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Profiling of the Bcl-2/Bcl-X_L-binding sites on type 1 IP_3 receptor

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ABSTRACT

Several members of the anti-apoptotic Bcl-2-protein family, including Bcl-2, Bcl-X_L and Mcl-1, directly bind and regulate the inositol 1,4,5-trisphosphate receptor (IP_3R), one of the two main intracellular Ca^{2+}-release channel types present in the endoplasmic reticulum. However, the molecular determinants underlying their binding to the IP_3R remained a matter of debate. One interaction site for Bcl-2 was proposed in the central part of the modulatory domain [Y.P. Rong, A.S. Aromolaran, G. Bultynck, F. Zhong, X. Li, K. McColl, S. Matsuyama, S. Herlitze, H.L. Roderick, M.D. Bootman, G.A. Mignery, J.B. Parys, H. De Smedt, C.W. Distelhorst, Targeting Bcl-2-IP3 receptor interaction to reverse Bcl-2's inhibition of apoptotic calcium signals, Mol. Cell 31 (2008) 255–265] and another site in the C-terminal domain of the IP_3R encompassing the sixth transmembrane domain, to which Bcl-2, Bcl-X_L and Mcl-1 can bind [E.F. Eckenrode, J. Yang, G.V. Velmurugan, J.K. Foskett, C. White, Apoptosis protection by Mcl-1 and Bcl-2 modulation of inositol 1,4,5-trisphosphate receptor-dependent Ca^{2+} signaling, J. Biol. Chem. 285 (2010) 13678–13684]. Here, we investigated and compared the binding of Bcl-2 and Bcl-X_L to both sites. Two different IP_3R domains were used for the C-terminal site: one lacking and one containing the sixth transmembrane domain. Our results show that elements preceding the C-terminal cytosolic tail located at the sixth transmembrane domain of IP_3R1 were critical for recruiting both Bcl-2 and Bcl-X_L to the C-terminal part of the IP_3R. Furthermore, consistent with our previous observations, Bcl-X_L bound with higher efficiency to the C-terminal part of the IP_3R and to a much lesser extent to the central, modulatory domain, while Bcl-2 targeted both sites with similar efficiencies. In conclusion, IP_3R harbors two different binding sites for anti-apoptotic Bcl-2 proteins, one in the central, modulatory domain and one in the C-terminal domain near the Ca^{2+}-channel pore.

1. Introduction

Intracellular Ca^{2+} signals originating from the endoplasmic reticulum (ER), the main intracellular Ca^{2+} store, control cell-survival and -death processes due to the close proximity between the ER and the mitochondria [1]. At the mitochondria-associated ER membranes (MAMs), there is physical contact between both organelles involving multi-protein complexes that participate in the privileged transfer of Ca^{2+} between them [2]. In the MAMs, the chaperone GRP75 links inositol 1,4,5-trisphosphate (IP_3) receptors (IP_3Rs), ER-located intracellular Ca^{2+}-release channels, to Ca^{2+}-permeable voltage-dependent anion channels, located in the mitochondrial outer membrane [3–5]. Several pro-survival and pro-death proteins regulate Ca^{2+} transfer between the ER and mitochondria [6,7]. Different members of the anti-apoptotic B-cell lymphoma 2 (Bcl-2) family, including Bcl-2, B-cell lymphoma extra large (Bcl-X_L) and myeloid cell leukemia sequence 1 (Mcl-1), control Ca^{2+} release from the ER. Different mechanisms were proposed, including a decrease in the steady-state ER Ca^{2+} levels [8–10] through a protein kinase A-dependent phosphorylation of the IP_3R [11] and a direct regulation of the Ca^{2+}-flux properties of the IP_3R through direct protein interactions [12–15]. Several groups provided evidence that anti-apoptotic Bcl-2 proteins directly bind and regulate IP_3R channels. However, the binding sites for Bcl-2 and Bcl-X_L on the IP_3R and the functional outcome of their interactions remained a matter of debate [16]. Foskett and co-workers reported that Bcl-X_L bound to the C-terminal part of the IP_3R containing the sixth transmembrane helix, thereby sensitizing IP_3R channels to low level of IP_3 and promoting pro-survival Ca^{2+}

Abbreviations: aa, amino acids; Bcl-2, B-cell lymphoma 2; Bcl-X_L, B-cell lymphoma extra large; ER, endoplasmic reticulum; GST, glutathione S-transferase; IP_3, inositol 1,4,5-trisphosphate; IP_3R, IP_3 receptor; Mcl-1, myeloid cell leukemia sequence 1; MAM, mitochondria-associated ER membrane; TMD, transmembrane domain.

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oscillations and mitochondrial bio-energetics [13,14]. The C-terminus of the IP_3R appeared to be a target not only for Bcl-X_L but also for Bcl-2 and Mcl-1 [17]. A different mechanism was proposed by C. Distelhorst in collaboration with our own group. We found that Bcl-2 bound to the central, modulatory domain of the IP_3R and suppressed IP_3R-channel activity, thereby preventing excessive pro-apoptotic Ca^{2+} transients [15]. Detailed molecular studies revealed the BH4 domain of Bcl-2 as sufficient and necessary for Bcl-2 binding to the IP_3R [16,18–20]. Furthermore, we showed that a critical difference between Bcl-2 and Bcl-X_L in the center of their respective BH4 domains may underpin the opposite outcome of Bcl-2 versus Bcl-X_L binding to IP_3R channels [16,21].

Here, we compared the Bcl-2/Bcl-X_L-binding properties of GST-fusion proteins of IP_3R1 covering (i) the central, modulatory domain (Domain3), (ii) the cytosolic C-terminal tail containing the distal 160 amino acids (Domain6) and (iii) the C-terminal domain encompassing the pore, including the putative pore helix, selectivity filter and the sixth transmembrane domain (TMD), and the C-terminal tail (TMD6 + Domain6). The first two proteins were developed before in our lab and used in previous studies [15,20,21]. For the third protein, we chose to develop exactly the same protein as the one used by Foskett and co-workers in their previous study [13]. Our experiments show that the C-terminal channel domain of the IP_3R is a target for Bcl-2/Bcl-X_L, demonstrating the presence of two differential binding sites for Bcl-2 proteins on IP_3R channels.

2. Materials and methods

2.1. Cloning, expression and purification of GST-constructs

Parental GST, GST-Domain3 (aa 923–1581) and GST-Domain6 (aa 2590–2749) were prepared as previously described [22]. The GST-fusion protein encompassing the sixth transmembrane domain and the C-terminal tail of IP_3R1, GST-TMD6 + Domain6 (aa 2512–2749 of mouse IP_3R1) was obtained by PCR amplification of the corresponding open reading frame (nucleotides 7536–8250 of mouse IP_3R1) using 5′-GCGGCGGGATCCGAGCTGCTCCCTGCC-GAAGAAACGG-3′ as forward primer and 5′-GCGGCGGAA TTCCTAGGCCGGCTGCTGTGGGTTGAC-3′ as reverse primer and cloning in the BamHI/EcoRI restriction site of the pGEX6p2 vector. The GST-TMD6 + Domain6 fusion protein was purified from BL21(DE3) *Escherichia coli* cells as described before [23], except that elution was done using a buffer containing 50 mM glutathione and 150 mM NaCl. Proteins were dialyzed using Slide-A-Lyzer cassettes with a cut-off of 10 kDa (Thermo Fisher Scientific, Pittsburg, PA). The protein concentration was determined using the Bradford assay (Sigma–Aldrich, Munich, Germany). After SDS–PAGE, the purity and quality of the purified GST-TMD6 + Domain6 was assessed via total protein gel staining using GelCode Blue Stain Reagent (Thermo Scientific, Rockford, IL) and Western-blotting analysis using anti-GST (dilution 1:2000, Invitrogen, Merelbeke, Belgium).

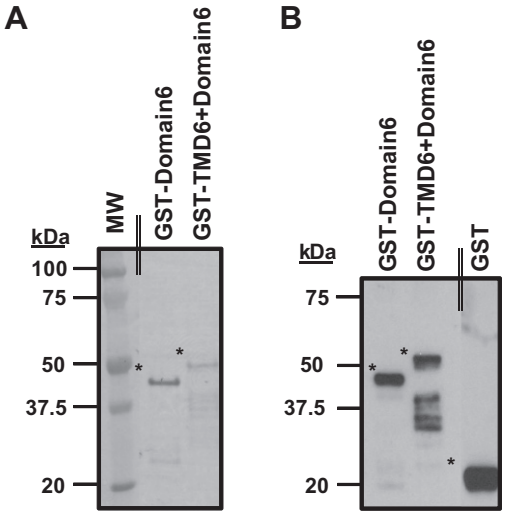

Fig. 1. Total protein staining and Western-blotting analysis of the purified GST-TMD6 + Domain6 fusion protein. (A) Total protein staining of the purified GST-Domain6 (~45 kDa protein) and GST-TMD6 + Domain6 (~50 kDa protein) obtained after purification using glutathione-Sepharose 4B beads and SDS–PAGE. Both full-length proteins are indicated with an asterisk. Similar amounts (2 μg) of both proteins were loaded. The first lane on the left shows the different molecular weight (MW) markers used as references and indicated on the left in kDa. (B) Western-blotting analysis of the purified GST-Domain6 and GST-TMD6 + Domain using an anti-GST antibody. Similar amounts of both proteins (500 ng) were loaded. Both full-length proteins are indicated with an asterisk. The double line on the Western blot indicates that lanes from another part of the same gel and exposure time were merged.

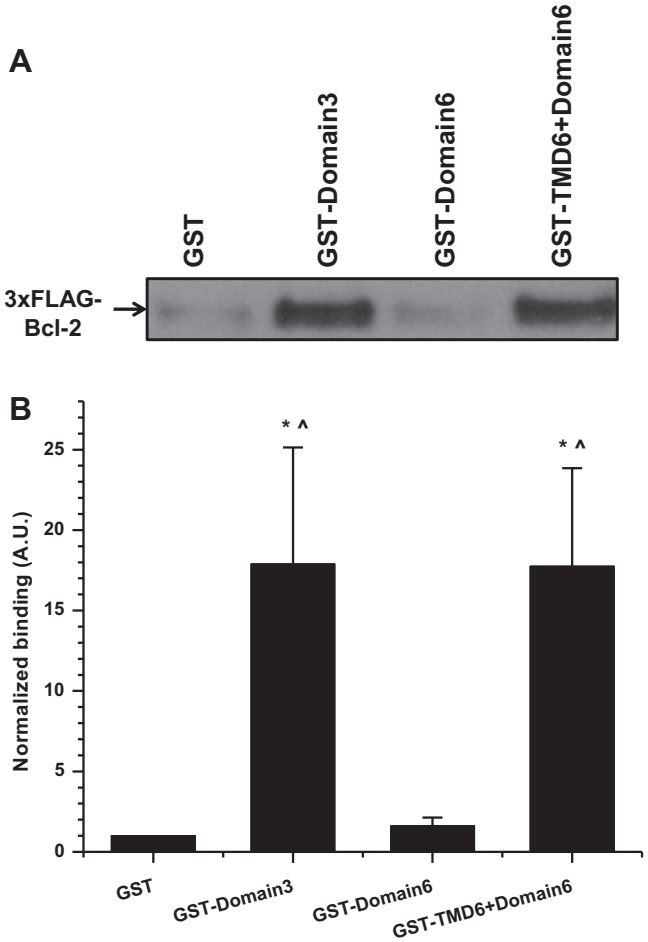

Fig. 2. GST-pull-down experiment showing the binding of 3×FLAG-Bcl-2 to the different purified GST-tagged IP_3R domains. (A) The amount of 3×FLAG-Bcl-2 retained by the different GST-fusion proteins was assessed via Western-blotting analysis using an anti-FLAG antibody. Parental GST was used as a negative control. The result of a representative GST-pull-down assay is shown out of three independent experiments. (B) The immunoreactive anti-FLAG signal was quantified using IMAGE-J and normalized to the signal obtained in the pull-down reactions using parental GST ($N = 3$). *indicates a significant difference ($p < 0.05$) compared to GST in a one-tailed *t*-test. ^A significant difference ($p < 0.05$) compared to GST-Domain6 in a one-tailed *t*-test.

2.2. GST-pull-down assays

Parental GST, GST-Domain3, GST-Domain6 and GST-TMD6 + Domain6 were used as bait in GST-pull-down assays to assess the binding of 3xFLAG-Bcl-2 or 3xFLAG-Bcl-X_L. The latter were expressed in COS-1 cells after transfection with 3xFLAG-MYC-pCMV-24 vectors (Sigma–Aldrich) encoding either Bcl-2 or Bcl-X_L. Three days after transfection, cell lysates were prepared using a buffer containing 25 mM Hepes, pH 7.5, 1% Triton X-100, 10% glycerol, 0.3 M NaCl, 1.5 mM $MgCl_2$, 1 mM DTT, 2 mM EDTA, 2 mM EGTA and protease inhibitor cocktail tablets (Roche, Basel, Switzerland). Protein concentrations were determined using Bradford analysis. Cell lysates (100 μg) were incubated for 1 h at 4 °C with equimolar amounts (~250 pmol/reaction) of the different purified GST-fusion proteins in a total reaction volume of 500 μl lysis buffer and captured using glutathione-Sepharose 4B beads (20 μl) for 1.5 h at 4 °C. The beads were washed 6 times with 500 μl reaction buffer and eluted in 40 μl of LDS sample buffer. These samples (15 μl) were analyzed on NuPAGE 4–12% Bis/Tris SDS–polyacrylamide gels using MES/SDS-running buffer (Invitrogen) and Western-blotting analysis was performed using anti-FLAG M2 antibody (dilution 1:6500, Sigma Aldrich).

3. Results and discussion

GST-TMD6 + Domain6 was purified and analyzed via SDS–PAGE along with GST-Domain6, developed and used in previous studies [15]. A total protein staining demonstrates the quality and purity of the purified GST-TMD6 + Domain6 (Fig. 1A). The presence of the full-length GST-TMD6 + Domain6 was confirmed using Western-blotting analysis using anti-GST antibody (Fig. 1B).

We then evaluated the Bcl-2-binding properties of GST-Domain6 and GST-TMD6 + Domain6 by performing semi-quantitative GST-pull-down assays with cell lysates obtained from COS-1 cells transiently transfected with 3xFLAG-tagged Bcl-2-encoding plasmids (Fig. 2A). Parental GST was used as a negative control, while GST-Domain3, which corresponds to the central, modulatory domain of IP_3R1 containing the previously identified Bcl-2-binding site [15], was used as positive control. Supplemental Fig. 1A shows a schematic representation of IP_3R1 with the different domains

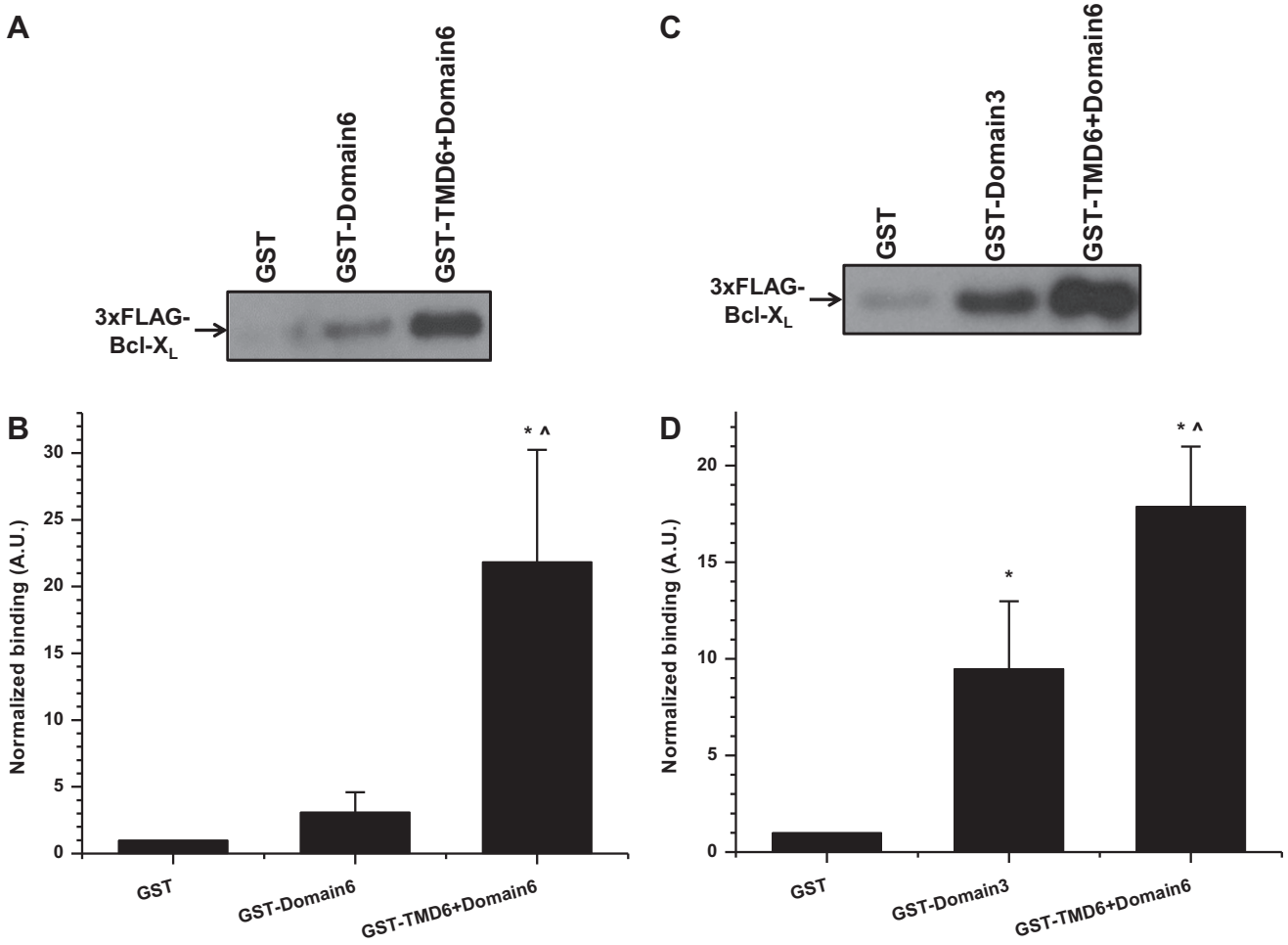

Fig. 3. GST-pull-down experiment showing the binding of 3×FLAG-Bcl-X_L to the different purified GST-tagged IP_3R domains. (A) The binding of 3×FLAG-Bcl-X_L to GST-Domain6 and GST-TMD6 + Domain6 was assessed via Western-blotting analysis using an anti-FLAG antibody. Parental GST was used a negative control. A typical experiment is shown out of three independent experiments. The double line on the Western blot indicates that lanes from another part of the same gel and exposure time were merged. (B) The immunoreactive anti-FLAG signal was quantified using IMAGE-J and normalized to the signal obtained in the pull-down reactions using parental GST ($N = 3$). *A significant difference ($p < 0.05$) compared to GST in a one-tailed t-test. ^A significant difference ($p < 0.05$) compared to GST-Domain6 in a one-tailed t-test. (C) The binding of 3×FLAG-Bcl-X_L to GST-TMD6 + Domain6 was compared to its binding to GST-Domain3. Parental GST was used as a negative control. A typical experiment is shown out of eight independent experiments. (D) The immunoreactive anti-FLAG signal was quantified using IMAGE-J and normalized to the signal obtained in the pull-down reactions using parental GST ($N = 8$). *indicates a significant difference ($p < 0.05$) compared to GST in a one-tailed t-test. ^A significant difference ($p < 0.05$) compared to GST-Domain3 in a one-tailed t-test.

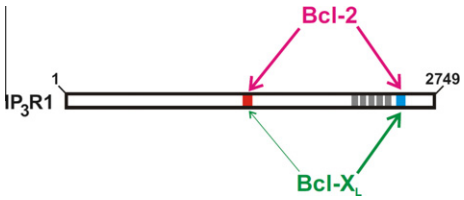

Fig. 4. Schematic representation of the IP_3R1 and its binding sites for Bcl-2 and Bcl-X_L. The IP_3R1 harbors two binding sites for Bcl-2 and Bcl-X_L. One binding site is located in the central, modulatory domain of the IP_3R1 (indicated in red) and one in the C-terminal channel domain including the sixth transmembrane domain (indicated in blue). Bcl-2 targets with similar efficiency both the central and C-terminal binding site in IP_3R1. In contrast, Bcl-X_L preferentially targets the C-terminal binding site in IP_3R1. These differences in IP_3R-binding profile between Bcl-2 and Bcl-X_L may underlie their different effects on Ca^{2+} fluxes through the IP_3R channel with Bcl-2 acting as an inhibitor and Bcl-X_L acting as an enhancer. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

used as GST-fusion proteins in this study. Supplemental Fig. 1B shows that similar amounts of GST-fusion proteins were present in the pull-downs. 3xFLAG-Bcl-2 binding to GST-Domain6 was not significantly higher than the binding of 3xFLAG-Bcl-2 to parental GST (Fig. 2B). In contrast, GST-TMD6 + Domain6 bound significantly higher amounts of 3xFLAG-Bcl-2. Furthermore, 3xFLAG-Bcl-2 bound to GST-Domain3 and GST-TMD6 + Domain6 with similar apparent affinities. These data indicate that elements preceding the cytosolic C-terminal tail of IP_3R1 are essential for Bcl-2 binding and that IP_3R1 contains two different Bcl-2-binding sites, which are targeted by Bcl-2 with comparable efficiencies.

A similar analysis was performed for 3xFLAG-tagged Bcl-X_L. Again, GST-pull-down assays revealed that binding of 3xFLAG-Bcl-X_L to GST-TMD6 + Domain6 was significantly higher than to GST-Domain6 and that binding of 3xFLAG-Bcl-X_L to GST-Domain6 was not significantly different from the binding to parental GST (Fig. 3A and B). This confirms that the sixth transmembrane domain of the IP_3R contains part of the interaction site for Bcl-X_L as well as for Bcl-2. In addition, we compared the binding of 3xFLAG-Bcl-X_L to the central, modulatory domain and the C-terminal domain of the IP_3R in a semi-quantitative manner (Fig. 3C and D). We found that 3xFLAG-Bcl-X_L binding to GST-Domain3 was significantly lower than the binding of 3xFLAG-Bcl-X_L to GST-TMD6 + Domain6. The latter data indicate that, different from Bcl-2, Bcl-X_L preferentially binds to the C-terminal binding domain of the IP_3R. This result is fully consistent with our previous study showing that the BH4 domain of Bcl-X_L is critically different from the BH4 domain of Bcl-2. BH4-Bcl-X_L binds with a much lower affinity to the central, modulatory domain of the IP_3R than BH4-Bcl-2, due to a critical single amino-acid modification in this domain (Asp11 versus Lys17) [21].

Thus, these experiments clearly indicate that the IP_3R harbors at least two different Bcl-2/Bcl-X_L-binding sites. The binding site in the C-terminal part of the IP_3R critically depends on elements preceding the C-terminal cytosolic tail, like the presence of the sixth transmembrane domain (Fig. 4). This is underpinned by a recent study by Eckenrode et al. showing that a GST-fusion protein containing the sixth transmembrane domain and the C-terminal tail of IP_3R1 binds various anti-apoptotic Bcl-2-family members with similar affinities [17]. Remarkably, Bcl-2 binds with equal efficiencies to both domains, while Bcl-X_L binds with significantly higher efficiency to the C-terminal domain than to the central, modulatory domain of IP_3R1. These differences in binding modes between Bcl-2 and Bcl-X_L may underlie their different properties with respect to the regulation of IP_3R channels. Bcl-2 may predominantly act as an effective inhibitor of excessive IP_3R-channel activity [24], whereas Bcl-X_L may predominantly operate as an enhancer of basal IP_3R-channel activity [13]. Thus, Bcl-XL by mainly targeting the a region close to the Ca^{2+}-channel pore which is also physically linked to the N-terminal IP_3-binding domain may facilitate the opening of the Ca^{2+}-channel pore upon binding of IP_3 [23,25]. Remarkably, a similar mechanism has recently been described for Bax Inhibitor-1 and IP_3Rs [26]. Furthermore, these data may also explain why Bcl-2 is a very effective regulator of the Ca^{2+}-flux properties of the IP_3R despite the relatively low affinity of the isolated BH4 domain to bind and regulate IP_3Rs. The presence of an additional binding site for Bcl-2 in the C-terminal part of the IP_3R may tether the BH4 domain of Bcl-2 in the close proximity of the central, modulatory domain. These findings are also in line with detailed molecular and modeling studies obtained for Bcl-2/ASPP2-protein complexes in which different Bcl-2 domains establish different contact sites for binding ASPP2, resulting in an overall high-affinity binding of Bcl-2 to ASPP2 [27]. Similarly as for the IP_3R, the BH4 domain of Bcl-2, which contains an additional positive charge (Lys17), has a higher affinity for ASPP2 than the BH4 domain of Bcl-X_L, which contains an additional negative charge (Asp11).

Collectively, these data in combination with previous observations [17] identify the sixth transmembrane domain of IP_3R1 as a critical component of the binding site for anti-apoptotic Bcl-2 proteins in the C-terminal part of the IP_3R channel. In addition, this binding site for anti-apoptotic Bcl-2 proteins may be more promiscuous for binding Bcl-2 and Bcl-X_L than the one in the central, modulatory domain that is more selective for binding Bcl-2 than Bcl-X_L through critical differences in the BH4-domain biology. Finally, differences in binding modes between various Bcl-2-family members targeting both binding sites with different affinities may underlie their distinct properties towards IP_3R-channel regulation, either inhibition (e.g. like for Bcl-2) or sensitization (like for Bcl-X_L).

Competing interests

The authors declare that there no competing interests.

Author contributions

GM and MB performed the experimental work. GB and GM designed the study, GB supervised the study. All authors were involved in analyzing and interpreting the data, GB prepared the manuscript in collaboration with the other authors.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.10.002.

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