Fusogenic Activity of Hepadnavirus Peptides Corresponding to Sequences Downstream of the Putative Cleavage Site

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Sequence homology between the amino-terminal region of the S protein of hepatitis B Virus (HBV) and known fusion peptides from retroviruses and paramyxoviruses led us to propose that this region might be equally involved in the initial infective steps of hepadnaviruses. In fact, we showed that a synthetic peptide corresponding to the N-terminus region of the S protein of HBV had membrane-interacting properties and was able to induce liposome fusion adopting an extended (β-sheet) conformation (Rodríguez-Crespo et al., 1996, 1995). We describe herein studies on the interaction of peptides derived from the N-terminal region of the S protein of duck (DHBV: Met-Ser-Gly-Thr-Phe-Gly-Gly-Ile-Leu-Ala-Gly-Leu-Ile-Gly-Leu-Leu) and woodchuck hepatitis B viruses (WHV: Met-Ser-Pro-Ser-Leu-Leu-Gly-Leu-Leu-Ala-Gly-Leu-Gln-Val-Val) with liposomes. These peptides were able to induce to a different extent aggregation, lipid mixing, and leakage of internal aqueous contents from both neutral and negatively charged phospholipid vesicles in a concentration-dependent and pH-independent manner. Fluorescence depolarization of 1,6-diphenyl-1,3,5-hexatriene-labeled vesicles indicated that both peptides become inserted into the hydrophobic core of the lipid bilayer. Circular dichroism studies indicated that the DHBV peptide adopts an extended conformation in the presence of lipids, whereas the WHV peptide displays a high content of α-helical conformation. Therefore, these results extend our previous findings obtained for human hepatitis B virus to other members of the hepadnavirus family and suggest that this region of the S protein is important in the initial steps of the infective cycle. © 1999 Academic Press

INTRODUCTION

Hepatitis B virus (HBV), the prototype member of the hepadnavirus family, is a small DNA-containing virus that infects human hepatocytes, eliciting important liver pathology (Ganem and Varmus, 1987; Ganem, 1996). Related hepadnaviruses have also been isolated from ducks (DHBV), herons (HHBV), ground and arctic squirrels (GSHV and ASHV) (Testut et al., 1996), woodchucks (WHB), and more recently woolly monkeys (WMHBV) (Lanford et al., 1998; see Ganem, 1996, for a review). The lack of an efficient in vitro infection system has hampered systematic studies on the early steps of the viral life cycle. Liver-derived cell lines are not readily infectable, and in addition to primary human hepatocytes not being easily accessible, the ability of HBV to infect these cells is poor and varies within donors and preparations (Mabit et al., 1996). In the case of human HBV, major efforts have been undertaken to identify the physiologically relevant hepatocyte receptors involved in virus binding and entry. Numerous candidates, such as endonexin-2, albumin receptors, transferrin receptors, or LDL receptors have been proposed (see Treichel et al., 1997, for a recent review), although none of them has been convincingly related to HBV infectivity.

On the other hand, the in vitro infection model of primary duck hepatocytes with DHBV has allowed systematic studies of the virus life cycle, since primary duck hepatocytes are easily obtained from domestic animals and conditions for maintenance have been described (Tuttleman et al., 1986). Using noninfectious subviral particles, it has been possible to show that DHBV binds to a cellular receptor on the hepatocyte surface via the preS domain of the viral L protein (Kuroki et al., 1994). This receptor has been identified as gp180, a transmembrane protein member of the carboxypeptidase D family (Kuroki et al., 1995; Tong et al., 1995; Eng et al., 1998). However, gp180 has been found abundantly in duck tissues that do not support DHBV infection (Kuroki et al., 1994; Tong et al., 1995) and transfection of LMH cells (a chicken hepatoma cell line capable of virus production but refractory to virus infection) with gp180 does not confer susceptibility to DHBV infection (Kuroki et al., 1995). Very recently, Schaller and co-workers have shown that gp180 is not detected on the hepatocyte cell surface, but concentrated in the Golgi apparatus instead, from which it functions by cycling to and from the plasma membrane (Breiner et al., 1998). These authors have also proposed that the initial binding of the preS domain of DHBV to

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gp180 must be followed by a species-specific interaction with some unknown cellular components that permits subsequent infection (Urban et al., 1998).

Following the binding to the appropriate receptor, one of the most important steps during virus infection is the fusion of the cellular and viral membranes. Recent three-dimensional data of several surface envelope glycoproteins of retroviruses, paramyxoviruses, and flaviviruses has confirmed that in most cases fusion proteins adopt rod-like structures where interweaved α-helices project the fusion peptides toward the cellular target (Skehel and Wiley, 1998, and references therein). This α-helical bundle motif that brings two membranes together is starting to be more widely recognized and has been found in proteins involved in neurotransmitter release as well as intracellular vesicle trafficking complexes.

In the light of sequence similarities between the hepadnavirus S protein N-terminal sequences and known fusion peptides from the retrovirus and paramyxovirus families, we proposed that this region might be equally involved in the coalescence of the viral and cellular membranes during infection (Rodríguez-Crespo et al., 1994). Furthermore, the heptad repeats necessary to form the coiled-coil structure described in other viruses have also been identified in hepadnaviruses (Rodríguez-Crespo et al., 1995). A synthetic peptide comprising this predicted fusion region of the S protein interacts with membranes, promoting liposome aggregation and fusion in a pH-dependent manner (Rodríguez-Crespo et al., 1995), and adopts an extended conformation during the process (Rodríguez-Crespo et al., 1996). In this same regard, evidence for the role of the N-terminal stretch of the S protein in fusion processes has been achieved from experiments showing that treatment of HBV virions with certain proteases removed the preS domains, allowing the virus to bind to HepG2 cells, fibroblasts, and HeLa cells (Gerlich et al., 1993). Digestion of virions with V8 protease cleaves the S sequence after a Glu residue at position 2, supposedly exposing the hydrophobic fusion peptide, resulting in productive infection of HepG2 cells. However, treatment with chymotrypsin results in cleavage of the S protein within the fusion peptide 14 amino acids downstream of the V8 site, thus abrogating infection (Lu et al., 1996). Assays with low concentrations of synthetic oligopeptides corresponding to this fusogenic sequence of HBV have confirmed the formation of HepG2 cell heterokaryon (fusion) in the presence of proteolytically processed virions (X. Lu, personal communication).

To ascertain whether the destabilizability properties observed for the human HBV fusion peptide can also be ascribed to other members of the hepadnavirus family, the 16-amino-acid oligopeptides corresponding to the N-terminal portions of both DHBV (representative of avian hepadnaviruses) and WHV (representative of small mammal hepadnaviruses) were synthesized and tested for lipid destabilization properties. We describe herein the interaction of both peptides with phospholipid vesicles as well as the structural features responsible for the interaction. These two new peptides interacted with lipids in a pH-independent manner and were more efficient in terms of fusion and lipid mixing than the HBV fusion peptide.

RESULTS

Vesicle aggregation

When the DHBV and WHV N-terminal peptides were incubated with phospholipid vesicles an increase in optical density at 360 nm was observed (Fig. 1). This must be due to the enlargement in vesicle size as a consequence of the aggregation or fusion induced by the interaction with the phospholipid molecules. Although only the results obtained at pH 7.3 are depicted, similar behavior was observed upon interaction of the peptides with the vesicles at pH 6.0 and 5.0 (data not shown). The DHBV N-terminal peptide was able to induce aggregation of vesicles composed of neutral phospholipids (PC) as well as asolectin vesicles, where acidic phospholipids are the main component (Fig. 1, left). However, this increase in the OD_{360} of the liposome suspension upon addition of the DHBV peptide was more evident in the case of asolectin vesicles in the concentration range tested (up to 30 μM). Under similar experimental conditions, the aggregation induced by the WHV N-terminal peptide was more limited for both PC and asolectin liposomes (Fig. 1, right). In fact, the effect of this peptide on PC vesicles was almost negligible.

![Graph showing the interaction of DHBV (left) and WHV (right) N-terminal peptides with phospholipid vesicles. To 1 ml of asolectin phospholipids (■) or PC (○) vesicles in medium buffer at pH 7.3, aliquots of peptide from a stock solution in DMSO were added. The optical density at 360 nm was measured after incubation of the mixture for 1 h at 37°C. Values of control samples containing corresponding amounts of DMSO were subtracted at each peptide concentration. The lipid concentration was 0.14 mM in both cases. The concentration of DMSO was kept below 1%. The results shown are representative of those obtained for at least three different experiments.](image-url)
Lipid mixing

The two N-terminal peptides were also capable of inducing phospholipid mixing between a population of vesicles containing the fluorescent probes (NBD-PE and Rh-PE) and probe-free vesicles (Struck et al., 1981). In this assay, mixing of phospholipids from labeled and unlabeled liposomes results in a separation of the donor–acceptor pair, with the concomitant decrease in energy transfer, thus reflecting accurately the degree of fusion (Düzgünès et al., 1987). The final extent of lipid mixing at pH 7.3 for asolectin phospholipids and egg phosphatidylcholine (PC) vesicles with increasing peptide concentrations is shown in Fig. 2. Both peptides were able to induce lipid mixing of both asolectin phospholipids and PC vesicles but to a different extent. Almost identical results were obtained in all cases when the assay was performed at pH 6.0 and 5.0 (data not shown). In the case of asolectin phospholipids, at a peptide concentration as low as 10 μM there was already a noticeable decrease of energy transfer and with both peptides saturation was observed at about 30 μM peptide. The decrease in acceptor surface density calculated according to the change in energy transfer was five- and eightfold for DHBV and WHV, respectively. These values indicate that, under the conditions studied, both peptides almost induced the complete fusion of vesicles where acidic phospholipids are present, since the mere aggregation would not result in such a change in energy transfer (Blumenthal et al., 1983). Lipid mixing of PC liposomes was less effective. In the peptide concentration range studied, DHBV N-terminal peptide induced a linear decrease of energy transfer, resulting in a fourfold reduction in acceptor surface density. When WHV peptide was added the decrease in energy transfer took place from 20 μM peptide and the reduction in acceptor surface density was only twofold. Hence, the N-terminal peptides were able to induce the partial fusion of vesicles composed of neutral liposomes.

Release of aqueous contents

The ability of the fusion peptides to destabilize the bilayer was also assessed by measuring the release of the aqueous contents of phospholipid vesicles. Liposome leakage was monitored by the dilution of coencapsulated 8-aminonaphthalene-1,3,6-trisulfonic acid (ANTS) and its collisional quencher p-xylenebis(pyridinium) bromide (DPX) that leads to a rapid increase in the ANTS fluorescence at 520 nm (Ellens et al., 1985). Figure 3 shows the leakage induced by the synthetic peptides added to an asolectin phospholipid suspension at pH 7.3. Both peptides were able to interact with the lipid bilayer and induce the release of the internal content of the vesicles in a concentration-dependent manner. No significant differences in the percentage of leakage were found between the three pH values tested: 7.3, 6.0, and 5.0 (data not shown). The peptide concentration needed to attain the maximum effect was substantially lower than that needed to induce maximum vesicle aggregation (Fig. 1) or fusion (Fig. 2). Complete release of the intravesicular content (100% leakage) was observed at 5 μM DHBV and 14 μM WHV peptide; the fluorescence intensity attained at these concentrations was similar to that obtained when the liposomes were lysed with the detergent Triton X-100.

CD studies of peptide conformation

Far UV circular dichroism was performed to analyze the conformational features responsible for the mem-
brane destabilizing properties. The spectrum of either peptide in trifluoroethanol displayed two minima at 220 and 205 nm which are indicative of a notable proportion of \( \alpha \)-helical structures (Fig. 4). In fact, deconvolution of these spectra by the CCA method (Perczel et al., 1991) renders 37% \( \alpha \)-helix, 24% \( \beta \)-sheet, and 39% nonordered structure for the DHBV peptide and 40% \( \alpha \)-helix, 18% \( \beta \)-sheet, and 42% nonordered structure for the WHV peptide.

The CD spectra of both peptides in aqueous solution at pH 7.3 are shown in Fig. 5. The spectra have two minima, centered at 222–225 nm and below 200 nm, which are characteristic of extended structures and nonordered conformations. However, in the presence of dimyristoylphosphatidylglycerol (DMPG) vesicles the secondary structure adopted by the peptides was altered significantly (Fig. 5, solid symbols). At a phospholipid:peptide molar ratio of 9:1 the CD spectrum of DHBV peptide exhibited a minimum at around 220 nm, characteristic of extended structures, whereas the CD spectrum of the WHV peptide displayed a minimum at 205 and a shoulder at 220 nm, characteristic of helical structures (Fig. 5). Incubation of either peptide at higher phospholipid:peptide molar ratios did not change the spectrum any further. Hence, upon interaction with phospholipids, the DHBV peptide restructured into a mostly \( \beta \)-sheet structure, whereas the WHV peptide adopted a structure in which the \( \alpha \)-helix predominates over the extended conformations. Deconvolution of the CD spectra of the peptides in the presence of DMPG vesicles using the CCA algorithm (Perczel et al., 1991) rendered 29% \( \alpha \)-helix structure, 57% \( \beta \)-sheet, and 14% nonordered structure for the DHBV peptide and 42% \( \alpha \)-helix structure, 0% \( \beta \)-sheet, and 58% nonordered structure for the WHV peptide. The CD spectra at pH 6.0 and 5.0 or in the presence of neutral phospholipid vesicles are virtually indistinguishable from those depicted in Fig. 5 (data not shown).

**Studies of peptide insertion into phospholipid bilayers**

To ascertain the existence of a hydrophobic component in the interaction of the N-terminal peptides with neutral and acidic phospholipids, their effect on the thermotropic behavior of these phospholipids has been studied. Accordingly, we determined the fluorescence polarization of the system after the probe DPH was used to label the hydrophobic core of the bilayers. The fluorescence depolarization of DPH-labeled dimyristoylphosphatidylcholine (DMPC) and dimyristoylphosphatidylserine (DMPS) vesicles with increasing temperatures in the absence and the presence of peptides at a phospholipid-peptide molar ratio of 12:1 at pH 7.3 are depicted in Fig. 6. Similar results were obtained at pH 6.0 and 5.0. Both peptides were able to interact with neutral and acidic phospholipids, although to a greater extent with the latter. The effect of the peptides on the DMPC transition temperature is almost negligible, but the amplitude of the transition is diminished in a concentration-dependent manner, due mainly to the restriction of the acyl chain mobility in the liquid crystal phase. Under the assay conditions, the DHBV peptide reduced the DMPC transition amplitude by 50%, whereas the reduction achieved by the WHV peptide was only 30% (Fig. 6, left). The DHBV caused a slight decrease in the transition temperature (from 36.5 to 34°C) of DMPS vesicles, while...
the WHV peptide had no effect on it. Both peptides decreased the transition amplitude by 70% (DHBV) and 80% (WHV), inducing an almost complete disappearance of the gel to liquid crystal transition (Fig. 6, right). Hence, the DHBV peptide is more effective than the WHV peptide in terms of disturbing neutral phospholipids, with the opposite being true for acidic phospholipids. In every case, the DHBV and WHV peptides perturb DMPC and DMPS thermotropic transitions more efficiently than the HBV fusion peptide (unpublished results). These effects are consistent with a model in which the peptides insert into the membrane interacting hydrophobically with the bilayers and restricting the mobility of the phospholipid acyl chains.

Electron microscopy studies

Incubation of the peptides with DMPS liposomes had an effect on the morphology and size of the phospholipid vesicles that can be assessed by electron microscopy after negative staining. As shown in Fig. 7A, the control DMPS liposomes had a regular shape and a average size of 110 ± 25 nm. However, at a peptide concentration of 12 μM, at which both lipid mixing and leakage of negatively charged vesicles were evident, the DHBV peptide induced aggregation and liposome fusion leading to the nearly complete disappearance of the original DMPS vesicles that instead adopted large irregular structures (320 ± 70 nm) (Fig. 7B). Interestingly, in the case of the WHV peptide (Fig. 7C), the DMPS liposomes were fragmented into smaller particles (40 ± 10 nm), in accordance with the diminished aggregation levels observed with this peptide (Fig. 1).

DISCUSSION

A great deal of information concerning the fusion of the viral and cellular plasma or endosomal membranes has come from the use of synthetic peptides representing the protein stretch able to interact and destabilize a lipid bilayer (for a recent review see Pecheur et al., 1999). In the case of HBV, and mainly due to the fact that primary human hepatocytes are not readily obtainable in a reproducibly infectable state (Mabit et al., 1996), the early steps of the viral life cycle are not yet understood.
Although great progress has been made with the hepadnaviral animal infection models, the exact sequence involved in the fusion step has not yet been identified. However, based on sequence homology, we have proposed that the N-terminal region of the S protein of HBV can be involved in the fusion of the virus with the host membrane (Rodríguez-Crespo et al., 1994). Subsequent studies performed with a synthetic oligopeptide derived from HBV established its ability to induce both fusion and leakage of phospholipid liposomes in a pH-dependent manner (Rodríguez-Crespo et al., 1995). Although this fusion peptide was flexible enough to adopt an α-helical structure when dissolved in trifluoroethanol, its fusion-competent conformation in the presence of lipids appeared to be the β-sheet (Rodríguez-Crespo et al., 1996). In order to extend these results to the other members of the hepadnavirus family, we have performed studies with synthetic oligopeptides corresponding the N-terminal regions of the S glycoproteins of DHBV and WHV. They are only partially homologous in sequence and lack both the Glu residue at position 2 and the Phe-Leu-Gly fusogenic motif (Table 1), present in numerous retroviral and paramyxoviral fusion peptides (White, 1990; Durell et al., 1997).

Both the DHBV and WHV N-terminal peptides were able to cause vesicle aggregation, lipid mixing, and the leakage of the vesicular internal contents in a concentration-dependent and pH-independent manner. Moreover, both peptides were significantly more potent in terms of membrane destabilization than their HBV counterpart. The HBV fusion peptide is able to interact specifically with anionic phospholipids promoting vesicle aggregation of asolectin but not of neutral phospholipids. However, the DHBV peptide produced a significant effect in both types of vesicles. More evident was the aggregation of asolectin but not of neutral phospholipids. A peptide concentration value close to 35 μM at pH 7.3 and 20 μM at pH 5.0 is needed to attain half of the maximal energy transfer value in the case of the HBV fusion peptide (Rodríguez-Crespo et al., 1995), while at pH 7.3, 12 μM DHBV or 8 μM WHV fusion peptides produced similar effects. On the other hand, the HBV peptide does not induce any decrease of energy transfer at either pH when using PC vesicles, while both DHBV and WHV were able to promote partial fusion of these vesicles.

However, the augmented interaction of these two peptides toward vesicles where negatively charged phospholipids are present when compared with PC vesicles also indicates that the electrostatic contribution of the peptide–lipid contacts plays a significant role in determining the studied properties.

The most marked difference when comparing the fusion peptides of DHBV and WHV with HBV is found in the leakage assay. With asolectin phospholipid liposomes, a concentration value close to 70 μM was needed in the case of the HBV fusion peptide in order to generate half of the maximal fluorescence signal at pH 7.3 (Rodríguez-Crespo et al., 1995). Concentrations as low as 1 μM (DHBV) or 4 μM (WHV) produce similar effects on identical vesicles. This increased destabilizing activity of the DHBV and WHV peptides must be a reflection of their subtle changes in the amino acid sequence. In this regard, it must be noted that the Phe and the Gly residues present in the well-characterized Phe-Leu-Gly tripeptide present in the HBV sequence are changed to Ile and Ala in the Ile-Leu-Ala (DHBV) and Leu and Ala in the Leu-Leu-Ala (WHV) tripeptides (Table 1). Although the Phe to Ile/Leu substitution at position 8 seems to be conservative enough to maintain a fusogenic activity, the substitution of the Gly by an Ala residue in DHBV and HHHBV has a somewhat special interest since this substitution in the fusion peptide of SIV enhanced syncytium formation in infected cells sixfold (Bosch et al., 1989).

CD studies have shown that the two peptides characterized in this work are able to adopt different conformations when challenged in different environments, i.e., TFE, aqueous solution, and phospholipid vesicles. Beyond the ability to interact with lipid membranes, one of the most important properties of fusion peptides is their flexibility as well as their capability to adopt different secondary structures (Durell et al., 1997; Davies et al., 1998). Perhaps, the most studied and better understood case would be the influenza hemagglutinin fusion peptide that, according to the crystallographic data, adopts a fold composed of sequential turns in the preactivated, neutral conformation (Wiley and Skehel, 1987) but inserts into lipid membranes adopting a mostly α-helical conformation (Lüneberg et al., 1995). This same plasticity is illustrated by the HIV fusion peptide that evolves from an
α-helix at high lipid/peptide ratios toward a β-strand at low lipid/protein ratios (Rafalski et al., 1990).

Fluorescence depolarization studies demonstrated that the two fusion peptides insert into the hydrophobic core of the phospholipid bilayer. Moreover, in the presence of DMPG liposomes each peptide seems to be able to adopt a different conformation. Thus, the DHBV fusion peptide adopts mostly an extended conformation (β-sheet), whereas the WHV fusion peptide displays a significant propensity to fold in an α-helical conformation. The presence of four Gly residues in the DHBV fusion peptide (Table 1) could be responsible for its low ability to adopt the more compact α-helical conformation. Correlation of the behavior of each of the peptides in the presence of liposomes with the secondary structure data led us to infer that the extended conformation of the DHBV peptide is more likely responsible for its ability to induce vesicle aggregation, very efficient leakage, and the massive appearance of fused membranes observed by electron microscopy photographs. On the other hand, the WHV peptide in the presence of lipids adopts a considerable percentage of α-helical structure, which could be responsible for its low aggregation and potent leakage capacities. As indicated by energy transfer, the insertion of the WHV peptide also induces complete and partial fusion of liposomes composed of asolectin phospholipids and PC, respectively. Therefore, the small vesicles which are observed by electron microscopy should result from the fragmentation of previously fused liposomes. Examples of viral fusion peptides that adopt either extended conformations (Epand et al., 1992; Davies et al., 1998) or α-helical structures (Lear and DeGrado, 1987; Rafalski et al., 1991; Lüneberg et al., 1995) are abundant in the literature.

The information concerning the fusion of HBV and hepatocyte membranes is still scarce and controversial. By means of a selective PCR technique it has been demonstrated that infection of primary human hepatocytes by HBV was still successful after preincubation of viral particles at low pH and not affected by the presence of lysosomotropic agents (Hagelstein et al., 1997). These results might suggest that viral passage through an acidic compartment is not required and fusion of HBV with the host cell membrane is a pH-independent event. In the case of DHBV, and in spite of the availability of an in vitro infection model, data describing pH-dependent and pH-independent viral fusion with the host cell have been presented. Thus, inhibition of DHBV infection by lysosomotropic agents such as chloroquine and ammonium chloride has been reported, suggesting a receptor-mediated endocytosis process followed by a pH-dependent fusogenic step in an acidic vacuole (Offensperger et al., 1991). However, other studies have pointed toward a completely different strategy of virus infection (Rigg and Schaller, 1992; Köck et al., 1996). The role of pH in initiating DHBV infection was investigated using ammonium chloride and monensin as lysosomotropic agents, carrying out control experiments with Semliki Forest Virus or Fowl Plague Virus, which are known to require the acidic pH of endosomes for infection. Conversely, these authors concluded that DHBV infection is not dependent on low pH and that internalization of DHBV occurred only between pH 6.8 and 8.0. Interestingly, uptake of DHBV by receptor-mediated endocytosis without passage through an acidic compartment has been postulated as the infection route (Köck et al., 1986). One of the key points in our data is the fact that the fusogenic activity of the DHBV and WHV peptides was similar at both neutral and acidic pH values. Neither fusion peptide displayed increased vesicle aggregation, fusion, or leakage at acidic pH values. These data, although indirect, would point to a pH-independent fusion mechanism. However, we have previously shown that the HBV S protein N-terminal peptide displayed increased fusogenic activity at acidic pH (Rodríguez-Crespo et al., 1995). Sequence comparison of the synthetic oligopeptides used in these studies reveal that the glutamic acid at position 2 of the HBV fusion peptide is absent in the other two viral sequences (Table 1). Partial protonation of this acidic residue at pH 5.0 together with the fact that similar secondary structures are adopted by the HBV peptide at neutral and acidic pH might explain the increased fusogenic activity of the HBV oligopeptide (Rodríguez-Crespo et al., 1995, 1996). Nevertheless, this Glu residue might be dispensable during the fusion process if in vivo a cellular protease is responsible for the generation of the fusion peptide by cleavage after the Glu residue. In this respect, it has been recently described that the cleavage of HBV virions by proteases immediately after this glutamic acid residue leads to the attachment to fibroblasts or HeLa cells, which would not interact with HBV under normal conditions (Lu et al., 1996). On the other hand, incubation of DHBV viral particles at low pH promotes translocation of internal preS domains that could facilitate its cleavage by proteases to generate a fusion peptide (Guo and Pugh, 1997).

In conclusion, S protein N-terminal peptides representative of all the hepadnavirus genera induce aggregation of phospholipid vesicles, close apposition of membranes, and destabilization of neutral and acidic phospholipid bilayers, the essential steps required for fusion (Bentz et al., 1983). Hence, this protein region might play an essential role in the fusogenic processes and may be considered an important target in preventing both HBV entry and infectivity.

**MATERIALS AND METHODS**

**Reagents**

DMPG, DMPS, DMPC, PC, N-(7-nitro-2,1,3-benzoxadiazol-4-yl)dimyristoylphosphatidylethanolamine (NBD-PE), and N-(lissamine rhodamine B sulfonyl)diacetylphosphatidylethanolamine (Rh-PE) were provided by Avanti Polar Lipids. Soybean asolectin phospholipids were purified from type II soybean asolectin (Sigma) by absorption
chromatography in an activated silica gel column in chloroform. The major components of asolectin phospholipids are: phosphatidylethanolamine (39%), phosphatidylcholine (24%), and phosphatidylserine (19%) (Papini et al., 1987). ANTS and DPX were obtained from Molecular Probes. Triton X-100 was purchased from Boehringer Mannheim. All other reagents were obtained from Merck and Sigma. All solvents were of HPLC grade.

Peptide synthesis

The peptides DHBV, Met-Ser-Gly-Thr-Phe-Gly-Gly-Ile-Leu-Ala-Gly-Leu-Leu-Leu-Leu, and WHV, Met-Ser-Pro-Ser-Ser-Leu-Gly-Leu-Leu-Ala-Gly-Leu-Gln-Val-Val, were synthesized as described above, was used as control. At the concentration used (0–70 mM) this peptide had no measurable effect on any of the assays performed.

Peptide synthesis

The peptides DHBV, Met-Ser-Gly-Thr-Phe-Gly-Gly-Ile-Leu-Ala-Gly-Leu-Leu-Leu-Leu-Leu, and WHV, Met-Ser-Pro-Ser-Ser-Leu-Gly-Leu-Leu-Ala-Gly-Leu-Gln-Val-Val, were synthesized as the C-terminal amide on an automated multiple peptide synthesizer (AMS 422, Abimed) using the solid-phase procedure and standard Fmoc chemistry in a base of 25 μmol. The synthesis was carried out on a N-9-Fmoc-DMP resin [4-(2′,4′-dimethoxyphenyl-Fmoc-aminomethyl)phenoxy resin (Novabiochem)] with Fmoc-protected amino acids activated in situ with PyBOP (benzotriazole-1-yl-oxy-tris-pyrrolidinophosphonium hexafluorophosphate) in the presence of N-methylmorpholine and 20% piperidine/dimethylformamide for deprotection. The protecting side chain groups were as follows: Gln (Trt), Ser, and Thr (tBu). Peptides were cleaved from the resin with 82.5% trifluoroacetic acid, 5% phenol, 5% H2O, 5% thioanisole, 2.5% ethane dithiol as scavengers (King et al., 1990), precipitated, and washed with cold methyl tert-butyl ether, water-extracted, lyophilized, and purified in reverse-phase HPLC using an Ultrasphere-ODS C18 column (10 × 150 mm) with a linear gradient water/35% acetonitrile in 0.1% trifluoroacetic acid. Purity of the peptides was assessed to be more than 95% by reverse-phase HPLC analysis on a Ultrasphere-ODS C18 column (4.6 × 150 mm). Amino acid analysis, performed on a Beckman 6300 amino acid analyzer, indicated the expected composition. Fast atom bombardment mass spectrometry yielded the following result: m/z 1519.9 and 1584.0 for DHBV and WHV, respectively (calculated 1518.9 and 1584.0). Due to the low solubility of the peptides in aqueous buffers, a 6 mg/ml stock solution in DMSO to a phospholipid vesicle suspension, in medium buffer at the appropriate pH, was measured on a Beckman DU-7 spectrophotometer after incubation for 1 h at 37°C. The concentration of DMSO was kept below 1%. Samples containing equal amounts of DMSO but in the absence of peptide were always used as a control. The final phospholipid concentration was 0.14 mM.

Lipid mixing assay

This was monitored by using the classical fluorescent probe dilution assay (Struck et al., 1981), in which the decrease in the efficiency of the fluorescence energy transfer between NBD-PE (energy donor) and Rh-PE (energy acceptor) incorporated into liposomes, as a consequence of lipid mixing, is monitored. Liposomes, in medium buffer at the appropriate pH, labeled with 1 mol% NBD-PE and either 0.8 or 0.9 mol% Rh-PE (depending on whether asolectin phospholipids or PC, respectively, were used), were mixed in a 1:9 molar ratio with unlabeled liposomes. Lipid mixing was initiated by addition of the peptide from a stock solution in TFE. The concentration of TFE was kept below 1%. This organic solvent has no effect on energy transfer values, as indicated by control samples containing TFE. The samples were incubated for 1 h at 37°C and the emission spectra were recorded in a SLM Aminco 8000 spectrofluorimeter, with the excitation wavelength set at 450 nm. Both the excitation and emission slits were set at 4 nm. The excitation polarizer was kept constant at 90° and the emission polarizer was kept constant at 0° to minimize dispersive interference. The efficiency of the energy transfer was calculated from the ratio of the emission intensities at 530 and 585 nm and the appropriate calibration curve. The final phospholipid concentration was 0.14 mM.

Release of aqueous contents

Leakage was determined by the ANTS/DPX assay (Ellens et al., 1985), which is based on the dequenching
of ANTS fluorescence caused by its dilution upon release of the aqueous contents of one vesicle population containing both ANTS and DPX. It was performed by coencapsulating 12.5 mM ANTS and 45 mM DPX with 20 mM NaCl, 10 mM Tris, pH 7.2, in asolectin phospholipid vesicles. The lipid film was vortexed and the vesicles were sonicated in a bath for 30 min. Afterward, the vesicles were subjected to four cycles of freeze-thawing in liquid nitrogen and passed at least four times through an Extruder apparatus (Lipex Biomembranes Inc.) with 100-nm polycarbonate filters (Costar). After the vesicles with the coencapsulated probe and quencher were formed, the whole sample was passed through a Sephadex G-75 column (Pharmacia) to separate the vesicles from the nonencapsulated material using medium buffer for elution (Düzgünès and Shavnin, 1992). The final phospholipid concentration in the assay was 0.14 mM and medium buffer at the appropriate pH value was used in all cases. Leakage was initiated by addition of the peptide from a stock solution in DMSO. The concentration of DMSO was kept below 1%, a value at which there was no effect on vesicle integrity. The fluorescence scale was set to 100% by addition of 0.5% Triton X-100 and 0% leakage was obtained measuring the fluorescence of the control vesicles after the addition of DMSO. The suspensions were excited at 385 nm and ANTS emission was monitored at 520 nm. Both the excitation and emission slits were set at 4 nm. The excitation and emission polarizers were kept constant at 90° and 0° to minimize dispersive interference.

Circular dichroism

Aliquots of peptide from a stock solution at 6 mg/ml in TFE were dried under a stream of nitrogen. These peptide films were dissolved in TFE and TFE/water mixtures or resuspended in medium buffer (100 mM NaCl, 5 mM Tris, 5 mM citrate, 5 mM MES, 1 mM EDTA) at the appropriate pH at a concentration of 120–140 μg/ml. The spectrum of the peptides in aqueous solution was recorded after elimination of peptide aggregates by centrifugation in a Beckman Microfuge E for 15 min. The CD was kept below 1%, a value at which there was no effect on vesicle integrity. The fluorescence scale was set to 100% by addition of 0.5% Triton X-100 and 0% leakage was obtained measuring the fluorescence of the control vesicles after the addition of DMSO. The suspensions were excited at 385 nm and ANTS emission was monitored at 520 nm. Both the excitation and emission slits were set at 4 nm. The excitation and emission polarizers were kept constant at 90° and 0° to minimize dispersive interference.

Fluorescence polarization

Fluorescence polarization measurements of 1,6-diphenyl-1,3,5-hexatriene (DPH) were made on a SLM 8000C spectrophoimeter equipped with 10-nm Glan-Thompson polarizers. DMPC and DMPS (0.14 mM), containing DPH at a ratio of 1 molecule of fluorophore for every 500 phospholipid molecules, was hydrated at the appropriate pH and extruded as indicated above. The peptide was added from a stock concentrated solution in DMSO. The concentration of DMSO was kept below 1.5%. Peptide-vesicle mixtures were incubated for 1 h at 37°C and then cooled. The excitation was set at 365 nm and emission was measured at 425 nm, after equilibration of the samples at the indicated temperature. The temperature in the cuvette was maintained with a PolyHub circulating water bath.

Electron microscopy

Samples of DMPS vesicles (obtained by extrusion through a 0.1-mm pore diameter polycarbonate filter) at 0.14 mM lipid concentration were incubated with DHBV or WHV fusion peptides at a final concentration of 12 μM for 1 h at 37°C. Subsequently, the lipid–peptide mixtures were applied to a glow-discharged 400-mesh Formvar–carbon-coated grid for 2 min. Excess fluid was drawn away with filter paper. Samples were then negatively stained with 2% (w/v) phosphotungstic acid at pH 7.0 and examined by using a Zeiss EM 902 (Jena, Germany) transmission electron microscope operating at 80 kV.

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