Biochimica et Biophysica Acta 1808 (2011) 2517–2526

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamem

Lipid composition modulates the interaction of peptides deriving from herpes simplex virus type I glycoproteins B and H with biomembranes

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ARTICLE INFO

Article history:
Received 24 March 2011
Received in revised form 1 July 2011
Accepted 12 July 2011
Available online 23 July 2011

Keywords:
Phospholipid
Cholesterol
Fusion peptide
Viral fusion
Herpes simplex virus
Electron paramagnetic resonance

ABSTRACT

Lipid membranes play a key role in the viral life cycle. Enveloped viruses particularly require a sequence of fusion and fission events between the viral envelope and the target membranes for entry into the cell and egress from it. These processes are controlled by one or more viral glycoproteins that undergo conformational changes favoring the necessary micro- and mesoscopic lipid re-arrangements. Multiple regions from these glycoproteins are thought to interact with the membranes, according to a concerted mechanism, in order to generate the distortion necessary for fusion. In this work, we perform an EPR study on the role played by the membrane composition in tuning the interaction between lipid bilayers and two peptides, gH626–644 and gB632–650, that are highly fusogenic fragments of the gH and gB glycoproteins of herpes simplex virus. Our results show that both peptides interact with lipid bilayers, perturbing the local lipid packing. gH626–644 localizes close to the hydrophilic bilayer surface, while gB632–650 penetrates deeply into the membrane. Chain perturbation by the peptides increases in the presence of charged phospholipids. Finally, cholesterol does not alter the ability of gB632–650 to penetrate deeply in the membrane, whereas it limits penetration of the gH626–644 peptide to the more external layer. The different modes of interaction result in a higher fusogenic ability of gB632–650 towards cholesterol-enriched membranes, as demonstrated by lipid mixing assays. These results suggest that the mechanism of action of the gH and gB glycoproteins is modulated by the properties and composition of the phospholipid bilayer.

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1. Introduction

Biological membranes consist of a lipid bilayer and associated proteins that may constitute up to 50% or more of the membrane content. The lipid bilayer was long considered as an "inert scaffold" solely with the role of a physical barrier between external and internal environments, whereas the membrane proteins were considered to be responsible for more specific membrane functions such as selective molecular transport, signal reception and transduction, and membrane–membrane interactions. Many recent studies have changed this concept, revealing that lipids participate actively in a variety of membrane processes, either directly or indirectly influencing the function of membrane proteins [1–3]. Lipids can affect protein structure and dynamics either via physicochemical characteristics of the membrane, such as elasticity, curvature, surface charge, hydration and the formation of domains, or by specific interactions involving the chemical structure, conformation and dynamics of the lipid head groups and acyl chains [4,5]. Consequently, much attention has been paid to the lipid composition of biomembranes, which includes a rich diversity of phospholipids, sterols and glycolipids [6].

Biomembranes are fundamental to the viral life cycle. Generally speaking, viruses infect cells by crossing the plasma membrane. Once viral replication is complete, new viruses are released to the extracellular medium by crossing the membrane in the reverse direction. For viruses that replicate their genomes in the nucleus of the infected cell, nuclear membranes also must be traversed. These

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doi:10.1016/j.bbamem.2011.07.012

events occur through a variety of molecular mechanisms, depending on the virus and the target cell. In the case of enveloped viruses (e.g., influenza virus, hepatitis C virus, human immunodeficiency virus, herpes virus), which possess a lipid membrane – the envelope – covering their protein capsid, viral entry and egress require a sequence of fusion and fission events between the viral envelope and the target cell membranes. These processes are controlled by one or more viral glycoproteins (fusion proteins) that undergo conformational changes favoring micro- and mesoscopic lipid re-arrangements [7–9]. The physical events necessary to membrane fusion induced by enveloped viruses are the exposure of hydrophobic peptides, loops or patches from one or more of the viral proteins, and their insertion into the cellular membrane to disrupt the normal organization of the lipids in their vicinity. Even though the exact mechanism of the protein–membrane interaction is still unknown, recent studies suggest that several membranotropic regions of the fusion proteins participate in the membrane distortion needed for fusion, as a result of their high propensity to partition into the membrane interface [10–12]. Indeed, the simple picture of a viral fusion protein interacting with the cell and the viral membranes by means of only two localized segments (the fusion peptide deputed to enter the target cell membrane and the transmembrane domain buried in the viral envelope, respectively), has given way to a more complex picture in which multiple regions from the viral proteins interact with membranes. The roles played by other regions not properly classified as fusion peptides could vary, from simply assisting canonical fusion peptides to adopt their correct structure and/or oligomeric state, to promote membrane apposition, to directly destabilize the viral and/or target membranes leading to fusion.

Similar to other membrane processes, there is increasing experimental evidence that lipids play a key role in membrane fusion and fission as chemically-defined molecules, affecting the protein activity. They may directly induce conformational changes in the protein, or create a local environment around the proteins that is favorable to their fusion capability [13].

Herpes simplex virus (HSV), unlike many enveloped viruses that induce fusion through the activity of a single viral fusion protein, requires four glycoproteins, glycoprotein B (gB), glycoprotein D (gD), glycoprotein H (gH), and glycoprotein L (gL), to execute fusion between the viral envelope and the plasma membrane of the target cell during the infection process [14]. The crystal structures of gB, gH and gL have been recently solved. The structures of the HSV-2 gH/gL complex reveal a boot-like structure which bears no structural homology to any known fusion protein [15]. However, there are several lines of evidence suggesting that gH is involved in membrane fusion [15–22]. At the same time, the structure of HSV-1 gB revealed a surprising structural homology with the postfusion structures of two known viral fusion proteins [23], indicating that gB is likely a fusion protein. Thus both gH and gB are expected to be key participants in a complex fusion event, the mechanism of which is still debated [20,23,24]. Interestingly, both proteins have also been found to promote fusion between the viral envelope and the nuclear membrane [17]. Several peptides matching a number of regions of the protein ectodomains have been shown to interact with membranes, and are proposed to play a role in the fusion process [11,19,21,25], probably forming a continuous tract of hydrophobic membrane-interacting surface that simultaneously or sequentially could destabilize apposing viral and cellular membranes during fusion.

In a previous study, we compared the behavior of peptides deriving from different domains of the gB and gH glycoproteins in order to discriminate transmembrane pore formation from other membrane perturbations. We found that two peptides, gH626–644 and gB632–650, selected on the basis of their ability to induce fusion of model membranes, interact strongly with lipid bilayers with an affinity constant similar to that of the HIV-gp41 fusion peptide [26]. Even though gH626–644 and gB632–650 are not unequivocally recognized as fusion peptides, our results indicated the possibility that they are involved in the fusion process. gH626–644 sequence comprises α-helices from gH helical domain II, while that of gB632–650 comprises a loop within the gB domain IV. Both sequences belong to stably folded globular domains and, in the solved crystal structures, while gH626–644 is only partially exposed to the solvent, gB632–650 is fully exposed. During the fusion process the lipid bilayers rearrangement is generally coupled to large-scale conformational changes of the fusion glycoproteins [27] so that it is possible that buried or partially exposed regions of both gH and gB become exposed to the cell and/or to the viral membrane.

In order to investigate the gH626–644 and gB632–650 interaction with phospholipid bilayers further, we decided to perform a systematic spin-label EPR study, focusing on the role played by the membrane lipid composition in controlling the peptide–membrane interaction. In particular, the effects of bilayer surface charge and inclusion of cholesterol are considered.

EPR spectroscopy with spin-labeled lipids has proved to be a fruitful experimental approach for studying the interactions of peripheral as well as integral proteins with membranes [28]. By this means, surface association can be distinguished from membrane penetration and transmembrane insertion, via the characteristic effects on lipid chain mobility that are registered in the spin-label spectrum [29,30]. EPR is well suited to this kind of investigation, because its dynamic sensitivity is optimally matched to the timescale of rotational motions of lipids in biological membranes. In the present work, we use EPR of spin-labeled lipids to investigate the effect of peptide–membrane interaction on bilayer structure and dynamics. Circular dichroism measurements are also performed to investigate the conformational preferences of the peptides when interacting with lipid membranes. Finally, we present some lipid mixing assays, performed to correlate the peptide–bilayer mode of interaction with the peptide fusogenic activity.

2. Experimental section

2.1. Materials

Fluorenylmethoxycarbonyl (Fmoc)-protected amino acids were from INBIOS (Pozzuoli, NA, Italy), and NovaSyn TGA resin was from Nova Biochem (Darmstadt, Germany). The reagents for solid-phase peptide synthesis (piperidine, pyridine) were from Fluka (Sigma-Aldrich, Milano, Italy); trifluoroacetic acid (TFA) and acetic anhydride were from Applied Bio systems (Foster City, CA, USA). DMF and CH_3CN were from LAB-SCAN (Dublin, Ireland). Dichloromethane and methanol, HPLC-grade solvents, were from Merck (Darmstadt, Germany). Phospholipids, dimyristoyl phosphatidylcholine and phosphatidylglycerol (DMPC and DMPG), as well as the fluorescent probes N-(7-nitro-benz-2-oxa-1,3-diazol-4-yl) phosphatidylethanolamine (NBD-PE) and N-(Lissaminerhodamine-B-sulfonyl)phosphatidylethanolamine (Rho-PE), were from Avanti Polar Lipids (Birmingham, AL, USA). Cholesterol (CHOL) and Triton X-100 were from Sigma (St. Louis, MO, USA). Spin-labeled phosphatidylcholines (*n*-PCSL) with the nitroxide group at different positions, *n*, in the *sn*-2 acyl chain, as well as spin-labeled phosphatidic acid, phosphatidylethanolamine, phosphatidylglycerol and phosphatidylserine with the nitroxide group at the fifth position of the *sn*-2 chain (5-PASL, 5-PESL, 5-PGSL, 5-PSSL, respectively) were synthesized as described in Marsh and Watts [28,31]. The spin-labels were stored at −20 °C in ethanol solutions at a concentration of 1 mg/mL.

2.2. Peptide synthesis

Peptides were synthesized by the solid-phase method using Fmoc chemistry and were purified as previously reported [25]. The purified peptides were shown to be homogeneous (>98%) by analytical HPLC. The peptides were further subjected to electrospray mass spectroscopy to confirm the expected molecular weights. Peptide sequences

are: gH626–644, GLASTLTRWAHYNALIRAF and gB632–650, PCTVGHRRYFTFGGGYVYF.

2.3. Sample preparation

Samples to be investigated by EPR spectroscopy contained lipid bilayer arranged as multi lamellar vesicles (MLVs), that constitute a good option to enhance the signal-to-noise ratio [28–31]. For selected samples, measurements of large unilamellar vesicles (LUVs) were also performed, and the registered spectra were found to coincide with those registered on MLVs with the same composition.

DMPC and DMPG multi lamellar vesicles (MLVs) were prepared for EPR spectroscopy as follows: 20 μg of lipid, dissolved in a CH_2Cl_2–methanol mixture (2:1 v/v), with 1% (wt/wt) of the spin-label, dissolved in ethanol, were mixed thoroughly, and a thin lipid film was produced by evaporating the solvents with dry nitrogen gas. Final traces of solvents were removed by subjecting the sample to vacuum desiccation for at least 3 h. The samples were then hydrated with 20 μL of 10 mM phosphate buffer, 137 mM NaCl, 2.7 mM KCl, pH 7.4 (PBS), warmed gently at 35 °C and repeatedly vortexed. The lipid suspension thus obtained was transferred into a 25 μL glass capillary.

MLVs of DMPC containing 20% by weight of CHOL, were prepared by the same procedure, mixing appropriate amounts of lipid and sterol solutions in CH_2Cl_2–methanol before evaporation of the organic solvents. Samples containing the peptide–lipid complex were prepared in a similar manner, except that the lipid film was hydrated directly with the peptide solution in PBS.

Large unilamellar vesicles (LUVs) were preferred for CD experiments, because of their lower scattering with respect to MLVs. In particular, peptide samples in lipids were prepared using the following protocol [32]: gH626–644 was first dissolved in TFE; immediately after preparation, the peptide solution was added to an equal volume of a chloroform solution containing the appropriate lipid concentration. Solutions were dried with a nitrogen gas stream and lyophilized overnight; the dry samples were rehydrated with deionized water to yield a final lipid concentration of 10 nM and 50 nM. Then the lipid suspension was freeze-thawed 6 times and then extruded 20 times through polycarbonate membranes with 0.1 μm diameter pores to produce LUVs containing the peptide. LUVs for lipid-mixing experiments were prepared by the same procedure.

2.4. EPR spectroscopy

Electron paramagnetic resonance spectra of lipid and lipid/peptide samples were recorded on a 9-GHz Bruker EMX EPR spectrometer with an ER 041 XK-D microwave bridge. Glass capillaries containing the samples were placed in a standard 4-mm quartz EPR-sample tube containing light silicone oil for thermal stability. The temperature of the sample was regulated and maintained constant during the measurement by blowing thermostated nitrogen gas through a quartz dewar. The instrumental settings were as follows: sweep width, 120 G; resolution, 1024 points; modulation frequency, 100 kHz; modulation amplitude, 1.0 G; time constant, 20.5 ms; incident power, 5.0 mW.

Several scans, typically 16, were accumulated to improve the signal-to-noise ratio. Values of the outer hyperfine splitting, $2A_{max}$, were determined by measuring the difference between the low-field maximum and the high-field minimum [33]. This parameter is a useful empirical measure of the lipid chain dynamics and order in both gel and fluid phases of lipid bilayers [34,35]. The main source of error in the $2A_{max}$-value is the uncertainty in composition of samples prepared by mixing a few microliters of the component solutions. For this reason, reproducibility of $2A_{max}$-determinations was estimated by evaluating independently prepared samples with the same nominal composition. It was found to be ± 0.2 G.

2.5. Circular dichroism spectroscopy

CD spectra were recorded using a Jasco J-715 spectropolarimeter in a 1.0 or 0.1 cm quartz cell at room temperature. The spectra are an average of 3 consecutive scans from 260 to 195 nm, recorded with a band width of 3 nm, a time constant of 16 s, and a scan rate of 10 nm/min. Spectra were recorded and corrected for the blank sample. Mean residue ellipticities (MRE) were calculated using the expression $MRE = Obsd/(lcn)$, where *Obsd* is the ellipticity measured in millidegrees, l is the path length of the cell in cm, c is the peptide concentration in mol/L, and n is the number of amino acid residues in the peptide. Solutions of gH626–644 with LUVs (0.1 mM) were prepared as described in the literature [32]. The measurements were performed at peptide/lipid ratios of 0.1 and 0.5 mol/mol.

2.6. Lipid mixing assays

Membrane lipid mixing was monitored using the resonance energy transfer assay (RET) reported by Struck et al. [36]. The assay is based on the dilution of the NBD-PE (donor) and Rho-PE (acceptor). Dilution due to membrane mixing results in an increase in NBD-PE fluorescence. Thus, we monitored the change in donor emission as aliquots of peptides were added to vesicles. Vesicles containing 0.6 mol% of each probe were mixed with unlabelled vesicles at a 1:4 ratio (final lipid concentration, 0.1 mM). Small volumes of peptides in dimethylsulfoxide (DMSO) were added; the final concentration of DMSO in the peptide solution was no higher than 2%. The NBD emission at 530 nm was followed with the excitation wavelength set at 465 nm. A cut off filter at 515 nm was used between the sample and the emission monochromator to avoid scattering interferences. The fluorescence scale was calibrated such that the zero level corresponded to the initial residual fluorescence of the labeled vesicles and the 100% value corresponding to complete mixing of all lipids in the system was set by the fluorescence intensity of vesicles upon the addition of Triton X-100 (0.05% v/v) at the same total lipid concentrations of the fusion assay. Lipid mixing experiments were repeated at least three times and results were averaged.

2.7. Thermodynamic calculations

Free energies of transfer and hydrophobic moments were calculated for the peptides according to the whole-residue scale for transfer from water to the interface, or to octanol [37]. Calculations were made by using the Membrane Protein Explorer package (MPEx) of Jayasinghe et al. [38]. Hydrophobic moments are defined according to Eisenberg [39].

3. Results

3.1. Peptide hydropathy

Using the Wimley-White octanol scale [37], transfer of the gH626–644 and gB632–650 peptides from water to the hydrocarbon core of the membrane is predicted to be strongly disfavored energetically. In contrast, transfer of both peptides from water to the polar–apolar interface of the membrane is predicted to be energetically favorable. On the Wimley-White interfacial hydropathy scale, the free energy of transfer from water is $\Delta G_{transf} = -8.7$ kJ mol^{-1} for gH626–644 and $\Delta G_{transf} = -13.5$ kJ mol^{-1} for gB632–650, if the N and C termini are charged and the peptides are in a random conformation. These values are augmented by an additional -14.1 kJ mol^{-1}, if the N and C termini are electroneutral. A further -1.7 kJ mol^{-1} per residue should be added when helix formation on surface association of the peptides is taken into account [40]. As found below from CD data (Section 3.7), this additional contribution is needed only in the case of gH626–644 interacting with DMPG bilayers, or with DMPC bilayers at high peptide/lipid ratio. In an

α-helical conformation, gH626–644 is predicted to have a hydrophobic moment of $\mu_H = 20.52$ kJ mol^{-1} on the interfacial scale, with the moment directed toward the tyrosine, Y637, of the peptide sequence. Summarizing, the free energy of transfer of gH626–644 from water to the membrane interface is predicted to lie within the range: $\Delta G_{transf} = -8.7$ to -54.5 kJ mol^{-1}, while the corresponding value for gB632–650 lies within -13.5 and -27.5 kJ mol^{-1}.

3.2. Interactions with phosphatidylcholine and phosphatidylglycerol membranes

Because the peptide–lipid interaction can depend upon the state (gel or fluid phase) of the lipid bilayer, we performed a systematic temperature dependence. The samples investigated were phosphatidylcholine spin-labeled on the 5-C atom of the *sn*-2 chain (5-PCSL) incorporated in DMPC membranes, and correspondingly spin-labeled phosphatidylglycerol (5-PGSL) in DMPG membranes, in the presence and absence of the peptides gH626–644 or gB632–650. Selected EPR spectra from the zwitterionic lipid samples are shown in Fig. 1; significant perturbations by the peptides are detected (compare solid and dashed lines). Fig. 2 shows the temperature dependences of the outer hyperfine splitting, $2A_{max}$, of 5-PCSL in DMPC membranes and of 5-PGSL in DMPG membranes. For DMPC alone (Fig. 2A, solid line), a sharp decrease in $2A_{max}$ is evident at ca. 25 °C, corresponding to the increase in lipid chain mobility on transition from the gel to the fluid phase of the DMPC bilayer. Addition of 1:1 wt/wt peptide (gH626–644 or gB632–650) with respect to lipid significantly affects the trend in $2A_{max}$ with temperature. Particularly in the fluid membrane phase, $2A_{max}$ is larger in the presence of peptide than in its absence, i.e., the mobility of the spin-labeled chains is decreased by interaction of the peptide with the membrane. The effect is stronger for the gB632–650 peptide (dotted line) than for the gH626–644 peptide (dashed line). Furthermore, the cooperativity of the DMPC chain-melting transition is reduced, while its position remains approximately the same. Also in this respect, the effect of gB632–650 is stronger than that of gH626–644. Fig. 2B shows the behavior of DMPG membranes in the presence and absence of the gH626–644 and gB632–650 peptides. The chain-melting transition of the charged lipid (at 22–24 °C) is less sharp than that of zwitterionic DMPC and becomes almost undetectable in the presence of the peptides. In the gel phase, the outer hyperfine splittings are less affected by the peptides, whereas in the fluid phase, a reduction of the spin-label mobility is very evident, particularly in the case of interaction with the gB632–650 peptide (dotted line). Comparison of Fig. 2A and B indicates that the lipid chain perturbation by interaction with the peptides is stronger for the anionic lipid than for the zwitterionic lipid.

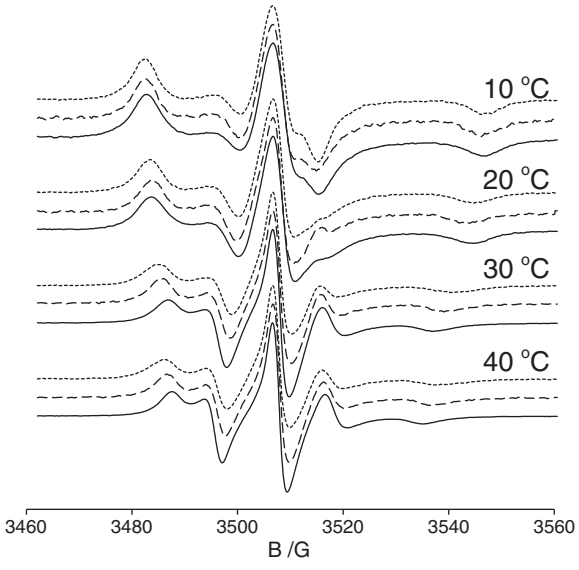

Fig. 1. EPR spectra of 5-PCSL in dimyristoyl phosphatidylcholine bilayer membranes, in the absence (continuous line) and presence of 1:1 wt/wt gH626–644 (dashed line) or gB632–650 (dotted line) peptides, at the temperatures indicated.

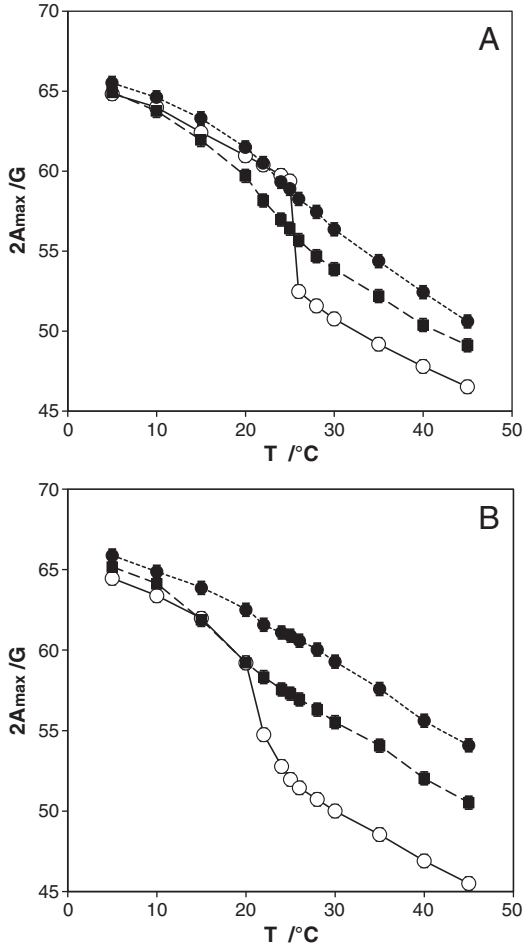

Fig. 2. Temperature dependence of the outer hyperfine splitting, $2A_{max}$, of 5-position phospholipid spin labels in phospholipid membranes in the absence (○, continuous line) and presence of 1:1 wt/wt gH626–644 (■, dashed line) or gB632–650 (●, dotted line) peptides. (A) 5-PCSL in dimyristoyl phosphatidylcholine bilayer membranes, and (B) 5-PGSL in dimyristoyl phosphatidylglycerol bilayer membranes.

3.3. Peptide–lipid titration

Fig. 3 shows the increase in outer hyperfine splitting, $2\Delta A_{max}$, of the 5-position spin-labeled lipids in DMPC and DMPG membranes with increasing peptide (gH626–644 or gB632–650) concentration. In all cases, a typical saturation binding is registered. The extent of change in $2A_{max}$ depends on the lipid, and for both peptides is higher in DMPG than in DMPC. On the other hand, the peptide/lipid weight ratio at which a constant value of $2A_{max}$ is reached is not dependent on the particular lipid.

The peptide/lipid ratios at which binding saturates are ~0.5 and ~0.8 wt/wt for gH626–644 and gB632–650, respectively. These values correspond to ca. 2 and 1 wt/wt lipid/peptide ratios. The stoichiometry of the interaction can be estimated by extrapolation of the increase in $2\Delta A_{max}$, on initial tight binding, to the saturation value of $2\Delta A_{max}$, as is shown in the figure. This gives a value of ~15 lipids per bound gH626–644 molecule, and ~5 lipids per bound gB632–650 molecule. These values are likely to be upper estimates because of a possible nonlinear dependence of $2\Delta A_{max}$ on peptide binding.

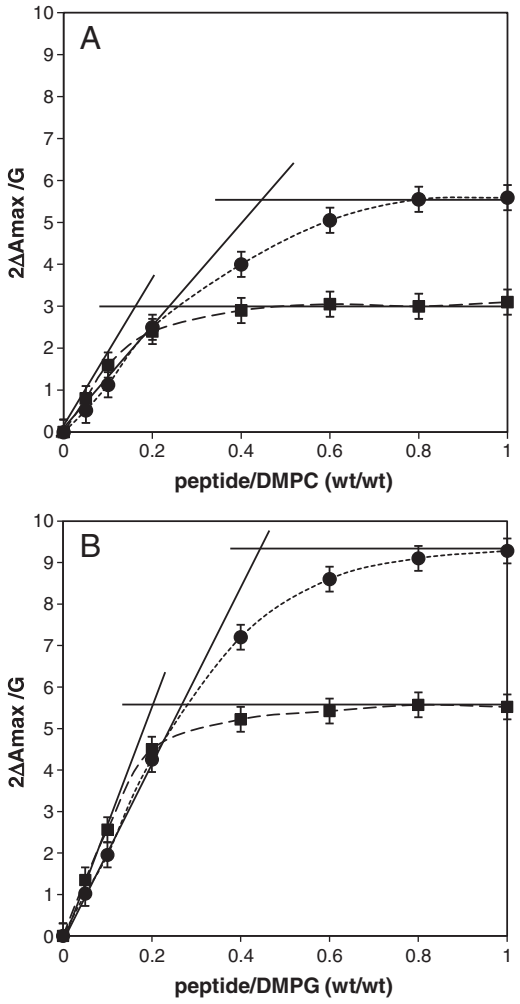

Fig. 3. Dependence of the increase, $2\Delta A_{max}$, in outer hyperfine splitting of 5-position phospholipid spin labels at 30 °C on the peptide/lipid ratio for gH626–644 (■, dashed line) or gB632–650 (●, dotted line) peptides. (A) 5-PCSL in dimyristoyl phosphatidylcholine bilayer membranes, and (B) 5-PGSL in dimyristoyl phosphatidylglycerol bilayer membranes.

3.4. Lipid chain flexibility profiles

Perturbation of the EPR spectra from spin-labels at different positions, *n*, in the *sn*-2 chain of the lipid by binding the gH626–644 or gB632–650 peptide was also investigated. Fig. 4 gives the EPR spectra of the *n*-PCSL phosphatidylcholine spin-label positional isomers in fluid DMPC bilayer membranes ($T = 30$ °C), in the presence and absence of the peptide at a peptide/lipid ratio of ~1 wt/wt. In the absence of peptide, the outer hyperfine splitting decreases progressively with increasing *n*, as the spin-label position is stepped down the chain towards the center of the membrane. As opposed to the clearly defined axially anisotropic spectra that are obtained for 5-PCSL towards the polar headgroup end of the chain, a narrow, three-line, quasi-isotropic spectrum is obtained for 14-PCSL that is positioned close to the terminal methyl region of the chain. This flexibility gradient in segmental chain mobility is a characteristic hallmark of the liquid-crystalline state of fluid phospholipid bilayers.

Binding of the gH626–644 or gB632–650 peptide significantly affects the spectra of all spin-label positional isomers. In the presence of gH626–644, the outer hyperfine splitting is increased at all spin-label chain positions, although the line-shape remains qualitatively similar. Fig. 5 shows the dependence of the outer hyperfine splitting, $2A_{max}$, on chain position, *n*, for the *n*-PCSL spin-labels in fluid DMPC membranes, with and without a saturating amount of bound peptide.

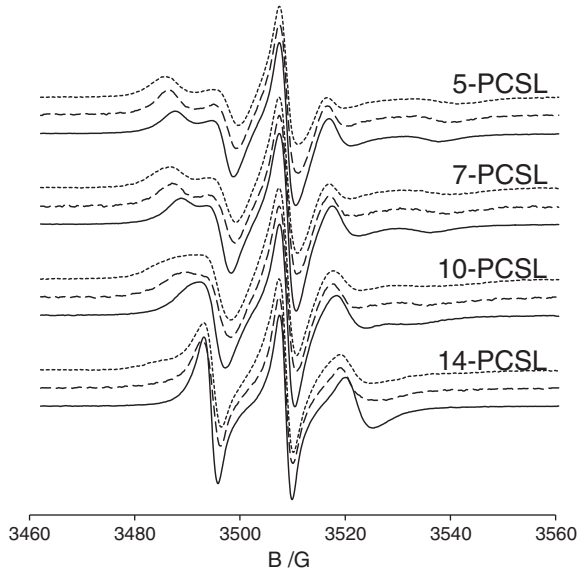

Fig. 4. EPR spectra of *n*-PCSL positional isomers of spin-labeled phosphatidylcholine in fluid-phase dimyristoyl phosphatidylcholine bilayer membranes, in the absence (continuous line) and presence of 1:1 wt/wt gH626–644 (dashed line) or gB632–650 (dotted line) peptides at 30 °C.

The gH626–644 and gB632–650 peptides increase $2A_{max}$ to roughly the same extent for the $n = 5$, 7 and 10 positions, whereas the increase in $2A_{max}$ of 14-PCSL is smaller for the gH626–644 peptide and greater for the gB632–650 peptide. This profile of perturbations in $2A_{max}$ indicates that the increase in lipid packing density, which is induced by surface association of the peptide, propagates down the acyl chains.

In the case of the gB632–650 peptide, a second, more motionally restricted component appears in the spectra of spin labels positioned toward the terminal methyl end of the chain (10-PCSL and even more clearly for 14-PCSL). This is evidence that the peptide penetrates appreciably into the membrane interior [41–43].

A set of experiments was also performed to evaluate the effect of both peptides on the lipid chain flexibility profile at lower peptide/lipid ratio (0.4 wt/wt, data not shown), obtaining variations that, although lower, are qualitatively similar to those observed at 1 wt/wt peptide/lipid ratio.

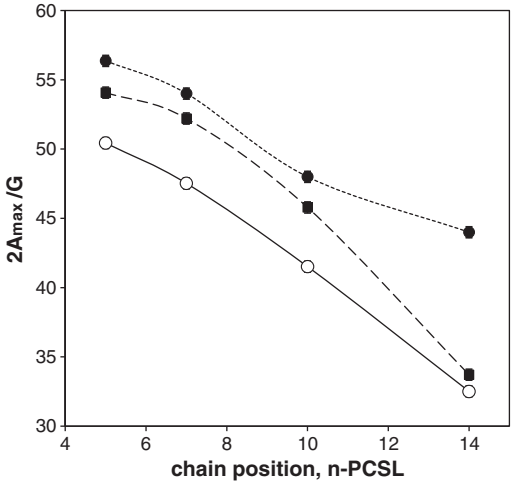

Fig. 5. Dependence on spin-label position, *n*, of the outer hyperfine splitting, $2A_{max}$, of the *n*-PCSL phosphatidylcholine spin labels in fluid-phase membranes of DMPC, in the absence (○, continuous line) and presence of 1:1 wt/wt gH626–644 (■, dashed line) or gB632–650 (●, dotted line) peptides. $T = 30$ °C.

3.5. Effect of inclusion of cholesterol in phospholipid membranes

We also investigated DMPC membranes containing cholesterol (CHOL) at 20% wt/wt. EPR spectra of *n*-PCSL in DMPC/CHOL lipid samples without peptide at 30 °C are shown by the solid lines in Fig. 6. Inspection of this figure shows large changes with respect to the spectra for DMPC membranes without cholesterol that are shown by the solid lines in Fig. 4. CHOL increases the spectral anisotropy, also for spin labels that are located deep in the bilayer inner core. This can be ascribed to the CHOL-induced transition of the bilayer from the liquid-disordered to the liquid-ordered state [44]. Addition of 1:1 wt/wt gH626–644 or gB632–650 peptide with respect to total lipid clearly perturbs the spectra of 5-PCSL in DMPC/CHOL membranes, gH626–644 (Fig. 6, dashed line) being more effective than gB632–650 (Fig. 6, dotted line). Interestingly, a different behavior is observed for the spectra of spin-labels further down the chain. Whereas gH626–644 perturbs the spectra of 5-PCSL and 7-PCSL in DMPC/CHOL membranes (Fig. 6), the spectra of 10-PCSL and 14-PCSL appear to be almost unperturbed. In contrast, perturbations by gB632–650 at 1:1 wt/wt with respect to lipid are evident for all label positions throughout the chain. These observations reflect the quantitative trends in the outer hyperfine splitting, $2A_{max}$, which are shown in Fig. 7, and indicate that the gB632–650 peptide (dotted line) penetrates appreciably into the DMPC/CHOL membrane interior, while the gH626–644 peptide (dashed line) adsorbs at the surface.

3.6. Selectivity of lipid–peptide interaction

The selectivity of interaction of different lipids with the gH626–644 or gB632–650 peptide bound to DMPC membranes was determined by using probe amounts of lipids spin labeled at the 5-C atom of the *sn*-2 chain [45,46]. Table 1 gives the values of the outer hyperfine splitting, $2A_{max}$, for the spin labels at 30 °C, in the presence and absence of 1:1 wt/wt peptide. For all spin-labeled lipids tested, $2A_{max}$ is greater for peptide-bound membranes than for membranes of the lipid alone. The increase in $2A_{max}$ differs, however, for the two peptides and for the different spin-labeled lipids. This reflects a selectivity of interaction of both gH626–644 and gB632–650 with the different lipid polar head groups. Table 1 gives the increase, $2\Delta A_{max}$, in outer hyperfine splitting

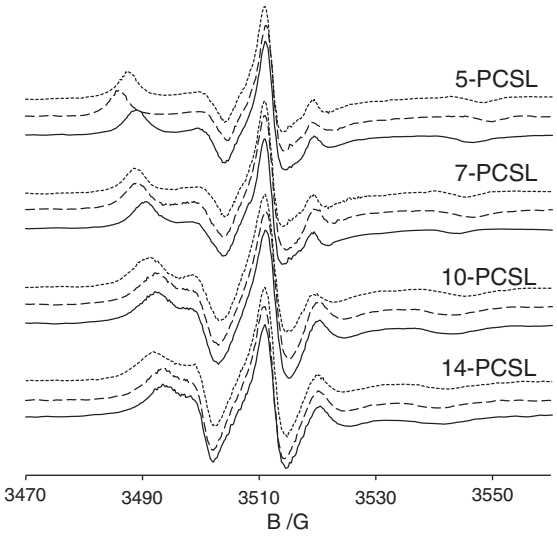

Fig. 6. EPR spectra of *n*-PCSL positional isomers of spin-labeled phosphatidylcholine in dimyristoyl phosphatidylcholine bilayer membranes containing 20 wt.% cholesterol, in the absence (continuous line) and presence of 1:1 wt/wt gH626–644 (dashed line) or gB632–650 (dotted line) peptides at 30 °C.

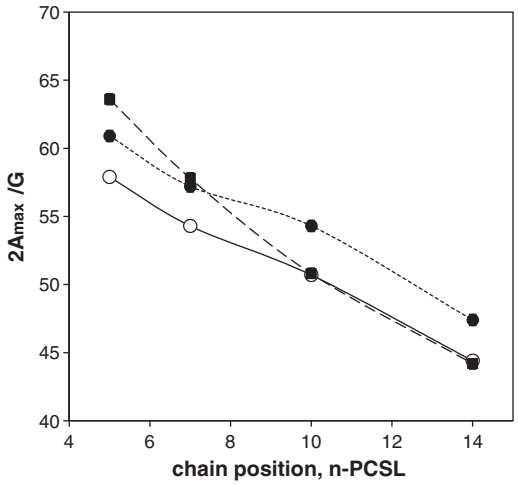

Fig. 7. Dependence on spin-label position, *n*, of the outer hyperfine splitting, $2A_{max}$, of the *n*-PCSL phosphatidylcholine spin labels in membranes of DMPC containing 20 wt.% cholesterol, in the absence (○, continuous line) and presence of 1:1 wt/wt gH626–644 (■, dashed line) or gB632–650 (●, dotted line) peptides. $T = 30$ °C.

for the peptide-bound membranes, relative to that for peptide-free membranes. For both peptides, the increase in $2A_{max}$ is larger for spin-labeled lipids with anionic head groups (5-PGSL, 5-PASL, and 5-PSSL) than for those bearing a zwitterionic head group (5-PESL and 5-PCSL). Furthermore, it is interesting to observe that, for each spin label, $2\Delta A_{max}$ is greater with the gB632–650 peptide than with the gH626–644 peptide.

3.7. Secondary structure of synthetic peptides

The secondary structure of the gH626–644 (Fig. 8) and gB632–650 peptides in buffer and bound to liposomes was determined by CD spectroscopy. As reported in the literature [47], fusion peptides can change their secondary structure at different peptide/lipid ratios. In particular, they may show a beta and/or oligomeric structure at high peptide/lipid ratios, while assuming an α-helical structure at low peptide/lipid ratios. The secondary structure measurements were therefore performed at two different peptide/lipid ratios; specifically, we used molar ratios of 0.1 (low peptide/lipid ratio) and of 0.5 (high peptide/lipid ratio). The 0.5 mol/mol peptide/lipid ratio was used because we observed high fusion activity for both peptides at this molar ratio.

All the CD spectra shown for gH626–644 in Fig. 8 indicate formation of substantial helical structure with DMPG, at low peptide/lipid ratio, whereas the spectra of this peptide with DMPC and DMPC/CHOL

Table 1
Outer hyperfine splittings ($2A_{max}$) of phospholipid probes, spin labeled at the fifth position of the *sn*-2 chain, incorporated in DMPC bilayers with and without the gH626–64 or gB632–650 peptides at 30 °C.

Spin label	$2A_{max}$ (G)			$2\Delta A_{max}$ (G)[a]	
	DMPC	DMPC + gH626–64	DMPC + gB632–650	DMPC + gH626–64	DMPC + gB632–650
5-PCSL	50.8	54.1	56.4	3.3	5.6
5-PGSL	52.1	55.8	58.3	3.7	6.2
5-PASL	52.3	56.1	58.4	3.8	6.1
5-PESL	52.4	55.8	58.2	3.4	5.8
5-PSSL	53.0	56.7	59.7	3.7	6.7

[a] ΔA_{max} is the difference in A_{max} with and without peptide.

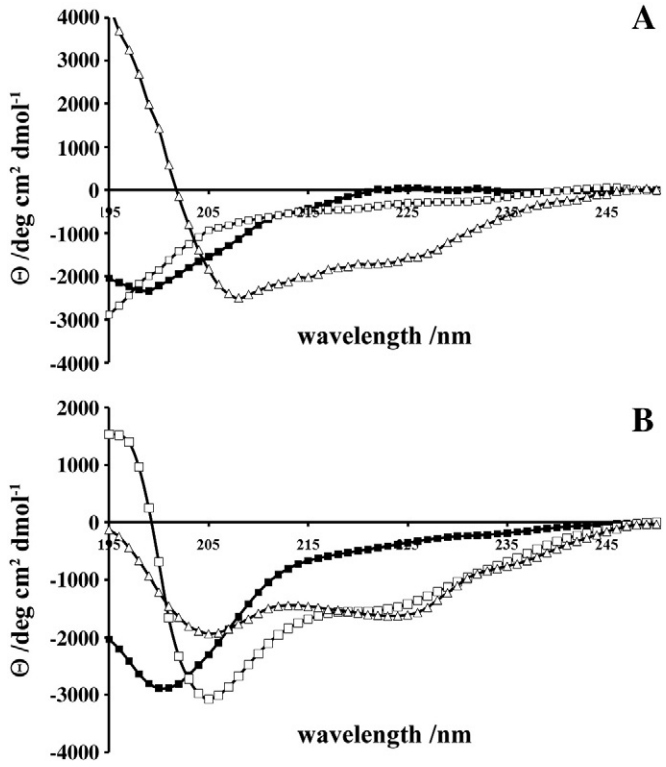

Fig. 8. CD spectra of gH626–644 10 μM (A) and 50 μM (B) in LUVs of DMPC (□), DMPG (△) and DMPC containing 20 wt.% cholesterol (■). $T = 30$ °C.

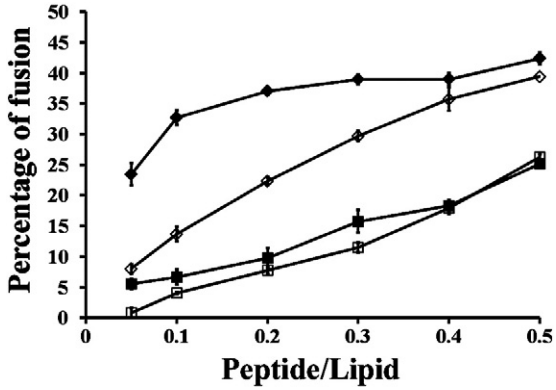

Fig. 9. Peptide-promoted membrane fusion of DMPC LUVs in the absence (□, gH626–644; ◊, gB632–650) and in the presence of 20 wt.% cholesterol (■, gH626–644; ♦, gB632–650) as determined by lipid mixing; peptide aliquots were added to 0.1 mM LUVs, containing 0.6% NBD-PE and 0.6% Rho-PE. The increase in fluorescence was measured after the addition of peptide aliquots; reduced Triton-X-100 (0.05% v/v) was referred to as 100% of fusion. In figure is reported the dose dependence of lipid mixing at 37 °C.

indicate a random conformation. At high peptide/lipid ratio, a gH626–644 tendency to assume a helical conformation is evident also with DMPC, while inclusion of cholesterol in the bilayer formulation keeps the peptide in a random conformation.

The ratio of ellipticities at 222 nm and 208 nm can be used to distinguish between monomeric and oligomeric states of helices [48]. When the ratio $\theta_{222}/\theta_{208}$ equals about 0.8, the peptide is monomeric, and when the ratio exceeds 1.0 it is oligomeric. The $\theta_{222}/\theta_{208}$ ratio for gH626–644 in DMPG is ~0.7 at low peptide/lipid ratio and ~0.9 at high ratio indicating that, with increasing peptide concentration, the monomer/oligomer equilibrium shifts from the monomeric to the aggregated state for this peptide associated with DMPG. In contrast, in DMPC liposome the peptide does not show a self-aggregative tendency.

The CD spectra of gB632–650 are not shown because we observed a strong tendency of the peptide to aggregate under all conditions tested here, and consequently we were unable to obtain a reproducible CD spectrum.

3.8. Lipid mixing assay

To investigate the fusogenicity of the selected peptides, NBD and Rho labeled PE were used as the donor and acceptor of fluorescence energy transfer. A population of LUVs labeled with both was mixed with a population of unlabeled LUVs and increasing amounts of peptides were added. Dilution of the fluorescent-labeled vesicles via membrane fusion induced by the peptide results in a reduction in the fluorescence energy transfer efficiency, hence dequenching of the donor fluorescence. The dependence of the extent of lipid mixing on the peptide to lipid molar ratio was analyzed, by adding increasing amounts of each peptide to a fixed amount of vesicles. In order to compare the activity of the two peptides, the percentage of lipid mixing as a function of the peptide to lipid molar ratio was calculated. Fig. 9 shows the results of lipid mixing assays in DMPC and DMPC/Chol for gH626–644 and gB636–650 at 37 °C. The peptides induced significant levels of fusion in both the types of LUVs, suggesting that they were able to interact with the bilayer also in this experimental conditions in accordance with previously reported data [11,18]. In particular, the fusion activity obtained for gB636–650 is always higher than that obtained for gH626–644. It is interesting to note that in presence of cholesterol 20%, we observe an increase in fusion activity at low peptide/lipid ratios for gB636–650, while there is no significant variation for gH626–644 in this experimental condition.

4. Discussion

Membrane fusion and fission, two key processes that occur during replication of enveloped viruses, require major rearrangements of the membrane microstructure, which results from a complex interplay between proteins and lipids [8,49]. All enveloped viruses display specialized proteins that, interacting transiently with the membranes, are able to modify the structural order of the lipid bilayers, eventually leading to their local bending. There is converging evidence that various protein domains other than the fusion peptide play an important role in membrane fusion, by means of these concerted mechanisms.

Viruses belonging to the herpes virus family possess a conserved core fusion machinery involving glycoprotein B (gB) and the non-covalently associated complex of glycoproteins H and L (gH/gL). Both glycoproteins gB and gH contain several hydrophobic domains that are believed to be involved in fusion, and synthetic peptides corresponding to these regions are able to associate with membranes and induce fusion of artificial liposomes. In the present work, we have focused on two peptides, gH626–644 and gB632–650, which are the most fusogenic fragments of the native proteins and interact strongly with lipid bilayers, as demonstrated previously [11,26]. When interacting with lipid membranes, gH626–644 can adopt a helical conformation, in which a hydrophobic face exposing the most bulky residues and an opposite hydrophilic face result in a marked amphipathic character [21], with a hydrophobic moment of $\mu_H = 20.52$ kJ mol^{-1} on the interfacial scale (see Section 3.1). On the other hand, analysis of the gB632–650 secondary structure in aqueous mixtures of trifluoroethanol reveals a tendency to adopt β-structure in hydrophobic environments [18].

The EPR results reported here show that both gH626–644 and gB632–650 peptides interact with lipid bilayers, perturbing the local

lipid packing. The spectra of *n*-PCSL in DMPC show that both peptides perturb the entire mobility profile of the lipid bilayer, from the membrane surface to deep in the interior. The mechanism of interaction depends on the specific peptide: gH626–644 tends to localize close to the bilayer hydrophilic surface, indirectly perturbing the acyl chain packing and dynamics, while gB632–650 penetrates deeply into the bilayer. This is shown by the EPR spectra of 14-PCSL in DMPC bilayers; these exhibit a narrow, three-line spectrum in the presence of gH626–644, while an additional motionally restricted component is resolved in the presence of the gB632–650 peptide [50,51]. Peptide–lipid titration further confirms the different behavior of the two peptides. Each molecule of gH626–644 interacts with a maximum of 15 lipids. On a per-residue basis, this value corresponds to ~0.8 lipids per amino acid, a stoichiometry similar to that obtained for two peptides derived from the fusion glycoprotein gp41 of FIV [52,53], whose mechanism of interaction with lipid bilayers has been established to be surface adsorption. In the case of gB632–650, peptide-lipid titration by EPR indicates that a single peptide molecule interacts with a smaller number of lipids (~5), as is expected in the case of peptide penetration in a transverse membrane orientation. Such a low value suggests possible association between gB632–650 molecules interacting with the lipid bilayer [54], thus explaining the problems encountered in registering CD spectra of this peptide in liposomal samples.

Despite the difference in mechanism of peptide–membrane interaction, both gH626–644 and gB632–650 show remarkable fusogenic capability. These evidences led us to further analyze the connection between the effects of the peptide on the bilayer structure and membrane fusion. The EPR spectra for both peptides show that the acyl chain packing and dynamics of the lipids are perturbed. Because both peptides remain confined to the lipid leaflet with which they come into contact [26], a difference in area is generated between the apposing lipid leaflets constituting the membrane bilayer. In consequence, the membrane will develop curvature to compensate for this area asymmetry, and this local deformation is likely to be fundamental to the membrane fusion process. In conclusion, our results point out that membrane fusion capability is not univocally related to a specific peptide location in the membrane, but rather to the resulting local perturbation of lipids arrangement.

For both peptides, membrane interaction is modulated by the lipid bilayer composition. Lipids can effect peptide/membrane interactions either by an indirect mechanism, i.e., by modifying membrane physical properties such as flexibility and surface charge density, or by a direct mechanism, in which some lipids show a preferential association with the peptide, hence promoting its interaction with the whole bilayer [13]. Both mechanisms were investigated in the present work and found to be effective for the two peptides. Comparison of the results obtained with DMPC and DMPG membranes shows that both peptides induce a stronger perturbation in packing and dynamics of negatively charged bilayers, relative to those of zwitterionic lipids. Indeed, experiments on the temperature dependence show qualitatively the same trends for the two lipid systems, but the changes in $2A_{max}$ induced by the peptides are much larger with DMPG. For example, the values of $2\Delta A_{max}$ for 5-PCSL in DMPG at 30 °C exceed those in DMPC by ~70%. The importance of the membrane surface charge arises from the net positive charge (+2, due to the presence of arginine residues) of each peptide. In particular, gH626–644 shows a pronounced tendency to assume amphipathic α-helical structure and to oligomerize in the presence of anionic lipids. However, the bilayer charge does not affect the positioning of the peptide relative to the bilayer, as indicated by the unchanged stoichiometry of peptide/lipid interaction.

Inclusion of cholesterol in the bilayer membrane affects the lipid chain ordering. At 30% mol% and a temperature of 30 °C, cholesterol induces transition of the DMPC bilayer from a liquid-disordered lamellar phase, L_α, to a liquid-ordered phase, L_o [44]. This is reflected by an increase in $2A_{max}$ of roughly 10 G for all spin labels. Especially, the EPR spectrum of 14-PCSL displays a clearly defined axially anisotropic lineshape in the presence of cholesterol, indicating that the inner core of the bilayer becomes more structured [55–57]. The changes in lipid order and mobility do not appear to change the ability of the gB632–650 peptide to penetrate deeply into the bilayer. In contrast, membrane perturbation by the gH626–644 peptide effectively propagates along the whole bilayer profile of DMPC, but in the presence of cholesterol is limited to the more external layer. Indeed, the EPR spectra of 10-PCSL and 14-PCSL are not significantly affected by the gH626–644 peptide in DMPC-cholesterol bilayers. These results well relates with those of the lipid mixing assays, showing a much higher fusogenic activity of gB632–650 in the presence of cholesterol. The CD spectra of the gH626–644 peptide indicate that inclusion of cholesterol in the bilayer formulation maintains the peptide in a random conformation even at a peptide/lipid ratio at which a helical conformation is detected in DMPC alone.

Besides the relevance of membrane surface charge and lipid chain ordering, our results also indicate a significant selectivity of different lipids for interaction with the peptides. Under certain assumptions, including those of fast lipid exchange on the spin-label EPR time scale, the increase in outer hyperfine splitting, ΔA_{max}, for different 5-position spin-labeled lipid species can be related to their relative association constants with the protein [45,46]. The lipid selectivity pattern for interaction with the gH626–644 peptide in DMPC is in the order: PA ≈ PG ≈ PS > PE ≈ PC, and that for the gB632–6509 peptide is PS > PG ≈ PA > PE ≈ PC (see Table 1). Thus, both peptides interact preferentially with anionic lipids. Moreover, the gB632–650 peptide shows a significant selectivity for the phosphatidylserine head group.

It is interesting to consider these results in the context of current studies on the role of the different HSV glycoproteins in the fusion events occurring during virus replication. HSV replicates its DNA in the nucleus of the infected cell and therefore virus egress requires crossing the nuclear envelope. Although this point is still debated, it seems that HSV nucleocapsids traverse the nuclear envelope by budding from the inner nuclear membrane into the perinuclear space and subsequently lose their membrane by fusing with the outer nuclear membrane. Consequently, at least two fusion events occur during virus replication: fusion of viral envelope with the plasma membrane on virus entry, and fusion of a primary envelope with the outer nuclear membrane on egress of the virus. The glycoproteins gB and gH participate in both events, but their mechanism of action is believed to be different in the two processes. Indeed, fusion with the plasma membrane requires cooperative action of both glycoproteins, whereas fusion with the nuclear membrane can be promoted independently by either gH or gB [17].

The lipid composition of nuclear membranes differs significantly from that of cytoplasmic membranes, having a much lower content of cholesterol, sphingomyelin and phosphatidylserine [58]. Because cholesterol and sphingomyelin are the main constituents of lipid rafts, it is to be expected that plasma membrane microstructure is much more heterogeneous than that of the nuclear membranes. Furthermore, while the net charge of lipid rafts is similar to the average membrane charge, these microdomains have been found to be enriched in phosphatidylserine relative to other anionic lipids [59]. Interestingly, membrane phosphatidylserine has been proposed recently to be a key regulator of protein localization in charged lipid bilayers [60].

Within this scheme, our results obtained with the two most fusogenic fragments of gH and gB suggest that the mechanism of action of these two glycoproteins is modulated by the phospholipid bilayer properties and composition. Indeed, the gB632–650 peptide inserts deeply in phospholipid bilayers, even those with a high content of cholesterol, and also its fusogenic activity is strongly enhanced in the presence of cholesterol. Moreover, this peptide also shows a significant selectivity towards phosphatidylserine. These findings are consistent with a propensity for the gB glycoprotein to interact with lipid rafts in

the cytoplasmic membrane, thus playing a fundamental role in viral entry into the cell. This conclusion agrees with the findings of Bender et al. [61], who observed that cholesterol is important during fusion with the plasma membrane, but plays no role once virus entry has occurred. It should be noted that glycoprotein gB is thought to interact with membranes via its fusion peptides, but that this interaction does not exclude other regions of gB, such as gB632–650, from providing the area of contact necessary for fusion during the conformational changes of the glycoprotein ectodomain.

On the other hand, the action of the gH626–644 peptide is less dependent on the state of the lipid membrane, and its membrane interaction ability decreases in the presence of cholesterol, suggesting that the gH glycoprotein exerts its destabilizing action on phospholipid bilayers by a different mechanism. Because both cholesterol-rich and cholesterol-poor domains are present in the plasma membrane of the cell, a concerted action of the gB and gH glycoproteins would be necessary for fusion of the membrane with the viral envelope. This is not the case for the nuclear membranes, which are mainly in the fluid-disordered phase, and thus can be induced to fuse by either the gB or the gH glycoprotein.

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