Contribution of Bcl-2 Phosphorylation to Bak Binding and Drug Resistance
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Abstract
Bcl-2 is phosphorylated on Ser70 after treatment of cells with spindle poisons. On the basis of effects observed in cells overexpressing Bcl-2 S70E or S70A mutants, various studies have concluded that Ser70 phosphorylation either enhances or diminishes Bcl-2 function. In the present study, the ability of phosphorylated Bcl-2, as well as the S70E and S70A mutants, to bind and neutralize proapoptotic Bcl-2 family members under cell-free conditions and in intact cells was examined in an attempt to resolve this controversy. Surface plasmon resonance indicated that phosphorylated Bcl-2, Bcl-2 S70E, and Bcl-2 S70A exhibit enhanced binding to Bim and Bak compared with unmodified Bcl-2. This enhanced binding reflected a readily detectable conformation change in the loop domain of Bcl-2. Furthermore, Bcl-2 S70E and S70A bound more Bak and Bim than wild-type Bcl-2 in pull-downs and afforded greater protection against several chemotherapeutic agents. Importantly, binding of endogenous Bcl-2 to Bim also increased during mitosis, when Bcl-2 is endogenously phosphorylated, and disruption of this mitotic Bcl-2/Bim binding with navitoclax or ABT-199, like Bcl-2 downregulation, enhanced the cytotoxicity of paclitaxel. Collectively, these results provide not only a mechanistic basis for the enhanced antiapoptotic activity of phosphorylated Bcl-2, but also an explanation for the ability of BH3 mimetics to enhance taxane sensitivity. Cancer Res; 73(23); 6998–7008. ©2013 AACR.

Introduction
Bcl-2 family members regulate apoptosis by controlling mitochondrial outer membrane permeabilization (1, 2). In particular, the proapoptotic multidomain Bcl-2 family members Bax and Bak are thought to directly permeabilize the outer mitochondrial membrane. Bax and Bak are held in check by binding to the antiapoptotic Bcl-2 family members Bcl-xL, Mcl-1, Bcl-w, and A1 as well as Bcl-2 itself. These interactions are further modulated by BH3-only proteins such as Bim, Puma, Bid, Noxa, Bik, Bad, and Bmf, which are activated by various cellular stresses and then facilitate apoptosis by neutralizing antiapoptotic family members and, at least in the case of Bim, Bid, and Noxa, by directly activating Bax and/or Bak.

Not all of the antiapoptotic Bcl-2 family members interact equally well with Bax and Bak. Even though the first article describing Bax showed that it could be pulled down with Bcl-2 (3), subsequent studies have reported that Bak binds Mcl-1 and Bcl-xL preferentially (4, 5). On the other hand, our previous studies demonstrated an equilibrium dissociation constant of ~150 nM/L for the Bcl-2/Bak complex and demonstrated that a sizable fraction of the total cellular Bak in certain lymphoid cell lines, particularly those with Bcl-2 overexpression, could be pulled down with endogenous Bcl-2 (6).

Additional experiments examined the potential role of phosphorylation in the protective effects of Bcl-2. Early studies not only identified Bcl-2 as a phosphoprotein (7) that is preferentially modified during mitosis (8, 9), but also suggested that paclitaxel treatment might inactivate Bcl-2 through this posttranslational modification (10, 11). In particular, it was reported that Bcl-2 S70A protected cells better than wild-type Bcl-2, raising the possibility that Bcl-2 Ser70 phosphorylation inhibits Bcl-2 function (12). In contrast, other studies have demonstrated that phosphomimetic mutants with glutamate substituted at amino acids Thr69, Ser70, and Ser87 also exhibit enhanced antiapoptotic effects, leading to the suggestion that phosphorylation enhances the activity of Bcl-2 (13, 14). Although it was suggested that these conflicting observations might reflect context-dependent effects of Bcl-2 phosphorylation (15), the mechanistic basis for either the proposed inactivation of Bcl-2 or its hyperactivation by phosphorylation remains unclear. Moreover, whereas several studies implicated cyclin-dependent kinase 1 (CDK1) as at least one possible Bcl-2 kinase (8, 16), other studies reported an inability of CDK1 to phosphorylate Bcl-2 in vitro (12, 17).

Previous studies have also shown that ABT-737 and navitoclax, which antagonize the effects of Bcl-2, Bcl-xL, and Bcl-w, dramatically sensitize a variety of cells to the cytotoxic effects of paclitaxel (18–22). These observations have led to at least two trials of navitoclax/taxane combinations (http://www.
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clinicaltrials.gov/). Largely on the basis of correlations between Bcl-xL expression and sensitivity to taxanes, it has also been suggested that the synergy between paclitaxel and ABT-737 or navitoclax reflects inhibition of Bcl-xL. The possibility that a form of Bcl-2 present mainly during mitosis plays an important role in taxane sensitivity has not been investigated.

To resolve these issues, the present study was designed to determine whether phosphorylation inhibits or enhances the antiapoptotic function of Bcl-2, to examine the mechanistic basis for the altered Bcl-2 function, and to assess the impact of Bcl-2 phosphorylation on anticancer drug sensitivity. Results of this analysis demonstrated that phosphorylated Bcl-2 or the S70E mutant not only bound Bak and Bim with higher affinity under cell-free conditions, but also sequestered more Bim and Bak in intact cells, leading to enhanced protection against apoptosis. Interestingly, the Bcl-2 S70A mutant afforded similar protection, supporting a model in which Bcl-2 phosphorylation drives Bcl-2 to a more active conformation rather than providing a charge modification required for interaction with a phosphoepitope-directed binding partner.

Materials and Methods

Materials

Reagents were obtained from the following suppliers: CM5 biosensor chips and Polysorbate 20 from GE Healthcare, Q-VD-OPh from SM Biochemicals, glutathione (GSH) and paclitaxel from Sigma, GSH-agarose and trypsin-TPCK from Thermo Scientific, navitoclax and ABT-199 from Chemietek, activated CDK1/cyclin B complex from Millipore, Ni2+–NTA-agarose from Novagen, and allophycocyanin (APC)-conjugated Annexin V from BD Biosciences. Antibodies to the following antigens were purchased from the indicated suppliers: Bcl-2 from BD Biosciences. Antibodies to the following antigens were purchased from the indicated suppliers: Bcl-2 and Bak from Dako; Bax, Bim, Bcl-xL, Mcl-1, green fluorescent protein (GFP), and gloceraldehyde phosphate dehydrogenase from Cell Signaling Technology; Bak and Ser70-Bcl-2 from Millipore; and actin (goat polyclonal) and Puma (rabbit polyclonal) from Santa Cruz Biotechnology. Anti-S peptide antibody was raised in our laboratory as described (23). The 26-mer Bim BH3 peptide was immobilized on a CM5 chip using a Biacore T200 biosensor.

Protein expression and purification

Plasmids encoding His6-tagged BakΔTM (GenBank BC004431; residues 1–186) in pET29b (+) and glutathione S-transferase (GST)–tagged Bcl-2ΔTM have been described previously (6). cDNA encoding Bcl-2ΔTM (GenBank BC027258; residues 1–219) was also cloned into pET29(−), generating the Bcl-2ΔTM protein with a C terminal His6-tag. Plasmids encoding Bcl-2 mutants were generated using site-directed mutagenesis. All plasmids were subjected to automated sequencing to verify the described alteration and confirm that no additional mutations were present.

To express tagged BakΔTM or Bcl-2ΔTM, plasmids were transformed into Escherichia coli BL21 by heat shock. After cells were grown to an optical density of 0.8, 1 mmol/L IPTG (isopropyl-β-D-thio-β-D-galactopyranoside) was added to induce protein synthesis at 18°C for 24 hours. Bacteria were then washed and sonicated on ice in TS buffer [150 mmol/L NaCl containing 10 mmol/L Tris-HCl (pH 7.4) and 1 mmol/L freshly added PMSF]. All further steps were performed at 4°C.

After His6-tagged proteins were applied to Ni2+–NTA-agarose, columns were washed with 20 volumes of TS buffer followed by 10 volumes of TS buffer containing 40 mmol/L imidazole and eluted with TS buffer containing 200 mmol/L imidazole. After GST-tagged proteins were incubated with GSH-agarose for 4 hours, beads were washed twice with 20–25 volumes of TS buffer and eluted with TS containing 20 mmol/L GSH for 30 minutes at 4°C.

Fast protein liquid chromatography

Bcl-2ΔTM-His6, either wild-type or mutant, was further purified by fast protein liquid chromatography (FPLC) on a mono Q column with buffer A (50 mmol/L Na2HPO4, pH 8.5) and buffer B (50 mmol/L Na2HPO4, 1 M NaCl, pH 8.5). The Bcl-2ΔTM-containing fractions were subjected to SDS-PAGE.

In vitro phosphorylation and mass spectrometry

To achieve a high degree of modification, 100 µg purified Bcl-2ΔTM-His6 was incubated with 5 µg purified CDK1/cyclin B complex at 30°C for 2 hours in buffer containing 1 mmol/L ATP, 1 mmol/L MgCl2, 150 mmol/L NaCl, and 20 mmol/L HEPES (pH 7.4). Aliquots of the reaction mixture were subjected to SDS-PAGE, transferred to nitrocellulose, stained with fast green, and blotted with anti-phospho-Ser70-Bcl-2 or subjected to liquid chromatography (LC) on an Agilent 1200 LC system coupled with electrospray ionization and time-of-flight mass spectrometry (MS) on an Agilent 6224 TOF mass spectrometer (Mayo Clinic Proteomics Core Facility, Rochester, MN). Alternatively, aliquots of the same sample were also subjected to trypsin digestion followed by nano-LC/MS-MS using an Eksigent nano-LC-2D high-performance liquid chromatography system (Eksigent, Dublin, CA) coupled with a Thermo Scientific Orbitrap Elite hybrid mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) to identify phosphorylation sites.

Trypsin digestion

After 2.5 µg Bcl-2ΔTM-His6, (wild-type, S70A, S70E) was incubated with 5 ng Trypsin-TPCK in HEPES buffer (150 mmol/L NaCl, 20 mmol/L HEPES, pH 7.4) for 30 minutes at 30°C, the reaction products were subjected to SDS-PAGE and stained with Coomassie blue G-250. An aliquot of trypsin-digested Bcl-2ΔTM-His6, S70A was subjected to liquid chromatography coupled with electrospray ionization and time-of-flight MS as described above to identify the two tryptic cleavage fragments.

Surface plasmon resonance

Proteins for surface plasmon resonance (SPR) were further purified by FPLC on Superdex S200, concentrated in a centrifugal concentrator (Centricon, Millipore), dialyzed against Biacore buffer [10 mmol/L HEPES (pH 7.4), 150 mmol/L NaCl, 0.05 mmol/L EDTA, and 0.005% (w/v) Polysorbate 20] and stored at 4°C for less than 48 hours before use. BakΔTM or 26-mer Bim BH3 peptide (24) was immobilized on a CM5 chip using a Biacore T200 biosensor.

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Binding assays were performed at 25°C using Biacore buffer containing GST or GST-Bcl-2ΔTM (wild-type or mutant) or Bcl-2ΔTM-His6 (phosphorylated or unmodified) injected at 30 μL/min for 1 or 2 minutes. Bound protein was allowed to dissociate in Biacore buffer at 30 μL/min for 10 minutes and then desorbed with 20 mmol/L Glycine (pH 2).

Binding kinetics were derived using BIA Evaluation software (Biacore).

Cell culture, transfection, and drug treatment
K562 and Jurkat cells were maintained at densities below 5 × 10^5 cells/mL in RPMI 1640 medium containing 10% heat-
inactivated FBS, 100 units/mL penicillin G, 100 μg/mL streptomycin, and 2 mM/L glutamine (Medium A), and passaged less than 3 months before being reinitiated from frozen stocks. Ovcar5 and Ovcar8 cells from the National Cancer Institute (Frederick, MD; ref. 25) or A2780 cells from T. Hamilton (Fox Chase Cancer Center, Philadelphia, PA) were maintained at less than 80% confluence in Medium A without (Ovcar5 and Ovcar8) or with (A2780) 10 μg/mL insulin. After cells were treated with paclitaxel in the absence or presence of navitoclax or ABT-199 for 24 or 48 hours as indicated in the individual figures, cells were harvested for assays of apoptosis, which were performed as previously described (6). All cell lines were authenticated by short tandem repeat profiling in the Mayo Advanced Genomics Technology Center, most recently in July 2012 and April 2013.

To compare the ability of various Bcl-2 constructs to protect cells, log-phase K562 cells were transiently transfected with plasmids encoding wild-type or mutant full-length Bcl-2 (40 μg), together with plasmid-encoding enhanced GFP (EGFP)-histone H2B (5 μg), using a BTX 830 square wave electroporator delivering a single 320-V pulse for 10 ms. After incubation to allow transgene exposure, cells were treated with drugs or solvents as indicated in the Figs. 4 and 5. At the completion of the incubation, cells were sedimented at 50 × g, reacted with APC-conjugated Annexin V, and analyzed on a Becton Dickinson FACS Canto II flow cytometer (Becton Dickinson) using the following lasers and filters: EGFP, 488-nm laser, 530/30 filter; APC, 633-nm laser, 660/20 filter. After collection of 20,000 events, APC-Annexin V binding to the EGFP-positive cells was determined using Becton Dickinson Cell Quest software.

To evaluate the effects of downregulating various Bcl-2 family members, log-phase K562 cells growing in antibiotic-free medium were electroporated with previously described siRNAs (6) targeting Bim, Puma, Bak, or Bax (1 μmol/L). To knock down antiapoptotic Bcl-2 family members, we used two previously described short hairpin RNAs (shRNAs) against Bcl-2 or Mcl-1 (6, 26), Bcl-xL (shRNA #1) generated by inserting the hairpin sequence of Huang and Sinicrope (26) into the pCMS5A plasmid, or Bcl-xL (shRNA #2) from Jin-san Zhang (Mayo Clinic, Rochester, MN). The targeted sequences were as follows: Bcl-2 #1: 5’-TGGATGACTGATCTGCAGC-3’, Bcl-2 #2: 5’-ATGGCCGACGCTGGGAGAACG-3’, Bcl-xL #1: 5’-CAGGGACAGCATATCAGAG-3’, Bcl-xL #2: 5’-GTGGAACGCTGAACTCTATGGGAACAAT-3’, Mcl-1 #1: 5’-CAGGGACAGCATATCAGAG-3’, Mcl-1 #2: 5’-GATTGTGACTCTATGGGAACAAT-3’. Beginning 24 hours after transfection, cells were treated with paclitaxel and/or navitoclax as indicated and assayed for Annexin V binding by flow microfluorimetry as described above.

Figure 2. Bcl-2 Ser70 modifications increase cellular resistance to Bak- or Bim-induced apoptosis. A, 24 hours after Jurkat cells were transiently transfected with S-tagged Bcl-2 [wild-type (wt), S70A, or S70E], cell lysates prepared in isotonic buffer containing 1% CHAPS were reacted with S protein-agarose to recover Bcl-2. Pull-downs and one fifth of the inputs were probed with the indicated antibodies. B and C, after transfection with 20 μg EGFP-C1 (control), EGFP-Bim-ΔN, or EGFP-Bak, together with 20 μg pSPN or pSPN encoding Bcl-2 (wild-type, S70A, or S70E), K562 cells were incubated for 24 hours in the presence or absence of 5 μmol/L QVD-OPh and stained with APC-Annexin V. Dot plots (B) from one experiment. Numbers at the right indicate the percentage of EGFP+ cells that are also Annexin V+. Summary of results from three independent experiments is shown in C. Error bars, ± SD. *P < 0.02 versus empty vector; & P < 0.03 (Bak) or P < 0.003 (Bim) versus wild-type Bcl-2 and P < 0.003 versus empty vector. C, right, cell lysates were subjected to SDS-PAGE and probed with antibodies to the indicated antigens to confirm equal transgene expression.
Pull-down assays

Using a 240-V pulse for 10 ms, log-phase Jurkat cells growing in antibiotic-free medium were transiently transfected with plasmids encoding full-length Bcl-2 (wild-type, S70A or S70E) fused at its N-terminus with the S peptide tag (6, 23). After 24 hours, cells were washed and lysed in CHAPS buffer [1% CHAPS, 1% glycerol, 150 mmol/L NaCl, 20 mmol/L HEPES (pH 7.5) containing 1 mmol/L freshly added PMSF, 10 μg/mL leupeptin, 10 μg/mL pepstatin, 100 mmol/L NaF, 10 mmol/L sodium pyrophosphate, 1 mmol/L sodium vanadate, and 20 nmol/L microcystin] at 4°C for 30 minutes. Following clarification at 14,000 x g, the supernatants were incubated with S protein-agarose for 24 hours at 4°C. Beads were then washed four times with isotonic wash buffer containing 1% (w/v) CHAPS; bound proteins were solubilized in SDS sample buffer, subjected to SDS-PAGE, and probed with the indicated antibodies. Bound antibodies were detected (27) using peroxidase-coupled secondary antibodies and enhanced chemiluminescence reagents (Thermo). The resulting X-ray images were imported into Photoshop on an Epson 4490 scanner, converted into PICT files without contrast adjustment, and assembled into figures in Canvas X.

To assess the effects of paclitaxel and navitoclax, K562 cells stably expressing S peptide–tagged Bcl-2 at levels equal to endogenous Bcl-2 were generated as previously described (28). Following treatment with 25 nmol/L paclitaxel and/or 1 mmol/L navitoclax for 18 hours, cells were washed with PBS, and lysed in CHAPS buffer. After lysates were clarified, aliquots containing 600 μg protein (assayed by the bicinchoninic acid method; ref. 29) were incubated with S protein-agarose for pull-downs as described in the preceding paragraph.

Statistical analysis

Graphs show summarized results from three independent experiments. Differences between treatments were analyzed by ANOVA using StatView5 (SAS). All P values stated in figure legends have been subjected to a Bonferroni correction (30).

Results

Bcl-2 Ser70 phosphorylation enhances Bcl-2/Bak and Bcl-2/Bim interactions

To assess the potential impact of Bcl-2 phosphorylation, purified Bcl-2 was treated with CDK1/cyclin B1 complex and assayed for binding to immobilized Bak using SPR, a well-established method for studying protein–protein interactions (31, 32). MS demonstrated that Bcl-2 was phosphorylated on Thr69, Ser70, Thr74, and Ser87 by CDK1. Under the conditions of the reactions, very little unmodified Bcl-2 remained (Fig. 1A and Supplementary Fig. S1). After CDK1/cyclin B-mediated modification, SPR indicated that Bcl-2 bound to Bak equally rapidly but dissociated more slowly than unmodified Bcl-2 (Fig. 1B). Compared with an equilibrium dissociation constant (K_D) of approximately 150 nmol/L for unphosphorylated Bcl-2 binding to Bak, phosphorylated Bcl-2 bound about 5-fold more tightly, although an accurate K_D could not be determined because the binding reflected multiple different phosphorylated species.

Figure 3. Bcl-2 S70A and S70E both diminish drug-induced apoptosis. Beginning 24 hours after Jurkat cells were transfected with 40 μg pSPN or S peptide–tagged Bcl-2 [wild-type (wt), S70A, or S70E] together with 5 μg plasmid encoding EGFP-Histone H2B, cells were treated with the indicated concentrations of etoposide for 7 hours (A and B) or tipifarnib for 72 hours (C), then stained with APC-Annexin V. The percentage of EGFP⁺ cells that were stained with Annexin V is indicated at the right of each dot plot in A and summarized in B. Error bars in B and C, ± SD of three independent experiments. B, right, cell lysates were subjected to SDS-PAGE and probed with the indicated antibodies to confirm equal expression of Bcl-2 constructs.
Further analysis demonstrated that mutation of Ser\textsuperscript{70}, a known site for phosphorylation by multiple kinases (33, 34), to the phosphomimetic amino acid glutamate also enhanced the affinity of purified Bcl-2 for Bak (Fig. 1C–F). Interestingly, mutation of Ser\textsuperscript{70} to alanine had a similar effect (Fig. 1E), as indicated by the slower dissociation of Bcl-2 S70E and S70A from Bak (Fig. 1F). These results raised the possibility that the effect of modification at this site does not require the introduction of a negative charge.
Figure 5. Navitoclax acts in part through Bcl-2 to enhance paclitaxel-induced apoptosis. A and B, 24 hours after K562 cells were treated with the indicated concentrations of paclitaxel with or without indicated concentrations of navitoclax, cells were stained with APC-Annexin V. Histograms from one experiment (A) and summarized results from three independent experiments (B) are shown. B, right, immunoblots probed with antibodies to the indicated antigen. C, 24 hours after K562 cells were transfected with control siRNA or siRNAs targeting Bim, Puma, Bak, or Bax together with EGFP-histone H2B plasmid, cells were treated with 25 nmol/L paclitaxel, 1 μmol/L navitoclax, or 25 nmol/L paclitaxel + 1 μmol/L navitoclax for another 24 hours, then stained with APC-Annexin V. &. \( P < 0.01 \) by ANOVA versus other treatments of cells transfected with control siRNA. /C3. \( P < 0.001 \) versus control siRNA treated with paclitaxel + navitoclax and \( P < 0.006 \) versus Puma siRNA or Bak siRNA treated with the combination. (Continued on the following page.)
In further studies, the effect of these mutations on binding to Bim, an important activator of apoptosis (35–37), was likewise examined. Wild-type Bcl-2 bound to Bim with a K_D of 7 nmol/L, whereas Bcl-2 S70E and Bcl-2 S70A bound with K_D of 0.1 and 1 nmol/L, respectively (Fig. 1G–I), again demonstrating that modified Bcl-2 has a higher affinity for the proapoptotic protein.

**Loop mutations alter Bcl-2 conformation**

Because Thr^{69}, Thr^{74}, and Ser^{77} are also phosphorylated by CDK1 in vitro (Supplementary Fig. S1), we examined the impact of mutating these residues to alanine or glutamate as well. As shown in Supplementary Fig. S2, each of these mutations also enhanced the binding of Bcl-2 to Bak under cell-free conditions. These results suggested that modification at any of the four sites is sufficient to affect binding and, because the altered function does not require introduction of a negative charge, that altered function might reflect a process that is independent of the generation of a phosphoepitope at a particular amino acid. Further experiments focused on Ser^{70}, the most intensively studied but also the most controversial phosphorylation site (see Introduction).

Because Ser^{70} is located in an unstructured region of the Bcl-2 protein, this portion of the protein has been deleted in previous constructs used to determine the Bcl-2 three-dimensional structure (18, 38, 39). Accordingly, it is impossible to assess the detailed effects of Ser^{70} mutations on Bcl-2 conformation. On the other hand, while purifying Bcl-2 S70A and S70E, we observed increased proteolysis of these proteins in bacterial lysates compared with wild-type Bcl-2 (Supplementary Fig. S3A). Moreover, when FPLC fractions containing full-length Bcl-2 S70A and S70E were subjected to limited proteolysis with trypsin, a procedure that has previously been used to assess changes in protein conformation (40), increased protease susceptibility was again observed (Supplementary Fig. S3B). MS indicated that the Bcl-2 fragment arising from this in vitro proteolysis has been preferentially cleaved at Arg^{28} (Supplementary Fig. S3C), providing evidence that Bcl-2 “flexible loop domain” is in a conformation that is relatively inaccessible to interaction with other proteins (as exemplified by trypsin) in wild-type Bcl-2 protein and is rendered more accessible by the S70E and S70A mutations.

**Ser^{70} modification increases Bak binding and survival in intact cells**

To determine whether the changes in binding detected under cell-free condition would also be observed in intact cells, plasmids encoding wild-type Bcl-2, Bcl-2 S70A, or Bcl-2 S70E were transiently transfected into Jurkat T-ALL cells, a cell line chosen because prior studies have demonstrated the constitutive presence of Bcl-2/Bak complexes in these cells (6). The three constructs were expressed equally (Fig. 2A, lanes 6–8), ruling out a major change in protein stability. Consistent with the results in Fig. 1, increased amounts of Bak and, to a smaller extent, Bim, were detected bound to Bcl-2 S70A and S70E compared with wild-type Bcl-2 (Fig. 2A, lanes 3 and 4 vs. 2).

To determine whether the increased affinity of Bcl-2 S70E for Bak and Bim resulted in increased cellular protection, Jurkat cells were transfected with empty vector, wild-type Bcl-2, or S70 mutants along with EGF-Bak or EGF-Bim{\textsubscript{EL}}. As indicated in Fig. 2B and C, and Supplementary Fig. S4, transfection with EGF-Bak induced apoptosis in 55% ± 7% (mean ± SD, 3 independent experiments) of cells cotransfected with empty vector. This was decreased to 40% ± 3% of cells by cotransfection with wild-type Bcl-2 but decreased further to 24% ± 3% by cotransfection with Bcl-2 S70E. Protection by Bcl-2 S70A was similar. Likewise, cotransfected Bcl-2 S70E and S70A protected cells more effectively than wild-type Bcl-2 when cells were transfected with EGF-Bim{\textsubscript{EL}} (Fig. 2C and Supplementary Fig. S4). Similar effects were also observed in other cells as well, including mouse embryo fibroblasts (Supplementary Fig. S5).

In further experiments, the ability of wild-type Bcl-2 versus Bcl-2 S70E to protect from drug-induced apoptosis was examined. When Jurkat cells were treated with 50 μmol/L etoposide, an agent previously shown to trigger apoptosis through the mitochondrial pathway (41), transfection with wild-type Bcl-2 diminished the apoptosis from 43% ± 5% to 33% ± 4% of transfected cells, whereas Bcl-2 S70A and S70E at similar levels (inset, Fig. 3B) further decreased apoptosis to 23% ± 1% and 21% ± 2% of transfected cells (Fig. 3B). Likewise, induction of apoptosis by the farnesyltransferase-inhibitor tipifarnib, an agent previously shown to induce apoptosis in lymphoid cells in a Bim- and Bak-dependent manner (41), was decreased more by Bcl-2 S70A and S70E than by wild-type Bcl-2 (Fig. 3C).

**Implications of Bcl-2 phosphorylation for paclitaxel/navitoclax synergy**

To further assess the impact of Bcl-2 phosphorylation, we examined conditions where Bcl-2 is phosphorylated in intact cells. In particular, treatment of K562 cells with 25 nmol/L paclitaxel, which induced mitotic arrest of more than 60% of the cells (Fig. 4A, left panel), caused near-maximal phosphorylation of Bcl-2 in situ (Fig. 4A, right panel). This paclitaxel-induced Bcl-2 phosphorylation rapidly diminished (Fig. 4B, lanes 5–8) after addition of the CDK inhibitor flavipiridol (42), suggesting that CDK1 plays a role in this modification in situ. After paclitaxel treatment, increased Bak was detected in Bcl-2

(Continued.)
immunoprecipitates (Fig. 4C, lane 2 vs. lane 1). Interestingly, total cellular BimEL diminished in this assay (Fig. 4C, lane 6), but the amount pulled down with Bcl-2 in paclitaxel-treated cells was unchanged (Fig. 4C, lane 2 vs. lane 1), suggesting an increase in affinity for Bim as well.

To assess the potential importance of this Bcl-2/Bim and Bcl-2/Bak binding, we examined the effect of Bcl-2 downregulation on paclitaxel-induced cell cycle changes and apoptosis. In these studies, Bcl-2 shRNA markedly enhanced the sensitivity of K562 cells to paclitaxel-induced apoptosis (Fig. 4D and Supplementary Figs. S6 and S7) without any discernible effect on paclitaxel-induced mitotic arrest (Fig. 4E). This sensitizing effect of Bcl-2 knockdown was particularly noticeable at low paclitaxel concentrations (Fig. 4D and Supplementary Fig. S7). Similar effects were observed with Bcl-xL shRNA (Fig. 4D and Supplementary Figs. S6 and S7), suggesting that Bcl-2 and Bcl-xL both inhibit apoptosis during paclitaxel treatment. In contrast, Mcl-1 shRNA had a much more limited effect on paclitaxel-induced apoptosis.

In further experiments, we examined the action of navitoclax, a BH3 mimetic that displaces proapoptotic Bcl-2 family members from Bcl-2 and Bcl-xL (43), in the absence and presence of paclitaxel. Paclitaxel by itself induced little apoptosis (Fig. 5A) that was Bim and Bax dependent (Fig. 5C). Similarly, in A2780 ovarian cancer cells, treatment with paclitaxel induced little apoptosis by itself, as did navitoclax, whereas the combination induced apoptosis in just under half of the cells (Fig. 5D and E), consistent with previous reports that ABT-737 or navitoclax sensitizes cells to paclitaxel (18). The increased affinity for Bcl-2 knockdown was partic-

Discussion

The present results demonstrate that phosphorylated Bcl-2 as well as Bcl-2 S70E and S70A exhibit increased affinity for the proapoptotic proteins Bak and Bim. Consistent with these observations, increased sequestration of Bak and Bim is observed in cells transfected with Bcl-2 S70E or S70A as well as in cells where Bcl-2 becomes endogenously phosphorylated during paclitaxel treatment. The importance of this enhanced binding between phosphorylated Bcl-2 and its proapoptotic binding partners is demonstrated not only by the increased ability of Bcl-2 S70E to protect against various chemotherapeutic agents, but also by the marked increase in paclitaxel sensitivity observed when Bcl-2 is downregulated by shRNA. These results contribute new insight into regulation of Bcl-2 and simultaneously provide an explanation for the incompletely understood synergy between paclitaxel and BH3 mimetics.

Previous investigations have found that Bcl-2 S70E affords increased protection from cytokine withdrawal-induced apoptosis compared with wild-type Bcl-2 (13, 14). The mechanism of this effect has been unclear. We have extended these results to drugs such as tipifarnib and etoposide (Figs. 3B and C). Our demonstration that Bcl-2 S70E, like phosphorylated Bcl-2, exhibits enhanced binding to Bak and Bim (Fig. 1) provides a mechanistic explanation for increased protection by Bcl-2 S70E relative to wild-type Bcl-2.

Previous studies also demonstrated that Bcl-2 is phosphorylated during mitosis (8–11). The molecular consequences of this phosphorylation were not completely understood. Instead, recent investigations focused on the role of Mcl-1 as a critical regulator of apoptosis during mitosis (45). In particular, degradation of Mcl-1 by the E3 ligase FBWX7 was reported to be critical for paclitaxel-induced apoptosis. On the other hand, our results in K562 cells showed that Mcl-1 downregulation has a limited effect on paclitaxel sensitivity (Fig. 4D and Supplementary Fig. S7). In contrast, Bcl-2 downregulation rendered K562 cells much more sensitive to paclitaxel-induced apoptosis, particularly at low drug concentrations (Fig. 4D and Supplementary Fig. S7). Further analysis demonstrated that Bcl-2 from paclitaxel-treated cells pulls down proportionately more Bak and Bim than Bcl-2 from interphase cells (Fig. 4C). The increased affinity of phosphorylated Bcl-2 for Bak and Bim in vitro (Fig. 1) provides a potential explanation for these observations.

In early descriptions of ABT-737 and navitoclax, impressive synergy between these Bcl-2/Bcl-xL antagonists and paclitaxel was reported (18, 43). We have likewise observed that navitoclax or ABT-199 increased paclitaxel-induced apoptosis in a variety of cell lines (Fig. 5 and Supplementary Fig. S8). Furthermore, we have demonstrated that the killing by the paclitaxel/navitoclax combination is markedly diminished by Bim knockdown (Fig. 5C). This synergy is not easily explained if Mcl-1 is the principal regulator of survival during mitosis but is readily explained if the mitotic phosphorylation of Bcl-2 renders this protein a particularly effective neutralizer of Bak and Bim during mitosis.

Previous studies have shown that Bcl-2 S70A, containing an alanine in place of Ser70, also protects cells from apoptosis (12). On the basis of these observations, along with the assumption that phosphorylation of Ser70 and replacement with alanine would have the opposite functional consequences, it was concluded that phosphorylation at Ser70 inactivates Bcl-2 (12). Accordingly, whether Ser70 phosphorylation enhances or diminishes Bcl-2 function has been controversial. Importantly, the effects of these modifications have not previously been addressed using purified Bcl-2 under cell-free conditions. The present study shows that Bcl-2 loop phosphorylation, replacement of Ser70 by glutamate, and replacement of Ser70 by alanine all increase the affinity of Bcl-2 for Bak and Bim under cell-free conditions (Fig. 1) as well as the ability of Bcl-2 to sequester these proteins in intact cells (Figs. 2 and 4C).
If one assumes that phosphorylation alters Bcl-2 function by altering the charge on Ser70, for example, by creating or destroying a site used for interaction with a binding partner, then the similar effects of alanine and glutamate substitutions are difficult to reconcile. On the other hand, if Ser70 is a hydrogen bonded to another residue in Bcl-2, then any alteration that disrupts this hydrogen bond, including phosphorylation or replacement of the serine, might exhibit the same effect. Consistent with this latter model, we observed that S70E and S70A mutations not only had similar effects on the affinity of Bcl-2 for binding partners (Figs. 1 and 2), but also altered the conformation of the Bcl-2 flexible loop domain in a similar fashion, as indicated by protease accessibility at Arg468 (Supplementary Fig. S3). Although the Thr69, Thr74, and Ser87 replacements of the serine, might exhibit the same effect.

Because this flexible loop domain has been deleted from all Bcl-2 constructs previously examined by nuclear magnetic resonance and X-ray crystallography, further structural studies are required to confirm the model described above and elucidate at the molecular level how these various modifications produce the same functional consequences.

In summary, results of the present study provide the first mechanistic explanation for the enhanced antiapoptotic effects of phosphorylated Bcl-2, demonstrate that endogenous Bcl-2 exhibits higher affinity for Bak and Bim when phosphorylated during mitosis, and provide new insight into the previously observed synergy between BH3 mimetics and paclitaxel. Interestingly, Bcl-xL is also phosphorylated by CDK1 during mitosis (46). Moreover, the Bcl-xL S62A mutant protects cells better than wild-type Bcl-xL against cyclin B-induced toxicity (46). Whether this phosphorylation reflects a similar mechanism for regulating Bcl-xL function during mitosis remains to be determined.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

References

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