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## Protein oligomerization mediated by the transmembrane carboxyl terminal domain of Bcl-XL

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### ARTICLE INFO

#### Article history:

Received 9 June 2010

Revised 28 July 2011

Accepted 9 August 2011

Available online 16 August 2011

Edited by Gianni Cesareni

#### Keywords:

Apoptosis

Mitochondria

Bcl-XL

Oligomerization

Transmembrane domain

### ABSTRACT

**Bcl-XL is a pro-survival member of the Bcl-2 family that can be found in the outer mitochondrial membrane and in soluble cytosolic homodimers. Bcl-XL can bind pro-apoptotic members of this family preventing them from activating the execution phase of apoptosis. Bcl-XL has been shown to homodimerize in different ways, although most binding and structural assays have been carried out in the absence of its carboxyl terminal transmembrane domain. We show here that this domain can by itself direct protein oligomerization, which could be related to its previously reported role in mitochondrial morphology alterations and apoptosis inhibition.**

#### Structured summary of protein interactions:

**Vamp2** physically interacts with **Vamp2** by blue native page (View interaction)

**Vamp2** physically interacts with **Vamp2** by cross-linking study (View interaction)

**Bcl-XL** physically interacts with **Bcl-XL** by blue native page (View interaction)

**Bcl-XL** physically interacts with **Bcl-XL** by cross-linking study (View interaction)

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### 1. Introduction

Apoptosis is a type of cell death characterized by the silent elimination of unnecessary or damaged cells, which is required for development and tissue homeostasis. Two major routes of apoptosis have been described: the extrinsic route is initiated at the cell surface and mediated by death receptors, like Fas-CD95 [1]; the intrinsic route is mediated by mitochondria [2], where many different signals are integrated and the final decision about the fate of the cell is made. Both routes are linked in some cells by Bid, a member of the Bcl-2 protein family [3]. Bcl-2 family members participate directly in the decision step [4]. Pro-apoptotic members of this family, Bax and Bak, have been shown to permeabilize the mitochondrial outer membrane (MOM), facilitating the release to the cytosol of

intermembrane space (IMS) proteins like cytochrome c [5], AIF [6] or Smac/DIABLO [7,8]. These proteins then activate a series of caspase-dependent or -independent mechanisms that dismantle the cell. A second mechanism for the release of apoptotic factors from the IMS involves permeability transition, which allows the entry of water in the matrix with subsequent burst of the MOM [9].

A complex interplay between pro-apoptotic and pro-survival Bcl-2 family members controls MOM permeabilization (MOMP), considered the point-of-no-return during apoptosis induction. Several models have been proposed to explain how Bcl-2 members regulate MOMP through interactions between pro-survival and pro-apoptotic proteins, both in solution and at the MOM [10]. Activation of these proteins involves conformational changes, specially relevant for Bax, which usually resides in the cytosol and requires a conformational change to expose hydrophobic domains required for membrane insertion, followed by further changes necessary for pore formation through oligomerization at the MOM [11].

Homodimerization of the pro-survival protein Bcl-XL in the cytosol involves a C-terminal membrane-targeting  $\alpha$  helix from one monomer and a hydrophobic groove on the other monomer [12], although other dimerization modes have been described [13–16]. Release of that helix from the hydrophobic groove allows its insertion into the MOM. Most interaction and structural studies

*Abbreviations:* PSAP/Mtch1, presenilin 1-associated protein/mitochondrial carrier homolog 1; MOM, mitochondrial outer membrane; IMS, intermembrane space; MOMP, mitochondrial outer membrane permeabilization; TMD, transmembrane domain; mRFP, monomeric red fluorescent protein; VAMP2, vesicle-associated membrane protein 2

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carried out with this protein employed deletion mutants lacking the C-terminal helix. This mutant can also bind membranes through its N-terminal domain [17], and Bax, Bcl-2 and Bcl-XL have been reported to insert  $\alpha$ -helices 5 and 6 into the MOM during apoptosis [18–20]. Many interaction and structural studies have been carried out in the absence of membranes, underestimating their role in protein interactions and conformational changes [17,21].

Since the C-terminal helix (transmembrane domain or TMD) of Bcl-XL and flanking sequences contain the necessary information for MOM targeting and insertion in a C-in N-out orientation [22], we used this domain to target fragments of PSAP/Mtch1 to the outer mitochondrial membrane [23]. PSAP was first identified as a presenilin-1 associated protein with homology to inner membrane mitochondrial carriers, and therefore is also known as mitochondrial carrier homolog 1 (Mtch1) [24]. The importance of its closest homolog, Mtch2, in Bid-induced apoptosis has been reported recently [25]. We reported that PSAP is a MOM protein with two pro-apoptotic domains [23]. Since apoptosis induction by these domains could depend on interactions with other proteins, we sought to analyze PSAP interactions by crosslinking. These assays suggested that the TMD of Bcl-XL could be involved in oligomerization, which was confirmed analyzing fusions to monomeric red fluorescent protein (mRFP).

## 2. Materials and methods

### 2.1. General reagents

All reagents were of molecular biology grade. Restriction enzymes were from Roche, Stratagene, Invitrogen, Fermentas and New England Biolabs; Accuprime Pfx DNA polymerase, custom-made primers and T4 DNA Ligase were from Invitrogen; Pfu polymerase, from Stratagene.

### 2.2. Construction of expression vectors

Expression vectors containing PSAP or Mtch2 sequences have been previously described [23]. pJAC295, expressing myc-mRFP-TMD Bcl-XL (39.1 kDa), was constructed by amplifying the mRFP sequence from another vector by PCR with primers containing Eco RI (mRFP<sub>Eco</sub>F: 5'-CTAGGATCGAATTCGGATGGCCTCTCCGAG-GACGT-3') and Hind III (mRFP<sub>Hind</sub>R: 5'-CTAGGATCAAGCTTGGC GCCGGTGGAGTGGCGGC-3') restriction sites. The digested PCR product was used to replace PSAP sequences in the vector expressing myc-PSAP65-112-Bcl (which contains the TMD of Bcl-XL) [23]. The resulting vector expresses mRFP preceded by a myc tag and followed by the sequence KLESRKQERFNRWFLTGMTVAGVLL GSLFSRK, where the first two amino acids (kl) correspond to the Hind III site and the remaining amino acids, to the TMD of Bcl-XL.

The same approach was used to construct vector pAOC2, expressing myc-mRFP-TMD VAMP2 (32.2 kDa), replacing PSAP sequences in a vector that expresses myc-PSAP39-168-Vamp (which contains the TMD of VAMP2) [23]. In this case, sequence KLLKRYWWKLNKMMIILGVICAILLIHIVYFSS followed mRFP. pAL2 (28.7 kDa), expressing myc-mRFP, was constructed by digesting pJAC295 with Hind III and Not I, to eliminate the TMD of Bcl-XL, blunting with Pfu polymerase, and re-ligating. Vectors were transformed into *E. coli* JM109 from Promega. Correct clones were confirmed by restriction digestion and sequencing. pAOC5, expressing myc-Bcl-XL (full length Bcl-XL preceded by a myc tag, 28.6 kDa), was constructed by carrying out a PCR on a vector containing the cDNA for Bcl-XL with primers BCLXLECOF (5'-CTAGGATCGAA TTCGAATGTCTCAGAGCAACAACCGGAGC-3') and BLCLXLNOTR (5'-CTAGGATCGCGGCCGCTCATTTCGACTGAAGAGTGAG-3'). The PCR product was digested with Eco RI and Not I and inserted into

pCMVMyc. pAOC6, expressing myc-BclXL- $\Delta$ TMD (Bcl-XL without its carboxyl terminal transmembrane domain, preceded by a myc tag, 26.7 kDa), was constructed by PCR with primers BCLXLECOF and BCLXLWOTMXHOR (5'-CTAGGATCCTCGAGGGCTGCTGCATT GTTCCCATAG-3'). The product was digested with Eco RI and Xho I and cloned into pCMVMyc. pAOC7, expressing myc-BclXL-TMD2Pit2 (BclXL preceded by a myc tag and with its carboxyl terminal domain replaced by the second transmembrane domain of Pit 2, 27.3 kDa) was constructed by doing PCR on vector pVLG60 (pEGFpN1-PSAP-DTM1-Pit2TMD2, see Lamarca et al. [26]) with primers Pit2TMD2XhoF (5'-CTAGGATCCTCGAGAGGCAGGCATGCA TTTAGCTTC-3') and Pit2TMD2NotR (5'-CATGGTACGCGGCCGC TCATTTGGCCTAGTAACACGGAG), the product was digested with Xho I and Not I and used to replace the equivalent fragment in vector pAOC6. The sequence of the second transmembrane domain of Pit 2 (TMD2Pit2) used is RQACILASIFETTGSVLLGAK. pAOC8, which expresses myc-BclXL-TMD3Pit2 (BclXL preceded by a myc tag and with its carboxyl terminal domain replaced by the third transmembrane domain of Pit 2, 27.3 kDa), was generated by PCR with primers Pit2TMD3XhoF (5'-CTAGGATCCTCGAGATGGCTGGGGAAGTT AGTGC-3') and Pit2TMD3NotR (5'-CTAGGATCGCGGCCGCTCACAGG AAGGAAGCAATCAGCT-3') in the same way as done with pAOC7, but using vector pVLG61 (pEGFpN1-PSAP-DTMI-Pit2TMD3, see Lamarca et al. [26]) as template for the PCR. The sequence of the third transmembrane domain of Pit2 (TMD3Pit2) used is MAGEVSAMVGSVAVWQLIASFL.

### 2.3. Cell culture and transfection

HEK293 cells and HeLa cells were cultured as previously described [26] and transfected using GeneJuice Reagent (Novagen) at a confluence of 80% in 24-well plates.

### 2.4. Crosslinking and immunoblotting

12 h post-transfection, cells were washed twice with 500  $\mu$ l phosphate-buffered saline (PBS) and resuspended in 180  $\mu$ l crosslinking buffer (20 mM sodium phosphate, 150 mM NaCl, pH 7.5) containing 0.2 mM phenyl-methyl-sulfonyl-fluoride (PMSF). To detect de effect of expression of chimeras on Bax and Bak oligomerization, 12 h post-transfection cells were treated with 30  $\mu$ M camptothecin for 24 h, and then processed as indicated. Samples were divided in two tubes, 10  $\mu$ l of a 25 mM solution in DMSO of the amine-reactive membrane-permeant crosslinker BSOCOES (Bis[2-(succinimidooxycarbonyloxy)ethyl]sulfone) (Pierce) was added to one tube and 10  $\mu$ l DMSO to the second tube, and incubated for 1 h or 20 min (for Bax and Bak) at room temperature with agitation. 5  $\mu$ l quenching solution (1 M Tris HCl, pH 7.5) was then added and samples agitated for 15 min at room temperature. Cells were recovered by centrifugation and analyzed using SDS-PAGE (Laemmli) followed by immunoblotting with anti-myc (Invitrogen), anti-PSAP MGAS [26], anti-Bax (Millipore) or anti-Bak (Millipore) antibodies after transfer onto polyvinylidene fluoride (PVDF) membranes (Invitrogen), as described [23]. Proteins were visualized by incubation with goat-anti mouse or goat anti-rabbit secondary antibodies conjugated to HRP and detected by ECL. Molecular mass markers were from Invitrogen (BenchMark Pre-Stained Protein Ladder) or Bio-Rad (Precision Plus Protein Standards).

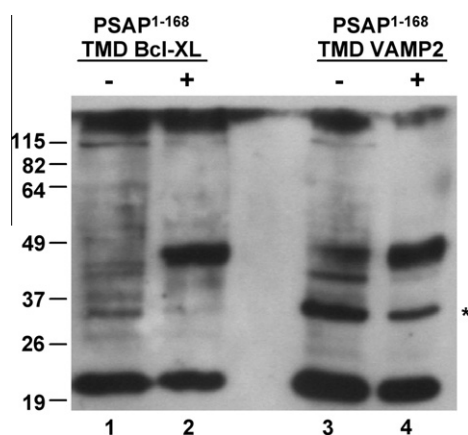
### 2.5. Blue-native electrophoresis (BN-PAGE)

BN-PAGE was carried out with digitonin-treated cells using the NativePAGE™ Novex® Bis-Tris Gel System from Invitrogen, 4–16% gradient gels, following instructions supplied by the manufacturer. 10  $\mu$ g protein was loaded per well. Native protein standards were from Invitrogen (NativeMark Unstained Protein Standard).

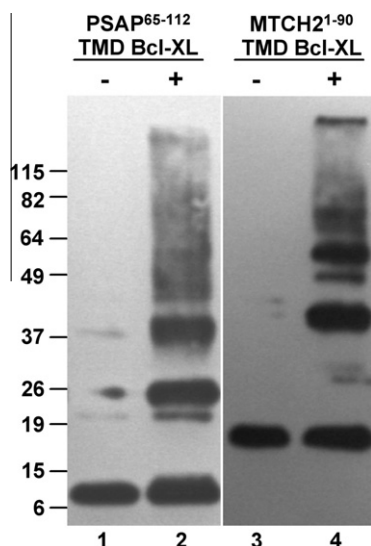
## 2.6. Cell viability and death assays

We used the Cell Proliferation Kit II (XTT) (Roche) as a quick method to measure cell death induced by camptothecin. HEK293 cells were cultured and transfected in 96-well plates, 12 h post-transfection cells were treated with 30  $\mu$ M camptothecin or DMSO and 24 h later they were incubated with XTT. Two independent assays were carried out in triplicate.

For trypan blue exclusion assays, HEK293 cells were cultured in 24-well plates until they reached 80% confluency and then transfected and treated with camptothecin or DMSO as indicated above. 24 h post-transfection wells were washed with PBS, which was transferred to tubes to avoid losing detached cells. Attached cells were trypsinized for 5 min and mixed with detached cells in PBS from the same well. Complete media, with serum, was added to inactivate trypsin. Cells were mixed with trypan blue (Roche) in a 1:1 ratio and counted using a hemacytometer. For each sample, four quadrants were scored twice, with cell numbers ranging



**Fig. 1.** Crosslinking of PSAP chimeras. Western blot of crosslinker-treated cells (+) or untreated (–) expressing PSAP1-168-TMD Bcl-XL or PSAP1-168-TMD VAMP2, separated on a 10% SDS gel and detected with anti-MGAS. Sizes of molecular mass markers are indicated on the left. Bands likely corresponding to heterodimers of PSAP1-168-TMD VAMP2 with endogenous VAMP2 are indicated with an asterisk on the right.

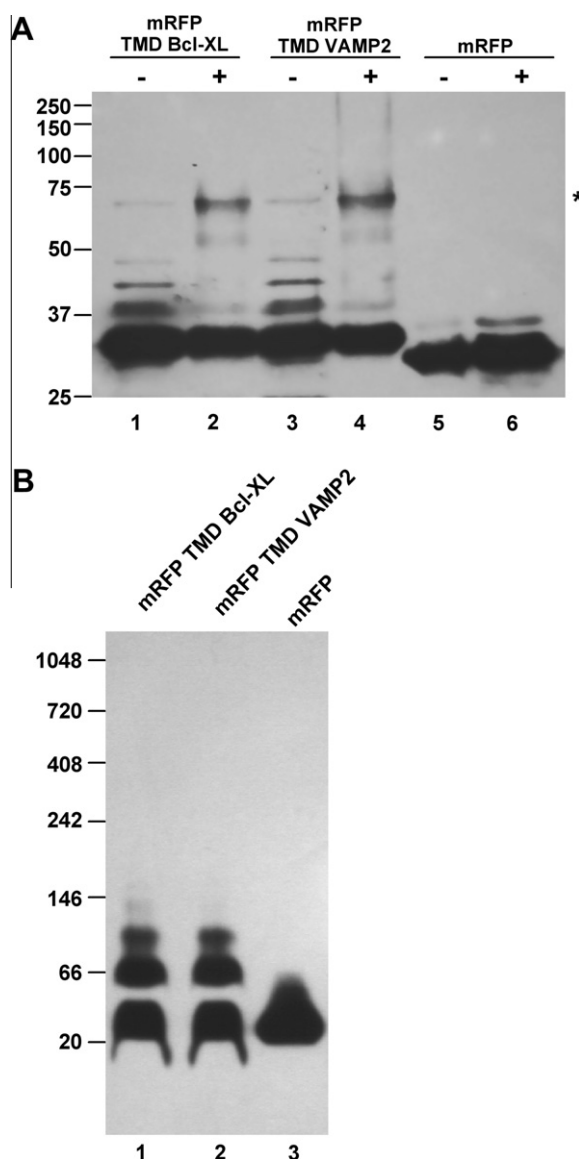


**Fig. 2.** Crosslinking of PSAP and Mch2 chimeras. Western blot of crosslinker-treated cells (+) or untreated (–) expressing myc-PSAP-65-112-TMD Bcl-XL or myc-Mch2-1-90-TMD Bcl-XL, separated on a 10% SDS gel and detected with anti-myc. Sizes of molecular mass markers are indicated on the left.

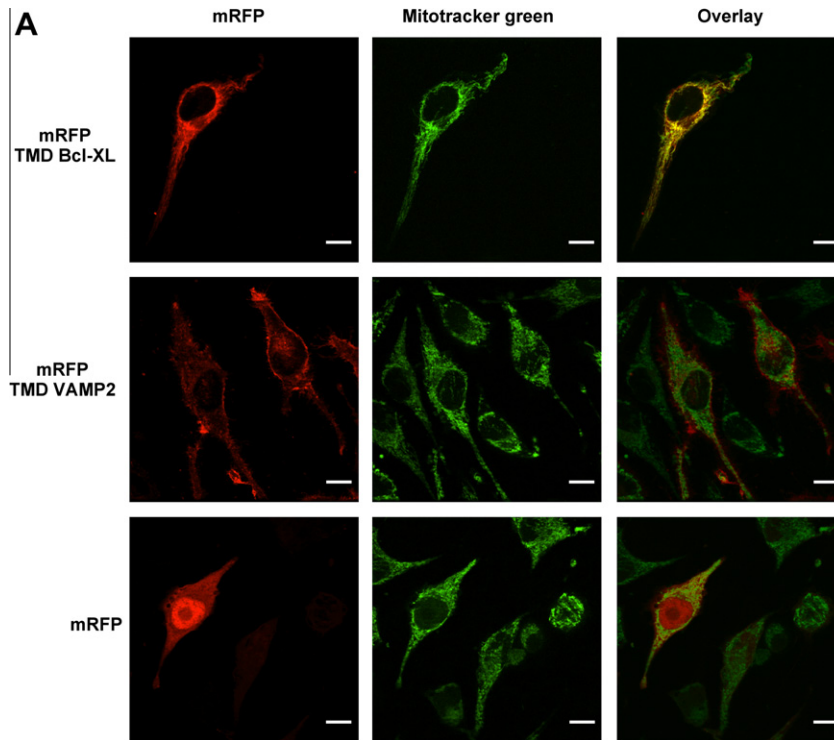
between 18 and 35 per quadrant. For one of the experiments an automatic cell counter (Countess from Invitrogen) was used, obtaining similar results as with manual counts. Each experiment was carried out in triplicates three independent times.

## 2.7. Immunocytochemistry

For subcellular localization of Bcl-XL chimeras, cells grown on round coverslides in 24-well plates were first incubated with 25 nM Mitotracker Red CMX-Ros (Invitrogen) for 30 min at 37 °C, the culture media replaced with fresh media and incubated again for 30 min at 37 °C. Cells were then fixed with a solution containing 50% culture media and 50% of a 3.7% formaldehyde solution in



**Fig. 3.** Crosslinking and blue-native electrophoresis of mRFP chimeras. (A) Western blot of crosslinker-treated (+) or untreated (–) cells expressing myc-mRFP-TM Bcl-XL, myc-mRFP-TMD VAMP2 or myc-mRFP alone, separated on a 10% SDS gel and detected with anti-myc. Sizes of molecular mass markers are indicated on the left. Bands corresponding to dimers (63.7 and 64.4 kDa) are indicated with an asterisk on the right. (B) Blue-native electrophoresis analysis of mRFP chimeras. Digitonin-solubilized proteins from cells expressing myc-mRFP-TMD Bcl-XL, myc-mRFP-TMD VAMP2 or myc-mRFP alone were separated on native 4–16% gradient gels, blotted and detected with anti-myc. Sizes of native molecular mass markers are indicated on the left.



**Fig. 4.** Subcellular localization of mRFP and Bcl-XL chimeras. (A) HeLa cells were grown on round coverslips, transfected with vectors encoding myc-mRFP-TMD Bcl-XL, myc-mRFP-TMD VAMP2 or myc-mRFP, incubated with Mitotracker green, fixed with formaldehyde and mounted for microscopy. (B) HeLa cells grown as in A and transfected with myc-BclXL, myc-BclXL- $\Delta$ TM, myc-BclXL-TMD2Pit or myc-BclXL-TMD3Pit, incubated with Mitotracker red CMX-Ros, with an anti-myc antibody, with goat anti-mouse Alexa fluor 488-conjugated antibody and mounted for microscopy. Images were acquired with a Leica DMI6000B inverted fluorescence microscope using structured illumination (Optigrid) with filters suitable for mRFP/Mitotracker Red and for Mitotracker green/Alexa fluor 488, and overlaid to show co-localization using software Metamorph. Bar is 10  $\mu$ m.

PBS for 5 min, followed by a 15-min incubation with 3.7% formaldehyde solution in PBS, and then blocked and permeabilized with a solution containing 1% BSA and 0.1% TX-100 in PBS for 1 h. After three 10-min washes with PBS, cells were incubated with an anti-myc antibody from Invitrogen at a 1:700 dilution for 2 h, washed with PBS and incubated with an Alexa Fluor 488-conjugated goat anti-mouse secondary antibody (Molecular Probes) for 1 h, washed again with PBS and mounted for microscopy using Fluoromount G (Southernbiotech).

Subcellular localization of mRFP chimeras was carried out as for Bcl-XL chimeras but using 100 nM Mitotracker Green.

### 2.8. Microscopy

We used a Leica DMI6000B inverted fluorescence microscope with structured illumination (Optigrid) and software Metamorph. For red fluorescent protein and Mitotracker red we used a BP560/40 excitation filter and a BP645/75 emission filter with a 595 nm dichroic, and for Mitotracker green and Alexa Fluor 488, we used a filter for GFP, excitation BP470/40, emission BP525/50 and 500 nm dichroic.

## 3. Results

In order to study PSAP function we generated several deletion mutants which were used to study mitochondrial import and induction of apoptosis by this protein [23]. We targeted PSAP fragments to the MOM by fusion to the TMD of Bcl-XL, demonstrating that MOM localization was required for apoptosis induction, since endoplasmic reticulum-targeting by fusion to the TMD of VAMP2 [27] did not induce apoptosis.

Since apoptosis induction by PSAP could be mediated by interactions with other proteins, we attempted to detect PSAP-interacting

proteins by crosslinking, using some of the above-mentioned chimeras. When HEK293 cells expressing the first 168 residues of PSAP fused to the TMD of Bcl-XL (PSAP<sup>1-168</sup>TMD Bcl-XL, 21 kDa) were treated with BSOCOES, a band was observed by western blot suggesting dimerization of the protein (Fig. 1, lane 2). Similar results were obtained with the protein containing the TMD of VAMP2 (PSAP<sup>1-168</sup>TMD VAMP2, 21.2 kDa). Since both proteins share the same PSAP sequences, we first assumed that PSAP was mediating dimerization. Nevertheless, a literature search indicated that the TMD of VAMP2 is responsible for its homo or heteromerization at the ER membrane [28], suggesting that the dimers observed in our experiments could depend on TMD instead of PSAP sequences. This interpretation could also explain a band migrating between those corresponding to the monomer (21.2 kDa) and dimer (42.4 kDa) in lanes 3 and 4 of Fig. 1, as a heterodimer between endogenous VAMP2 (12.6 kDa) and PSAP<sup>1-168</sup>TMD VAMP2 (expected molecular mass: 33.8 kDa, marked with an asterisk in Fig. 1). Note that some of the bands are also present in the absence of crosslinker, suggesting strong interactions.

When similar constructs containing shorter PSAP fragments were used, oligomerization was observed. Fig. 2 shows the results obtained with a construct containing residues 65–112 preceded by a myc tag and followed by the TMD of Bcl-XL (11.2 kDa). Crosslinking of a protein chimera containing Mtch2 sequences (16 kDa) instead of PSAP sequences (lanes 3 and 4 in Fig. 2) also produced oligomers.

Our constructs contained parts of PSAP or Mtch2 and the TMD of Bcl-XL, proteins that can all be inserted into the MOM, therefore we could not rule out a mixed effect due to different sequences from either protein. In fact, whether dimers or oligomers were observed depended on the sequences attached to the TMD (Fig. 1 and Fig. 2). In order to clarify this subject, we fused the TMD of Bcl-XL or that of VAMP2 to monomeric red fluorescent protein



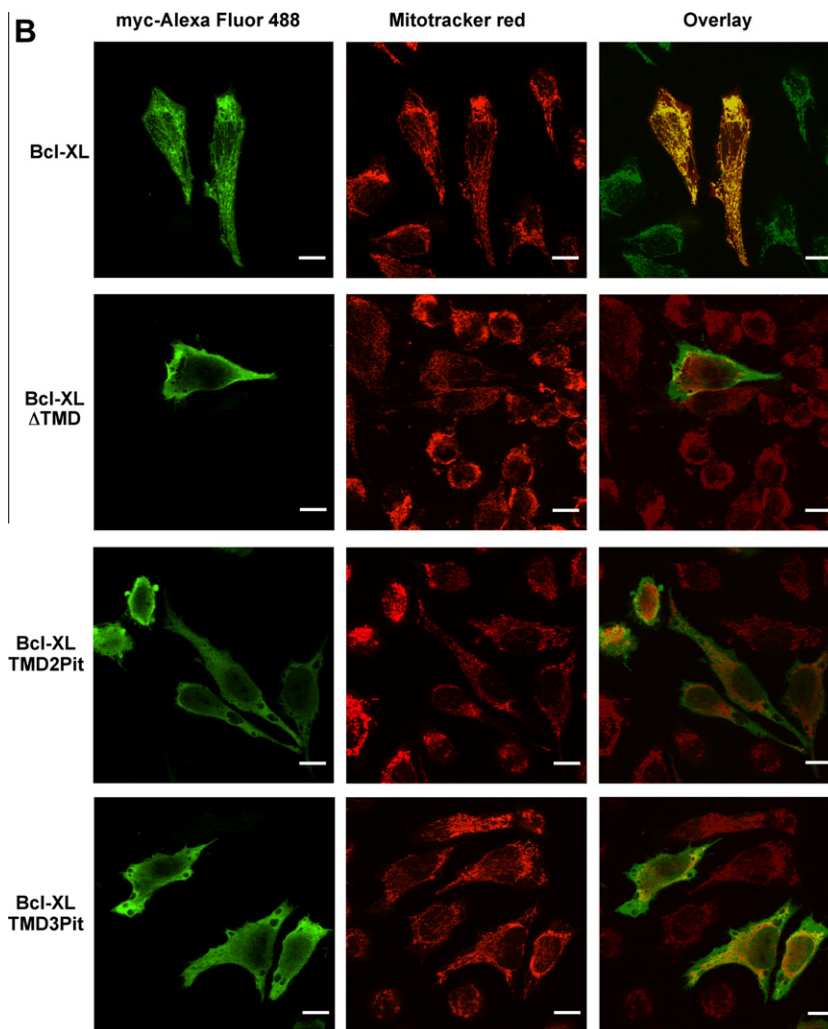


Fig. 4 (continued)

(mRFP) preceded by a myc tag for detection. We used myc-mRFP (28.7 kDa) as control. Crosslinking assays indicated that similar complexes could be detected when each TMD was fused to mRFP (Fig. 3A), whereas myc-mRFP remained as a monomer, therefore indicating that the TMDs were responsible for the dimers observed. The contribution of PSAP sequences to dimers and oligomers shown in Fig. 1 and 2 is under investigation and will be reported elsewhere.

In order to analyze these interactions with a different technique, we used blue-native electrophoresis (Fig. 3B), confirming that fusion of mRFP to either the TMD of Bcl-XL or the TMD of VAMP2 induced oligomerization of the protein. The pattern obtained indicated the presence of oligomers up to pentamers, although less abundant (or less stable) than the dimers. These data clearly indicate the involvement of the TMD of Bcl-XL in self-association, an event that had not been reported previously. The fact that only dimers could be observed upon crosslinking could be due to the location of the reacting amino groups in the oligomers. Crosslinking covalently fixes those interactions where reacting groups (amino groups in this case) are localized in close proximity, and uncrosslinked molecules are separated later during denaturing electrophoresis. Blue native electrophoresis does not rely on specific reactive groups but on the overall stability of protein complexes where individual molecules are held together by non-covalent bonds. It could be that, in our complexes, reacting amino groups are only located closely between monomers of a

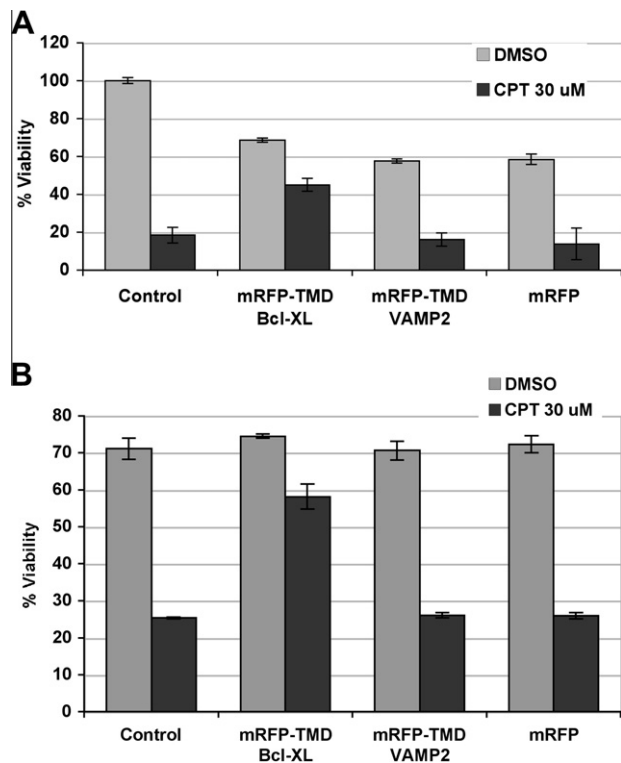
single dimer, and dimers associate in oligomers in such a way that adjacent dimers cannot be crosslinked with each other. This could explain why dimers are mainly observed upon crosslinking whereas oligomers are observed after blue-native electrophoresis.

We analyzed the subcellular localization of our chimeras by fluorescence microscopy, using Mitotracker green to label mitochondria. As expected, mRFP-TMD Bcl-XL localized to mitochondria, mRFP-TMD VAMP2 localized to membranes and mRFP distributed evenly throughout the cell (Fig. 4A).

The TMD of Bcl-XL fused to yellow fluorescent protein has been reported to alter mitochondrial morphology and to moderately protect cells against staurosporine-induced apoptosis [29]. In order to find out if our mRFP-TMD Bcl-XL chimera also protected cells against camptothecin-induced cell death we carried out cell viability assays using XTT, which were later confirmed by trypan blue exclusion assays. The results, shown in Fig 5, clearly indicated that the TMD of Bcl-XL fused to mRFP protected cells from death, whereas the TMD of VAMP2 fused to mRFP or mRFP alone did not.

Since Bcl-XL can prevent Bax and Bak oligomerization, we analyzed the state of oligomerization of these two proapoptotic proteins upon transfection of cells with mRFP chimeras and induction of apoptosis with camptothecin. We did not see any effect on Bax and Bak oligomerization in cells transfected with either mRFP-TMD Bcl-XL, mRFP-TMD VAMP2 or mRFP alone (Fig 6).

Even though Bcl-XL is able to dimerize in absence of its TMD under some conditions, we analyzed how the absence of its TMD

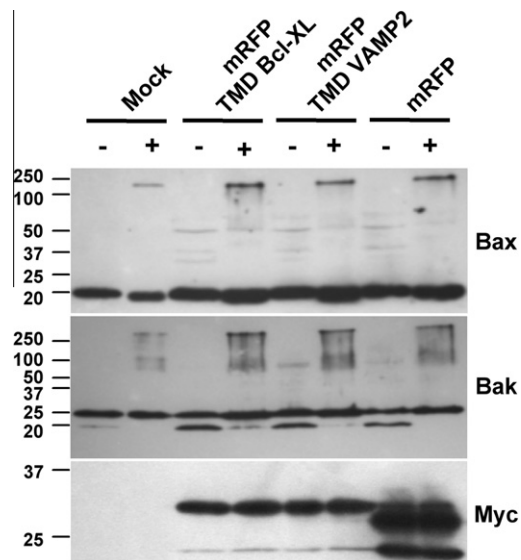


**Fig. 5.** Protection of mRFP-TMD Bcl-XL against camptothecin-induced cell death. HEK293 cells transfected with myc-mRFP-TMD Bcl-XL, myc-mRFP-TMD VAMP2 or myc-mRFP alone were treated with DMSO or with 30  $\mu$ M camptothecin for 24 h starting 12 h post-transfection. (A) Cells were analyzed using the Cell Proliferation Kit II (XTT) (Roche). Results of two independent assays carried out in triplicates are shown. (B) Cells were analyzed by trypan blue exclusion. Results of three independent assays carried out in triplicates are shown. Error bars indicate standard deviation.

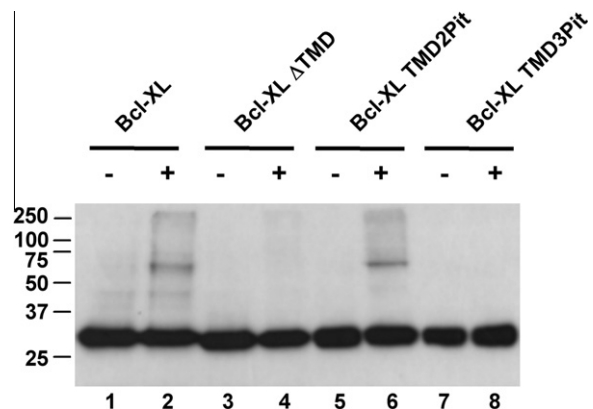
or the presence of a TMD from an unrelated protein affected its dimerization. For this, we constructed a new vector to express Bcl-XL without its TMD, as well as another vector encoding Bcl-XL with its TMD replaced by the second TMD of Pit 2, a plasma membrane sodium transporter [30]. We expressed these constructs in cells and carried out crosslinking assays using BSOCOES. Under these conditions, we could detect dimers of full-length Bcl-XL (Fig 7, lane 2) and no dimers of Bcl-XL lacking its TMD (Fig 7, lane 4). Curiously, Bcl-XL with its TMD replaced by the second TMD of Pit2 was also able to dimerize (Fig 7, lane 6).

Analysis of the sequences of the TMD of Bcl-XL and the second TMD of Pit 2 revealed the presence of the sequence motif GXXXXG (where G is glycine and X, any amino acid), in tandem in Bcl-XL (GMTVAGVLLG) and alone in the TMD of Pit2 (GSVLLG). Note that five out of six amino acids are conserved between sequence GVVLLG in Bcl-XL and sequence GSVLLG in the second TMD of Pit2. Since the sequence motif GXXXXG had been reported to be involved in dimerization of the BH3-only Bcl-2 family member BNIP3 through its TMD, and also in other proteins [31–38], which is very similar to the motifs found in the TMD of Bcl-XL and the second TMD of Pit2, we constructed a new vector where the TMD of Bcl-XL was replaced by the third TMD of Pit2, which lacks sequence GXXXXG. Crosslinking assays with this new chimera indicated that it was unable to dimerize (Fig 7, lane 8).

We analyzed the subcellular localization of these chimeras, with myc-BclXL localizing to mitochondria as expected and the remaining chimeras showing a diffuse localization in the cell (Fig 4B). This also indicates that sequences involved in dimerization and mitochondrial targeting within the TMD of Bcl-XL are different, since



**Fig. 6.** Effect of overexpression of chimeras in Bax and Bak oligomerization. HEK293 cells were transfected with vectors encoding myc-mRFP-TMD Bcl-XL, myc-mRFP-TMD VAMP2 or myc-mRFP, treated with 30  $\mu$ M camptothecin for 24 h and the effect on Bax and Bak oligomerization analyzed by crosslinking with BSOCOES. Fractions from each sample were analyzed by western blot using antibodies against Bax, Bak or myc (to detect overexpressed proteins). Sizes of molecular mass markers run alongside are indicated on the left in kDa. –, without BSOCOES; +, with BSOCOES.



**Fig. 7.** Effect of different TMDs in Bcl-XL dimerization. Full length Bcl-XL (Bcl-XL), Bcl-XL without its carboxyl terminal TMD (Bcl-XL  $\Delta$ TMD), Bcl-XL with the second TMD of Pit 2 (Bcl-XL TMD2Pit) or Bcl-XL with the third TMD of Pit 2 (Bcl-XL TMD3Pit) all preceded by a myc tag, were overexpressed in HEK293 cells, treated with the crosslinker BSOCOES and analyzed by western blot with an anti-myc antibody. –, without BSOCOES; +, with BSOCOES.

the second TMD of Pit2 is able to induce oligomerization but not mitochondrial localization.

#### 4. Discussion

We have shown that the transmembrane C-terminal domain of Bcl-XL can directly participate in oligomerization. This is a mode of self-association unreported for Bcl-XL. Bcl-2 family members control their activities through a complex network of protein-protein interactions, posttranslational modifications, transcriptional control or protein degradation [11]. Conformational changes induced upon heteromeric or homomeric protein interactions are very important for their activities.

Bcl-XL has been shown to dimerize by different mechanisms under several conditions [12–17]. Many of these studies, as well

as structural studies, used Bcl-XL mutants lacking the C-terminal TMD under non-physiological assay conditions, although its presence is likely to affect the overall fold of the protein, conformational changes and protein interactions.

Bcl-XL has been reported to regulate apoptosis by heterodimerization-dependent and -independent mechanisms. The former implies binding to pro-apoptotic proteins; the latter, could depend, at least in part, on the formation of an ion channel that can counteract the effects of MOM permeabilization by proteins like Bax or Bak [39]. Interestingly, C-terminal cleavage products of Bcl-XL can form a pore large enough to allow cytochrome c release [40], changing a pro-survival protein into a pro-apoptotic one, what has been also described for Bcl-2 upon binding of a Nur77-derived peptide [41]. Zheng et al. [29] reported changes in mitochondrial morphology and protection against apoptosis mediated by a YFP-TM Bcl-XL fusion protein, suggesting that this TMD could be required for a bioenergetic function of Bcl-XL distinct from BH3 domain sequestration. Our results suggest that oligomerization mediated by the TMD of Bcl-XL could be involved in these activities.

The TM domains of other Bcl-2 family members have been also reported to be involved in protein dimerization, like the TMD of BH3-only protein BNIP3 [37] or the TMD of Bax [42]. In the case of BNIP3, the sequence GXXXG has been shown to directly participate in dimerization. Interestingly, a similar sequence is present in the TMD of Bcl-XL, but in tandem (GMTVAGVLLG: GMTVAG and GVLLG), with one more amino acid between the two glycines (GXXXG). The presence of sequence GXXXG in tandem had been previously described in other proteins [35,38]. Curiously, a very similar sequence containing that motif is also present in the second TMD of Pit2 (GSVLLG), which we used to replace the TMD of BclXL expecting to block dimerization (before we analyzed the TMD sequences), and Bcl-XL with that TMD also dimerizes. BclXL with its TMD replaced by the third TMD of Pit2, which does not contain that sequence motif, does not dimerize, strongly suggesting the importance of motif GXXXG in dimerization.

Sequence GXXXG was first reported to be involved in TMD dimerization by Engelman and coworkers [34,43]. Furthermore, within these motifs, residues V and L are also usually present close to G residues, as is the case for the TMD of Bcl-XL and the second TMD of Pit2. Sequence specificity in the dimerization of transmembrane alpha-helices was first reported by Lemmon et al. [31], reporting later that the pattern LxxGVxxGVxxT is involved in dimerization [44]. Furthermore, the GXXXG motif has been involved in the formation of a membrane channel by the *Helicobacter pylori* vacuolating toxin [38], where this motif appears in three tandem repeats. This motif has also been found in several other proteins, like subunits e [45] and g [46] of yeast mitochondrial ATP synthase, APH-1 [47], ABCG2 [48], the SARS coronavirus spike protein (where it is involved in trimerization) [49], prion protein [50], amyloid precursor protein [51], the two-peptide bacteriocin lactococcin G [52], carnitine palmitoyltransferase 1A [53], ErbB [54], protein FliH and its Type III secretion homologue Yscl [55], the Japanese encephalitis virus precursor membrane (prM) protein [56], the PufX polypeptide of *Rhodospirillum rubrum* RC-LH1 photosynthetic complex [57], human organic anion transporter 1 (hOAT1) [58], VDAC1 [59] and the NS4B protein of hepatitis c virus [60]. A review about some of these proteins can be seen in Senes et al. [61].

Although in the TMD of Bcl-XL the two glycines are separated by four residues instead of 3 as in the GXXXG motif, our results strongly suggest that this motif is mediating interaction of Bcl-XL molecules through their TMDs, since the second TMD of Pit2, with 5 out of 6 conserved residues within that GXXXG motif also allows dimerization of the protein, whereas the third TMD of Pit2, which lacks that motif, does not dimerize when fused to Bcl-XL.

In summary, we have shown that the TMD of Bcl-XL is involved in protein dimerization and that motif GXXXG is very likely responsible for this interaction. This will most likely have important implications in Bcl-XL function.

## Acknowledgements

We thank Dr. Ester Perales and Dr. Patricio Fernández-Silva for their help with blue-native electrophoresis. We thank Drs. Isabel Marzo, Javier Sancho and Ramón Hurtado for critical reading. We thank Rubén Calvo for his help with cell culture. This work was funded by grants BFU2006-07026 from the Spanish Ministry of Education, BFU2009-11800 from the Spanish Ministry of Science and Innovation and UZ2010 BIO-03 from the University of Zaragoza to J.A.C. A.O. is the recipient of a Ph.D. Fellowship from the Banco Santander/Universidad de Zaragoza. A.L.M. was a recipient of a short-term fellowship from the Universidad Nacional Autónoma de México-CONACYT.

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