The Structure of the C-Terminal Domain of the Pro-Apoptotic Protein Bak and Its Interaction with Model Membranes

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ABSTRACT Bak is a pro-apoptotic protein widely distributed in different cell types that is associated with the mitochondrial outer membrane, apparently through a C-terminal hydrophobic domain. We used infrared spectroscopy to study the secondary structure of a synthetic peptide ($^{+}{}_{3}$HN-^{188}ILNVLVVLGVVLLGQFVVRRFFKS^{211}-COO^{-}) with the same sequence as the C-terminal domain of Bak. The spectrum of this peptide in D_2O buffer shows an amide I′ band with a maximum at 1636 cm^{-1}, which clearly indicates the predominance of an extended β-structure in aqueous solvent. However, the peptide incorporated in multilamellar dimyristoylphosphatidylcholine (DMPC) membranes shows a different amide I′ band spectrum, with a maximum at 1658 cm^{-1}, indicating a predominantly α-helical structure induced by its interaction with the membrane. It was observed that through differential scanning calorimetry the transition of the phospholipid model membrane was broadened in the presence of the peptide. Fluorescence polarization of 1,6-diphenyl-1,3,5-hexatriene (DPH) in fluid DMPC vesicles showed that increasing concentrations of the peptide produced increased polarization values, which is compatible with the peptide being inserted into the membrane. High concentrations of the peptide considerably broaden the phase transition of DMPC multilamellar vesicles, and DPH polarization increased, especially at temperatures above the T_c transition temperature of the pure phospholipid. The addition of peptide destabilized unilamellar vesicles and released encapsulated carboxyfluorescein. These results indicate that this domain is able to insert itself into membranes, where it adopts an α-helical structure and considerably perturbs the physical properties of the membrane.

INTRODUCTION

The Bcl-2 family of proteins is known to be related to the control of cell apoptosis. This family consists of the following three different subfamilies: 1) anti-apoptotic members, such as Bcl-2 and Bcl-x_L, showing sequence homology in Bcl-2 homology 1 (BH1), BH2, BH3 and, in most cases, BH4 domains; 2) pro-apoptotic members, such as Bax and Bak, which share homology with Bcl-2 at BH1, BH2, and BH3; and 3) pro-apoptotic proteins, which only share homology with Bcl-2 in the BH3 domain, including Bid, Bik, and Bim (Adams and Cory, 1998; Tsujimoto and Shimizu, 2000), and Fig. 1 shows a comparison of these different proteins. When Bak (for Bcl-2 homologous antagonist/killer) was cloned in two different ways (Farrow et al., 1995; Chittenden et al., 1995b; Kiefer et al., 1995), it shared 25, 22, and 19% amino acid sequence identity with Bcl-2, Bcl-x_L, and Bax, respectively. In particular, Bak shares 53% amino acid sequence with Bcl-2 in the BH1 and BH2 domains. In addition, Bak, like Bcl-2, contains a hydrophobic transmembrane domain at its carboxy terminus, indicating that it may exist as an integral membrane protein (Farrow et al., 1995; Chittenden et al., 1995b; Kiefer et al., 1995).

Heterodimerization between members of the Bcl-2 family of proteins is a key event in the regulation of programmed cell death. Mutational and structural analysis have indicated that the BH1 and BH2 (and probably the BH3) domains are

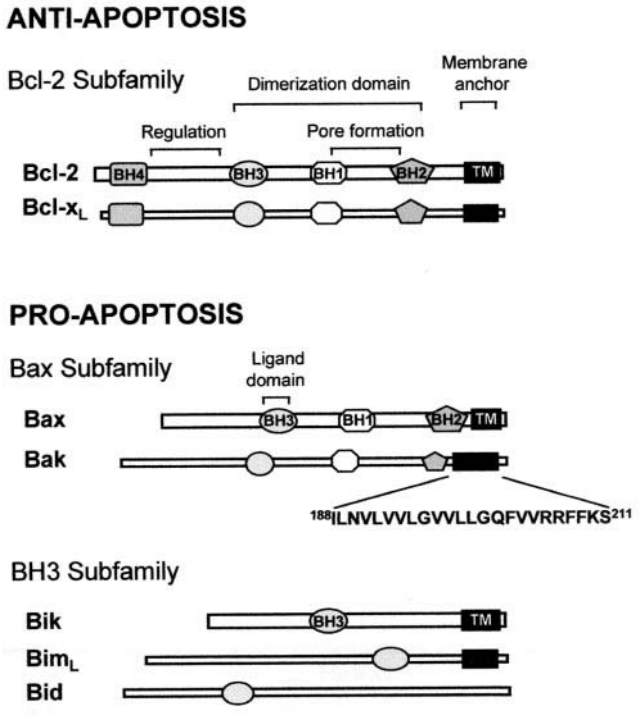

FIGURE 1 Representation of some of the Bcl-2 family members. Three subfamilies are indicated: the Bcl-2 subfamily promotes cell survival, whereas the Bax and BH3 subfamilies facilitate apoptosis. BH1 to BH4 are conserved sequence motifs. TM is the transmembrane domain.

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essential for the dimerization of anti-apoptotic proteins with pro-apoptotic proteins, and hence inhibit their pro-apoptotic activity (Sattler et al., 1997). It has been proposed that the BH3 domain of proteins like Bak and Bax may be sufficient to prevent the normal ability of Bcl-2 to suppress apoptosis (Boyd et al., 1995; Chittenden et al., 1995a; Zha et al., 1996; Hunter and Parslow, 1996; Cosulich et al., 1997; Holinger et al., 1999). It has also been observed that Bax and Bak may interact with the permeability transition pore of mitochondria to induce permeability transition with the loss of membrane potential ($\Delta\psi$), release of cytochrome *c* (Marzo et al., 1998; Narita et al., 1998; Rossé et al., 1998; Shimizu et al., 1999; Brenner et al., 2000) and subsequent activation of caspase and apoptosis, the BH3 domain being essential for such interactions. However, other pro-apoptotic proteins, such as Bid and Bik, which only possess a BH3 domain, produce apoptosis after the release of cytochrome *c* but do not open the transition pore and so do not lead to the loss of membrane potential (Shimizu and Tsujimoto, 2000). In addition, truncated Bid may allosterically activate Bak to produce intramembranous oligomerization, giving rise to a pore for cytochrome *c* efflux (Wei et al., 2000). Note that in the last mechanism, the pore is formed by Bid and Bak proteins without the participation of the proteins involved in the mitochondrial transition pore mentioned above, which may explain why the transition pore is not involved in the action of Bid/Bak, as mentioned above. The number of possible ways in which apoptosis is produced may be further increased if we take into account that Bak BH3 peptides antagonize the Bcl-x_L function and induce apoptosis by means of a cytochrome *c*-independent activation of caspases through caspase activating factors such as Apaf-1, a mechanism which is not associated to the loss of membrane potential (Holinger et al., 1999).

Since the 3D structure of Bak is not known and because this protein has not yet been purified, it is difficult to understand the exact function of its domains. In particular, it is difficult to identify the function of its hydrophobic C-terminal domain, which, it has been suggested by analogy with Bcl-2, may act as a membrane anchor (Farrow et al., 1995; Chittenden et al., 1995b; Kiefer et al., 1995), meaning that Bak exists as an integral membrane protein. Nevertheless, some experiments have been carried out with a truncated form of Bak (Narita et al., 1998; Priault et al., 1999), which has been seen to show pro-apoptotic activity interacting with the membrane transition pore, and so this domain may serve as a mere anchor or targeting domain. However, even if this is the case, it would be useful to understand the interaction of Bak with membranes, because the life or death of a cell may depend on the correct targeting or anchoring of this protein.

In this paper we report our investigations into the secondary structure of the C-terminal domain of Bak, its interaction with model membranes, and its ability to disrupt their barrier properties. It was found that this domain changes its secondary structure when it interacts with the membrane and may insert itself into the membrane, resulting in disruption. It also showed a strong potential to release encapsulated carboxyfluorescein, all of which points to the membrane intrinsic character of this domain and to its ability to form pores in the membrane.

MATERIALS AND METHODS

Materials

The synthetic peptide encompassing residues 188–211 of Bak ($^{+}{}_{3}$HN-^{188}ILNVLVVLGVVLLGQFVVRRFFKS^{211}-COO^{-}) was obtained from Genemed (San Francisco, CA) and judged pure (>95%) according to HPLC and MALDITOF spectroscopy. 1,2-Dimyristoyl-*sn*-glycero-3-phosphatidylcholine (DMPC) and egg yolk phosphatidylcholine (EYPC) were obtained from Avanti Polar Lipids (Alabaster, AL). Deuterium oxide (D_2O), 1,6-diphenyl-1,3,5-hexatriene (DPH), and 2,2,2-trifluoroethanol (TFE) were purchased from Sigma Chemical Co. (Madrid, Spain). The other solvents were from Merck (Darmstadt, Germany) and all other reagents used were of analytical grade.

Transmission infrared spectroscopy

The infrared spectra were obtained by using a Bruker Vector 22 Fourier transform infrared spectrometer equipped with a liquid nitrogen-cooled MCT detector. Each spectrum was obtained by collecting 500 interferograms with a nominal resolution of 4 cm^{-1} and triangular apodization using the sample shuttle accessory to average background spectra between sample spectra over the same time period. The spectrometer was continuously purged with dry air.

Samples were prepared from DMPC and Bak C-terminal domain stocks dissolved in chloroform/methanol (1:1, v/v). Bak C-terminal domain (0.18 μmol) with phospholipid, at a peptide/phospholipid molar ratio of 0.1, was dried under a stream of N_2, free of O_2, and the last traces of solvents were removed by evaporation under high vacuum for a further 3 h. Then, 200 μl of TFE were added and the sample was vortexed vigorously and dried as described before. Samples were then hydrated in 100 μl of D_2O buffer (10 mM Hepes pD 7.4, 0.1 mM EDTA) and dispersed with vigorous vortex mixing in the liquid-crystalline phase to form multilamellar vesicles (MLV) for 1 h. Next, they were centrifuged at 13,200 $\times$ *g* for 25 min. The phospholipid phase at the top of the solution and the supernatant phase were separated from the pellet and centrifuged again at 13,200 $\times$ *g* for 25 min to obtain the highest degree of the phospholipid phase separation from the supernatant. The phospholipid phase containing bound peptide (25 μl) was then transferred to a Specac 20710 cell equipped with CaF_2 windows and 25-μm Teflon spacers (Specac, Kent, UK). Samples containing pure peptide (without phospholipid) were dried from TFE as when mixed with DMPC, and then hydrated in 25 μl D_2O buffer (10 mM Hepes, 0.1 mM EDTA, pD 7.4), vortexed for 1 h, and transferred to a Specac 20710 cell, as described above.

For temperature studies, samples were prepared as described before with DMPC in the absence or presence of Bak (0.18 μmol) at peptide/phospholipid molar ratios of 0.1 and 0.05. They were then equilibrated at the starting temperature for 30 min before acquisition and scanned between 5 and 49°C at 2°C intervals, with a 2-min delay between each consecutive scan with a water bath interfaced to the spectrometer computer. Fourier transform infrared (FTIR) spectra were obtained in a Philips PU9800 Fourier transform infrared spectrometer equipped with a deuterated triglycine sulfate detector. Each spectrum was obtained by collecting 128 interferograms with a nominal resolution of 2 cm^{-1} and triangular apodization using the sample shuttle accessory to average background spectra between sample spectra over the same time period.

Spectral subtraction was performed interactively using the Spectra-Calc program (Galactic Industries Corp., Salem, NH). The spectra were subjected to deconvolution and second derivation using the same software. Deconvolution was carried out using a γ factor of 2 and a smoothing factor of 0.4. Both deconvolution and derivation gave the number and position, as well as an estimation of the bandwidth and intensity of the bands, making up the amide I′ region. Data treatment and band decomposition of the original amide I′ have been described previously (Arrondo et al., 1989, 1994). The fractional areas of bands in the amide I′ region were calculated from the final fitted band areas.

Differential scanning calorimetry

Calorimetric measurements were performed in a Microcal MC-2 differential scanning calorimeter (Microcal Inc., Northampton, MA). All heating scans were recorded at the same rate of 60°C min^{-1} in the temperature interval from 5 to 40°C. Before starting each scan, the samples were equilibrated in the calorimetric cell for 15 min at 5°C. Appropriate amounts of peptide and 1 mg of DMPC of the respective stock solutions in chloroform/methanol (1:1 v/v) were dried under a stream of N_2 and stored under vacuum for 3 h. Then, 200 μl of TFE were added, vigorously vortexed, and dried as described before. The samples were then dispersed in 0.1 mM EDTA, 10 mM Hepes, pH 7.4 buffer, hydrated in the liquid-crystalline phase with vigorous vortex mixing to form MLV. Finally, a reference-containing buffer and sample were placed into the calorimetric cells for the measurement and the results were plotted as a function of the peptide/lipid molar ratios. The MicroCal Origin softwares were used for data acquisition and analysis. The exact lipid concentration was determined according to the method of Böttcher et al. (1961) and values are normalized according to the maximum enthalpy value obtained (6.96 kcal/mol).

Leakage of liposome contents

The leakage of liposome contents to the external medium was kinetically monitored by measuring the release of the 5(6)-carboxyfluorescein (CF) trapped inside the vesicles (Weinstein et al., 1977; Rex, 1996; Rex and Schwarz, 1998; Simon and Gear, 1998).

To prepare large unilamellar vesicles (LUV) with a diameter of 100 nm, we used the extrusion technique (Mayer et al., 1986). Ten μmol of EYPC and as tracer, [^{3}H]-PC at a concentration of ~900 cpm/nmol in chloroform/methanol (1:1) were dried under N_2. The last traces of solvents were removed by a further 3 h evaporation under high vacuum. Lipid was then hydrated in a 50 mM Mes pH 7.4, 50 mM K_2SO_4 buffer, containing 50 mM purified CF (Rex, 1996) and dispersed by vortexing to form MLV. Subsequently, the suspension was extruded 10 times through two stacked 100-nm pore size polycarbonate membranes (Millipore Inc., Bedford, MA). The external dye was separated from the vesicles by gel filtration over a Sephadex G-25 (1 × 20) column and eluted with a 50 mM Mes pH 7.4, 100 mM K_2SO_4 buffer. Vesicles were separated from larger particles and untrapped CF eluting in the void volume of the column. To obtain a homogeneous preparation, only the top fractions of the LUV elution peak were collected and pooled. The total lipid content was quantified by liquid scintillation counting after and before the extrusion and gel filtration. The increase in CF fluorescence upon CF release was followed using a Fluoromax-3 from Jobin Yvon (Longjumeau, France) fluorescence spectrophotometer with excitation at 490 nm (5-nm slit width) and emission intensity was monitored at 520 nm (15-nm slit width). Measurements were made in a thermostatted quartz cuvette with constant stirring at 25°C. A small volume of vesicles was diluted in the buffer (50 mM Mes pH 7.4, 100 mM K_2SO_4) to yield a total volume of 2 ml at a final phospholipid concentration of 6 μM. A baseline measurement of the fluorescence of vesicles alone was made for 10 min. In our experimental conditions the spontaneous leakage rate was <5%. Then, Bak C-terminal peptide dissolved in TFE at 2 mg/ml or 0.5 mg/ml, or TFE vehicle was added. Fluorescence was measured for 15 min, and then a small volume of concentrated Triton X-100 (0.5% final, w/v) was added to determine the maximum fluorescence attainable under conditions of totally solubilized vesicles. The addition of 4 μl TFE did not produce any substantial leakage. The percentage of carboxyfluorescein released was determined by the following equation: $[(F_{t=15\ min} - F_0)/(F_{max} - F_0)] \times 100$, where $F_{t=15min}$ is the maximum intensity 15 min after addition of peptide or TFE, F_0 is the intensity of vesicles alone at time 0, and F_{max} is the intensity after addition of Triton X-100.

Fluorescence polarization

Multilamellar vesicles of DMPC were prepared with appropriate amounts of Bak C-terminal domain, using stock solutions in chloroform/methanol (1:1, v/v) to give the required peptide/phospholipid molar ratios. Phospholipid concentration was 0.02 μmol. The fluorescent probe DPH was prepared in tetrahydrofuran and added to the organic solvent solution before drying to give a probe/lipid molar ratio of 1:500. Then samples were dried and subsequently 100 μl TFE were added and vortexed vigorously, dried under a stream of nitrogen, and stored under vacuum for 3 h to totally remove the organic solvent. Samples containing phospholipid and peptide were then hydrated in 70 μl filtered buffer (10 mM Hepes, 0.1 mM EDTA, pD 7.4) and dispersed with vigorous vortex mixing in the liquid-crystalline phase to form MLVs.

DPH fluorescence polarization was measured in a Hitachi F-4500 fluorescence spectrophotometer equipped with a polarization accessory. Excitation and emission wavelengths were 350 and 452 nm, respectively, with an excitation and emission slit width of 5 nm. Cuvette temperature was monitored continuously by means of a thermistor probe.

Fluorescence polarization data were analyzed according to Hoffmann et al. (1981). Essentially, a normalized polarization $P'(c)$ is defined for a given peptide/lipid molar ratio (c) as: $P'(c) = [P(c) - P(0)]/[P_{max} - P(0)]$, in which P_{max} is the value of $P(c)$ at very high peptide concentrations, $P(c)$ is the observed polarization value, and $P(0)$ is the DPH polarization in the absence of protein. Then, by fitting $\ln[1 - P'(c)]$ to a suitable straight line it was found that $P'(c) \approx 1 - e^{-Mx}$, in which M is a number corresponding approximately to the maximum number of lipid chains that could fit around an isolated intrinsic molecule in each half of the bilayer (Hoffmann et al., 1981; Soloaga et al., 1999; Azpiazu et al., 1993), and x may be either $x = c$ for a transmembrane protein or $x = c/(2 - c)$ for a protein occupying only one of the monolayers. Thus, by fitting the experimental results to a theoretical $P'(c)$ versus (c) curve and if M is independently known by other procedures, for example DSC, transmembrane integral proteins can be distinguished from non-transmembrane ones.

RESULTS

Secondary structure of the peptide as studied by infrared spectroscopy

When Bak C-terminal domain peptide was prepared in D_2O buffer in the absence of phospholipids, the amide I′ band of the infrared spectrum showed a maximum at 1636 cm^{-1} (Fig. 2 *A*), a frequency which indicates the predominance of β-sheet in its secondary structure (Krimm and Bandekar, 1986; Susi and Byler, 1987; Arrondo et al., 1989, 1994; Fabian et al., 1992; González et al., 1997; Zhang et al., 1998). The amide I′ band was decomposed as shown in Fig. 2 *B*. The number and initial position of the component bands were obtained from band-narrowed spectra by Fourier deconvolution and derivation. The corresponding parameters, band position, percentage area, and assignment of each

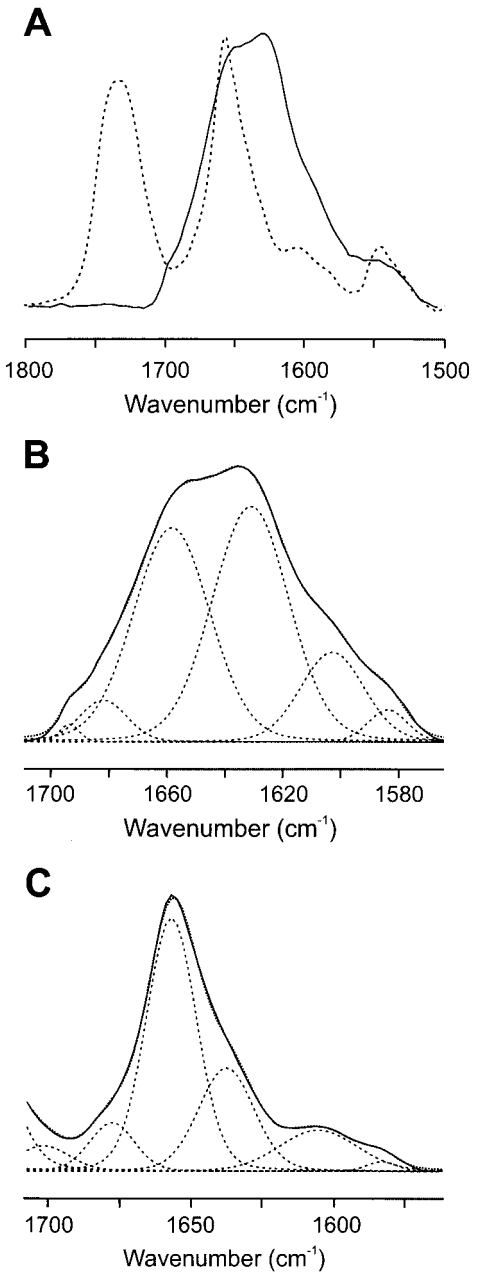

FIGURE 2 (*A*) FTIR spectrum of the Bak C-terminal domain in the absence (*solid line*) and the presence (*dashed line*) of multilamellar vesicles containing DMPC at a peptide/phospholipid molar ratio of 0.1 in D_2O buffer. (*B*) FTIR spectrum of the Bak C-terminal domain in the absence of membranes (*solid line*) and in the presence (*C*) of multilamellar vesicles containing DMPC at a peptide/phospholipid molar ratio of 0.1 in D_2O buffer (*solid line*) with the fitted component bands (*dashed line*). The position of the individual bands was obtained from the resolution-enhanced spectrum. The parameters corresponding to the component bands are reflected in Table 1. The dotted line represents the curve-fitted spectrum.

spectral component are shown in Table 1. It can be seen that the spectrum exhibited four component bands in the 1700–1600 cm^{-1} region, although the band with the lowest frequency (1602 cm^{-1}) cannot be attributed to peptide bonds but rather to amino-acidic side chains. The quantitative contribution of the other three bands to the amide I′ contour was obtained by band curve-fitting of the original spectrum. The component with the maximum contribution (50%) was located at 1632 cm^{-1}, which probably corresponds to intramolecular C═O vibrations of peptidyl bonds within β-pleated sheets (Krimm and Bandekar, 1986; Susi and Byler, 1987; Arrondo et al., 1989, 1993, 1994; Fabian et al., 1992; Arrondo and Goñi, 1999). The component at 1659 amounted to 45%, and can be attributed to α-helix (Arrondo et al., 1993; Arrondo and Goñi, 1999). Finally, the component at 1682, corresponding to only 5%, can be assigned to turns (Surewicz et al., 1990; Fabian et al., 1993; Muga et al., 1993; González et al., 1997; Zhang et al., 1998).

TABLE 1 FTIR parameters of the amide I′ band components of the Bak C-terminal domain in the absence or presence of multilamellar vesicles containing DMPC in 10 mM Hepes and 0.1 mM EDTA-D_2O buffer (pD 7.4)

Peptide			Peptide + DMPC		
Position* (cm^{-1})	Assignment	Area† (%)	Position* (cm^{-1})	Assignment	Area† (%)
1682	Turns	5	1678	Turns	10
1659	α-Helix	45	1657	α-Helix	62
1632	β-Pleated sheet	50	1638	β-Pleated sheet	28

*Peak position of the amide I′ band components.
†Percentage area of the band components of amide I′. The areas corresponding to side chain contributions located at 1615-1600 cm^{-1} have not been considered.

The infrared spectrum was quite different when Bak C-terminal domain was resuspended in the presence of multilamellar vesicles of DMPC, in which case the maximum of the amide I′ band was found at 1656 cm^{-1}, which is indicative of a predominant α-helical structure (Arrondo et al., 1993; Arrondo and Goñi, 1999) (Fig. 2 *C*). The 1800–1700 region corresponds to the carbonyl ester band coming mainly from the phospholipid acyl chains. Band decomposition was carried out as described above for the peptide in the absence of phospholipid, and it can be observed that the main band (62%) was now centered at 1657 cm^{-1}, indicating the predominance of an α-helical structure (Arrondo et al., 1993; Arrondo and Goñi, 1999). The component at 1637 cm^{-1} (representing 28%) can be attributed to β-sheets (Krimm and Bandekar, 1986; Susi and Byler, 1987; Arrondo et al., 1989, 1993, 1994; Fabian et al., 1992; Arrondo and Goñi, 1999). The high-frequency component at 1678 cm^{-1} can be assigned to turns (Surewicz et al., 1990; Fabian et al., 1993; Muga et al., 1993; González et al., 1997; Zhang et al., 1998) and corresponded to 10% of the total area of the amide I′ band, which is assigned to peptidyl bonds (i.e., excluding components from amino-acidic side chains).

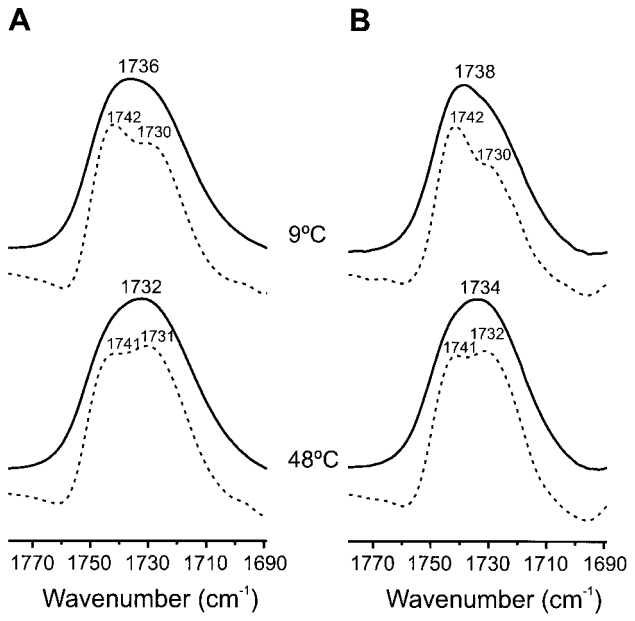

FIGURE 3 FTIR spectra of DMPC in the absence (*A*) and in the presence (*B*) of the Bak C-terminal domain at a peptide/DMPC molar ratio of 0.1. Original (*solid line*) and its deconvolution (*dashed line*) spectra are represented at different temperatures as indicated, showing the carbonyl stretching region. The maxima of peaks or subcomponents are given.

The effect of Bak C-terminal domain on the stretching vibration of the C═O ester of DMPC

The thermotropic phase behavior of pure DMPC and in the presence of Bak C-terminal domain was also examined by monitoring the temperature dependence of the stretching vibration peak arising from the C═O ester of the phospholipid, which provides information on the state of the lipid-water interface of the membrane. In Fig. 3 the deconvolutions of the spectra are presented. The C═O peak from the pure DMPC sample (Fig. 3 *A*) at 9°C, i.e., below the phase transition temperature, showed a broad band which can be deconvolved into two components, one at 1742 cm^{-1} and the other at 1731 cm^{-1}. The frequencies of these two components were not affected by temperature and remained the same at 47°C, i.e., above the phase transition temperature, but their relative intensities changed, as has been reported for other phospholipids (Mantsch and McElhaney, 1991). Whereas at temperatures below the phase transition, the 1742 cm^{-1} component was more intense than the 1731 cm^{-1} component, the contrary was observed at temperatures above the phase transition. As has been shown by a number of authors (Blume et al., 1988; Hübner and Mantsch, 1991; Mantsch and McElhaney, 1991; Lewis et al., 1994), the component occurring at the highest frequency can be attributed to unhydrated C═O groups, whereas the other component is assigned to hydrated C═O groups. Therefore, a higher proportion of C═O groups are hydrated at temperatures above the gel-to-liquid-crystalline phase transition temperature than below, as has been shown in the case of other diester phospholipids (Mantsch and McElhaney, 1991). The incorporation of peptide to give a Bak C-terminal domain/DMPC molar ratio of 0.1 (Fig. 3 *B*) resulted in a clearly narrower C═O vibration peak at 9°C. The half-bandwidth of 35.7 cm^{-1} obtained for pure DMPC at 9°C fell to 32.6 cm^{-1} at the same temperature in the presence of the peptide. Similarly, at 48°C, the band corresponding to pure DMPC has a width of 38.5 cm^{-1}, compared with 34.6 cm^{-1} in the presence of the C-terminal domain of Bak. These changes in width, and those described above for the frequency of the peak maximum, can be explained by a quantitative increase in the importance of the subcomponent with the highest frequency, which is attributed to unhydrated C═O groups. It seems, then, that the presence of the peptide induces a certain increase in the proportion of unhydrated carbonyl ester groups of the phospholipid molecules.

Differential scanning microcalorimetry

Pure DMPC multilamellar vesicles and vesicles containing different concentrations of Bak C-terminal domain peptide were studied by DSC with the aim of obtaining information on the way in which this peptide interacts with the membrane. Fig. 4 *A* shows that the presence of a low peptide content, as in a 0.01 peptide/DMPC molar ratio, already occasioned the disappearance of the pretransition and the widening of the main transition so that the onset took place at ~20.4°C instead of 23°C, which is the onset temperature of the main transition for pure DMPC. This peak was centered at 22.5°C. At 0.02 molar ratio the transition was further widened and the onset was located at 18.5°C, and the transition peak was clearly asymmetric with the maximum of the peak centered at 21.2°C. At 0.05 molar ratio the transition peak was quite wide with the onset at ~11.1°C. At 0.1 molar ratio the transition was very smeared and the onset was at 12.5°C. The pattern of variation of the transition peak with increasing concentrations of peptide indicated a strong effect on the transition, which became increasingly less cooperative and wider and with a lower ΔH, which decreased linearly with peptide/DMPC molar ratio (Fig. 4 *B*). Extrapolation of this plot gives us the point at which ΔH becomes zero. This is usually interpreted as the average number of phospholipid molecules that are prevented from undergoing the thermotropic transition per peptide molecule. In our case, the corresponding data are about five phospholipid molecules per one peptide molecule.

Fluorescence probe polarization spectroscopy

To obtain more information on the interaction of Bak C-terminal domain peptide with membranes, experiments

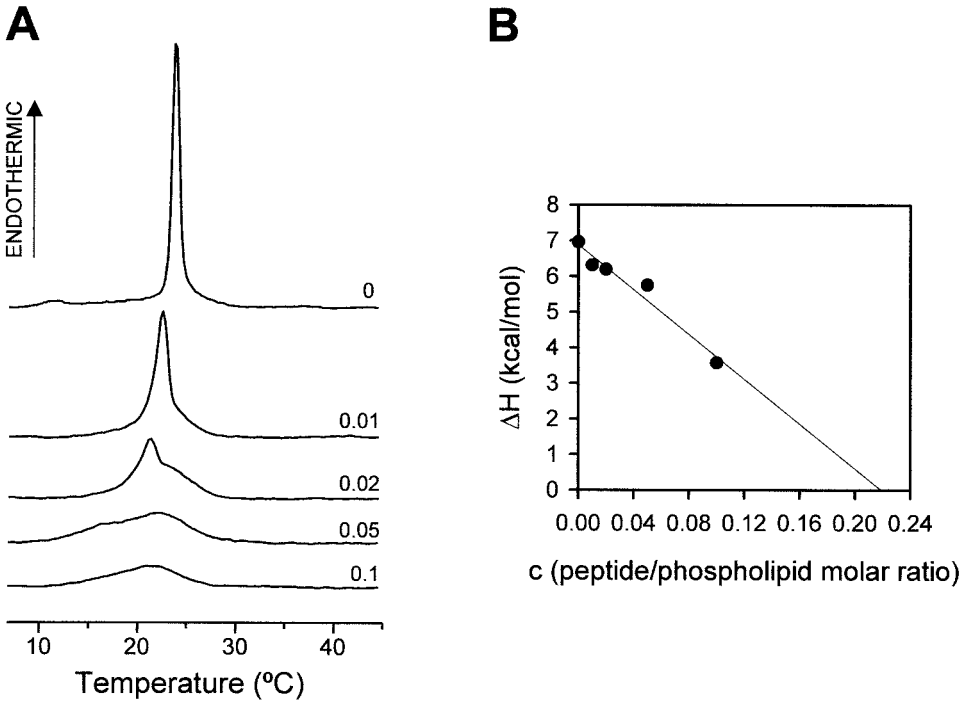

FIGURE 4 (*A*) DSC heating thermograms of aqueous dispersions of DMPC (MLV) with the Bak C-terminal domain peptide obtained at different peptide/DMPC molar ratios as indicated. (*B*) Enthalpy change associated with the main gel-fluid phase transition of DMPC in phospholipid-peptide mixtures, as a function of peptide/lipid molar ratio shown in the DSC thermograms of this figure. The value of peptide/lipid molar ratio at which the phase transition enthalpy reaches zero was extrapolated to a value of 0.22, corresponding to a lipid/peptide molar ratio of ~5:1.

based on changes in fluorescence polarization of the probe DPH were carried out.

DPH fluorescence polarization was first studied at different temperatures to monitor the influence of the Bak C-terminal domain peptide on the phase transition of DMPC vesicles in which it was incorporated (Fig. 5). The presence of the peptide at a peptide/DMPC molar ratio of 0.05 resulted in a broadening of the phase transition of the pure phospholipid, this effect being clear from the increased degree of polarization above of the phase transition temperature. This pattern was also observed for a sample containing a peptide/DMPC molar ratio of 0.1, the phase transition being further broadened and polarization values increasing above the phase transition, which indicated a considerable increase in apparent membrane microviscosity in the presence of the peptide.

The phase transition for DPH polarization was also studied at a fixed temperature of 30°C so that the DMPC membrane was in a fluid state. Fig. 6 shows that increasing concentrations of the peptide produced increasing polarization values, which is in agreement with the effects reported for other intrinsic molecules, such as Ca^{2+}-ATPase (Gómez-Fernández et al., 1980), cholesterol and cytochrome *c* oxidase (Hoffmann et al., 1981), bacteriorhodopsin (Alonso et al., 1982), myelin apolipoprotein (Goñi et al., 1988), Pf-1 viral protein (Azpiazu et al., 1993), α-hemolysin (Soloaga et al., 1999) and, more recently, with the C-terminal domain of Bcl-2 (Martínez-Senac et al., 2000). The experimental data were fitted to exponential lines according to the model of Hoffmann et al. (1981). The lines in Fig. 6 correspond to the $P'(c) = 1 - e^{-Mx}$ equation for the M range indicated, assuming $x = c$ (transmembrane protein)

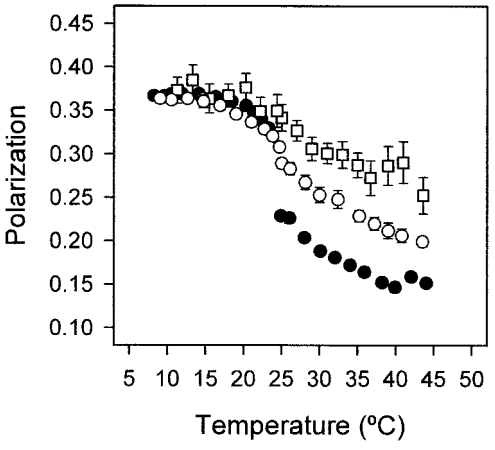

FIGURE 5 Fluorescence polarization as a function of temperature of DPH in DMPC (MLV) (10 μM) (probe/lipid molar ratio of 1:500) in the absence (●) or in the presence of the Bak C-terminal domain at 0.05 (○) and 0.1 (□) peptide/DMPC molar ratios. Experiments were carried out at constant stirring. Two independent experiments were carried out with 25 determinations for each experiment.

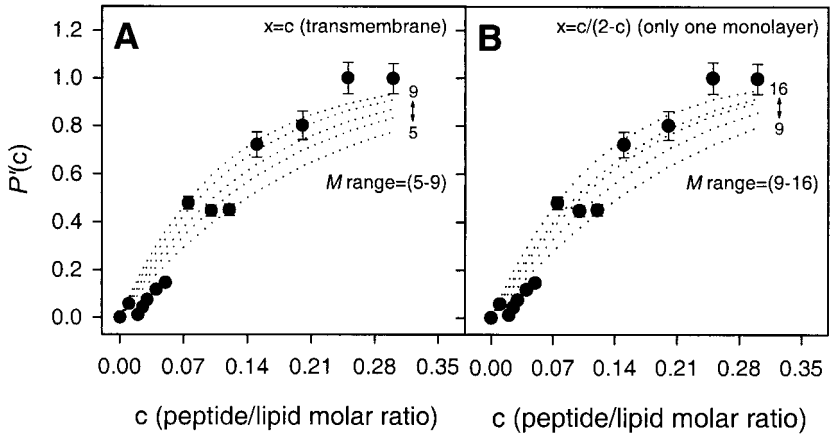

FIGURE 6 Normalized polarization $P'(c)$ of DPH fluorescence in DMPC (MLV) (10 μM) (probe/lipid molar ratio of 1:500) with the Bak C-terminal domain at increasing molar fractions (c). The experimental data were fitted to exponential lines that correspond to the $P'(c) = 1 - e^{-Mx}$ equation, where M is a number corresponding approximately to the maximum number of lipid chains that could fit around an isolated intrinsic molecule in each half of the bilayer, assuming (*A*) $x = c$ (transmembrane model) with an M range between 5 and 9 and (*B*) $x = c/(2 - c)$ (peptide occupying only one of the monolayers) with an M range between 9 and 16. Experiments were carried out at 30°C with constant stirring. Measurements were made in two independent experiments with 25 determinations for each experiment.

or $x = c/(2 - c)$ (protein occupying only one of the monolayers) (see Materials and Methods). The $x = c$ assumption (Fig. 6 *A*) fits the data to a curve obtaining an M range between 5 and 9. Using the $x = c/(2 - c)$ assumption (Fig. 6 *B*), an M range between 9 and 16 has been obtained. Since the value $M \approx 5$ was found from the DSC data, it may be concluded that the $x = c$ assumption (transmembrane protein with M range = 5–9) is the best fit to experimental data and hence a number of phospholipid molecules close to 5 are perturbed by each C-terminal domain peptide of Bak.

Leakage of CF induced by Bak C-terminal domain peptide

To further assess the insertion of the peptide in membranes, the release of encapsulated CF trapped inside LUVs was monitored at increasing concentrations of peptide. EYPC vesicles at 25°C, i.e., in the fluid state, were used in these experiments rather than the DMPC used in the other experiments reported in this work, because DMPC vesicles rapidly release the dye even in the absence of peptide, as has been reported by others (Bramhall et al., 1987). Fig. 7 shows how the peptide released up to 95% of the probe within 15 min of the addition of the peptide at a peptide/lipid molar ratio of 0.04. This result indicates that the peptide may disrupt the barrier properties of the phospholipid bilayer by inserting itself into this membrane, thus forming very conductive pores.

DISCUSSION

In this paper we have used a number of physical techniques to study the structure and interaction of the C-terminal domain of Bak with model membranes, and the first conclusion that can be reached from the results obtained is that this peptide is able to incorporate into phospholipid vesicles as an integral molecule. It should be considered that the C-terminal domain of Bak is considerably hydrophobic and the phospholipid used to prepare the model membranes is phosphatidylcholine. This phospholipid is zwitterionic and very well known to lack the capacity to attract proteins from outside the membrane (Chapman, 1993; Hernández-Caselles et al., 1993). By this reason if an interaction is detected be-

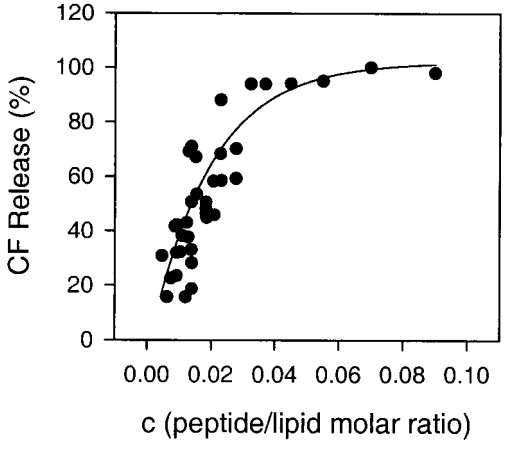

FIGURE 7 Leakage of carboxyfluorescein from EYPC (LUV) induced by the Bak C-terminal domain. The percentage of carboxyfluorescein released after 15 min of incubation with increasing concentrations of peptide was plotted as a function of the peptide/lipid molar ratio (c); 100% of leakage was established by lysing LUV with Triton X-100 (0.5%, w/v).

tween this peptide and a phosphatidylcholine membrane this interaction is most probably of hydrophobic type.

The infrared study clearly shows that the C-terminal domain of Bak significantly changes its secondary structure in the presence of phospholipid vesicles. Whereas the extended β-structure was predominant in D_2O buffer solution, the α-helical structure became the most important (62%), when the peptide was resuspended with DMPC vesicles. There are many known examples of the folding of membrane-active peptides induced by lipid membranes (Kaiser and Kezdy, 1984, 1987; Schwyzer, 1995; White and Wimley, 1998; Silvestro and Axelsen, 2000). Recently, it has been shown that some peptides do not require anionic lipids to fold or to exhibit permeabilizing membrane activity, e.g., magainin and cecropin A (Wieprecht et al., 1999) and the C-terminal domain of Bcl-2 (Martínez-Senac et al., 2000). It appears, then, that the C-terminal domain of Bak also folds and permeabilizes membranes without the need of anionic phospholipids. In this case, hydrophobic interactions must be important, probably due to the clearly hydrophobic nature of this peptide, which favors its insertion into the membrane. It is interesting to underline, in this context, the remarkable hydrophobicity of this peptide compared with the C-terminal domain of Bcl-2, the former being 71% composed of hydrophobic amino acids and the second 55%. Despite this, when the two sequences are aligned they share 78% homology and 28% identity, suggesting that these peptides probably form a special group of signaling-localization peptides belonging to apoptotic-related proteins. Note that whereas hydrophobic peptides will adopt transmembrane dispositions (Azpiazu et al., 1993; Arkin et al., 1995; Zhang et al., 1995) other peptides with amphipathic structure have been reported to remain bound to the surface (Ishiguro et al., 1993; Gazit et al., 1996; Ghosh et al., 1998; Oren et al., 1999; Hong et al., 1999; Ben-Efraim et al., 1999).

It was deduced from DSC that the peptide broadens the phase transition of DMPC without significantly shifting the center of this transition. Although when hydrophobic mismatches exist the phase transition may be considerably shifted, the shift may be not so important when the mismatch with the fatty acyl chains of the phospholipids is not large (Mouritsen and Bloom, 1993; Chapman et al., 1979; Soloaga et al., 1999). Since we are using DMPC and a peptide with a hydrophobic stretch sufficiently long to span the membrane, an important hydrophobic mismatch should not be expected. In addition, the study of the C═O stretching indicated that the peptide modified the lipid-water interface as it increased the proportion of dehydrated ester carbonyls of the phospholipid.

It was also observed that increasing peptide concentrations increased the polarization of the fluorescent probe DPH. Steady-state fluorescence polarization provides a time-averaged indication of the mobility of a fluorophore. DPH is a hydrophobic fluorescent molecule that, when incorporated into a lipid bilayer, is located in the hydrophobic region. In pure lipid bilayers DPH is freely mobile and its fluorescence is minimally polarized. The presence of intrinsic membrane proteins may restrict the mobility of the probe, therefore increasing its polarization. The polarization of diphenylhexatriene fluorescence has been studied to understand protein-induced changes in lipid dynamics (Gómez-Fernández et al., 1980; Azpiazu et al., 1993; Soloaga et al., 1999). It is deduced from the increase in polarization caused by the C-terminal domain of Bak that its presence increased the apparent order of the membrane by restricting the mobility of the probe molecules. This effect is the same as that found for many intrinsic molecules (Gómez-Fernández et al., 1980; Hoffmann et al., 1981; Azpiazu et al., 1993; Soloaga et al., 1999; Martínez-Senac et al., 2000) and is compatible with the notion that this peptide becomes a membrane integral molecule in the presence of phospholipid vesicles. In addition to this, quantitative results arising from differential scanning calorimetry and fluorescence polarization studies provide strong support to the hypothesis of the transmembrane character of the C-terminal domain of Bak. In fact, both techniques can provide a figure of the average number of lipids that are perturbed by the presence of the peptide, so that they appear to be removed from the gel-fluid phospholipid transition. In our case, and given that phosphatidylcholine was the only type of phospholipid present in the membrane and the results obtained with the other biophysical techniques, the most likely explanation is that the peptide is incorporated into the membrane in a transmembrane disposition. However, in some cases of interaction of peptides with model membranes a variation in polarization is observed where the interaction is basically electrostatic, as in the case of polylysine and phosphatidylglycerol (Houbre et al., 1988).

A study of the phase transition of DMPC both in the absence and in the presence of peptide by steady-state fluorescence polarization of DPH confirmed the results detected by DSC, namely that the peptide produced a broadening of the phase transition without essentially modifying the temperature at which the center of the transition was taking place. In addition, it was found that the peptide increased the polarization values of DPH mainly at temperatures above the phase transition. By combining data obtained from DSC and fluorescence polarization of DPH we found that the experimental data are better fitted to a model in which the peptide occupies a transmembrane disposition. These observations again support the membrane intrinsic nature of the peptide.

Insertion of the peptide into the model membranes was also confirmed by experiments that showed carboxyfluorescein leakage from phospholipid vesicles induced by the C-terminal domain of Bak, which indicates that the peptide penetrates the lipid hydrophobic core, breaking the barrier properties of the membrane, although it is not clear whether this effect is related to the physiological effect of Bak on the

biological membranes. This concentration-dependent permeabilization was probably due to a pore formed by the aggregation of peptide molecules, similar to what we previously suggested for the Bcl-2 C-terminal domain (Martínez-Senac et al., 2000).

The role of the C-terminal domain of Bak in the function of this protein is far from clear at the present moment. It might be that this domain will act simply as an anchor or targeting signal, but no experiments have been carried out so far to adequately test this possibility, as only truncated forms of the protein lacking this domain have been used (Narita et al., 1998; Priault et al., 1999). These experiments indicated that the protein has the capacity of opening the permeability transition pore of mitochondria. Nevertheless, given the tendency of these proteins to oligomerize with other members of the family, they could be located in membranes even if they lacked the anchor domain. In addition, the information described above does not preclude that this domain may contribute to the pro-apoptotic activity of Bak, whether it forms a pore itself (together with Bid), or even if the activity of full-Bak, which has been very poorly studied, is different from that of truncated Bak. It has been proposed that Bax, which belongs to the same subfamily as Bak, forms channels that render the membrane permeable for cytochrome *c* (Basañez et al., 1999; Nouraini et al., 2000), that its insertion and oligomerization in the membrane is promoted by Bid (Eskes et al., 2000), and that the C-terminal hydrophobic domain is a key element for the insertion of the protein in outer mitochondrial membranes in vivo (Goping et al., 1998; Nechushtan et al., 1999).

In summary, we have found that the peptide which imitates the C-terminal domain of Bak interacts with model phospholipid membranes, altering their physical properties and making them leaky to carboxyfluorescein (hence indicating the insertion of Bak into the membrane). At the same time, the peptide is folded by its interaction with the membrane to adopt a secondary structure in which α-helix is predominant. These results confirm that this domain may act as an anchor for the Bak protein in the membrane, and also that Bak may eventually help to create a pore through the outer mitochondrial membrane.

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