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The interaction of the Bax C-terminal domain with membranes is influenced by the presence of negatively charged phospholipids

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ABSTRACT

The C-terminal domain of the pro-apoptotic protein Bax (Bax-C) is supposed to act as a membrane anchor motif when Bax is activated leading to programmed cell death. A synthetic peptide which imitates this domain has been used to study the mechanism of peptide–phospholipid interaction. We have used static and MAS-NMR techniques to show that the interaction of Bax-C with membranes is modulated by the presence of a negatively charged phospholipid like phosphatidylglycerol. Bax-C slightly shifted upfield the ^{31}P resonances coming from phosphatidylglycerol and phosphatidylcholine. However the width of the resonance peaks was considerably higher when phosphatidylglycerol was present. Bax-C substantially decreased the T_1 relaxation times of phosphatidylglycerol and those of phosphatidylcholine when mixtured with phosphatidylglycerol, but T_1 values were not decreased when phosphatidylcholine was the only phospholipid present in the membrane. ^{13}C-MAS-NMR showed that T_1 values were decreased when Bax-C was incorporated into the lipid vesicles and this reduction affected similarly to carbons located in different regions of the membrane when the only phospholipid present was phosphatidylcholine. However, when phosphatidylglycerol was also present, the decrease in T_1 affected considerably more to some carbons in the polar region. These results indicate that Bax-C interacts differently with the polar part of the membrane depending on whether phosphatidylglycerol is present or not, suggesting that an electrostatic interaction of Bax-C with the membrane determines the location of this domain. Fluorescence spectroscopy showed that the Trp residues of Bax-C were placed in a microenvironment more hydrophobic and less accessible to quenching by acrylamide when phosphatidylglycerol was present.

1. Introduction

Bax is a protein which belongs to the Bcl-2 family and is implicated in the regulation of cell apoptosis. Members of this family are characterized for possesing BH domains, in some cases several of them, and they can be either anti- or pro-apoptotic proteins. In general, the anti-apoptotic members (e.g. Bcl-2, Bcl-x_L, Mcl-1, Bcl-w from mammals and Ced-9 from *Caenorhabditis elegans*) display sequence homology in all four BH domains, whereas the pro-apoptotic members (e.g. Bax, Bak and Bok) have homologous BH1–3 domains. Pro-apoptotic Bcl-2 family members, upon activation by apoptotic stimuli, are capable of forming heterodimers with anti-apoptotic members.

Most of the available evidences suggest that the BH regions mediate direct interactions between members of the protein family and that these interactions are critical for protein function [1,2]. Bax and Bak, control commitment to apoptosis by regulating mitochondrial outer membrane permeabilization [3].

In addition to BH domains, many of these proteins contain a hydrophobic transmembrane domain at their carboxy terminus. This C-terminal domain is supposedly responsible for membrane localization, for example in the outer membrane of mitochondria, the nucleus or in the endoplasmic reticulum membranes [4–9], indicating that these proteins may exist as integral membrane proteins [10–12]. The pattern of membrane localization differs among pro- and anti-apoptotic members. In this sense, Bcl-2 has been shown to reside on the cytoplasmic face of the mitochondrial outer membrane, whereas Bax or Bid are found mainly in the cytosol or as membrane-peripherals in healthy living cells, and only interact with the mitochondrial membrane when they are activated [13–16]. In all cases the C-terminal segments were shown to fold back onto a groove on the structures, similarly to Bax [13].

In unstressed cells, Bax resides inert in the cytosol, with its C-terminal domain sequestered within the hydrophobic surface groove [17]. Only when the sequestered C-terminal domain is released as a consequence of changes occurring at the N-terminus of the molecule, the protein will associtate to the target membrane. This activation of Bax has been described to occur by interaction with tBid which is already membrane-bound and will culminate in membrane

Abbreviations: CS, chemical shift; CSA, chemical shift anisotropy; FWHH, full width at half height; MLV, lipid multilamellar vesicles; MAS, magic angle spinning; POPC, 1-palmitoyl-2-oleoyl-*sn*-3-phosphocholine; POPG, 1-palmitoyl-2-oleoyl-*sn*-3-phospho-rac-glycerol; TFE, 2,2,2-trifluoroethanol

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permeabilization by Bax [18]. Permeabilization requires the alteration of the membrane by the formation of channels which may be formed by helices 5 and 6 [19].

Previous work from this laboratory has shown that the C-terminal domains of Bcl-2 [20], Bax [21] and Bak [22] interact with membranes, by association with model lipid vesicles. In addition, in the case of the C-terminal domains from pro-apoptotic Bak and anti-apoptotic Bcl-2 proteins, the lipid composition may modulate their secondary structure and their orientation with respect to the lipid bilayer [23]. It is not known whether the C-terminal domain of Bax is just anchoring the protein in the membrane or if it participating in the formation of pores. It has been observed, at least *in vitro*, that the C-terminal domains of Bcl-2, Bax and Bak form pores that release encapsulated 6-carboxyfluorescein [24]. It is very interesting that the C-terminal domain of Bax adopts a predominantly α-helical structure by interaction with negatively charged phospholipids such as phosphatidylglycerol [25].

In this paper we try to shed light on the way in which Bax-C, after leaving its interaction with other parts of Bax, may get inserted into the membrane and the conformation adopted when membrane-bound. It should be noted that this domain posses an amino acid sequence (NH_3^+-^{169}TWQTVTIFVAGVLTASLTIWKKMG^{192}-COO^-) with two Trp residues flanking a 19-residue hydrophobic sequence and two Lys residues near the C-end, very characteristic of a domain with the ability to insert in a membrane.

We have used different NMR techniques to investigate the mode of interaction of the C-terminal domain of Bax (Bax-C) with membranes and the effect of incorporating negatively charged phospholipids, such as POPG, on peptide–lipid interactions. Results indicate that Bax-C may have electrostatic interactions with the negatively charged phospholipids but it also interacts with the hydrophobic part of the membrane. Furthermore, this membrane interaction with the membrane will differ depending on the presence or absence of POPG, possibly because electrostatic interactions are a prerequisite for a correct insertion in the membrane.

2. Materials and methods

2.1. Materials

1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-[phospho-*rac*-(1-glycerol)] (POPG) were purchased from Avanti Polar Lipids (Alabaster, AL, USA). The synthetic Bax C-terminal domain peptide (Bax-C) including residues 169–192 of Bax (NH_3^+-^{169}TWQTVTIFVAGVLTASLTIWKKMG^{192}-COO^-) was obtained from Peptide Protein Research Ltd. (Wickham, Hampshire, UK). 2,2,2-Trifluoroethanol (TFE) was from Sigma (Madrid, Spain). All other reagents and solvents were commercial samples of the highest purity.

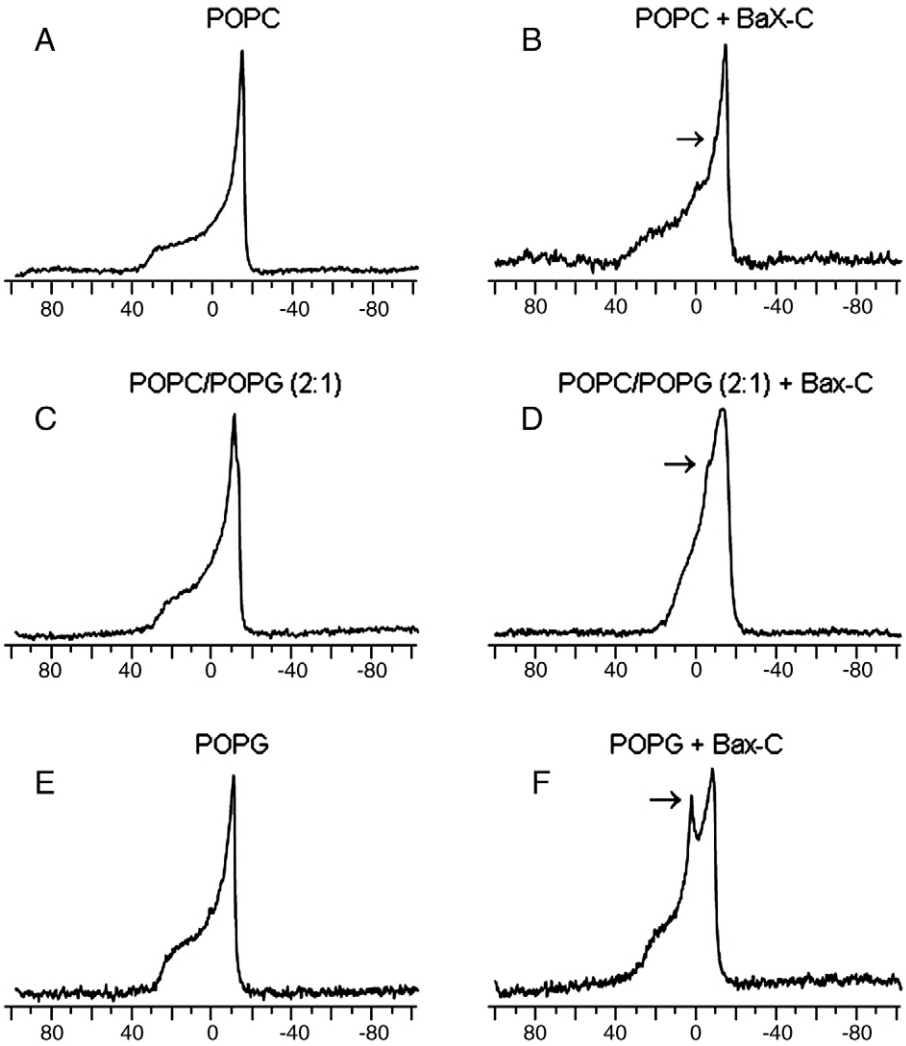

Fig. 1. Static ^{31}P NMR spectra of the different phospholipid mixtures in the presence (right panels) and in the absence (left panels) of Bax-C. The spectra were collected at a temperature of 25 °C. Arrows point to the isotropic peaks.

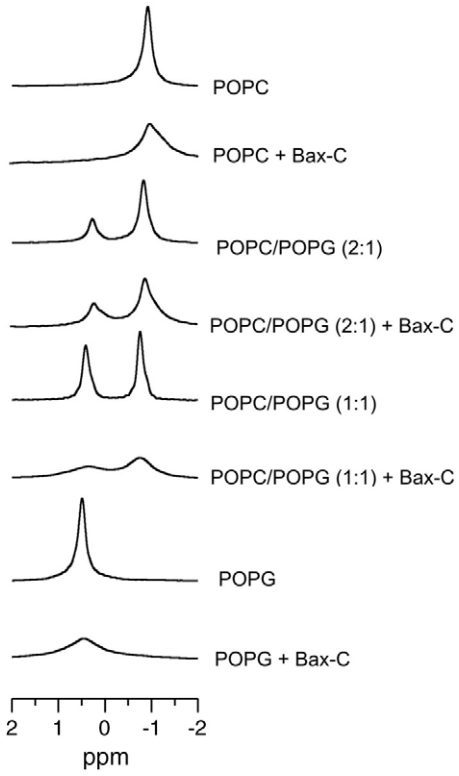

Fig. 2. ^{31}P MAS-NMR spectra of different POPC and POPG mixtures (MLVs) in the presence and in the absence of Bax-C. A spinning speed of 4 kHz was applied to obtain the spectra and the temperature was 25 °C.

2.2. NMR sample preparation

POPC, POPC/POPG (2:1), POPC/POPG (1:1) and POPG in solution of chloroform in the presence and in the absence of Bax-C dissolved in trifluoroethanol were dried under a stream of oxygen-free N_2 gas. Residual chloroform was removed under high vacuum for at least 3 h. Further 200 μl of trifluoroethanol were added, the samples were mixed, and the solvent was removed again by N_2 and high vacuum. When the peptide was present, the final lipid/peptide molar ratio was 10:1. To obtain good quality spectra, a total amount of 24 mg of phospholipids and an appropriate amount of Bax-C were used. Finally, the samples were resuspended in 50 μl of 10 mM Hepes, pH 7.4, 1 mM DTT and multilamellar vesicles (MLVs) were produced by vigorous vortexing. In order to check that the samples were well homogenized an alternative procedure was employed in some cases and samples were subjected to 3 cycles of rapid freezing and thawing but the results obtained were identical when doing this additional step and without it (not shown).

2.3. NMR spectroscopy

^{31}P and ^{13}C MAS-NMR spectra were obtained using a Bruker Avance 600 instrument (Bruker, Etlingen, Germany) operating at a frequency of 600.1 MHz for the proton and of 242.9 and 150.9 MHz for ^{31}P and ^{13}C respectively. A PH MAS Rotor ZrO2 BL_4 and a double-resonance 4 mm MAS broadband probe from Bruker were used for the observation of both nuclei under CW high power proton decoupling using a field strength, during acquisition, of 50 kHz. The spectra were obtained at a temperature of 25 °C and at a spinning speed of 4 kHz. A spectral width of 19,380 (^{31}P) or 31,746 (^{13}C) Hz, with 1352 (^{31}P) or 2156 (^{13}C) data points, an acquisition time of 0.034 s and a recycle time of 5 (^{31}P) or 4 (^{13}C) s were used. To obtain the one-dimensional MAS ^{31}P and ^{13}C spectra, 5 and 5.75 μs pulse respectively were used with gated decoupling ^{1}H. The T_1 experiments were performed using the inversion-recovery method as previously described [26], with ^{1}H decoupling and 10 different delay times at raising temperatures from 10 °C to 40 °C with 5 °C steps for ^{31}P from POPC/POPG (1:1) and POPG in the presence and absence of Bax-C, from 5 °C to 45 °C with 10 °C steps for ^{31}P from POPC and POPC with Bax-C and only at 25 °C for ^{13}C.

The same samples and the same spectrometer operating at the same frequencies were also used to collect static ^{31}P-NMR spectra. The samples were placed into 5 mm OD NMR glass tubes and the spectra were recorded at 25 °C. All spectra were obtained in the presence of a gated broad band proton decoupling (5 W input power during acquisition time), and accumulated free inductive decays were obtained from up to 8000 scans. A spectral width of 48,536 Hz, a memory of 48,536 data points, a 2 s interpulse time, and a 90° radio frequency pulse (11 μs) were used with inverse gated decoupling ^{1}H. Prior to Fourier transformation, an exponential multiplication was applied, resulting in a 100 Hz line broadening.

DMFIT programme [27] was used to calculate the percentage of isotropic component in samples studied using static ^{31}P-NMR.

2.4. Fluorescence spectroscopy

The steady state fluorescence emission spectra of Bax-C was measured using a Fluoromax-3 fluorescence spectrometer (Jobin Yvon, Longjumeau, France) equipped with a thermostated quartz cuvette with constant stirring at 25 °C. 0.48 μmol of POPC, POPC/POPG (1:1) and POPG in solution of chloroform in the absence and presence of an appropriate amount of Bax-C dissolved in TFE and a solution of Bax-C without lipids were dried under a stream of N_2 oxygen-free and then under high vacuum for at least 2 h. MLVs were generated by hydrating the sample with 3 ml of the same buffer used for NMR experiments and vortexing. Emission spectra were collected between 320 and 400 nm, exciting at a wavelength of 285 nm with a bandwidth of 5 nm. In all cases background intensities due to the buffer and the lipids emission were subtracted from the samples spectra.

Acrylamide quenching experiments were carried out using the same spectrometer, cuvette and conditions described above for the steady state fluorescence emission experiments. Samples were prepared as described above except that the MLVs were finally sonicated to obtain small unilamellar vesicles (SUVs). Trp fluorescence was detected between 320 and 400 nm and the excitation λ was 290 nm. Fluorescence was quenched by the progressive addition of small aliquots of a 7.5 M acrylamide solution to obtain increasing concentrations ranging from 0 to 0.5 M. Results were elaborated and plotted according to the modified Stern–Volmer equation proposed by Lehrer [28].

3. Results

3.1. Static ^{31}P-NMR-NMR

The phase behavior of aqueous dispersions of the phospholipids in the absence and in the presence of Bax-C was studied using static

Table 1
^{31}P MAS-NMR isotropic chemical shift and full width at half height (FWHH) of POPC and POPG.

	^{31}P from POPC		^{31}P from POPG	
	Chemical shift (ppm)	FWHH (ppm)	Chemical shift (ppm)	FWHH (ppm)
POPC	−0.86	0.21		
POPC + Bax-C	−0.93	0.42		
POPC/POPG (2:1)	−0.79	0.21	0.31	0.23
POPC/POPG (2:1) + Bax-C	−0.84	0.27	0.25	0.60
POPC/POPG (1:1)	−0.72	0.16	0.45	0.22
POPC/POPG (1:1) + Bax-C	−0.73	0.59	0.38	1.01
POPG			0.48	0.19
POPG + Bax-C			0.45	0.91

The values are of different type of POPC/POPG mixtures with and without Bax-C.

(anisotropic) ^{31}P-NMR at 25 °C and results are depicted in Fig. 1. The ^{31}P-NMR of pure POPC was asymmetric with a high-field peak and a low-field shoulder, characteristic of an axially symmetrical shift tensor and consistent with the arrangement of the phospholipids in a bilayer configuration, characteristic of MLV in the L_α phase. The corresponding CSA was 45 ppm (Fig. 1A). The addition of Bax-C induced some changes since CSA was now 42 ppm (Fig. 1B), indicating a certain disordering of the lipid headgroup. In addition, an increase in the intensity of the centre region of the spectrum and a narrowing of the spectrum was observed which may indicate a reduction in the diameter of the MLV [29]. Finally a very small isotropic peak was observed.

The lineshape of the spectrum corresponding to the mixture POPC/POPG (2:1 molar ratio), again indicated the presence of MLV in a L_α phase. Since a mixture with two phospholipids was present here, the observed spectrum is a superposition of the spectra corresponding to each one of these two phospholipids. However, it is noteworthy that only a small shoulder was noted on the high-field peak indicating that although the two spectra are not precisely identical they cannot be resolved in the static case. CSA was 34 ppm (Fig. 1C) which is a clearly lower value than that observed for pure POPC indicating a direct interaction between the polar groups of POPC and POPG as it has been already observed and discussed (see for example [30]). In the presence of Bax-C a further reduction in the width of the spectrum was observed with a CSA of 28 ppm (Fig. 1D). This indicated a disordering of the polar part of the membrane and a reduction in the size of the vesicles as a consequence of the incorporation of the peptide, being in agreement with previous laser light scattering studies [24]. It is also remarkable that an isotropic peak is now clearly visible, indicating that t he interaction with POPG may lead to the formation of small vesicles or to the formation of non-lamellar phases in the membrane such as lipidic particles. The isotropic component was 8% of the total spectrum.

In the case of pure POPG the spectrum showed again a pattern characteristic of MLV in a L_α phase but with a lineshape indicative of vesicles with lower size than those of pure POPC, as pointed out by the increase in the intensity of the lower field peak and the decrease in width, with a CSA of 33 ppm. In the presence of Bax-C the spectrum observed showed a more reduced width and a CSA of 28 ppm (Fig. 1E). It should be underlined that a clear isotropic peak was now present, indicating that the interaction between Bax-C and POPG leads to either very small vesicles or to non-isotropic phases embedded in MLV of conventional size. The isotropic component amounted to 16% of the total spectrum.

3.2. ^{31}P-MAS-NMR

In order to better discern the effect of Bax-C on the phospholipids, high resolution ^{31}P-MAS-NMR was used. Fig. 2 shows the spectra obtained from different phospholipid mixtures and the characteristics of the different peaks are summarized in Table 1. Resonances from the individual bilayer components were well resolved. In this way pure POPC spectrum showed an isotropic resonance at −0.86 ppm and pure POPG an isotropic resonance at 0.48 ppm.

When the membrane studied contained mixtures of POPC and POPG it was possible to see separate resonances coming for each one of the lipids and the sizes of the peaks reflected the composition. It was observed that both resonances moved downfield as the fraction of negatively charged lipid was increased (Table 1) these changes being in agreement with other previously published for phosphatidylcholine/phosphatidylglycerol mixture [31,32]. The shifts were from 0.48 ppm (pure POPG) to 0.45 ppm when an equimolar sample was studied and to 0.31 ppm when the molar ratio of POPC/POPG was 2:1. Similarly, whereas the ^{31}P-resonance corresponding to POPC appeared at −0.86 ppm when the pure POPC sample was studied, it moved to −0.79 for the POPC/POPG (2:1 molar ratio) sample and to −0.72 for the sample with equimolar ratio. Since both phosphates undergo changes in their CS of the same sign, a common response to a change in the membrane surface charge must be inferred.

These CS were moved upfield by the presence of Bax-C as it could be expected to result from a partial charge screening between negative lipid head groups and positive amino acids of the peptide as shown previously for other systems [31,32]. However the effect was modest (Table 1), possibly indicating that the positively charged residues are not in close proximity to the negatively charged phosphates of POPG molecules. In this way, pure POPG spectrum showed an isotropic peak with a CS of 0.48 ppm which moved to 0.45 ppm in the presence of Bax-C. In the case of pure POPC, CS was −0.86 ppm in the absence and −0.93 ppm in the presence of the peptide. In mixtures POPC/POPG (Table 1) the same trend was observed, so that for POPC/POPG (2:1 molar ratio), ^{31}P-POPC CS was −0.79 ppm in the absence of Bax-C and −0.84 ppm in its presence, whereas ^{31}P-POPG CS was 0.31 ppm in the absence and 0.25 ppm in the presence of Bax-C. Finally, spectra corresponding to POPC/POPG with 1:1 molar ratio, produced ^{31}P-POPC CS of −0.72 ppm in the absence and −0.73 ppm in the presence of Bax-C, whereas for ^{31}P-POPG CS was 0.45 ppm in the absence and 0.38 ppm in the presence of Bax-C.

More important was the considerable widening in FWHH (Table 1) which increased from 0.21 ppm (pure POPC) to 0.42 ppm in the

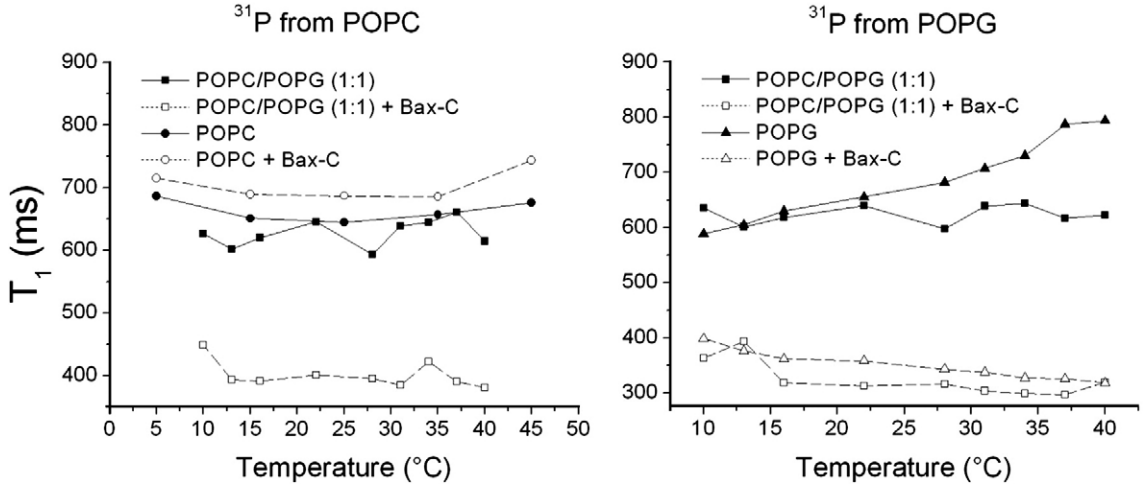

Fig. 3. ^{31}P spin-lattice relaxation times (T_1) of POPC (left panel) and POPG (right panel) in different mixtures in the presence and in the absence of Bax-C. T_1 values are reported as a function of temperature between 5 °C and 45 °C with 10 °C increments for POPC and POPC+ Bax-C and between 10 °C and 40 °C with 5 °C increments for the other samples.

presence of Bax-C, indicating a decrease in phospholipid mobility. A widening of this type has previously been observed for dimyristoyl-phosphatidylglycerol in the presence of the $A\beta_{1-40}$ peptide, when it was also interpreted as a restriction in the mobility of the phospholipid [32] and for 1-palmitoyl-2-oleoyl-*sn*-3-phosphatidic acid in the presence of the C2 domain of PKCε where a mobility restriction was also deduced [33]. In the case of pure POPG, FWHH was 0.19 ppm in the absence and 0.91 ppm in the presence of Bax-C indicating a very substantial reduction in mobility.

Table 1 also shows that for the POPC/POPG (1:1 molar ratio) the ^{31}P-POPC exhibited a FWHH of 0.16 ppm in the absence and 0.59 ppm in the presence of Bax-C, whereas ^{31}P-POPG showed 0.22 ppm in the absence and 1.01 ppm in the presence of Bax-C.

3.3. ^{31}P-relaxation

The ^{31}P spin-lattice relaxation times, T_1, of each phospholipid in the membrane were simultaneously determined from the high resolution MAS-NMR spectra, which were obtained at different temperatures (Fig. 3).

The addition of Bax-C to pure POPC did not substantially modify T_1 at any temperature (Fig. 3), and only a modest increase was observed, probably indicating that the peptide–lipid interaction slightly reduces the axial rotational motion which could make the T_1 relaxation mechanism less efficient [30].

When the sample POPC/POPG (1:1 molar ratio) was studied, the ^{31}P-POPC resonance (Fig. 3A) T_1 values were considerably lower in the

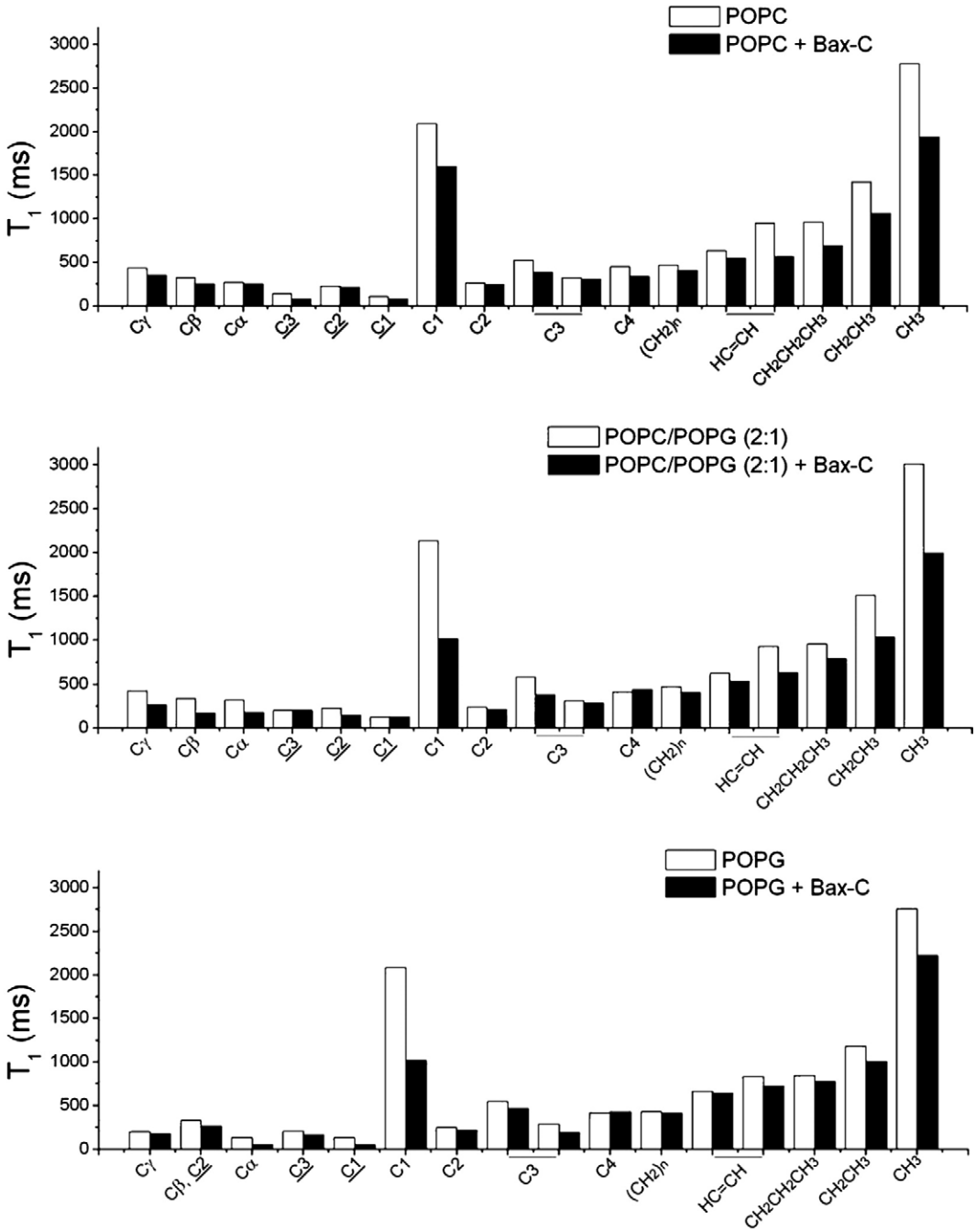

Fig. 4. ^{13}C spin-lattice relaxation times in the laboratory frame (T_1) for POPC (top), POPC/POPG (2:1) (middle) and POPG (bottom) MLVs in the presence and absence of Bax-C. The different carbon sites of the phospholipids are depicted in the abscissa.

Table 2
^{13}C MAS-NMR isotropic chemical shift and spin-lattice relaxation times (CT_1) for POPC in the presence and absence of Bax-C.

	Assignment	POPC			
		Without Bax-C		With Bax-C	
		Chemical shift (ppm)	CT_1 (ms)	Chemical shift (ppm)	CT_1 (ms)
Polar head group	Cγ	54.6	435.4	54.5	352.2
	Cβ	66.5	326.0	66.5	251.5
	Cα	60.0	269.9	60.0	254.7
Interfacial region	*C3*	64.2	139.7	64.9	78.9
	C2	71.2	226.7	71.0	217.0
	C1	63.6	108.3	63.5	78.9
Hydrocarbon chains	C1	174.0	2091.0	174.0	1603.8
	C2	34.6	267.3	34.5	249.1
	C3	26.1	525.2	26.0	386.8
	C3	25.6	322.8	25.5	305.7
	C4	27.8	452.6	27.7	339.6
	$(CH_2)_n$	30.8	467.3	30.4	408.8
	HC = CH	130.2	638.1	130.1	547.2
	HC = CH	128.4	951.9	128.3	566.0
	$CH_2CH_2CH_3$	32.6	963.3	32.5	691.8
	CH_2CH_3	23.2	1421.0	23.2	1062.3
	CH_3	14.4	2779.0	14.4	1949.7

Table 4
^{13}C MAS-NMR isotropic chemical shift and spin-lattice relaxation times (CT_1) for POPC/POPG (2:1) in the presence and absence of Bax-C.

	Assignment	POPC/POPG (2:1)			
		Without Bax-C		With Bax-C	
		Chemical shift (ppm)	CT_1 (ms)	Chemical shift (ppm)	CT_1 (ms)
Polar head group	Cγ	54.8	424.9	54.6	265.6
	Cβ	66.8	335.7	66.5	170.8
	Cα	60.3	316.0	60.1	179.8
Interfacial region	*C3*	64.2	198.6	64.2	200.0
	C2	71.4	226.2	71.2	143.4
	C1	63.8	127.5	63.1	130.1
Hydrocarbon chains	C1	174.2	2133.0	174.1	1014.0
	C2	34.8	240.5	34.6	211.3
	C3	26.3	584.9	26.1	381.6
	C3	25.8	309.6	25.6	281.7
	C4	28.1	412.1	27.8	439.1
	$(CH_2)_n$	31.0	470.7	30.5	405.5
	HC = CH	130.4	623.8	130.2	535.2
	HC = CH	128.7	928.9	128.5	632.7
	$CH_2CH_2CH_3$	32.8	957.0	32.6	789.7
	CH_2CH_3	23.5	1511.0	23.2	1030.0
	CH_3	14.6	3009.0	14.5	1991.0

presence of Bax-C, going down from values around 600 ms in the absence to 400 ms in the presence of Bax-C. Similar results were observed for the ^{31}P-POPG both in pure POPG and in POPC/POPG (1:1 molar ratio), with a remarkable decrease in T_1 (Fig. 3). This reduction in T_1 would be the result of the presence of the protein that is interacting electrostatically with the negatively charged surface of the membrane.

3.4. ^{13}C-MAS-NMR

The ^{13}C-MAS-NMR spectra of pure POPC in the absence and in the presence of Bax-C were obtained (not shown) and each sample produced a well resolved ^{13}C NMR spectrum, in which there are three main spectral regions: the carbonyl groups (near 170 ppm), the double bond (near 130 ppm) the polar head group and the glycerol backbone (55–72 ppm), and the aliphatic acyl chains of the phospholipid molecule (10–40 ppm). Most of the carbons in the POPC molecule were assigned with the base of previously reported spectra, and the chemical shift values are very close to those found by Forbes et al. [34] and Haberkorn et al. [35]. In the presence of Bax-C, most of the resonances appeared with the same CS, and the only relatively minor modification in the spectrum was the widening of the doublet corresponding to carbons 1 and 3 of glycerol (not shown).

^{13}C-MAS-NMR spectra of pure POPG in the absence and in the presence of Bax-C were also obtained and each sample gave a well resolved ^{13}C NMR spectrum in which, similarly to what was described above for the POPC spectrum, three main spectral regions can be distinguished, the carbonyl groups (near 170 ppm), the double bond (near 130 ppm), the glycerol group making the polar head group and the glycerol backbone (60–72 ppm), and the aliphatic acyl chains of the phospholipid molecule (10–40 ppm). Most of the carbons in the POPG molecule can be assigned in this spectrum (not shown). The assignments of the hydrophobic parts are based on previously reported spectra mentioned above for POPC and the polar parts on the work by Lau et al. [36]. The chemical shift values were very close to those previously reported. In the presence of Bax-C, most of the resonances appeared with the same CS, and the only relatively small modification in the spectrum was the widening of the doublet corresponding to *C1* and .Cγ (not shown).

The ^{13}C-MAS-NMR spectra of the mixture POPC/POPG in the absence and in the presence of Bax-C were similar to those of pure POPC and POPC/Bax-C respectively (not shown).

Table 3
^{13}C MAS-NMR isotropic chemical shift and spin-lattice relaxation times (CT_1) for POPG in the presence and absence of Bax-C.

	Assignment	POPG			
		Without Bax-C		With Bax-C	
		Chemical shift (ppm)	CT_1 (ms)	Chemical shift (ppm)	CT_1 (ms)
Polar head group	Cγ	63.1	201.5	63.1	176.1
	Cβ	71.6	329.8	71.6	261.0
	Cα	64.5	133.0	64.5	50.0
Interfacial region	*C3*	67.3	205.4	67.3	165.2
	C2	71.6	329.8	71.6	261.0
	C1	63.8	130.8	63.7	50.0
Hydrocarbon chains	C1	174.4	2083.0	174.3	1016.0
	C2	34.7	249.3	34.7	215.3
	C3	26.2	546.4	26.2	467.7
	C3	25.7	290.8	25.7	190.0
	C4	27.9	421.5	27.9	428.3
	$(CH_2)_n$	30.9	433.1	30.6	412.2
	HC = CH	130.4	666.8	130.3	649.7
	HC = CH	128.6	833.9	128.5	728.0
	$CH_2CH_2CH_3$	32.8	847.2	32.8	774.6
	CH_2CH_3	23.4	1181.0	23.4	1008.0
	CH_3	14.6	2757.0	14.6	2223.0

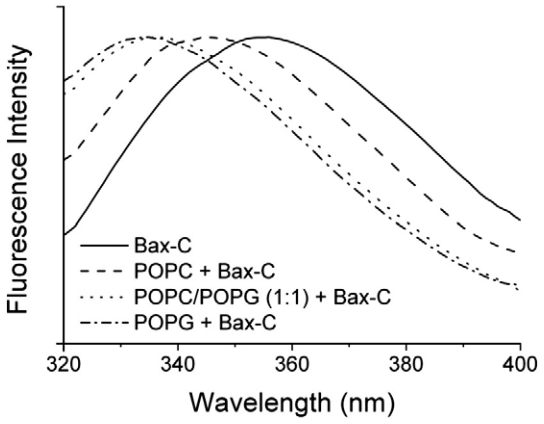

Fig. 5. Fluorescence emission spectra for Bax-C alone and in the presence of POPC, POPC/POPG (1:1) and POPG MLVs. The excitation wavelength was 285 nm and the temperature 25 °C. All spectra were normalized at the emission maximum after subtracting the contribution from buffer and lipid vesicles.

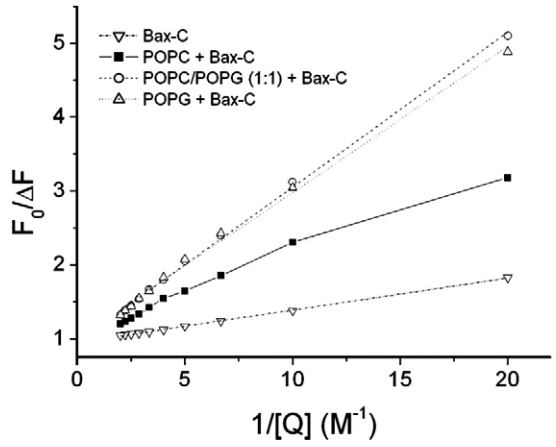

Fig. 6. Modified Stern–Volmer plot of tryptophan fluorescence quenching of Bax-C by acrylamide. Fluorescence quenching of Bax-C in the presence of different lipid SUVs: POPC, POPC/POPG (1:1) and POPG.

3.5. CT_1 relaxation time measurements

The spin-lattice relaxation times in the laboratory frame (T_1) is dominated by dipolar interactions between the ^{13}C and bonded protons. In the fast motional regime, appropriate for the fluid state of lipid bilayers, the more rapid the motion, the longer will be the T_1. Carbons without bonded protons often have longer T_1 values.

Fig. 4 shows that CT_1 corresponding to all phospholipid carbons decreased in the presence of Bax-C although some of them decreased more significantly than others. In the case of POPC, there was a fall in T_1 corresponding to the hydrophobic portions of the phospholipid molecule (see Table 2) such as the terminal methyl group, the carbons just preceding the last one and the carbons located in the centre of the hydrophobic core as those forming double bonds. Drops in T_1 were also observed in carbons corresponding to the polar part.

A bigger effect of Bax-C on the C1 (carbonyl ester) was observed in the samples containing POPG, namely pure POPG (Table 3) and POPC/POPG (2:1 molar ratio) (Table 4), indicating a reduction in the motion occasioned by the presence of Bax-C and its interaction with the polar part of glycerol, presumably by electrostatic interaction.

3.6. Fluorescence spectroscopy

Fluorescence spectroscopy based on the two Trp residues of Bax-C was carried out to confirm the different location of these residues and hence the different location of the peptide, as a function of the lipid composition of the membrane. Fig. 5 shows that the maximum of the emission spectrum was shifted towards lower wavelengths when POPG was present, and the emission maximum shifted from 355 nm in Bax-C in solution and in the absence of lipids, to 345 nm in the presence of POPC, 336 nm with POPC/POPG (1:1 molar ratio) and 334 nm with pure POPG.

Acrylamide quenching (Fig. 6) showed that the Trp residues of Bax-C were less accessible to the fluorescence quencher when POPG was present in the membrane, so that, according with the modified Stern–Volmer plot, K_Q was about 5 M^{-1} in the presence of a POPC/POPG mixture (1:1 molar ratio) or pure POPG. However the accessibility was bigger in the presence of pure POPC, and even bigger for Bax-C in the absence of phospholipids with a K_Q of 33 M^{-1}. These results clearly indicate a change in the location of the Trp residues in the presence of POPG. These results were compatible with those described above after the emission maxima, suggesting a more hydrophobic microenvironment for the Trp residues in the presence of POPG. It is also interesting that a deviation from linearity was observed for Bax-C in the presence of pure POPC indicating different locations for the Trp residues.

4. Discussion

In healthy cells pro-apoptotic Bax exists in soluble form in the cytosol. In this state the C-terminal domain is tucked within the hydrophobic groove, but when the cell machinery is prompted for apoptosis, the C-terminal domain inserts in the mitochondrial outer membrane [2]. Little is known above the role played by the C-terminal domain of Bax, whether it is just a hydrophobic anchor or a signal peptide which must be recognized by a receptor in the target membrane or a contributor to membrane permeabilization. The amino acid sequence of this domain is very interesting since it comprises a hydrophobic stretch of 19 residues which begins and ends with a Trp residue (spanning from residue 170 to 188) plus a C-terminal stretch of 4 residues which contains 2 Lys. If we predict that each one of the Trp residues will be located at the lipid–water interface as it has been observed in many other cases [37] the 19mer peptide stretch which includes the segment from one Trp to the other will need to be straight and perpendicular to the membrane plane to fully span the membrane. We observed in a previous work that this was the case when the insertion of Bax-C in membranes was studied, but this was true only in the presence of phospholipids with negative charge [25]. Our results show that the interaction of Bax-C with model membranes varies depending on the presence or absence of negatively charged phospholipids, such as POPG. This is illustrated by the fluorescence spectra, showing a shift towards the blue and a more restricted access to acrylamide quenching in the presence of POPG with respect to POPC or Bax-C alone. These results indicate that Trp residues are located in a less polar environment in the presence of POPG. Very interesting is that when Bax-C was studied in the presence of pure POPC a deviation from linearity in the quenching plot was observed indicating that not all Trp residues were equally accessible to the quencher. Note that a transmembrane location of the peptide implies a similar accessibility for all Trp residues.

When static ^{31}P-NMR was used, Bax-C introduced disorder in the polar part in all the compositions studied as detected by a reduction in

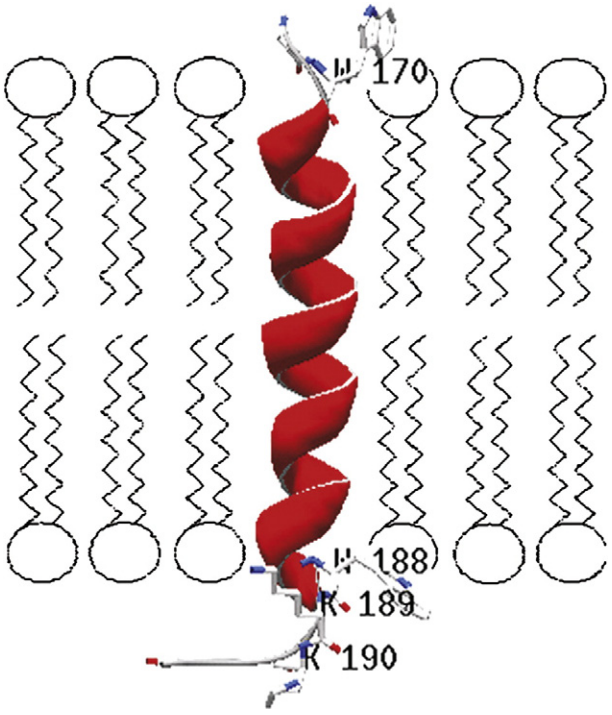

Fig. 7. Schematic representation of the orientation of Bax-C peptide inserted into the membrane. Bax-C lies perpendicular to the membrane normal with the two Trp residues at the lipid–water interface and the two Lys protruding. Trp and Lys residues are shown as CPK sticks.

CSA, including pure POPC, but especially when POPG was present. In the presence of POPG it was possible to detect the appearance of isotropic peaks which may arise from vesicular or micellar structures within the bilayers [38], or for very small vesicles induced upon peptide binding, or as a result of motional or orientational averaging without involvement of non-bilayer structures [39].

^{31}P-MAS-NMR showed some upfield shifts induced by the presence of Bax-C, although the shifts were modest (0.07 ppm as the most). In other electrostatic interactions values of up to 0.5 ppm between extrinsic peptides or proteins and negatively charged membranes have been reported [31,33]. Upfield shifts of CS can be interpreted as a compensation of the negative surface charge by the positively charged peptide and the possible reason might be that the positive charges are not located in the immediate vicinity of the phosphorus atoms. This could be the case if K-189 and K-190 are out of the membrane. However, the width of the peaks increased substantially more in the presence of the peptide than in its absence, and this applied to both the ^{31}P from POPG and from POPC, but especially in the presence of POPG, indicating that the peptide imposes restrictions in the motion of both phospholipids but more for POPG. Since the width of the peaks is inversely related with T_2 relaxation times, this means that a reduction in the slowest motion of the polar head group is imposed by the presence of Bax-C [40] and this applies to both POPG and POPC when both phospholipids are present but the effect was bigger for POPG. Note that once POPG is present, the effect affects both POPC and POPG and this suggest that the presence of POPG is decisive to determine the location of the peptide with respect to the membrane.

A considerable reduction in T_1 of the phosphorus nuclei corresponding to pure POPG was also observed. Similar results have already been seen for the binding of basic proteins to membranes containing negatively charged phospholipids. For example, this type of effect was observed for cytochrome *c*, when it was interpreted as being caused by restrictions of the headgroup motion that lower the spectral density of fast motions (on the 10^{-9} s timescale) of the phosphorus atom, as a consequence of binding to the protein [40]. Other basic proteins, which have been shown to reduce the T_1 values of ^{31}P of negatively charged phospholipids are poli-(L-lysine), myelin basic protein, lysozyme or ribonuclease [40] and the C2 domain of PKCε [33].

When pure POPC was studied, Bax-C did not reduce T_1 values but, on the contrary, it slightly increased them, confirming that the interaction of the peptide with membranes changed in the presence of POPG. However it should be underlined that in POPC/POPG mixtures a reduction in T_1 corresponding to both phospholipids was observed. Other previous authors have also reported that when positively charged proteins interact with membranes containing a mixture of a negatively charged phospholipid and a zwitterionic phospholipid, T_1 of both are affected [41,42], probably indicating that peptides induced a slower molecular motion of all phospholipids.

^{13}C-NMR showed that bigger reductions were produced by Bax-C on CT_1 when POPG was present. In the case of POPC the reduction similarly affected the polar and the hydrophobic part. When POPG was present the main change was that Bax-C induced a bigger reduction in CT_1 of some carbons of the polar part of the phospholipids like C1 (carbonyl ester) and *C1* (carbon 1 of glycerol) indicating a restriction in mobility occasioned by the peptide.

The fluorescence spectra and the quenching experiment also confirmed that, in the presence of POPG, the Trp residues are located in a more hydrophobic location, as it could be the membrane interface, whereas in the absence of POPG, Trp residues may be forming part of aggregated peptides or of peptides adopting β-pleated sheet structure.

Taken together, these results and our previous observations indicating that the presence of negatively charged phospholipids (like POPG) modulated the secondary structure and membrane orientation of Bax-C [25], suggest that this peptide inserts differently in the membrane in the presence and in the absence of POPG. When POPG is present, the peptide probably establishes electrostatic interactions with the membrane and afterwards it gets inserted into the membrane, adopting a predominantly α-helical structure in a nearly perpendicular orientation [25]. Bax-C locates each Trp residue at a lipid–water interface, with the two Lys residues protruding from the membrane (see Fig. 7). As a consequence of this location, the mobility of the ^{31}P nuclei belonging to POPG and to POPC are reduced and also the carbons belonging to the polar part of the membrane, and less so other carbon atoms located in the hydrophobic zone. This location of the Trp residues justifies the shift towards low wavelength in the fluorescence spectra and the acrylamide quenching restriction observed when POPG was present.

However, when POPG is not present, and the only membrane phospholipid is POPC, Bax-C shows an important percentage of secondary structure as β-pleated sheet and it is randomly oriented [25]. In this case, Bax-C produced less effect on the motions of the phosphorus nuclei and the reduction of the motions of the carbons was occurring in the polar part and in the hydrophobic part of the membrane. It seems that the Trp is not located in the hydrophobic/hydrophilic interface of the membrane and their fluorescence maxima occurred at a higher wavelength because the Trp residues are more accessible to water, as also shown by the fluorescence quenching experiment. It may be speculated that the peptides are forming aggregates, in which the Trp residues are accessible to water and acrylamide.

If it is admitted that Bax-C is nearly perpendicular to the plane of the membrane, the only polar residue found within the hydrophobic stretch of Bax-C is Ser-184. It has been shown that this residue is quite important to determine mitochondrial localization of Bax, so that mutation S184K led to a diffuse cytoplasmic localization of Bax [43] and we have also shown that mutation S184K led to a very reduced capacity to release carboxyfluorescein trapped in liposomes [24]. Ser is one of the polar residues most often found in transmembrane segments and it has been suggested that their presence in these segments may be explained by their ability to form hydrogen bonds with adjacent carbonyl hydrogens [44] and these small side chains may be associated to tight packing of helices [45] which allows one to suspect that the C-terminal domain may really be associated with other parts of Bax even when inserted in the membrane. Their ability to form pores on liposomes [24] is another indication of their possible implication in more roles than just being an anchor.

In conclusion, our results suggest that when Bax senses the signal indicating that it must insert into the membrane, and a conformational change unleashes the C-terminal part from the hydrophobic groove, this domains needs to interact with negatively charged phospholipids as a first step before getting inserted in to the membrane. The location adopted depends on the presence of these negatively charged phospholipids.

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