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Molecular basis for the interplay of apoptosis and proliferation mediated by Bcl-xL:Bim interactions in pancreatic cancer cells

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ABSTRACT

A major mechanism through which cancer cells avoid apoptosis is by promoting the association of anti-apoptotic members of the pro-survival Bcl-2 protein family (like Bcl-2 and Bcl-xL) with BH_3 domain-only proteins (like Bim and Bid). Apoptosis and cell proliferation have been shown to be linked for many cancers but the molecular basis for this link is far from understood. We have identified the Bcl-xL:Bim protein–protein interface as a direct regulator of proliferation and apoptosis in pancreatic cancer cells. We were able to predict and subsequently verify experimentally the effect of various Bcl-xL single-point mutants (at the position A142) on binding to Bim by structural analysis and computational modeling of the inter-residue interactions at the Bcl-xL:Bim protein–protein interface. The mutants A142N, A142Q, and A142Y decreased binding of Bim to Bcl-xL and A142S increased this binding. The Bcl-xL mutants, with decreased affinity for Bim, caused an increase in apoptosis and a corresponding decrease in cell proliferation. However, we could prevent these effects by introducing a small interfering RNA (siRNA) targeted at Bim. These results show a novel role played by the Bcl-xL:Bim interaction in regulating proliferation of pancreatic cancer cells at the expense of apoptosis. This study presents a physiologically relevant model of the Bcl-xL:Bim interface that can be used for rational therapeutic design for the inhibition of proliferation and cancer cell resistance to apoptosis.

1. Introduction

Apoptosis is a programmed cell death mechanism used by metazoa to get rid of damaged, infected or redundant cells, so this process is necessary for the homeostasis of organisms from early embryonic development to adult stage and in disease pathologies [1,2]. Apoptosis is downregulated in cancer as well as autoimmune diseases and upregulated in degenerative diseases [1].

During the past decade, significant progress has been achieved in understanding the molecular mechanisms of apoptosis in cancer [3]. The information obtained suggests that the commitment to apoptosis occurs through mitochondrial depolarization and release of mitochondrial pro-apoptotic factors resulting in activation of caspases, a unique family of cysteine proteases. These pathways are all orchestrated by the Bcl-2 (B-cell lymphoma 2) family of proteins [4], where different subfamilies not only control apoptosis [5], but also other mechanisms of programmed cell death (autophagy, necrosis) [6] and cell proliferation [7]. The Bcl-2 family of proteins has been investigated intensively for understanding their mechanistic roles in apoptosis. Less is known about their mechanistic roles in proliferation.

The Bcl-2 protein family is especially important for cancer because chemotherapeutic agents usually activate cell death involving permeabilization of the mitochondrial outer membrane leading to the release of cytochrome c and other apoptogenic factors into the cytosol [4,8,9]. Pro-survival Bcl-2 proteins such as Bcl-2, Bcl-xL and Mcl-1 have the potential to hetero-dimerize with pro-apoptotic members such as Bax, Bak, Bim and Puma through binding of the exposed BH3 helix in a groove (dimerization domain) on the surface of anti-apoptotic members [4,10]. A major hypothesis in the field is that pro-survival members sequester pro-apoptotic members by hetero-dimerization resulting in the apoptosis resistant state. The potential for therapeutics targeted to Bcl-2 protein interactions is very high considering the central importance of the Bcl-2 protein family in regulating the survival and death of cancer cells [4].

It is expected that apoptosis should be coupled to cell proliferation and a vast body of literature has looked at this coupling directly or indirectly. It has been shown that apoptosis and

Abbreviations: Bcl-2, B-cell lymphoma 2; BH, Bcl-2 homology; siRNA, small interfering RNA.

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proliferation utilize mechanistically distinct signaling pathways [6], though these pathways may intersect. Mathematical models have also been proposed that attempt to tease out the balance between apoptosis and proliferation using limited experimental data [11]. Multiple studies have also looked directly at the effect of small molecules on apoptosis and proliferation in different cancers [12–16]. This is enough to suggest that the coupling between apoptosis and cell proliferation is complex and expected to be different in different cancers.

The Bcl-2 protein family has been directly implicated in cell proliferation, which is not simply due to its anti-apoptotic function [7]. Different members of the Bcl-2 family have been shown to play a role in modulating both apoptosis and cell proliferation [17–21], but given the large number of proteins in this family that interact with each other [4] and the complex coupling between apoptosis and cell proliferation, no simple model has emerged to describe this coupling even for specific cancers.

Structures for most Bcl-2 family members have already been determined experimentally using X-ray or NMR techniques. Many structures are also available for protein–protein complexes, e.g., Bcl-xL: Bad (PDBid=2bzw), Bcl-xL:Bak peptide (1bxl), Bcl-xL:Bim peptide (1pq1), Mcl-1:Noxa (2jm6), Mcl-1:Bim (2nl9). These structures provide a very good starting point for our computational method to determine intra-family interactions at the atomic level and for predicting interactions between proteins whose complexed structures are not available yet.

This study focuses on the interaction between Bcl-xL and Bim, two of the members of the Bcl-2 family and how this single interaction can directly affect apoptosis and cell proliferation in pancreatic cancer. We developed a computational model representing the physiologically relevant dynamic interaction between these two proteins and used the model to predict different Bcl-xL mutants with increased and decreased binding for Bim. We then validated these predictions experimentally by transfecting cancer cells with mutants to determine the effect of the alterations in the Bcl-xL:Bim interaction on proliferation and apoptosis in pancreatic cancer.

2. Materials and methods

2.1. Computational procedures

2.1.1. Relaxing the protein–protein complex

To understand the functional role of Bcl-xL:Bim protein–protein interaction, we first validated that the crystal structure of this complex is a physiologically relevant model of the interaction. For this validation, we started with the crystal structure of this complex from the PDB database (pdbid: 1pq1) and first relaxed it through conjugate gradient minimization using the Dreiding force field [22].

2.1.2. Computational analysis of protein–protein binding interaction

The protein–protein interaction surface in the Bcl-xL:Bim was used to identify residues on both Bcl-xL_L and Bim that line the interaction interface. Each of these residues from Bcl-xL were used in an energy analysis to calculate their interaction energy with Bim using the Dreiding force field. Corresponding analysis was done for the interface residues from Bim, whose interaction energy was calculated with Bcl-xL. The corresponding table of interaction energies is included as Table S1 in the supplementary information. Based on this analysis and the visual inspection of the Bcl-xL:Bim interface, we selected the Alanine residue (A142) in Bcl-xL_L as the one to mutate in order to have the greatest impact the Bcl-xL:Bim interaction. This residue points directly at Bim (Fig. 1A), has a low interaction energy with Bim (Table S1) and can be used to modulate the interaction between the two proteins. The premise was that if we can predict single-point mutations at the A142 site, designed to increase or decrease binding between the two proteins, and confirm it with experiments then we would have validated the relaxed (minimized) crystal structure as a good model for apoptosis and proliferation related functional studies.

2.1.3. Virtual mutation screening

The A142 position in Bcl-xL was computationally mutated to 19 other amino acids using the following protocol. All residues on Bcl-xL and Bim proteins in the Bcl-xL:Bim complex that are within 5.0 Å of the A142 position are selected. The new mutant residue at position 142 and previously selected residues are all optimized at the same time to find the most optimal side chain orientations for all these residues. This rotamer optimization step is performed for all 19 mutants at position 142 and the WT complex by using the SCREAM (Side-Chain Rotamer ExcitAtion Method) program [23]. SCREAM uses a library of residue conformations ranging from a CRMS diversity of 0.4 to 1.6 Å in conjunction with a Monte Carlo sampling algorithm using full valence, hydrogen bond and electrostatic interactions, but special van der Waals potentials that reduce somewhat the penalty for contacts that are slightly too short while retaining the normal attractive interactions at full strength. This altered vdW potential enables the use of a discrete rotamer library. The resulting structures are relaxed using 25 steps of conjugate

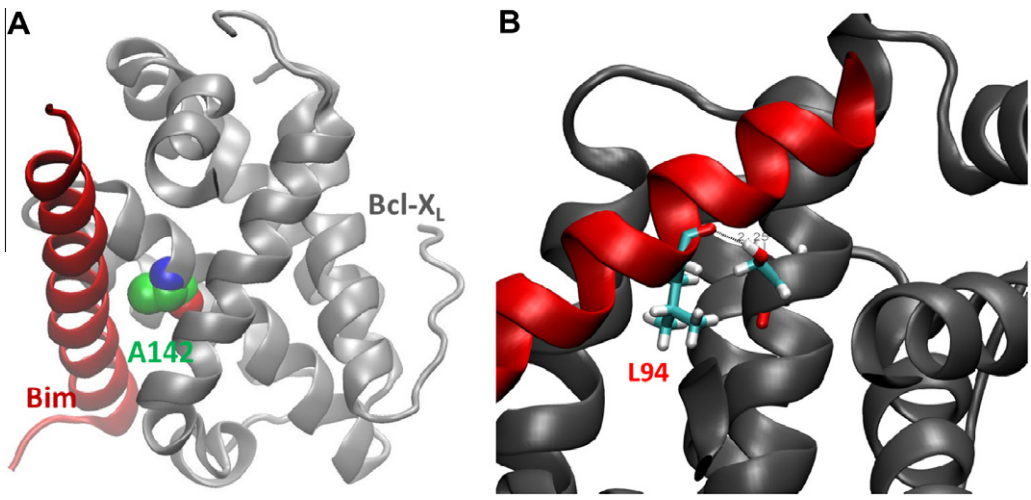

Fig. 1. (A) Bcl-xL:Bim complex from PDBid:1pq1. Residue A142 on Bcl-xL is shown with van der Waals spheres. (B) A142S mutant makes a hydrogen bond with backbone of L94 on Bim.

gradient minimization using the Dreiding force field with normal vdW potential, which is able to relax most of the close contacts enabled by SCREAM. The resulting complexes are used to get the binding energy between Bcl-xL and Bim using the Dreiding force field and the following expression:

$$E = E(\text{Bcl-xL} : \text{Bim}) - E(\text{Bcl-xL}) - E(\text{Bim}) \quad (1)$$

2.2. Experimental procedures

2.2.1. Reagents

Antibodies against Bim and Bcl-xL were from Cell Signaling (Beverly, MA). All other chemicals were from Sigma–Aldrich (St. Louis, MO).

2.2.2. Cell culture

Human pancreatic ductal adenocarcinoma cells, the poorly differentiated MIA PaCa-2 (CRL-1420) line, was obtained from the American Type Culture Collection (Manassas, VA). MIA PaCa-2 cells were grown in 1/1 DMEM/F-12 medium (Gibco Invitrogen Corporation, Grand Island, NY) supplemented with 15% FBS, 4 mM L-glutamine, and antibiotic/antimicotic solution (Omega Scientific, Tarzana, CA). Cells were maintained at 37 °C in a humidified atmosphere containing 5% CO_2 and used between passages 3 and 9. For the experiments, MIA PaCa-2 cells were cultured for 48 h.

2.2.3. Plasmids construction

Bcl-xL wild type (wt) plasmid was synthesized by DNA amplification by PCR using human cDNA as template and cloning into the pcDNA3.1 vector (Genescript, Piscataway, NJ). Bcl-xL mutants were synthesized by inducing point mutation in the Bcl-xL wt plasmid (Genescript, Piscataway, NJ).

2.2.4. Transfections

Transient transfections of MIA PaCa-2 cells were performed using the electroporation Amaxa System Nucleofector™ (Amaxa Inc., Gaithersburg, MD) according to the manufacturer protocol. 2 μg of each plasmid was applied to 2 million cells. Control cells were transfected with the empty vector pcDNA3.1 (Ambion, Foster City, CA).

2.2.5. In vitro proliferation

Proliferation was assessed by measuring the level of ^{3}H–thymidine incorporation into DNA in cells cultured for 48 h [24].

2.2.6. Measurement of apoptosis

Internucleosomal DNA fragmentation in cultured cells was measured by using Cell Death Detection ELISA^{Plus} kit (Roche Molecular Biochemicals, Manheim, Germany) according to the manufacturer's instructions [25].

2.2.7. Western blot analysis

Cells were lysed in radioimmune precipitation phosphorylation buffer (50 mM NaCl, 50 mM Tris/HCl, pH 7.2, 1% deoxycholic acid, 1% Triton X-100, 0.1% SDS, 10 mM $Na_2HPO_4 + NaH_2PO_4$, 100 mM NaF, 2 mM Na_3VO_4, 80 μM glycerophosphate, 20% glycerol, 1 mM phenylmethylsulfonyl fluoride (PMSF), 5 μg/ml each of pepstatin, leupeptin, chymostatin, antipain, and aprotinin), sonicated, and centrifuged for 15 min at 16,000*g* at 4 °C. Proteins were separated by SDS–PAGE and electrophoretically transferred to nitrocellulose or PVDF membranes. Nonspecific binding was blocked for 1 h with 5% bovine serum albumin or nonfat dry milk in Tris-buffered saline (4 mM Tris base, 100 mM NaCl, pH 7.5) containing 0.05% Tween 20. Membranes were incubated with primary antibody for 2 h and then for 1 h with peroxidase-conjugated secondary antibody. Blots were developed using the SuperSignal chemiluminescent substrate (Pierce).

2.2.8. Immunoprecipitation

Cells were collected, washed twice in a buffer containing 20 mM Tris (pH 7.5) and 10 mM DTT, and then resuspended in a lysis buffer (50 mM Tris–HCl, 150 mM NaCl, 2 mM EGTA, 10 μg/ml each of leupeptin and aprotinin, 1 mM PMSF, 1% IGEPAL CA-630), and sonicated for 30 s. The lysates were clarified by centrifugation, and 1 mg of protein was subjected to immunoprecipitation with the indicated antibodies at room temperature according to the Catch and Release immunoprecipitation kit (Upstate Biotech Millipore, Temecula, CA) [26]. In this and other assays, protein concentration was measured by the Bradford assay (Bio-Rad Laboratories).

3. Results

3.1. Validation of physiologically relevant structure for the Bcl-xL:Bim complex

As described in the methods section, the residue A142 was selected to predict mutations that will modulate the binding between Bcl-xL and Bim in the complex. This residue was mutated to 19 other amino acids, the local side chains optimized, and the resulting complex minimized as described in the methods section. The interaction energy between Bcl-xL and Bim was obtained for each of these mutants using the Dreiding force field [22]. This energy is shown in Table 1 relative to the interaction energy in the WT complex. The table also breaks up the interaction energy into its Coulombic, van der Waals and hydrogen bond (H-Bond) components, as the Dreiding force field contains a hydrogen bond term.

As seen in Table 1, the mutants containing amino acids with charged (R, K, D, E) and bulky (W, Y) side chains lead to the greatest modulation of the binding energy between Bcl-xL and Bim. In order to experimentally test these predictions to validate the structure, we selected one mutant A142S that increased binding energy. Of note, there were three mutants (A142S, A142R, A142K) that significantly increased binding energy, but we wanted to avoid mutations based on charged residues, so A142R and A142K were not used for experimental testing. The A142S mutant actually gains hydrogen bond forces between the serine hydroxyl hydrogen and

Table 1
Predicted energies of interaction (in kcal/mol) between Bcl-xL mutants and Bim, relative to that for WT Bcl-xL.

Mutant	Energy	Coulomb	H-bond	vdW
WT	0.0	0.0	0.0	0.0
A142C	−0.7	−0.3	0.0	−0.4
A142D	19.2	16.0	0.0	3.2
A142E	20.9	15.8	0.1	5.0
A142F	19.3	0.7	0.2	18.3
A142G	1.5	0.0	0.0	1.5
A142H	12.8	−1.1	0.1	13.8
A142I	7.0	0.8	0.1	6.1
A142K	−2.6	−20.6	0.2	17.8
A142L	8.5	0.7	0.1	7.6
A142M	8.0	0.5	0.2	7.3
A142N	3.0	0.5	0.1	2.4
A142P	2.1	0.5	0.2	1.4
A142Q	3.8	−1.5	−5.0	10.3
A142R	−8.0	−16.8	0.2	8.6
A142S	−3.0	−0.6	−3.3	0.9
A142T	0.3	0.1	−1.1	1.3
A142V	4.6	0.4	0.1	4.1
A142W	20.9	0.8	0.4	19.7
A142Y	24.1	1.6	0.3	22.3

the backbone carbonyl of the L94 residue on Bim protein as shown in Fig. 1B. This increased binding energy is also demonstrated in Table 1 which shows a −3.3 kcal/mol hydrogen bond contribution dominating the overall interaction energy gain of −3.0 kcal/mol, as a serine amino acid side chain is closest in size to the wild-type alanine side chain.

The three single-point mutations (A142Q, A142Y, A142 N) were also selected for experimental testing that were predicted to decrease binding of Bcl-xL to Bim. The relative predicted ordering of binding interaction between Bcl-xL and Bim is the following:

A142S > WT > A142N > A142Q > A142Y

This relative ordering is based on a purely enthalpic contribution to binding interaction between Bcl-xL and Bim, so entropic change upon Bcl-xL mutant binding to Bim (relative to WT) is being ignored as an approximation. These mutants were experimentally tested for Bcl-xL:Bim binding and the results are presented in Fig. 2A. Mutants A142Q, Y and N showed decrease in the level of Bim binding. Whereas, mutant A142S showed increase in Bim binding confirming the predicted trend for the mutants.

3.2. Functional consequences of Bcl-xL:Bim binding

The structurally validated Bcl-xL:Bim complex and four mutants were studied for their effect on cell proliferation and apoptosis. First, we found that transfection of MIA PaCa-2 cells with Bcl-xL WT stimulated cell proliferation (Fig. 2B). The mutant A142S, which, showed increase in Bcl-xL:Bim binding stimulated proliferation at a level similar to Bcl-xL WT plasmid. Further, transfection with mutants A142Q and A142Y, which, decreased Bim binding to Bcl-xL prevented the stimulation of proliferation induced by Bcl-xL WT (Fig. 2B). Bim inhibition using Bim siRNA prevented the pro-proliferation effect of Bcl-xL WT and A142S mutant indicating that Bim is needed for inducing proliferation of MIA PaCa-2 cells through interacting with Bcl-xL (Fig. 2B).

Differently from proliferation, Bcl-xL WT and A142S mutant transfections did not affect apoptosis (Fig. 2C). However, transfection with A142Q, A142Y and A142 N mutants stimulated apoptosis. Figs. 2B and C show a strong correlation between the effects of Bcl-xL mutations on proliferation and apoptosis.

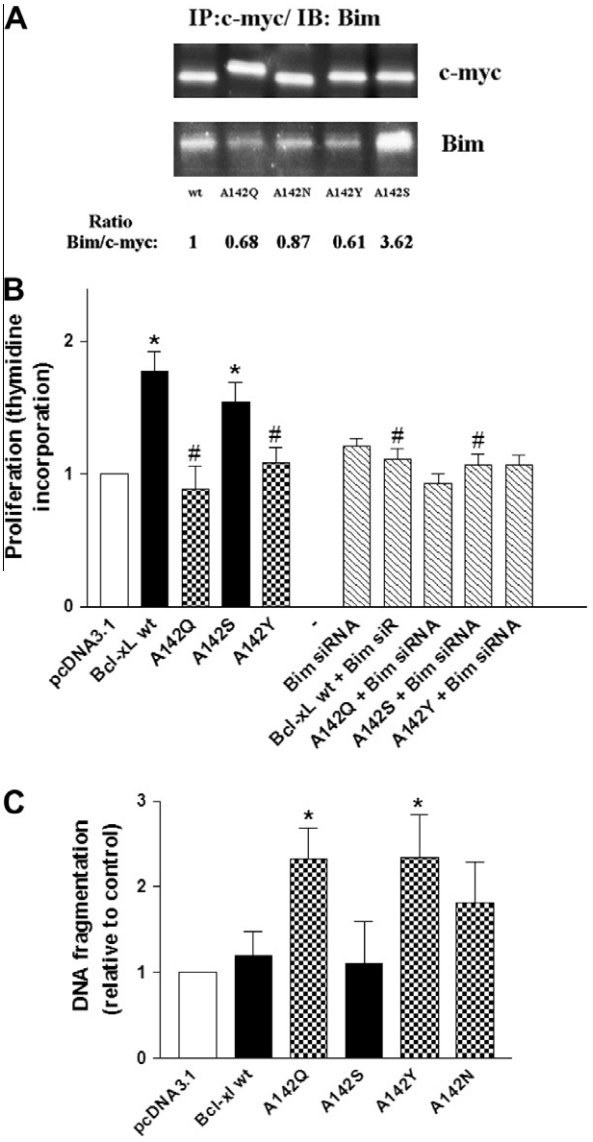

Fig. 2. (A) A142S mutation increases the level of Bim binding to Bcl-xL. Mutations A142Q/Y/N decrease binding of Bim to Bcl-xL MIA PaCa-2 pancreatic cancer cells were transfected using electroporation and cultured for 48 h. Binding of Bim to Bcl-xL was assessed by measuring the level of Bim present in Bcl-xL immunoprecipitate by Western. (B) Bim siRNA prevented the increase in proliferation induced by Bcl-xL wt and Bcl-xL A142S suggesting that Bim is necessary for the pro-proliferation effect of Bcl-xL. MIA PaCa-2 pancreatic cancer cells were transfected using electroporation and cultured for 48 h. Proliferation was assessed by measuring H-Thymidine incorporation into DNA. *, $p < 0.05$ *versus* pcDNA3.1; #, $p < 0.05$ *versus* Bcl-xL WT. (C) Bcl-xL wt and A142S did not affect apoptosis; whereas, A142Q/Y/N stimulated apoptosis. MIA PaCa-2 pancreatic cancer cells were transfected using electroporation and cultured for 48 h. Apoptosis was assessed by measuring DNA fragmentation by ELISA. *, $p < 0.05$ *versus* pcDNA3.1.

4. Discussion

The interaction of Bcl-xL with Bim has been shown to be one of the important interactions in the Bcl-2 family that affects apoptosis [4]. Bcl-xL or Bim knockout experiments do not explore the effect of binding strength between the two protein partners on apoptosis or another cellular function of interest.

The availability of three forms of this protein–protein complex allows for a functional evaluation of the role played by this binding in apoptosis and cell proliferation. The overall study provides a valuable tool to characterize the effect of protein–protein interactions on cellular function that doesn't rely on simply knocking out one of the partners in the interaction, which provides much less insight. It uses a chemically modulated (via mutations) binding gradient to truly assess the role of the interaction strength between the protein partners (Bcl-xL and Bim) on apoptosis and cell proliferation. This molecular level understanding of the Bcl-xL:Bim interaction also provides a promising approach for tackling pancreatic cancer and other cancers as any therapeutic agent targeting the Bcl-xL:Bim binding interface on Bcl-xL will be capable of causing apoptosis while preventing cell proliferation.

Systematic mutational analysis of protein–protein interaction surfaces has been normally used to assess the effect of protein–protein binding on function for other protein–protein systems [27]. This is a very resource-intensive exercise to characterize the protein–protein interface. A more efficient method is the one presented here where structural predictions guide the experiments to first find mutants that lead to a mutant protein–protein complexes with differential binding. Then these mutants are used in functional studies to characterize the importance on this binding strength on function.

Table 1 and Fig. 1A show results for Bcl-xL mutants that exhibit differential binding towards Bim. Four mutants were selected to sample the protein–protein binding energy landscape. The A142S Bcl-xL mutant exhibited increased binding for Bim (relative to WT Bcl-xL), and the other three mutants (A142N, A142Q, A142Y)

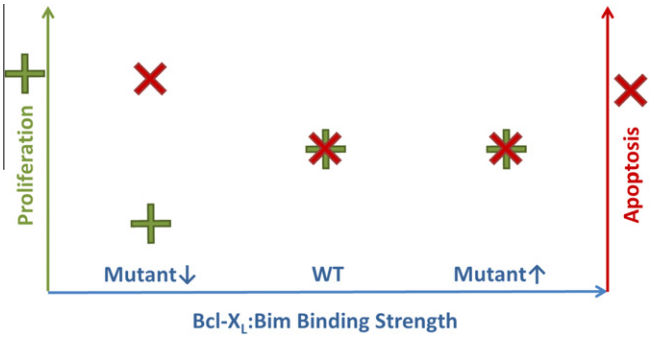

Fig. 3. Model summarizing the effect of Bcl-xL:Bim interaction strength on apoptosis (green plus) and proliferation (red cross) for Mutant↓ of Bcl-xL (that decreases Bcl-xL:Bim binding) and Mutant↑ of Bcl-xL (that increases Bcl-xL:Bim binding) relative to WT Bcl-xL (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article).

exhibited decreased relative binding for Bim (relative to WT Bcl-xL). The serine residue is the closest in size to alanine (except glycine) among the 20 amino acids, so it had the best chance to fit sterically in the protein–protein interface. In addition, it gained a hydrogen bonding interaction with the backbone carbonyl oxygen of L94 residue on Bim, which allowed Bcl-xL to increase its binding strength for Bim. All other residues are larger than serine and produced a net repulsive interaction with Bim. These results were confirmed experimentally and show that we can chemically alter (using mutations) protein–protein interactions in a predictive manner resulting in protein–protein complexes displaying a range of relative binding affinities. This in turn enabled the characterization of protein–protein binding strength on cellular functions of interest to us like apoptosis and proliferation.

We found that transfection of the cancer cells with Bcl-xL mutants with decreased binding affinity for Bim relative to WT Bcl-xL (A142Q and A142Y) show decreased proliferation than WT or A142S mutant. This differential effect on proliferation is abolished upon the use of Bim siRNA. This data strongly suggests that direct interaction of Bcl-xL with Bim is responsible for the regulation of cell proliferation. Transfection of cancer cells with mutants with decreased Bcl-xL:Bim binding (A142 N, A142Q, A142Y) resulted in greater apoptosis rates whereas A142S and WT showed similar apoptosis levels.

This data is cumulatively summarized in Fig. 3 through a model, which shows that decreasing the binding of Bcl-xL to Bim provides a double benefit of promoting apoptosis and reducing pancreatic cancer cell proliferation. In addition, it shows that transfections with A142S which increased binding to Bim did not result in significant effects on apoptosis or proliferation compared to transfection with WT. We speculate that this occurred because transfection with WT itself increased cellular expression of Bcl-xL sufficiently to effect maximal responses on downstream signaling for proliferation and apoptosis.

The Bcl-2 family of proteins has been shown to be a key player in apoptotic pathways. This study shows that at least in pancreatic cancer cells, a key Bcl-2 family interaction (Bcl-xL:Bim) can also directly control proliferation. These findings make the Bcl-xL:Bim complex and the binding interface a promising therapeutic target for pancreatic cancer, whose disruption will provide a dual benefit of increased apoptosis and decreased cell proliferation. Cell proliferation can be caused by numerous biochemical signaling pathways. How these pathways interact with Bcl-xL or Bim is currently being investigated.

This study also exemplifies the use of computational predictions to guide biological investigations, as it not only provides vital structural insights but also testing a handful of predicted mutations consumes much less resources than doing multiple-residue scanning mutagenesis making efficient use of experimental resources.

Acknowledgments

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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.2012.05.032.

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