interaction with viral nucleocapsids and in other intracellular functions by the internalized region of the preS1 domain in infected cells (Bruss & Vieluf, 1995; Hildt et al., 1996; Klingmuller & Schaller, 1993; Neurath et al., 1986, Rotthmann et al., 1998; Ryu et al., 2000).

The topology models for HBV surface antigens described above depict no overall differences in the structure of the S domain of L and M compared with S. However, evidence to support this hypothesis has been lacking. In this paper, we provide the first experimental evidence for the existence of structural differences between the S domain of L and the S protein. In addition, we have examined the intracellular distribution of the HBV surface proteins and their interaction with each other.

Methods

**Cell culture.** COS-7, HepG2 and HuH7 cells were cultured in Dulbecco’s modified essential medium (DMEM) supplemented with 10% foetal calf serum, 5% non-essential amino acids (Gibco) 100 units/ml penicillin–streptomycin and 10 mM glutamine (Gibco).

**Generation of plasmid constructs and recombinant vaccinia viruses expressing HBV surface proteins.** The genes encoding the HBV surface proteins were cloned immediately downstream from a strong synthetic late vaccinia virus promoter into the transfer vector pMJ601 (Davison & Moss, 1990) as described below. The portion of the HBV genome encoding L was amplified from the serum of a patient infected with HBV (subtype adw) by PCR. The resulting PCR product carrying the L open reading frame (ORF) was cloned into pMJ601. The nucleotide sequence encoding the M ORF was excised from the L construct described above using appropriate restriction enzymes and inserted into pMJ601. The S gene was subcloned from plasmid pRKS/HbsAg (a kind gift from W. F. Carman, Glasgow, UK) into pMJ601. The HBV sequences in pMJ601 were inserted into the thymidine kinase gene of vaccinia virus strain WR by homologous recombination, and the recombinant viruses Lb, M and S protein, respectively, were isolated as described (Davison & Moss, 1990). The nucleotide sequence encoding the HBV preS1 domain (aa 1–108) of L was cloned into the bacterial expression vector pQE30 (Qiagen). The resulting plasmid, pQES1, carries sequences encoding aa MRRGSHHHHHGS followed by aa 1–108 representing the HBV preS1 domain. This protein was expressed in *E. coli*, purified, and used to immunize rabbits for generation of antiserum.

**Immunoprecipitations.** Cells were infected at an m.o.i. of 10 p.f.u. per cell with recombinant vaccinia viruses. Where required, cells were incubated with 10 µg/ml tunicamycin for 3 h prior to infection to inhibit glycosylation. For radiolabelling, cells were washed with PBS at 5 h post-infection and incubated in methionine-free Eagle’s medium containing 50 µCi/ml [35S]methionine for 16 h. The culture supernatant was aspirated and clarified at 13000 r.p.m. in a benchtop centrifuge to remove cellular debris. The clarified medium containing radiolabelled proteins was adjusted to 20 mM Tris–HCl, pH 7.4, 150 mM NaCl; 1 mM EDTA, 0.5% Triton X-100 (final concentrations) and used in immunoprecipitation assays described below. Cell monolayers were washed three times with PBS and the cells were dislodged and pelleted by brief centrifugation. The cells were resuspended in lysis buffer (see above) and incubated on ice for 30 min. The cell nuclei were removed by centrifugation and the lysates used for immunoprecipitation of proteins as follows. The clarified and radiolabelled medium of cell lysate was incubated with appropriate monoclonal or polyclonal antibodies at 4°C overnight. The immune complexes were then precipitated using protein A-Sepharose. Following washing with lysis buffer, the immune complexes were released from protein A-Sepharose using SDS–PAGE denaturation buffer and boiled for 3 min. Samples were fractionated by SDS–PAGE (10% polyacrylamide) and the labelled proteins detected using a Bio–Rad Personal FX phosphorimag.
Differences in S domain of HBV L to S protein

Fig. 1. Recognition of HBV surface antigens by various anti-S MAbs. HepG2 cells were infected with wild-type vaccinia virus strain WR (A, E, I, M) or recombinant virus vL (B, F, J, N), vM (C, G, K, O) or vS (D, H, L, P). At 24 h post-infection, the cells were fixed and probed with anti-S MAbs H53 (A–D), H35 (E–H), H166 (I–L), or 6B1 (M–P) followed by anti-mouse IgG–FITC conjugate, and analysed by confocal microscopy.

- **Confocal microscopy.** Cells were grown on coverslips to 50–60% confluence prior to experimental procedures. They were infected with vaccinia virus strain WR or recombinant viruses expressing HBV surface proteins at a multiplicity of 0.5 p.f.u. per cell and incubated overnight at 37 °C. The cell monolayers were washed with PBS, fixed with methanol at −20 °C and washed with PBS containing 0.05% Tween 20 (PBST). The permeabilized cells were then incubated with appropriate primary antibodies described in the text. The bound antibodies were detected with appropriate secondary antibodies conjugated to FITC (Sigma) or FluorLink Cy5 (Amersham). Concanavalin A–FITC (Sigma) was used at appropriate dilution to stain internal cellular membranes. The coverslips carrying the labelled cells were mounted on glass slides with a drop of Citifluor anti-fade reagent, and examined with a Zeiss laser scanning microscope; the images were analysed using the LSM5150 software.
Results of the cell while L had a more uniform cytoplasmic distribution. The distribution of M and S was markedly distinct from that of L: anti-S MAb 6B1 showed that this antibody was able to recognize S by immunoprecipitation and in immunofluorescence assays but not by Western blotting, indicating that it recognizes a conformation-dependent epitope in S (data not shown). The anti-S MAbs H35 and H53 also recognize discontinuous epitopes on S, whereas MAb H166 interacts with a linear epitope [for data on H35, H53 and H166 see Chen et al., (1996)]. The reactivity of the various anti-S MAbs was compared by immunofluorescent staining and confocal microscopy. HepG2 cells were infected with recombinant vaccinia viruses vL, vM and vS, respectively. Expression of the individual viral proteins in recombinant vaccinia virus-infected cells was verified using specific antibodies in immunofluorescence, Western blot and immunoprecipitation assays (data not shown). Our initial characterization of MAb 6B1 showed that this antibody was able to recognize S by immunoprecipitation and in immunofluorescence assays but not by Western blotting, indicating that it recognizes a conformation-dependent epitope in S (data not shown). The anti-S MAbs H35 and H53 also recognize discontinuous epitopes on S, whereas MAb H166 interacts with a linear epitope [for data on H35, H53 and H166 see Chen et al., (1996)]. The reactivity of the various anti-S MAbs was compared by immunofluorescent staining and confocal microscopy. HepG2 cells were infected with recombinant vaccinia viruses vL, vM and vS, and fixed after 24 h. The cells were incubated with different anti-S MAbs and the bound antibodies detected by FITC-conjugated anti-mouse IgG. As expected, MAbs H53, H35 and H166 recognized not only S, but also M and L proteins (Fig. 1, panels B–D, F–H, J–L). MAb 6B1, on the other hand, recognized S and M, but interestingly failed to detect L (Fig. 1, panels N–P). In all cases, the reactivity of the MAbs to wild-type vaccinia strain WR-infected negative controls was minimal (panels A, E, I and M). The above results show that MAb 6B1 is able to distinguish between the S protein and the S domain present in L, thus providing evidence of a structural difference in the S domain of L in comparison with S. It therefore constitutes a valuable immunological reagent to study the properties of these proteins in expressing cells.

Intracellular localization of HBV surface antigens

Data presented in Fig. 1 showed that the intracellular distribution of M and S was markedly distinct from that of L: M and S both localized in a matrix-like pattern in the cytoplasm of the cell while L had a more uniform cytoplasmic distribution. Recent work has shown that L protein, when expressed in a heterologous system, is retained in the ER, and co-localizes with p53, a protein known to be located in the ER–Golgi intermediate compartment (ERGIC) (Xu et al., 1997). To evaluate the intracellular localization of the surface antigens expressed by our recombinant vaccinia viruses and to compare it with previously published data, we performed confocal microscopy of HepG2 cells infected separately with vL, vM or vS, or co-infected with vL and vS. As shown in Fig. 2, L localized predominantly in the perinuclear compartment (panel C), whereas the ERGIC p53 protein appeared to be localized as large spots again in the perinuclear area, although some weak staining was also seen throughout the cytoplasm (panel A). In keeping with the findings of Xu et al. (1997), superimposition of the two images showed that ERGIC marker p53 co-localized or co-compartmentalized in part with the L protein (visible as the yellow colour in panel B). Furthermore, the bulk of L did not co-localize with the lectin concanavalin A (Con A), a marker for internal membranes (Fig. 2, panels D–F). Con A has a highly specific affinity for carbohydrates with terminal α-D-mannosyl and α-D-glucosyl residues present on glycoproteins. Thus, it stains specifically the glycoproteins present in internal membranes (mainly the rough ER) which have such sugar residues. In contrast, co-staining of cells infected with vM or vS with Con A and MAb 2-12F2 (anti-M) or MAb 6B1 (anti-S), respectively, showed that both M and S were distributed throughout the cytoplasm (Fig. 2, panels J and L), and they co-localized with intracellular membranes (panels H and K). These data are consistent with published observations that S and M are not retained in the ER, but rather are secreted from expressing cells probably through the exocytic pathway (Huovila et al., 1992, Molnar-Kimber et al., 1988).

Co-expression of L and S

L has been shown to form mixed disulfide-linked dimers with M and S, probably through the region containing signal II and downstream sequences carrying cysteine residues required for covalent linkages (Wunderlich & Bruss, 1996). To our knowledge the intracellular distribution of the HBV surface antigens in co-expressing cells has not been comprehensively investigated. The difficulty in performing this sort of analysis is largely due to the unavailability of appropriate immunological reagents. For example, due to the presence of the S domain in the L and M proteins, all anti-S MAbs analysed to date are expected to recognize all three surface antigens. Since our anti-S MAb 6B1 distinguishes between S and L, we sought to examine the intracellular distribution of L and S proteins in...
co-expressing cells. HepG2 cells were co-infected at low multiplicity with recombinant vaccinia viruses vL and vS, fixed after 24 h, and incubated with the anti-L rabbit polyclonal antiserum R143 and anti-S MAb 6B1. The bound antibodies were detected by FluoroLink Cy5-labelled goat anti-rabbit IgG and FITC-conjugated anti-mouse IgG. In Fig. 2, panels (M)–(O) show three cells each expressing L (open arrow, panel O), S (solid arrow, panel M), or L plus S (solid arrowhead, panel O). The cell infected with vL alone expressed L, which had a perinuclear distribution (panel O). As expected, L in this cell was not detected by the anti-S MAb 6B1 (panel M), which nevertheless readily identified the S protein with a matrix-like distribution in the cell infected with vS alone (panel M, solid arrow). In the cell co-infected with vL and vS, both L and S proteins were detected by the respective antibodies (arrowhead, panels M and O). Superimposition of the two images showed L and S co-localized in the co-expressing cells (arrowhead, panel N). Interestingly, the intracellular distribution of L when co-expressed with S changed dramatically – from the ERGIC compartment to the membrane-associated localization observed with S in panels (J)–(L) of Fig. 2. Our immunofluorescence data shown in Fig. 2 (panels M–O) indicate that L and S proteins interact with each other and are consistent with the results of Wunderlich & Bruss, (1996) who identified an interaction using two-dimensional SDS–PAGE.

To confirm the interaction between L and S, we performed immunoprecipitation assays. HepG2 cells were infected with vaccinia viruses (m.o.i. of 10 p.f.u. per cell) and the expressed proteins labelled with \[^{35}S\]methionine. Radiolabelled proteins from infected cell lysates and medium were immunoprecipitated using MAb 6B1 or anti-preS1 polyclonal antiserum R143 followed by SDS–PAGE. The anti-preS1 antiserum R143 readily immunoprecipitated the 42 kDa L from vL-infected cells (Fig. 3 A, lane 2). Not surprisingly, L was not found in the medium of vL-infected cells (Fig. 3 A, lane 6). Interestingly, both L and the two forms of S proteins were co-immunoprecipitated by R143 from the medium as well as lysates of cells co-infected with vL + vS (Fig. 3 A, lanes 7 and 3, respectively), indicating that the interaction between these proteins is required for secretion of L from the cells. These results are consistent with our immunofluorescence data, which showed that both L and S co-localize in the cytoplasm.

As shown in Fig. 3 (B), MAb 6B1 specifically immunoprecipitated the characteristic 28 and 25 kDa bands representing glycosylated and non-glycosylated S proteins, re-
respectively, from both the lysates and medium of cells infected with V S alone (lanes 4 and 8) or co-infected with vL + V S (lanes 3 and 7). MAb 6B1 recognized, albeit very weakly, the L protein synthesized only in vL-, but not in vL + V S-infected cells (Fig. 3B, lanes 2 and 3, respectively). This indicated that the weak recognition of L in immunoprecipitation assay by MAb 6B1 is probably due to high levels of this protein produced in V L-infected cells relative to that in co-infected cells (Fig. 3A, compare lanes 2 and 3). Alternatively, the small amounts of L recognized by MAb 6B1 could represent immature, misfolded species. Thus, taken together, our confocal microscopy and immunoprecipitation data show that this conformation-sensitive anti-S MAb is unable to recognize the S domain of mature L. Contrary to our expectation, MAb 6B1 did not co-immunoprecipitate L with S from the lysate or medium of cells co-expressing L and S (Fig. 3B, lanes 3 and 7, respectively), indicating that the MAb 6B1 epitope may be occluded or altered upon heterodimerization of L and S.

Discussion

To date, the biogenesis of HBV surface antigens has been studied largely by biochemical means. Surprisingly little information on the intracellular distributions of these proteins exists, particularly in co-expressing cells. This is probably due to the lack of appropriate reagents that are able to distinguish between different surface antigens. Whereas anti-L and anti-M antibodies recognize epitopes within the respective unique preS1 or preS2 regions can readily distinguish between L, M and S proteins, the anti-S MAbs are generally expected to react with all three proteins. An example of this is shown in Fig. 1 where three known anti-S MAbs (H35, H53 and H166) recognized L, M and S in an immunofluorescence assay. These three MAbs are known to recognize non-overlapping epitopes within the ‘a’ determinant of S; MAbs H35 and H53 bind to discontinuous epitopes, whereas H166 interacts with a continuous epitope (Chen et al., 1996). To our knowledge, an anti-S MAb incapable of recognizing L or M proteins has not as yet been reported. Here we investigated the properties of a previously uncharacterized antibody, MAb 6B1, which recognizes a conformation-sensitive epitope in the S protein of HBV. In contrast to MAbs H35, H53 and H166, MAb 6B1 failed to recognize the S domain of L (but not of M), strongly indicating that conformational differences exist in the S region of L compared to that of M or S.

In agreement with previously published observations (Cheng et al., 1986; Chisari et al., 1987; Persing et al., 1986; Xu et al., 1997), our confocal microscopy and immunoprecipitation data show that L localizes predominantly to the ERGIC compartment and is not secreted. In keeping with their secretory characteristic (Huvila et al., 1992; Molnar-Kimber et al., 1988), the intracellular distribution of S and M proteins was distinct from that of L in that both S and M co-localized with intracellular membranes, as defined by co-staining with Con A.

The unique characteristic of the anti-S MAb 6B1 allowed us to investigate the intracellular distribution of L and S proteins. In co-expressing cells, L re-localized from the ERGIC to the membrane-associated S protein indicating that these proteins may interact with each other. Presumably this interaction occurs via the disulfide linkages in the transmembrane regions of the S domains and is required for secretion of L. This was confirmed in immunoprecipitation assays where the anti-preS1 antiseraum specifically co-immunoprecipitated S from the medium and lysates of cells co-expressing L and S. These results are consistent with those published previously where the surface antigens have been shown to form heteromultimeric complexes upon co-expression (Cheng et al., 1986; Chisari et al., 1987; Molnar-Kimber et al., 1988; Wunderlich & Bruss, 1996). Interestingly, the anti-S MAb 6B1 failed to co-immunoprecipitate S–L complex from co-expressing cells, indicating that the recognition of S by MAb 6B1 is abrogated when S and L are interacting. This suggests that the binding site of the MAb 6B1 may be occluded or altered (possibly due to conformational changes) when S is interacting with L protein.

To our knowledge, this is the first report that describes an immunological reagent capable of selectively discriminant S from the S domain of L (but not M). The recognition by MAb 6B1 of the conformational epitope on S is not dependent on glycosylation. When expressed in the presence of tunicamycin, S, but not L, was efficiently detected by the antibody both in immunofluorescence and immunoprecipitation assays (data not shown). Thus, the ability of MAb 6B1 to recognize S but not L is not due to possible differential glycosylation of the two proteins. The topology models for HBV surface antigens proposed by various groups do not predict overall differences in the S domain of L and M to the S protein (Bruss et al., 1996a; Gerlich et al., 1993; Prange & Streeck, 1995; Stirk et al., 1992). However, based on the results presented here, we postulate that the S domain in L has a different topology to the S protein. The topological differences are likely due to a rearrangement of the transmembrane regions in the S domain of L, resulting in a difference in the conformational epitopes displayed by L, and therefore resulting in non-recognition by MAb 6B1. It is possible that the insertion of the preS1 domain into the lipid bilayer at Gly-2 via the myristic acid moiety (Bruss et al., 1996b) may affect the spatial arrangement of the transmembrane regions, therefore contributing to a difference in the conformation of S and L upon interaction of these two proteins. It is also feasible that the rearrangement of the transmembrane α-helices in S and L differ, but the association of the disulfide linkages forming the intermolecular bonds remains conserved to allow oligomerization of the envelope proteins. It is unlikely that the failure of MAb 6B1 to recognize L is due to occlusion of its epitope by the preS region, since in this case the dual topology of L would ensure MAb 6B1 recognition of at least 50% of the L molecules. Interestingly, Paulij et al. (1999) recently identified a unique anti-S mono-
clonal antibody epitope within the so-called transmembrane domain III, leading the authors to propose that this domain may not span the membrane at all, but rather is exposed in the lumen. More recently, using protease protection and immunological assays, Grgcic et al. (2000) showed that the presumed cytoplasmic loop between transmembrane 1 and 2 of duck HBV is actually membrane embedded and protrudes to the particle surface.

In conclusion, numerous studies on the biosynthesis and maturation of hepadnaviral surface antigens have been performed over the years, leading researchers to propose topology models of these proteins. However, in the absence of any X-ray crystallographic data to support a model of the structure, the nature of the presentation of these proteins on the virion surface remains unclear. The availability of MAbs 6B1, with its novel characteristics, should further facilitate the study of the conformational nature of these proteins, without the use of potentially disruptive techniques such as the insertion of epitope tags or fluorescent protein domains into the surface antigens.

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