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Interaction of preS domains of hepatitis B virus with phospholipid vesicles

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ABSTRACT

The role of preS domains of the hepatitis B virus (HBV) envelope proteins in the first steps of viral infection has been restricted to their implication in virus attachment to a putative hepatocyte receptor. In order to explore a fusion activity in these regions, we used recombinant preS domains to characterize their interaction with liposomes. Binding experiments carried out with NBD-labeled proteins indicated that preS were able to interact in a monomeric way with acidic phospholipid vesicles, being the partition coefficient similar to that described for peptides which can insert deeply into bilayers. Fluorescence depolarization of DPH-labeled vesicles confirmed the specificity for negative charged phospholipids. Upon interaction the proteins induced aggregation, lipid mixing and release of internal contents of acidic vesicles at both acid and neutral pH in a concentration-dependent manner. Taken together, all these data indicate that preS domains are able to insert into the hydrophobic core of the bilayer. Moreover, the insertion resulted in a protein conformational change which increased the helical content. Therefore all these results suggest that, besides their participation in the recognition of a cellular receptor, the preS domains could be involved in the fusion mechanism of HBV with the plasma membrane of target cells.

1. Introduction

Hepatitis B virus (HBV) is a small enveloped DNA virus that belongs to the hepadnaviridae family. It causes persistent infection of hepatocytes, resulting in some cases in the development of chronic hepatitis and hepatocellular carcinoma. Despite representing a worldwide health problem, the mechanisms of attachment and entry of HBV into target cells are still poorly understood.

The envelope proteins of HBV are assumed to play key roles in the binding and fusion of target cells. The virus possesses three surface proteins known as small (S), medium (M) and large (L) that are translated from a single open reading frame at three different translational start codons. Thus, all three proteins share 226 amino acids (the complete S protein) at the carboxy-terminus; the M protein possesses an extension of 55 amino acids, termed as preS2 region, at the N-terminus of S protein, while the L protein is composed of the entire M protein prolonged at the N-terminus by the preS1 region, which consists of 108 in the HBV subtype known as ayw or 119 residues in the other three main subtypes adw, ayr and adr (Fig. 1). The preS1 and preS2 regions, known together as preS domains, have been implicated in the binding of the virus to hepatocytes [1, 2]. Several receptors for HBV have been proposed to interact with different regions of preS domains [3, 4] although none of them have been proven to function in viral entry. Nowadays a generally accepted view is the involvement of preS1 in attachment to hepatocytes, being the viral entry activity of this region solely dependent on the integrity of its first 75 amino acids [5]. Moreover, recent studies suggested that lipoprotein lipase (LPL) may play a role in the initiation of HBV infection via interaction with the N-terminal part of preS1 [6].

Regarding the role of HBV envelope proteins in the fusion step, a putative fusion peptide sequence of 16 amino acids at the N-terminus of S protein has been described [7]. A synthetic peptide comprising this predicted fusion region was shown to interact with model membranes, promoting liposome destabilization in a pH-dependent manner [8], and adopting an extended conformation during the process [9]. Evidence for the role of the N-terminal S peptide in fusion has been obtained after treatment of HBV virions with V8 protease, an enzyme that cleaves the S sequence at position 2, removing the preS domains and exposing the hydrophobic fusion peptide; intact HBV hardly infected HepG2 cells, but V8 protease-digested HBV particles efficiently infected and proliferated in these human hepatoblastoma cell lines [10]. The destabilization properties observed for the HBV fusion peptide can be extended to other members of the hepadnavirus family, as demonstrated using oligopeptides corresponding to the N-terminal portion of duck and woodchuck hepatitis B viruses (DHBV

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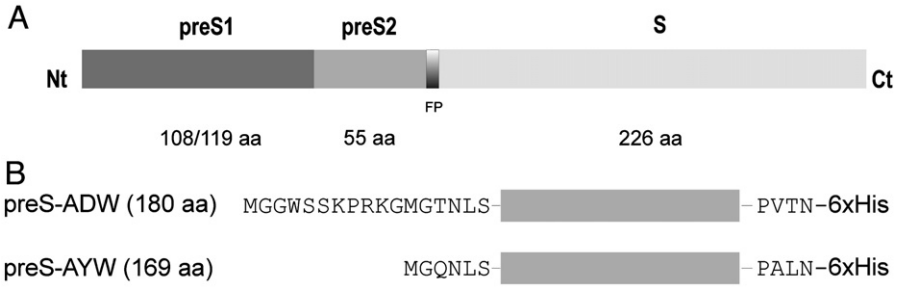

Fig. 1. Schematic representation of HBV Large surface protein and preS domains. (A) The S, preS1 and preS2 domains are depicted. The position of the putative fusion peptide (FP), at the N-terminal of S region, is also indicated. (B) The amino acid sequence of the N and C-terminal regions of adw and ayw preS domains are showed. The 6xHis at the C-terminal indicates the position of the His tag added to the amino acid sequence.

and WHV, respectively) [11]. Likewise, V8 protease-digested WHV particles induced infectivity towards human HepG2 cells [12], confirming that the exposure of this consensus fusion motif is important in hepadnavirus entry.

Despite their location at the surface of natural virions, the preS domains have never been directly involved in the fusion process, being their role in viral entry only associated with the attachment of HBV particles to possible cellular receptors. With the aim to explore the fusogenic capabilities of these regions, two recombinant preS domains from adw and ayw subtypes, produced in *E. coli* cells as previously described [13], were used in membrane interaction studies. In this paper we describe that preS domains are able to interact with acidic phospholipid vesicles and to destabilize these membrane model systems, and hence, could contribute, together with the N-terminal S peptide, to the fusion of viral and cellular membranes.

2. Materials and methods

2.1. Reagents

N-(7-nitro-2,1,3-benzoxadiazol-4-yl)-dimyristoylphosphatidylethanolamine (NBD-PE), N-(lissamine rhodamine B sulfonyl)-diacylphosphatidylethanolamine (Rh-PE), egg phosphatidylglycerol (PG), and dimyristoylphosphatidylglycerol (DMPG) were provided by Avanti Polar Lipids. 8-Aminonaphtalene-1,3,6-trisulfonic acid (ANTS), p-xylenebis(pyridinium) bromide (DPX) and 1,6-diphenyl-1,3,5-hexatriene (DPH) and 4-fluoro-7-nitrobenz-2-oxa-1,3-diazole (NBD-F) were purchased 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.

2.2. Cloning, expression, purification and labeling of preS domains

The cDNAs coding for preS domains of subtypes adw and ayw were cloned as described previously in expression vectors that add six-histidine sequences at the carboxy-terminal end of each protein [13]. *E. coli* strains BL21 (DE3) and HMS174 (DE3) were transformed with the recombinant plasmids pT7-7-preS-his-adw and pET-3d-preS-his-ayw respectively and isopropyl -D-thiogalactopiranosyde (IPTG) was added to a final concentration of 0.5 mM to induce protein expression. Both recombinant proteins were purified using a single affinity-chromatography step in Sepharose CL-6B Ni-nitrilotriacetic acid (NTA) column (Qiagen), following procedures previously described, giving rise to highly pure and stable 20–25 mg of preS-his-ayw and 35–40 mg of preS-his-adw from 1 L of culture media [13].

Fluorescent labeling of the N-terminus of the proteins was achieved following the procedure described by Rapaport and Shai [14]. Briefly, preS proteins were incubated at pH 6.8 with a ten molar excess of NBD-F for 4 h at room temperature. Unbound NBD-F was removed by means of a PD-10 column. The labeling of the protein could be monitored by the appearance of a maximum at 467 in the absorbance spectrum, which was used to determine the labeling ratio. The NBD and protein concentrations were determined by using 20,000 M^{-1} cm^{-1} as the molar extinction coefficient of NBD-PE and amino acid analysis, respectively.

2.3. Vesicle preparation

In all cases a lipid film was obtained by drying a chloroform: methanol (2:1) solution of the lipid under a current of nitrogen and this film was further kept under vacuum for 4–5 h to completely remove the organic solvent. The phospholipids were resuspended at a concentration of 1 mg/ml in medium buffer (100 mM NaCl, 5 mM MES, 5 mM sodium citrate, 5 mM Tris, 1 mM EDTA) at the appropriate pH value and incubated for 1 h at 37 °C and eventually vigorously vortexed. This suspension was sonicated in a bath sonicator (Branson 1200) and was subsequently subjected to nineteen cycles of extrusion in a Liposo Fast-Basic extruder apparatus (Avestin, Inc.) with 100-nm polycarbonate filters (Costar). When encapsulation was required, an additional step of five freeze-thawing cycles was included after the sonication process.

2.4. Binding experiments

Binding experiments were conducted as previously described [14]. In order to determine the degree of NBD-preS-his association with phospholipid vesicles, PC or PG vesicles were added to a fixed amount of labeled protein (0.15 μM) in medium buffer at the desired pH and incubated at 37 °C. The fluorescence intensity at different lipid/protein molar ratios was registered in a SLM AMINCO 8000C spectrofluorimeter (SLM Instruments), with excitation and emission wavelengths set at 468 and 530 nm, respectively. In all cases, fluorescence from control vesicles in the absence of labeled protein was subtracted. In order to obtain the partition coefficient, data were analyzed using the equation [14, 15]:

$$X_b = K_p \cdot C_f$$

where X_b is the molar ratio of bound protein per total lipid, K_p corresponds to the partition coefficient and C_f represents the equilibrium concentration of free protein in solution. It was assumed that proteins only partitioned over the outer leaflet of vesicles. Therefore, X_b values were corrected as $\underline{X}_b^* = X_b/0.6$ and the data analyzed as:

$$X_b^* = K_p^* \cdot C_f$$

Values of the corrected partition coefficient, K_p^*, were determined from the initial slopes of the binding isotherms.

In order to calculate X_b, we estimated F_∞, the fluorescence signal obtained with a saturating phospholipid concentration by extrapolating from a double reciprocal plot of F (total protein fluorescence) versus C_L (total lipid concentration). At every phospholipid

concentration, the fraction of bound protein can be calculated by the formula:

$$f_b = (F - F_0)/(F_\infty - F_0)$$

where F_0 represents the fluorescence of unbound protein and F_∞ the fluorescence of bound protein.

2.5. Fluorescence polarization

Fluorescence polarization measurements of the probe 1,6-diphenyl-1,3,5-hexatriene (DPH) were taken in the SLM AMINCO 8000C spectrofluorimeter by using 10 mm Glan-Thompson polarizers. DMPG and DMPC vesicles (0.14 mM) were prepared as indicated above containing DPH at a weight ratio of 1:500. The protein-vesicle mixtures were incubated for 30 min 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 circulating water bath.

2.6. Vesicle aggregation

The increase in the optical density at 360 nm (OD_{360}) produced by addition of preS proteins to a phospholipid vesicle suspension, in medium buffer at the appropriated pH, was measured on a Beckman DU-7 spectrophotometer after incubation for 1 h at 37 °C. Values of control samples containing only vesicles and only protein were subtracted at each protein concentration. The final phospholipid concentration was kept at 0.14 mM.

2.7. Release of aqueous contents

Leakage was determined by the ANTS/DPX assay [16], which is based on the dequenching of ANTS fluorescence caused by its dilution upon release of the aqueous contents of one vesicle preparation containing both ANTS and DPX. It was performed by coencapsulating 12.5 mM ANTS and 45 mM DPX in 10 mM Tris, 20 mM NaCl, pH 7.2, in phospholipid vesicles. The lipid film was hydrated as described previously and the vesicles were sonicated 30 min. Afterwards the vesicles were subjected to five cycles of freeze-thawing in liquid nitrogen an passed 15 times through a Liposo Fast-Basic extruder apparatus (Avestin, 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 non encapsulated material using medium buffer for elution. Assays were performed in medium buffer at the appropriated pH, at a phospholipid concentration of 0.1–0.14 mM in medium buffer at the appropriated pH by incubating with different amounts of proteins for 1 h at 37 °C and measuring in the SLM Aminco 8000C spectrofluorimeter. The excitation wavelength was set at 385 nm and the ANTS emission was monitored at 520 nm. Both the excitation and emission slits were set at 4 mm. The excitation and emission polarizers were kept constant at 90° and 0°, respectively, to minimize interference due to dispersion. The fluorescence scale was set to 100% by addition of 0.5% Triton X-100, and 0% leakage was obtained measuring the fluorescence of control vesicles without protein.

2.8. Lipid mixing assay

Lipid mixing was monitored by using the classical fluorescent probe dilution assay [17], 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 measured. Liposomes, in medium buffer at the appropriated pH, labeled with 1 mol% NBD-PE and 1 mol% Rh-PE were mixed with unlabeled liposomes in a 1:9 molar ratio. After incubation of liposomes with the preS domains at different concentrations for 1 h at 37 °C, emission spectra were recorded with the excitation wavelength set at 450 nm. Both the excitation and emission slits were set at 4 mm. 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 590 nm and the appropriated calibration curve. The final phospholipid concentration was 0.14 mM. The organic solvent itself had no effect on the efficiency of the energy transfer.

2.9. Electron microscopy

Samples of egg PG vesicles (obtained by extrusion through a 0.1 mm pore diameter polycarbonate filter) were incubated with preS proteins at different lipid and protein concentrations for 1 h at 37 °C. Subsequently, the lipid–protein mixtures were applied to a glow-discharged 400-mesh Formvar-carbon-coated grid for 2 min. Grids were washed with deionized water and with PBS, and excess fluid was drawn away with filter paper. Samples were then negatively stained for 5 min with 2% phosphotungstic acid at pH 7.0 and examined under a Zeiss EM 902 (Jena, Germany) transmission electron microscope operating at 80 kV.

2.10. Circular dichroism

CD measurements were carried out on a Jasco spectropolarimeter, model J-715. All the measurements were carried out at 25 °C with cells thermostated with a Neslab RTE-111 water bath. The spectra were taken in medium buffer at the desired pH and at a protein concentration of 0.10 mg/ml. The pathlength was 1 mm. Five scans were averaged for each measurement and the contribution of the buffer was always subtracted. The spectra were calculated by using 110 as the mean residue molecular mass and the results are expressed in terms of residue molar ellipticity in deg cm^2 $dmol^{-1}$. The secondary structure of the protein was evaluated by computer fit of the dichroism spectra according to Convex Constraint Analysis (CCA) [18]. This method relies on an algorithm that calculates the contribution of the secondary structure elements that give rise to the original spectral curve without referring to spectra from model systems.

3. Results

3.1. Binding studies

The two domains used through this work are representative of all the HBV subtypes. They have been previously characterized [13]. The results presented are those obtained with preS domains which have been tagged with a 6xHis sequence at their C-terminal end (Fig. 1). However, similar interacting and destabilization properties have been observed for a preS domain which does not have the His tag. The reaction of preS domains with NBD-F led to the addition of the NBD moiety to the polypeptide chain. From the absorbance spectrum it can be calculated that approximately the protein was labeled in a 1:1 ratio. Taking into account that the reaction was carried out at pH 6.8, it is likely that the α-amino group, and not the Lys side chains, is the main labeling target. The NBD fluorophore has been employed in binding studies since its fluorescence spectrum reflects the environment in which the NBD group is located [14, 19]. Emission spectra of NBD-labeled preS proteins were recorded in buffer or upon interaction with PC or PG Large Unilamellar Vesicles (LUV). The wavelength of the maximum of the emission spectrum of labeled proteins under these conditions is shown in Table 1. In solution both proteins exhibited emission maxima centered at 544 nm, which is consistent with

Table 1
Fluorescence emission maxima of NBD-labeled preS domains in solution or in the presence of PC and PG vesicles and corrected partition coefficients (K_p^*) determined from the initial slopes of the binding isotherms, as indicated in the Materials and methods section

	pH	Emission maxima			K_p^* (M^{-1})×10^{-5}	
		Buffer	PG_h	PC_h	PG_h	PC_h
NBD-preS-his-ayw	7.0	544	522	534	0.1	–
	5.0	544	522	534	2.9	0.11
NBD-preS-his-adw	7.0	544	522	534	0.047	–
	5.0	544	520	534	3.0	0.036

previously reported emission wavelength for NBD derivatives [20]. Upon interaction with negatively charged phospholipids, and under saturating conditions to avoid the contribution of the free protein to the emission spectrum, these maxima were shifted to 522 nm. The observed blue shift reflects a relocation of the NBD group in a more hydrophobic environment. In the presence of neutral phospholipids there is a smaller change in the position of the maximum (Table 1) which could reflect a more shallow location of the protein within the bilayer [14].

In order to calculate the extent of binding, the labeled proteins, at a final concentration of 0.15 μM, were titrated with increasing amounts of PG or PC vesicles. This protein concentration was low enough to avoid aggregation of vesicles. Moreover, control experiments were performed by titrating unlabeled proteins with the same concentration of vesicles. The measured increase in fluorescence at 530 nm, after subtracting the contribution of the control samples, was plotted against the phospholipid concentration (Fig. 2). After incubation with PG_h, there is a significant increase in fluorescence intensity either at pH 7.0 or pH 5.0 (Fig. 2), although the effect is higher at acidic pH. From these data binding isotherms were obtained (Fig. 2, inset) as described in Materials and methods section. The partition coefficients, reflecting the binding constants, were calculated as the slopes of these lines (Table 1). The values of K_p^* determined in the presence of acidic phospholipids at pH 5.0, 3×10^5 M^{-1}, were similar to those described for labeled peptides which insert into phospholipid bilayers [14]. However, as expected from the fluorescence increments, the coefficients obtained at pH 7.0 were two orders of magnitude lower. On the other hand, when neutral phospholipids were used in these experiments, no increase in F_{530} values were obtained at pH 7.0 (data not shown) and thus no partition coefficients could be determined while at pH 5.0 the calculated K_p^* were similar to those observed with acidic phospholipids at neutral pH (Table 1).

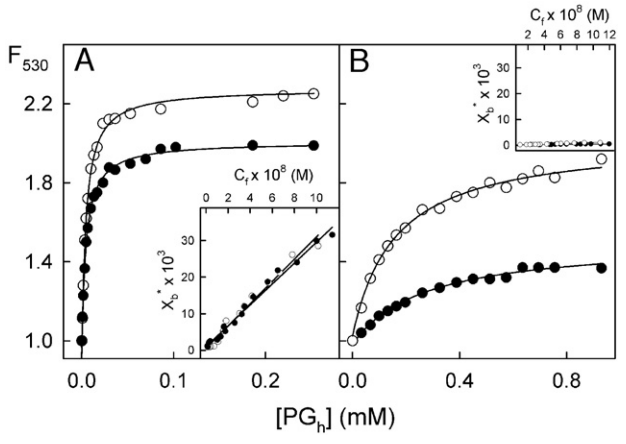

Fig. 2. Increase in fluorescence of NBD-preS-his proteins upon titration with PG_h vesicles. Labeled preS-his-ayw (●) and preS-his-adw (○) proteins (0.15 μM) were added to PG_h vesicles at different lipid concentrations in medium buffer at pH 5.0 (A) or 7.0 (B). The mixtures were incubated at 37 °C for 2 min and fluorescence intensity was measured at 530 nm. Insets represent the binding isotherms obtained from the increments of NBD fluorescence intensities. Values of X_b^* and C_f were calculated as indicated under Materials and methods. The results shown are representative of those obtained for three different experiments.

3.2. Fluorescence polarization

To ascertain the existence of a hydrophobic component in the interaction of the preS proteins with acidic and neutral phospholipids, their effect on the thermotropic behavior of DMPG and DMPC vesicles has been studied by measuring the fluorescence polarization of these liposomes labeled in the hydrophobic core of the bilayer with the fluorescent probe DPH. Fig. 3 shows the fluorescence depolarization of DPH-labeled DMPG vesicles with increasing temperatures in the presence of preS-his proteins adw (A, B) or ayw (C, D) at different protein/phospholipid molar ratios at pH 7.0 (A, C) and 5.0 (B, D). The addition of preS-his proteins to DMPG vesicles induced a decrease in the amplitude of the transition in a concentration-dependent manner, without affecting the temperature of the transition. This effect was observed almost exclusively at temperatures above the transition temperature, indicating that the proteins affected mainly the acyl chains in the liquid–crystal phase, inducing a higher order in the chain packing. At low protein concentrations the effect was slightly higher at acidic pH. However, when protein concentration increased, the effects were nearly indistinguishable at both pHs. As observed in all the insets of Fig. 3, the fluorescence polarization measured at 37 °C increased linearly up to a protein to lipid ratio of 0.045, remaining constant from this point. The fact that the amplitude of phase transition was mainly the only modified parameter reveals the importance of the hydrophobic component in the interaction of preS domains with phospholipids. On the other hand, no effects in the transition curve of DMPC vesicles were observed (data not shown).

When studies were performed using the fluorescent probe TMA-DPH, the results obtained were very similar to those described above for DPH (data not shown).

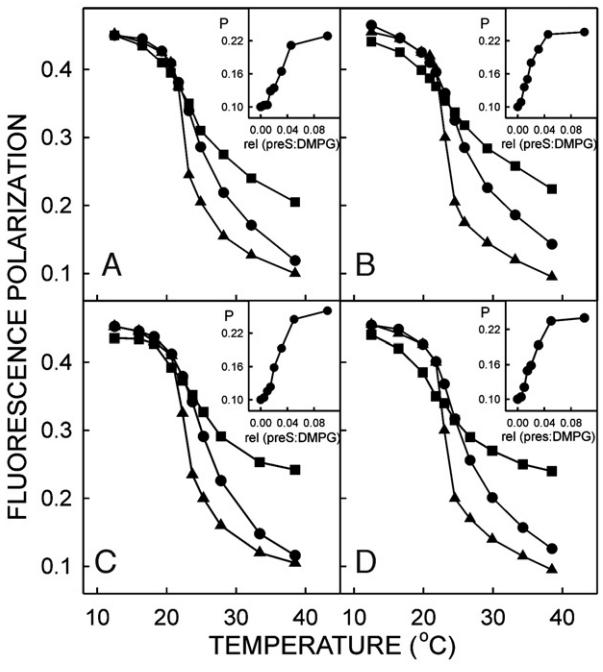

Fig. 3. Temperature dependence of the fluorescence polarization of DPH-labeled DMPG liposomes. Vesicles were incubated with different concentrations of preS-his-adw (A, B) or preS-his-ayw (C, D) proteins for 30 min at 37 °C in medium buffer at pH 7.0 (A, C) or 5.0 (B, D). After cooling, polarization was measured at the indicated temperature. The phospholipid concentration was 0.14 mM and the preS/lipid molar ratios employed were (▲) 0.0, (●) 0.01 and (■) 0.045. Insets represent fluorescence polarization at 37 °C as a function of protein/phospholipid ratio. The results shown are representative of those obtained for three different experiments.

3.3. Vesicle aggregation

Vesicle aggregation was monitored by measuring the increment of the optical density at 360 nm (ΔOD_{360}) of PG_h liposomes as a result of the increase in vesicle size upon incubation with different concentrations of preS-his proteins. Fig. 4 shows the results obtained with preS-his adw, being those with preS-his ayw very similar. The ΔOD_{360} value increased up to a protein concentration of 6–8 μM and then remained constant. This effect was observed both at pH 7.0 and 5.0 although ΔOD_{360} was slightly higher at acidic pH. On the other hand, when preS-his were incubated with neutral phospholipids, the increase of OD_{360} at any pH value was almost negligible (data not shown).

Vesicle aggregation was also measured at different PG concentrations and maintaining a constant protein to lipid molar ratio of 1:75 (Fig. 4, inset). As it was expected, OD_{360} augmented almost linearly up to a lipid concentration of 1.5 mM and remained constant up to 2.0 mM. However, at 2.6 mM PG (34.6 μM of preS-his adw) the optical density diminished to values even lower than the corresponding control, indicating a possible disruption of the vesicles. These results were similar both at pH 7.0 and 5.0 and using either preS-his-adw or preS-his-ayw.

3.4. Lipid mixing

Mixing of phospholipid vesicles was followed by the decrease in resonance energy transfer (RET) between the fluorescent probes NBD-PE and Rh-PE incorporated into a lipid matrix [17], which accurately reflects the degree of fusion [21]. As observed in Fig. 5A preS domains of both ayw and adw subtypes were able to induce lipid mixing when using PG vesicles, both at pH 7.0 and 5.0. Although at low protein concentration the decrease of RET was more pronounced at acidic pH, the results obtained at both pH values made equal as the protein concentration was increased. At either pH value %RET decreased from 72, in the absence of protein, to 18–20 at 10 μM preS-his. These values correspond to a dilution in acceptor surface density of approximately eightfold. Since the mere aggregation of the vesicles would not result in such a big change in energy transfer [22], it could be concluded that, under the conditions studied, preS domains induce the complete fusion of vesicles.

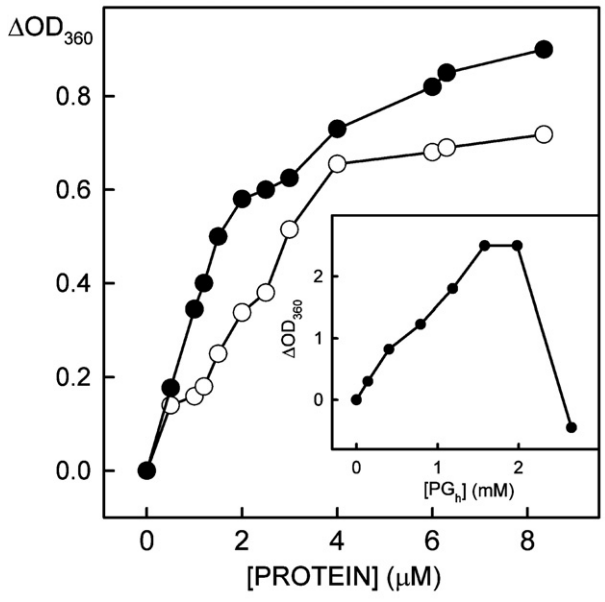

Fig. 4. Aggregation of egg PG phospholipid vesicles induced by preS-his-adw protein. The optical density at 360 nm (ΔOD_{360}) was measured after incubation of vesicles (0.14 mM) in medium buffer at pH 7.0 (○) and 5.0 (●) with proteins at different concentrations. Values of control samples containing only PG liposomes were subtracted. Inset represents the ΔOD_{360} as a function of PG concentration at a constant protein/lipid ratio of 1:75 at pH 7.0. The results shown are representative of those obtained for at least three different experiments.

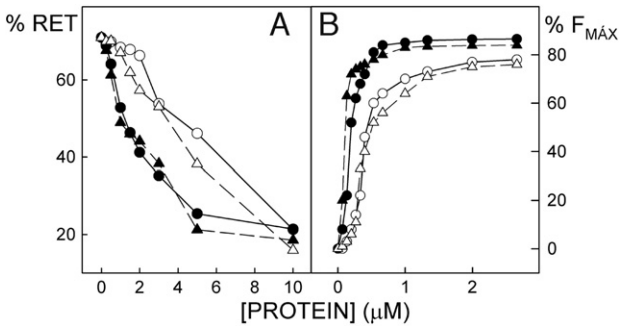

Fig. 5. Lipid mixing (A) and leakage of ANTS/DPX (B) induced by preS proteins. (A) Increasing concentrations of preS-his-adw (△) or preS-his-ayw (○) were added to a 1:9 mixture of labeled (NBD-PE 1% and Rh-PE 1%) and unlabeled PG vesicles hydrated in medium buffer at pH 7.0 (empty symbols) and 5.0 (filled symbols). The Resonance Energy Transfer (RET) between NBD-PE and Rh-PE was calculated as indicated in Materials and methods. (B) Increasing concentrations of preS-his-adw (△) or preS-his-ayw (○) were added to egg PG vesicles loaded with ANTS and DPX in medium buffer at pH 7.0 (empty symbols) and 5.0 (filled symbols). The mixtures were incubated at 37 °C for 1 h and the fluorescence intensity was measured at 520 nm. Maxima fluorescence was obtained upon addition of 0.5% Triton X-100. The results shown are representative of those obtained for at least three different experiments.

3.5. Release of aqueous contents

The ability of preS proteins to destabilize the lipid bilayer was also assessed by determining the release of aqueous content of phospholipid vesicles. Liposome leakage was monitored by measuring the increase in ANTS fluorescence at 520 nm [23]. Fig. 5B shows the leakage induced by ayw and adw preS-his domains when added to PG vesicles. Both proteins were able to induce the release of internal contents of the vesicles in a concentration-dependent manner. The maximum effect was attained at 0.5–1.0 μM of protein, concentrations much lower than those needed to induce vesicle aggregation or lipid mixing (5–10 μM). On the other hand, at low protein concentrations leakage was slightly higher at acidic pH, being the pH dependence of the lipid destabilization lower than that observed in the aggregation and lipid mixing assays. The maximum fluorescence reached with either protein, 75–85%, is similar to that described for other proteins and did not attain the value obtained when liposomes were lysed with the detergent Triton X-100 (100% leakage).

3.6. Electron microscopy studies

Incubation of preS-his domains with PG liposomes had effects on the morphology and size of the phospholipid vesicles as can be observed by electron microscopy after negative staining with sodium phosphotungstate. Fig. 6 shows the results obtained with PG vesicles in the presence of preS-his adw at pH 7.0. Fig. 6A indicates that control PG vesicles had a homogeneous size of 100–130 nm. Addition of protein at a concentration of 5.3 μM induced aggregation and fusion of liposomes, leading to the disappearance of the original half moon typical staining of control PG vesicles that instead adopted large aggregated structures of 250–420 nm (Fig. 6B). Furthermore, in some cases interaction with preS-his protein induced formation of filaments (Fig. 6C). When lipid and protein concentrations were increased, maintaining unchanged the molar ratio, the PG vesicles were fragmented into smaller, 30–60 nm, particles (Fig. 6D); this observation is in accordance with the aggregation studies (Fig. 4, inset), and indicates that the protein was able to induce the vesicle disruption under these conditions.

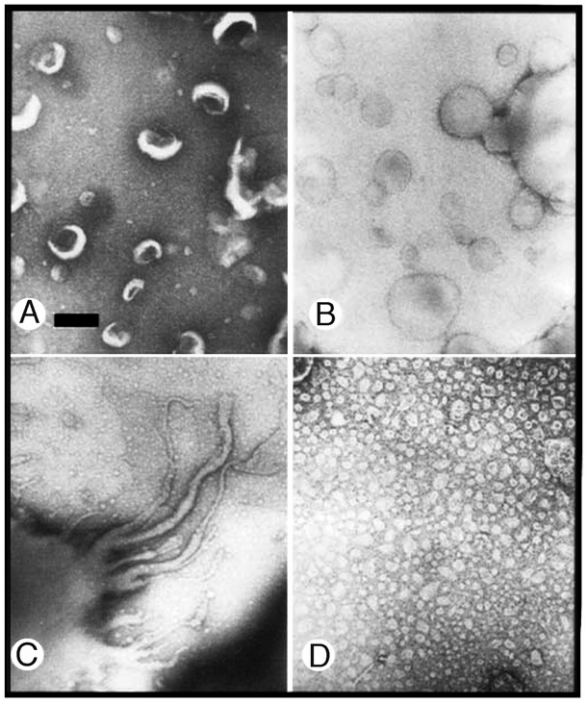

Fig. 6. Electron micrographs of egg PG vesicles in the presence of preS-his-adw protein. PG liposomes were incubated with the protein at pH 7.0 for 1 h at 37 °C and the mixtures were transferred to glow-discharged Formvar-carbon-coated grids. Afterwards, they were negatively stained with sodium phosphotungstate. (A) Egg PG vesicles (0.14 mM) in the absence of protein. (B–D) PG-preS mixtures at a 1:75 molar lipid/protein ratio; (B, C) [PG]=0.4 mM; (D) [PG]=2.65 mM. The bar indicates 150 nm.

3.7. Circular dichroism

The interaction and insertion of preS domains into the hydrophobic core of phospholipid vesicles brought about a conformational change which could be assessed by circular dichroism. Thus, Fig. 7 shows the CD spectra of adw preS-his at pH 7.0 (Fig. 7A) and 5.0 (Fig. 7B) both alone and in the presence of egg PG. At either pH, the CD spectrum of preS domains is characteristic of a protein with a high content of non-regular structure. However, in the presence of acidic phospholipids there is an increase in the ellipticity values together with a shift of the minimum from 200 to 205 nm and the appearance of a shoulder at 225 nm, characteristic of helical structure. In fact, deconvolution of CD spectrum by CCA method indicated that the percentage of helical content increased from 0 to 10% with the concomitant decrease of non-regular structures. The results obtained with the ayw subtype were similar (data not shown). However, no changes in the CD spectrum were observed in the presence of neutral phospholipids.

4. Discussion

Based on a previously reported method which relies on the changes of the fluorescence properties of NBD-labeled peptides or proteins [14], it can be assessed that preS domains interact with phospholipid vesicles. The addition of increasing concentrations of phospholipids to NBD-labeled proteins led to a shift in the fluorescence emission maximum of NBD as well as to an increase in the fluorescence intensity at 530 nm. As described for other peptides, the first data gives information about the location of the protein in the bilayer after the interaction [14]. Thus, in the presence of acidic phospholipids the maximum was shifted to 520–522 nm, both at pH 7.0 and 5.0, indicating that the NBD was located in a more hydrophobic environment, similarly to what has been described for peptides deeply inserted into the bilayer (λ_{max}=518–528 nm). However, in the presence of neutral phospholipids the position of the emission maximum (λ_{max}=534 nm) was analogous to that observed for peptides interacting with the bilayer at the surface [14]. On the other hand, binding isotherms, derived from the increments in fluorescence intensities, provide information about the mechanism of the interaction [15, 24]. Thus, the straight lines obtained in all cases denoted that the proteins interact with the bilayer in a monomeric form, being unnecessary the formation of oligomers to elicit lipid–protein interactions. Furthermore, crosslinking experiments with the bifunctional reagent bis-(sulfosuccinimidyl)suberate indicate that when preS domains interact with acidic phospholipids no oligomers of defined structure are formed since only a small fraction of dimers and trimers is observed (data not shown). On the other hand, the interaction coefficients were calculated from the slopes of the lines. Thus, in the presence of acidic phospholipids at pH 5.0 the values achieved with both preS domains were of the order of magnitude of those described for peptides having a strong interaction with the bilayer, such as the transmembrane peptides or even peptides forming pores in the membrane [14, 19, 25]. However, the constant reached at pH 7.0 was significantly lower, indicating a much lower interaction between the protein and the phospholipids although NBD was located in a similar hydrophobic environment to that at pH 5.0. When neutral lipids were used no partition coefficients could be calculated at pH 7.0 and the values obtained from the increment in fluorescence intensities at pH 5.0 were similar to those obtained with PG at pH 7.0. These strong differences observed with both types of phospholipids reveal the importance of electrostatic interactions in the binding of preS domains to lipid vesicles as expected for two basic proteins which undergo an increase in positive net charge from 3–5 at pH 7.0 to 12–15 at pH 5.0.

Fluorescence depolarization studies confirmed the above mentioned specificity. The observed decrease of the transition amplitude together with the maintenance of the transition temperature are typical effects of integral membrane proteins [26], suggesting that preS domains are able to insert into the lipid bilayer as a consequence of their interaction with acidic phospholipids. Moreover, assuming that the NBD labeling was mainly produced in the α-amino group, the amino terminal region of preS domains should, at least, be the area inserted into the bilayer. When fluorescence depolarization was plotted against the protein to lipid ratio, a linear increase was obtained until a relationship of 0.045. This value would indicate that each molecule of protein prevented an average of 22 phospholipid molecules to undergo a phase transition. The use of DPH, a fluorescent probe that gives information of the deep core regions of the bilayer [27], indicates that insertion is stabilized by hydrophobic interactions between the acyl chains and the protein. However, data from TMA-

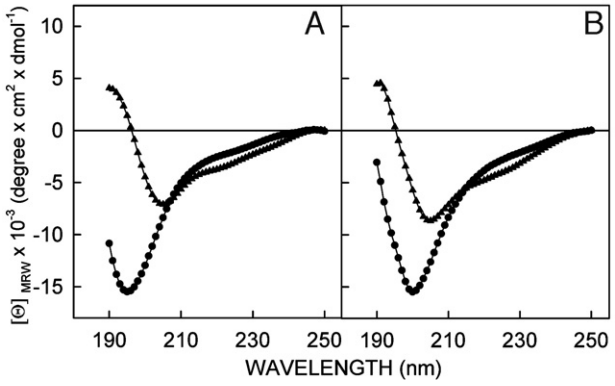

Fig. 7. CD spectra of preS-his-adw protein incubated for 1 h at 37 °C with egg PG vesicles. The circular dichroism spectra were recorded both in the absence (▲) and the presence (●) of egg PG liposomes, at pH 7.0 (A) and 5.0 (B). The protein concentration was 0.1 mg/ml, and the lipid/protein molar ratio was kept at 20:1.

DPH fluorescence depolarization, a probe partitioning into surface regions of the bilayer [28], indicate that an electrostatic component must also be involved in the interaction. Thus, the insertion of preS domains into the bilayer would take place in two steps: a first one governed by electrostatic interaction between the phospholipid polar head and the preS domain which is intrinsically basic, and a second step driven by hydrophobic interactions which leads to the insertion of the protein into the bilayer. When the first step does not take place, such as when neutral phospholipids are used, the protein does not insert into the bilayer. Moreover, this mechanism of interaction would explain the differences observed with acid phospholipids at pH 7.0 and 5.0. The increment in positive charge as the pH is diminished would favor the initial step and hence the insertion into the bilayer. It is worth noting that these differences cannot be attributed to a conformational change of the protein but rather to a modification in its ionic state since the spectroscopic properties at pH 5.0 are coincident with those previously described at pH 7.0 [13].

As it comes about for other viral fusogenic proteins such as the influenza virus haemagglutinin [29] or the vesicular stomatitis virus haemagglutinin [30], preS domains were able to destabilize model membrane systems. Thus, the interaction with negatively charged vesicles induced their aggregation. However, these effects were not observed when neutral phospholipid vesicles were used, indicating that the weak interaction that takes place between the proteins and these type of lipids, assessed by the NBD-labeling studies, was not able to promote the vesicle–vesicle contacts necessary to form the aggregates. Moreover, in the case of acid phospholipids, lipid mixing studies indicate that the aggregation process leads to fusion. The maximum degree of aggregation and lipid mixing was attained at a protein concentration of 5–10 μM. However preS domains were able to disrupt the physical integrity of negatively charged phospholipid vesicles at a considerably lower concentration (1 μM), giving rise to the release of the aqueous contents. Electron microscopy studies revealed that the average size of vesicles increased from 100–130 nm to 250–430 nm after their aggregation and fusion induced by preS domains. On the other hand, under the conditions at which OD_{360} diminished to values even lower than those obtained in the absence of protein, electron microscopy revealed a particle size of 30–60 nm, also lower than the size of control vesicles, indicating that preS domains have the ability to induce the fragmentation of previously fused liposomes. This effect has been also described for some other proteins, as the myelin basic protein [31], and for peptides, as the WHBV fusogenic peptide [11]. It has been suggested that membrane tubulation and vesiculation are a consequence of insertion of the protein into the outer leaflet of the bilayers, producing an excess of area of that leaflet and therefore a concomitant increase in curvature [32].

The destabilization properties mentioned above show some pH dependence. At low protein concentrations these membrane perturbing properties increased as the pH was decreased, revealing, once again, the importance of the electrostatic interaction between the protein and the phospholipid polar head group. This dependence would support the hypothesis that fusion of HBV with the hepatocyte should take place in acidic vesicles, just after a receptor-mediated endocytosis process. However, the fact that destabilization was produced at both pHs and that the observed differences were mostly canceled at higher protein concentrations, would be in accordance with a pH-independent viral infection model, since in cases of viruses infecting cells in a pH-dependent manner no destabilizing effect at neutral pH should be observed at all. The physiological significance of the increase in the fusogenic properties at acidic pH is not clear, but it has been also observed in other viruses entering the cell by fusion with its plasmatic membrane at neutral pH, as it is the HIV [33]. On the other hand, studies performed with the glycoprotein HA of the pH-dependent influenza virus demonstrated that, in the presence of liposomes, the decrease of pH promoted a conformational change in the protein consisting in the exposure of the fusogenic peptide that results in the binding of the protein to the vesicles; however, if this protein was incubated at acidic pH in the absence of membranes, an irreversible conformational change was produced, resulting in a drastic binding decrease [34]. The results obtained herein with preS domains were unchanged no matter the pH was adjusted just before or after the addition of the phospholipid vesicles. Thus, these results might indicate that entry of hepatitis B virus into hepatocytes would be pH-independent.

On the other hand, interaction with liposomes also involves structural alterations in the preS domains. CD spectra in the presence of acidic phospholipid vesicles are indicative of a conformational change as a consequence of the interaction which increased the helical content of the protein. Although CCA method is based on globular proteins and hence the results obtained with protein–lipid systems cannot be taken as absolutely precise, the change in the shape of the spectra is enough to infer such a conformational change. The increase on helical content gives no clue on the viral fusion mechanism since both α-helix and β-sheet have been shown to take part in the fusion steps of other viruses [35, 36].

In summary, the results reported in this work point to the insertion of HBV preS domains into the deep hydrophobic region of the membrane inducing perturbing properties similar to those described for the N-terminal S peptide [8]. Furthermore, the interaction properties should be adscribed to the polypeptide chain and not to the His tag since a purified preS domain which does not contain the tag possesses similar destabilizing properties. It has been postulated that the mechanism by which fusion proteins facilitate the formation of fusion intermediates is a complex process involving several membranotropic segments [37]. Moreover, the involvement of different regions of a protein on the membrane fusion process has indeed been proposed for various enveloped virus harboring additional membrane fusion motifs [38], and more recently, for the Hepatitis C virus [39, 40]. Hence, in the case of HBV, it could be possible that different segments of the surface proteins, located in preS domains and in the N-terminal portion of the S polypeptide, might contribute to membrane fusion acting either simultaneously or at different stages.

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