TABLE 1 Fluorescence emission maxima (λ_{max}) and surface partitioning constants (k^*_p) exhibited by NBD-labeled peptides in the presence of phospholipid vesicles

Peptide designation	pH	λ_{max} (nm)				$K^*_p \times 10^{-4}$ (M^{-1})		
		Buffer	PC	PA	PG	PC	PA	PG
ShB-NBD	7	555	551	531	531	No binding	77.1 (±11.6)	2.80 (±1.10)
ShBL7E-NBD	7	554	551	533	534	No binding	3.05 (±1.45)	0.72 (±0.20)
ShB-NBD	8.5	552	—	530	536	—	1.95 (±0.65)	0.64 (0.09)
ShBL7E-NBD	8.5	553	—	536	552	—	0.62 (±0.025)	≥0.05

as large as that produced by the ShB peptide (pK_a ~7.7), which seems consistent with the experiments using fluorescently labeled peptides in which the surface partition coefficients estimated for the interaction of the ShB peptide with PA vesicles were always higher than those corresponding to the ShB-L7E peptide.

Differential scanning calorimetric studies of peptide insertion into lipid bilayers

The possible implication of the hydrophobic domains of the phospholipid bilayers in the association of the peptides with the vesicles was investigated by DSC using synthetic dimirystoyl derivatives of the different phospholipids, which have convenient phase transition temperatures at 23.5°C, 23.3°C, and 49.6°C for DMPC, DMPG, and DMPA, respectively. In these experiments, peptide insertion into the lipid bilayer and, therefore, its interaction with the phospholipid acyl chains are expected to prevent part of the phospholipid molecules from undergoing the phase transition characteristic of the "pure" phospholipid species and thus decrease the phase transition enthalpy in a peptide concentration-dependent manner (Papahadjopoulos et al., 1975). We observed that neither the ShB nor the ShB-L7E peptides cause any effects on the transition temperature or the transition enthalpy of DMPC vesicles (data not shown). On the other hand, confronting the ShB peptide to anionic phospholipid vesicles (Fig. 2, *A* and *B*) causes a large, concentration-dependent decrease in the transition enthalpy, which is slightly more pronounced in DMPG than in DMPA vesicles, without modifying the phase transition temperature. A plot of the observed changes in the transition enthalpies versus the peptide/phospholipid molar ratios used in these studies (Fig. 2 *C*) predicts that each ShB peptide molecule prevents an average of three to four anionic phospholipid molecules from undergoing the phase transition. As for the mutant ShB-L7E peptide, its effects on the DMPA or DMPG phase transition enthalpies are negligible compared to those of the ShB peptide (Fig. 2, *A* and *B*).

DMPG vesicles were also used to study whether the observed peptide insertion into the phospholipid bilayer is pH-dependent. DMPG was chosen for these studies because it lacks chemical groups whose titration within the pH region of interest could interfere with the peptide insertion phenomena determined by DSC. Fig. 3 shows that, indeed, the ability of the ShB peptide to insert into the hydrophobic domains of the anionic bilayer is strongly pH-dependent. A maximum decrease in the DMPG phase transition enthalpy in the presence of the ShB peptide can be observed at pH 6.0, whereas increasing the pH to 8.5 or beyond renders the ShB peptide incapable of inserting into the phospholipid bilayer. In spite of the observed lack of peptide insertion under the latter conditions, peptide binding experiments, carried out at pH 8.5 using NBD-labeled peptides as described above, demonstrate that the ShB peptide still binds to anionic phospholipid vesicles, although with a much lower affinity (Table 1). As for the mutant ShB-L7E peptide, its effects on the DMPG phase transition enthalpies are negligible at all of the pHs explored (Fig. 3).

Fourier transform infrared spectroscopy studies of peptide conformation

Fig. 4 shows the infrared amide I region of the spectra corresponding to the ShB and ShB-L7E peptides in the presence of different phospholipid vesicles. Either peptide in the presence of PC vesicles (Fig. 4, *A* and *B*) (as well as in plain buffers in the absence of lipids) exhibits a bell-shaped amide I band with a maximum centered at 1645 cm^{-1} characteristic of nonordered protein structures. The similarities observed between the spectra of the peptides in solution and in the presence of PC vesicles is consistent with the lack of binding of either peptide to the zwitterionic PC vesicles. On the other hand, the spectra of the ShB peptide in the presence of either PA or PG vesicles exhibit a very prominent amide I component at 1623 cm^{-1} and a smaller one at 1689 cm^{-1} (lower traces in Fig. 4, *C* and *E*), which have been related to the adoption of a strongly hydrogen-bonded structure (Demel et al., 1990). Such a 1623 cm^{-1} component in the ShB spectra appears readily and has approximately the same relative importance regardless of the peptide concentration or the peptide-to-phospholipid molar ratio used in the FTIR experiments (ranging from 1.2 to 10 mg/ml and from 2 to 60 (by mole), respectively; Fernandez-Ballester et al., 1995). Even though they are similar, the ShB spectra taken in the presence of PG or PA vesicles differ in that the characteristic 1623 cm^{-1} component is more heat-stable in the former, because at temperatures as high as 70°C, the absorbance at 1623 cm^{-1} seen in the ShB/PG samples is still maintained at no less than 65% of that observed at room temperature, whereas that seen in the presence of PA vesicles decreases more

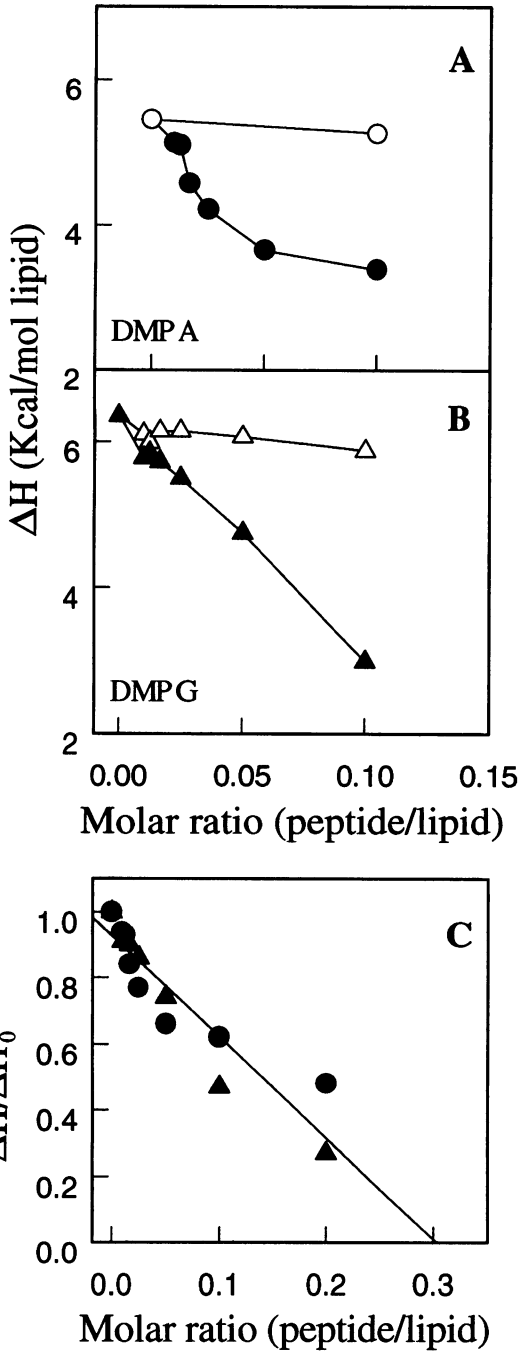

FIGURE 2 Changes in the gel to liquid crystal phase transition enthalpies of DMPA (*A*, ○, ●) or DMPG (*B*, △, ▲) large multilamellar vesicles in the presence of increasing concentrations of either ShB (●, ▲) or ShB-L7E (○, △) peptides, as determined by differential scanning calorimetry of the indicated phospholipid/peptide mixtures in 10 mM HEPES, pH 7.0, 100 mM NaCl. Error bars are within the size of the symbols used to plot the experimental results. In the absence of added peptides, the estimated phase transition enthalpies in vesicles made from pure DMPA or DMPG were 5.4 and 6.5 kcal/mol of phospholipid, respectively. (*C*) Plot of the observed $\Delta H/\Delta H_0$ from above (transition enthalpies of either DMPA (●) or DMPG (▲) determined in the presence (ΔH) and in the absence (ΔH_0) of added ShB peptide) versus the ShB peptide/phospholipid ratios. In spite of the heterogeneity arising from the use of data from DMPA and DMPG samples altogether, a linear extrapolation of such plot indicates that the addition of ShB peptide in this sample at a peptide/phospholipid ratio of ~0.3 will completely avoid the occurrence of the cooperative lipid phase transition.

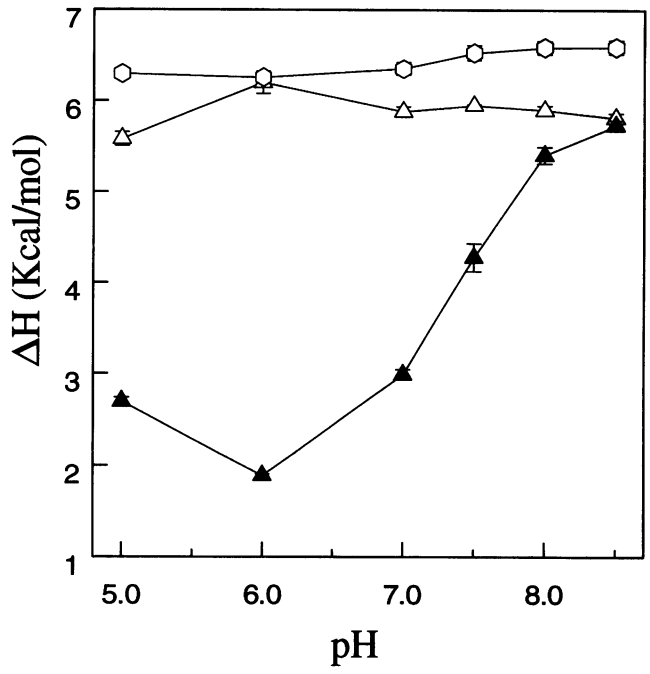

FIGURE 3 pH dependence of the observed gel to liquid crystal phase transition enthalpy of large multilamellar DMPG vesicles, as determined by differential scanning calorimetry. Symbols correspond to DMPG vesicles alone (○) and in the presence of either ShB (▲) or ShB-L7E (△) peptides, both at a peptide/DMPG molar ratio of 0.1. The buffers used at each of the indicated pHs were: pH 5.0, 25 mM citrate, 50 mM phosphate, 20 mM NaCl, 130 mM KCl; pH 6.0, 10 mM 2-(*N*-morpholino)ethanesulfonic acid, 20 mM NaCl, 130 mM KCl; pH 7.0, 10 mM phosphate, 20 mM NaCl, 130 mM KCl; pH 7.5, 10 mM phosphate, 20 mM NaCl, 130 mM KCl; pH 8.0, 10 mM *N*-(2-hydroxyethyl) piperazine-*N'*-3-propanesulfonic acid Epps, 20 mM NaCl, 130 mM KCl; pH 8.5, 10 mM Epps, 20 mM NaCl, 130 mM KCl.

markedly with increasing temperature (Fig. 4, *C* and *E*, middle traces). Other properties attesting to a remarkable conformational stability exhibited by the ShB peptide in the presence of anionic phospholipid vesicles are that the absorbance at 1623 cm^{-1} 1) recovers quite efficiently upon cooling of previously heat-denatured samples (Fig. 4, *C* and *E*, upper traces); 2) is fairly insensitive to increasing the ionic strength of the aqueous media, unless the vesicles are preincubated, before the addition of the peptide, with NaCl at more than 1 M, which efficiently prevents the conformational event; 3) is insensitive to the presence of low concentrations of nonanionic detergents such as octyl-glucoside, unless the vesicles are completely disrupted by the detergents at concentrations above their critical micellar concentration; and 4) is insensitive to the presence of millimolar concentrations of EDTA (data not shown).

As for the mutant ShB-L7E peptide (Fig. 4, *D* and *F*), the 1623 cm^{-1} structural component can be observed only in PG vesicles (Fig. 4 *F*), at a moderately high peptide concentration and only in the virtual absence of EDTA (below ~20 μM), and even then it is much less heat-stable than the component seen in the ShB peptide under similar conditions