Bax activation by the BH3-only protein Puma promotes cell dependence on antiapoptotic Bcl-2 family members

Tristan Gallenne,[1,2] Fabien Gautier,[1,2] Lisa Oliver,[1,2] Eric Hervouet,[1,2] Belinda Noël,[1,2] John A. Hickman,[3] Olivier Geneste,[3] Pierre-François Cartron,[1,2] François M. Vallette,[1,2] Stephen Manon,[4] and Philippe Juin[1,2]

[1]Institut National de la Santé et de la Recherche Médicale Unité Mixte de Recherche 892, Département de Recherche en Cancérologie, F-44035 Nantes, Cedex 01, France
[2]Faculté de Médecine, Université de Nantes, F-44035 Nantes, Cedex 01, France
[3]Institut de Recherche Servier, Département Cancer, 78290 Croissy sur Seine, France
[4]Centre National de la Recherche Scientifique Unité Mixte de Recherche 5095, Université de Bordeaux 2, F-33077 Bordeaux, France

It is still unclear whether the BH3-only protein Puma (p53 up-regulated modulator of apoptosis) can prime cells to death and render antiapoptotic BH3-binding Bcl-2 homologues necessary for survival through its ability to directly interact with proapoptotic Bax and activate it. In this study, we provide further evidence, using cell-free assays, that the BH3 domain of Puma binds Bax at an activation site that comprises the first helix of Bax. We also show that, in yeast, Puma interacts with Bax and triggers its killing activity when Bcl-2 homologues are absent but not when Bcl-xL is expressed. Finally, endogenous Puma is involved in the apoptotic response of human colorectal cancer cells to the Bcl-2/Bcl-xL inhibitor ABT-737, even in conditions where the expression of Mcl-1 is down-regulated. Thus, Puma is competent to trigger Bax activity by itself, thereby promoting cellular dependence on prosurvival Bcl-2 family members.

Introduction

The Bcl-2 family of proteins plays a major role in regulating apoptosis (Adams and Cory, 2007). Mammalian antiapoptotic members include Bcl-2, Bcl-xL, or Mcl-1 and display sequence conservation throughout four Bcl-2 homology domains (BH1–4). They oppose the multidomain proapoptotic proteins such as Bax and Bak, which share BH1, -2, and -3 in common with Bcl-2, and the BH3-only proteins (e.g., Bid, Bim, Puma [p53 up-regulated modulator of apoptosis], Bad, and Noxa; Puthalakath and Strasser, 2002). The resistance of murine cells lacking both Bax and Bak to cell death induction by multiple stimuli, including to BH3-only proteins, implies that antiapoptotic Bcl-2 homologues favor survival by antagonizing the recruitment by death signals and/or the activity of Bax/Bak (Adams and Cory, 2007). This prosurvival activity relies in great part on the ability of Bcl-2 homologues to engage the BH3 domains of Bax, Bak, or BH3-only proteins (Petros et al., 2004). Thus, the mechanisms through which Bcl-2 homologues allow survival is linked to those through which BH3-only proteins induce apoptosis upstream of Bax/Bak.

One model for BH3-induced apoptosis proposes that releasing Bax/Bak from survival Bcl-2 homologues is sufficient to promote cell death (Willis et al., 2005, 2007; Adams and Cory, 2007). This is achieved when the BH3-binding sites of diverse Bcl-2 homologues, which slightly differ in structure (Petros et al., 2004; Chen et al., 2005; Certo et al., 2006), are occupied by promiscuous BH3-only proteins (such as Bid, Bim, or Puma) or by the combination of more selective ones (such as Bad or Noxa). This model does not fully integrate the notion that native Bax is essentially inert and that substantial conformational changes are required for this protein to kill cells (Lalier et al., 2007a): induction of Bax-dependent apoptosis must rely on some Bax-activating signals. Another model for BH3-induced apoptosis proposes that such signals are provided by some BH3-only proteins: death agonists such as Bid, Bim, and

Correspondence to Philippe Juin: Philippe.Juin@univ-nantes.fr

Abbreviations used in this paper: BdGBM, Bax-deficient GBM; BeGBM, Bax-expressing GBM; GBM, glioblastoma multiforme; Hα1-Cter, Hα1 C terminus; Puma, p53 up-regulated modulator of apoptosis; scr, scramble.

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possibly Puma harbor a BH3 domain that can promote ligand-induced activation of Bax, and other survival antagonist BH3-only proteins (Bad and Noxa) allow this process to occur by preventing Bcl-2 homologues to sequester death agonists (Wang et al., 1996; Kuwana et al., 2002, 2005; Letai, et al., 2002; Cartron et al., 2004a). The validity of this model has also been discussed, as it has been difficult to demonstrate the interaction between endogenous purported death agonist BH3-only proteins and Bax during the course of cell death.

Whether certain BH3-only proteins are factors that, independently from antiapoptotic Bcl-2 homologues, prime cells to Bax-dependent cell death is a key question regarding the biology of human cancer cells because (a) the apoptotic response of these cells, including to BH3-only proteins, generally depends in great part on Bax and much less on Bak to be efficient and (b) Bcl-2 homologues are highly expressed in these cells and contribute to their aberrant survival (Letai, 2008). These data, together with the recent development of a small molecule inhibitor of the BH3-binding activity of Bcl-xL and Bcl-2 (ABT-737; Oltersdorf et al., 2005), make timely the identification of proteins that contribute to induction of cell death induced by inhibition of Bcl-2 homologues.

Puma is an essential mediator of p53-dependent and -independent apoptosis in vivo (Jeffers et al., 2003; Villunger et al., 2003). We showed that Puma, and its BH3 domain in particular, might promote ligand-induced activation of Bax (Cartron et al., 2004a). This has been debated since then (Certo et al., 2006; Willis et al., 2007), even though recent evidence has suggested that Puma is a potent activator of cellular Bax and might function downstream of survival antagonist BH3-only proteins Bad and Noxa (Kim et al., 2006). In this study, we have analyzed the nature of the interaction between the BH3 domain of Puma and Bax, and we have investigated whether Puma exerts a Bax-activating function independently from Bcl-2 homologues and whether, consistently, Puma plays an active role when death is triggered in human cancer cells by inhibition of these survival proteins.

Results

The BH3 domain of Puma physically and functionally interacts with Bax

We previously identified Puma as a binding partner of the first α helix of Bax (Bax Hα1; residues 20–37) from a bacterial two-hybrid screen, and subsequent two-hybrid assays showed that the sole BH3 region of Puma (residues 127–150) interacts with Bax Hα1 and full-length Bax (Cartron et al., 2004a). To investigate whether Puma BH3 can interact with cellular Bax, we used a biotinylated 24-mer peptide (Biot-Puma^{BH3}) that encompasses this domain. Pull-down assays after incubation of 1 µM Biot-Puma^{BH3} with 500-µg lysates from glioblastoma cells expressing endogenous Bax (Bax-expressing glioblastoma multiforme [GBM; BeGBM]) showed that this peptide bound to endogenous Bcl-xL and also to Bax. Both interactions were inhibited by an excess of nonbiotinylated peptide (Fig. S1 A). In the absence of competing peptide, ∼2% of the initial Bax and 10% of initial Bcl-xL were pulled down by Biot-Puma^{BH3} (unpublished data).

In two-hybrid assays, the interaction between Puma (or its sole BH3 domain) and Bax (or its first α helix) is prevented by the alteration within Bax of an aspartate at position 33 (i.e., in the C-terminal end of Bax Hα1) into an Ala (Cartron et al., 2004a). To confirm a role for this residue in the interaction of cellular Bax with Puma BH3, we used cell lysates made from originally Bax-deficient GBM (BdGBM) cells that were engineered to express wild-type Bax, the BaxD33A variant, or the BH3-mutated BaxK64A variant as a control (Cartron et al., 2004a). Wild-type Bax and BaxK64A but not BaxD33A were pulled down by Biot-Puma^{BH3} despite the comparable expression of each protein in the lysates used (Fig. 1 A). Thus, D33 is involved in the interaction of Puma BH3 with Bax. Incidentally, this also demonstrates the specificity of the interaction evaluated by this pull-down assay.

In an independent approach to map regions within the N-terminal end of Bax involved in its interaction with Puma BH3, we performed a linear peptide scan derived from epitope mapping (Brix et al., 1999). This approach helped to define the molecular motifs involved in the interaction between Bax and its mitochondrial receptor, TOM22 (Bellot et al., 2007). It was based on the use of nitrocellulose membranes on which 12-mer peptides, overlapping by 10 residues and covering the sequence of Bax comprised between residues 1–106, are covalently bound. Membranes were incubated with Biot-Puma^{BH3}, and the presence of peptides bound to each Bax peptide was revealed using avidin-coupled peroxidase (Fig. 1 B). Peptides comprising residues 25–36, 27–38, and 29–40, which are located in the C-terminal end of Bax Hα1, were strongly labeled by Biot-Puma^{BH3}. Another region, which encompasses residues 89–100 and map to Bax Hα4, showed some, albeit weaker, interaction with Biot-Puma^{BH3}, but most other peptides exhibited no detectable interaction. Collectively, these data reveal a preferential interaction between Puma BH3 and the C-terminal end of Bax Hα1.

The aforementioned Puma BH3–Bax Hα1 interaction recalls our previous study showing that a peptide encompassing the first helix of Bax (Hα1^{Bax}) could prevent the interaction between Puma and in vitro–translated Bax (Cartron et al., 2004a). Likewise, an excess of this 20-mer peptide but not that of a mutant peptide bearing the D33A mutation prevented the interaction between Biot-Puma^{BH3} and cellular Bax in pull-down assays (Fig. S1 B). We inferred from Pepscan analysis that contiguous residues at the C-terminal end of Bax Hα1 might suffice to interact with the Puma BH3 domain and that a shorter peptide encompassing these residues might also prevent the interaction between Puma BH3 and cellular Bax. Thus, we analyzed the effects of a 7-mer peptide (Hα1 C terminus [Hα1-Cter]; residues 31–37) in pull-down assays. 10 µM of this peptide inhibited the interaction between Biot-Puma^{BH3} and Bax from BeGBM lysates (Fig. 1 C). Of note, it also inhibited the ability of Biot-Puma^{BH3} to interact with Bax present in the cytosolic fraction of immortalized rat fibroblasts, whereas a mutant 7-mer peptide carrying the D33A mutation or other 7-mer peptides comprising adjacent residues 25–31 or residues 92–98 did not (unpublished data).

We used Hα1-Cter as a tool to investigate whether the interaction of Puma BH3 with Bax accounts for the ability of the

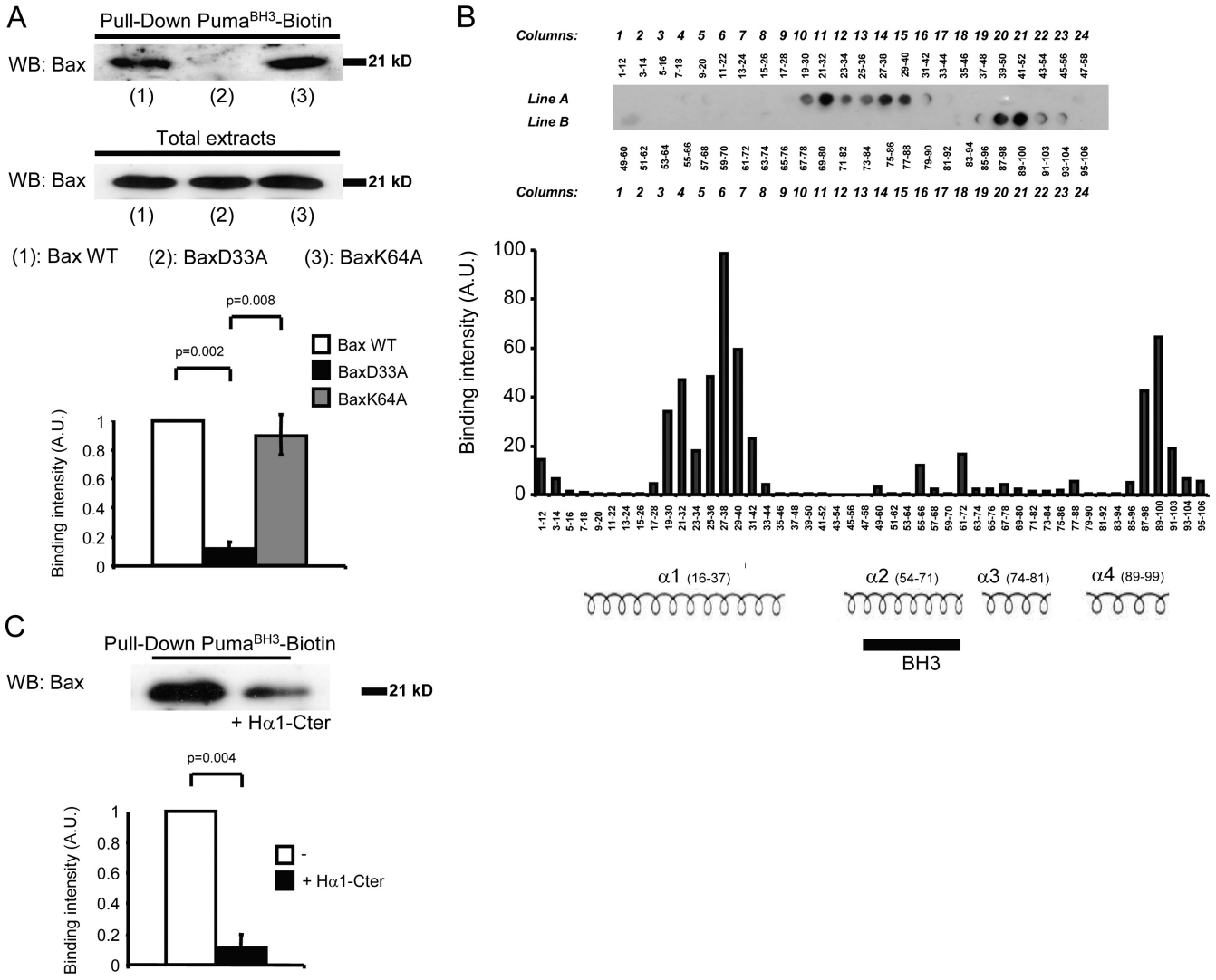

Figure 1. **Role of the C-terminal end of Bax Hα1 in the interaction of Bax with the BH3 domain of Puma.** (A) Interaction of cellular Bax with a Puma BH3 peptide. Lysates from BdGBM(Bax-α), BdGBM(BaxD33A), and BdGBM(BaxK64A) cells expressing comparable amounts of Bax were used. Bax molecules pulled down from these lysates by biotinylated Puma^{BH3} were evaluated by Western blotting. (bottom) In each independent experiment, the amount of pulled down Bax was quantitated by densitometry and normalized to the maximum intensity. (B) Pepscan analysis. Interaction of biotinylated Puma^{BH3} with nitrocellulose membranes, on which were spotted 48 12-mer peptides covering the indicated sequence of Bax, was analyzed as described in Materials and methods. The top panel shows one representative autoradiogram. Peptides range from A1, which is the 12-mer peptide encompassing residues 1–12, to B24, which is the 12-mer peptide encompassing residues 95–106. In the bottom panel, the mean binding intensities (which were evaluated by densitometry and normalized to the maximum binding intensity) for three independent experiments using three different membranes are represented for each peptide. The positions of helices 1–4 (as defined in Suzuki et al. [2000]) are shown for illustration. (C) Interaction of cellular Bax with Puma^{BH3} in the presence of Bax Hα1-Cter peptide. Pull-down experiments were performed as in A, using lysates from BeGBM cells. Where indicated, 10 μM Bax Hα1-Cter was added to lysates before pull-down. (A and C) Data are mean ± SEM of three independent experiments. P-values were assessed using a Student's *t* test. A.U., arbitrary unit; WB, Western blot; WT, wild type.

former to promote the activity of the latter. We have shown that Puma^{BH3} can induce an apoptotic-like change of conformation in in vitro–translated Bax and trigger its mitochondrial activity, whereas nonactivator peptides such as Bad^{BH3} do not (Cartron et al., 2004a). In dose-response experiments, we detected no differences between the efficiency of Puma^{BH3} and that of other purported activator BH3 peptides (such as Bid^{BH3}) to impact on Bax conformation and activity (Fig. S1 C). Hα1-Cter inhibited, in a dose-dependent manner, this cell-free effect of Puma^{BH3} on Bax, indicating that it relies on a physical interaction between the two molecules (Fig. S1 D). The reason why an impact of Puma^{BH3} on Bax activity has been difficult to detect in other cell-free systems (Kuwana et al., 2005; Certo et al., 2006) remains to be elucidated. In an integrated approach to measure Bax apoptogenicity, we also measured induction of cell death by the microinjection of recombinant Bax, which had been preincubated with various peptides. Experiments were performed in human glioblastoma cells in which Bax expression was selectively knocked down by RNA interference (GBM shBax; Manero et al., 2006) to avoid interference of endogenous Bax. Consistent with previous studies (Cartron et al., 2003, 2004a), 100 nM Puma^{BH3} only induced weak, background cell death 6 h after its microinjection in GBM shBax, whereas it induced significant cell death amounts in control cells (GBM scramble

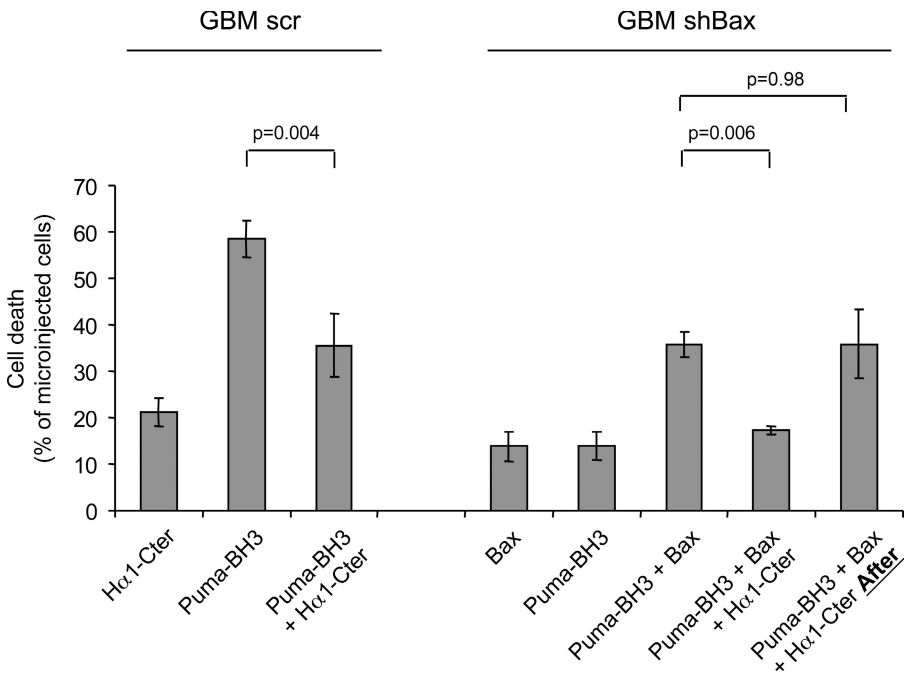

Figure 2. **Functional cooperation between Puma^{BH3} and Bax.** The indicated GBM cells were microinjected with combinations of 100 nM Puma^{BH3}, 1 µM Hα1-Cter, and/or 100 nM recombinant Bax together with a fluorescent microinjection marker (0.5% FITC–dextran 40S). Where indicated, Hα1-Cter was incubated with Puma^{BH3} before incubation with Bax or after Puma^{BH3} was incubated with Bax. The percentage of properly microinjected (i.e., fluorescent) cells exhibiting morphological features of cell death was assayed 6 h after microinjection. Data are mean ± SEM of at least three independent experiments. P-values were assessed using a Student's *t* test.

[scr]; Fig. 2). Although microinjection of 100 nM recombinant Bax by itself was mostly inefficient at inducing cell death in GBM shBax cells, microinjection of 100 nM Bax that had been preincubated with 100 nM Puma^{BH3} (for 60 min at 37°C) did promote significant cell death (Fig. 2). The ability to trigger Bax induction of cell death was also found for Bid^{BH3}, but it was not observed for Bad^{BH3} (Fig. S1 E), indicating that this short-term system assays the apoptogenicity of the injectate at the time of microinjection. Hα1-Cter diminished the ability of Puma^{BH3} to promote cell death in Bax-expressing, GBM scr cells (Fig. 2). Moreover, when this peptide was added to Puma^{BH3} before incubation with recombinant Bax and microinjection in GBM shBax cells, no significant cell death could be detected, indicating that enhancement of Bax activity by Puma^{BH3} was inhibited by Hα1-Cter (Fig. 2). Importantly, when Hα1-Cter was added to the microinjection preparation after Bax had been incubated with Puma^{BH3}, the resulting mixture proved as efficient at inducing cell death in GBM shBax cells as the mixture without Hα1-Cter (Fig. 2). Thus, in this system, Hα1-Cter exerts an inhibitory effect during the incubation of Puma^{BH3} with Bax, but it has no effect on the cell response to the mixture. In contrast, recombinant Bcl-xL prevented Puma^{BH3} activation of Bax when added not only before but also after incubation of the peptide with Bax, supporting the idea that Bcl-xL can exert control over both ligand-induced Bax activation and Bax activity (Fig. S1 F). Importantly, microinjected Hα1-Cter also prevented induction of cell death by overexpression of Puma in GBM cells, and Puma-dependent induction of cell death by Adriamycin in HCT116 cells (Fig. S2). These observations, together with our previous study showing that ectopic wild-type Bax-α or BaxK64A but not ectopic BaxD33A restores the sensitivity of Bax-deficient glioblastoma cells to Puma and Puma^{BH3} (Cartron et al., 2002), are in strong agreement with the notion that the interaction of Puma BH3 with Bax Hα1 enhances Bax activity and promotes cell death.

Puma cooperates with Bax to promote yeast cell death in a Bcl-xL–sensitive manner

To investigate whether Puma and Bax can physically interact and functionally cooperate to promote cell death in the absence of Bcl-2 homologues, we analyzed the effects of their expression in yeast, which express no recognized Bcl-2 family proteins. When expressed in yeast, full-length untagged human Bax remains cytosolic and induces neither cytochrome *c* release nor cell death (Priault et al., 2003; Arokium et al., 2004). Nevertheless, conformational changes of the protein caused by substitution of critical residues can promote mitochondrial translocation and the subsequent release of cytochrome *c* (Arokium et al., 2004). We generated yeast strains that express galactose-inducible Bax, Puma, or both. Although the induction of Puma only (not depicted) or that of wild-type Bax only (Fig. 3 A) had no detectable effect on yeast viability, the coexpression of Bax and Puma induced yeast cell death (Fig. 3 A) and cytochrome *c* release (Fig. 3 C). Cell death induced by Puma and Bax, as previously reported for that induced by active substituted Bax mutants (Arokium et al., 2004), was prevented by the expression of Bcl-xL (Fig. 3 A).

To evaluate whether the functional cooperation between Puma and Bax involved Bax Hα1, we then used yeast strains expressing galactose-inducible BaxD33A. This mutant promoted some cytochrome *c* release and cell death by itself (Fig. 3, B and C), most likely owing to a partial unfolding induced by the mutation (Cartron et al., 2005; Arokium et al., 2007). Nevertheless, the deleterious effects of BaxD33A in yeast cells were not stimulated by the coexpression of Puma, even at early time points after induction (Fig. 3, B and C).

Coimmunoprecipitation experiments were performed to investigate whether the functional cooperation between Puma and Bax coincided with a physical interaction between these two proteins. Wild-type Bax and Puma interacted with each

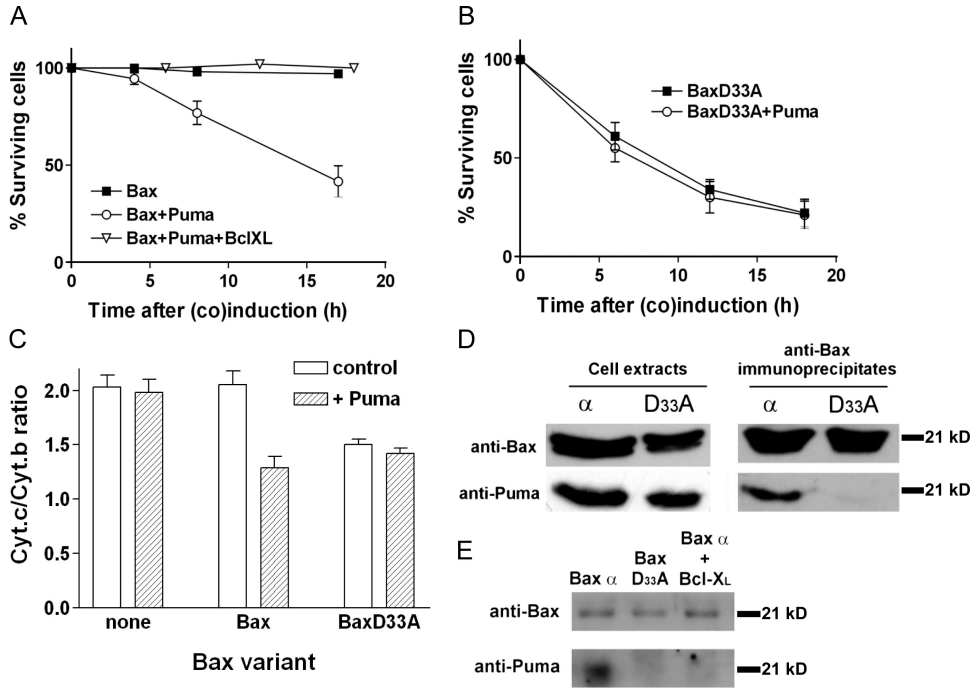

Figure 3. **Interaction of Bax and Puma in yeast.** (A) Yeast cell survival after Bax, Puma, and Bcl-xL coexpression. Induction of Bax, Puma, and/or Bcl-xL in the corresponding yeast cells and survival assays at the indicated time after induction were performed as described in Materials and methods. (B) Yeast cell survival after BaxD33A and Puma coexpression. The experiment was performed as in A. (C) Cytochrome *c* release after wild-type Bax, BaxD33A, and Puma coexpression. Cytochrome (Cyt.) content in mitochondria isolated from the indicated yeast cells 14 h after induction was measured as described in Materials and methods. (D) Puma interaction with wild-type Bax and BaxD33A in yeast. Extracts from cells coexpressing Bax (or BaxD33A) and Puma were immunoprecipitated with anti-Bax antibody, and immunoprecipitates were analyzed for the presence of Bax and Puma by Western blotting. (E) Effect of Bcl-xL on Puma–Bax interactions in yeast. Extracts from cells coexpressing Bax and Puma, BaxD33A and Puma, or Bax, Puma, and Bcl-xL were treated as in D. (A–C) Data are mean ± SEM of three independent experiments.

other in a yeast context, whereas BaxD33A and Puma could not (Fig. 3 D). Moreover, Bcl-xL expression inhibited this interaction (Fig. 3 E). Thus, Puma and Bax physically interact and functionally cooperate by a Bax Hα1–dependent, Bcl-xL–sensitive process, even in a cellular context that lacks known equivalents of the mammalian apoptotic machinery.

Puma plays a role in Bax-dependant apoptosis induced by inhibition or down-regulation of Bcl-xL

We investigated whether the aforementioned activation of Bax by Puma could take place in mammalian cells. As this activation involves the BH3 domain of Puma and is prevented by Bcl-xL, we reasoned that its occurrence in mammalian cells would define Puma as an actor of cell death that ensues when Bcl-2 homologues are inhibited. Thus, we analyzed whether Puma plays a role when cell death is induced by ABT-737, a potent inhibitor of the BH3-binding activity of Bcl-xL and Bcl-2 (Oltersdorf et al., 2005), using the human colorectal cancer cell line HCT116 $p21^{-/-}$ and its isogenic counterparts in which genetic ablation of Puma or Bax was performed ($p21^{-/-}Puma^{-/-}$ and $p21^{-/-}Bax^{-/-}$; Fig. 4 A).

We analyzed the response of control, $Puma^{-/-}$, and $Bax^{-/-}$ cells to 24-h treatment with increasing concentrations (1–10 μM) of ABT-737. As shown in Fig. 4 B, ABT-737 induced dose-dependent cell death in control cells. $Bax^{-/-}$ cells were completely resistant to this short-term treatment at all concentrations tested, further underscoring the importance of Bax in the apoptotic response of these human cancer cells. $Puma^{-/-}$ cells exhibited some cell death response at high concentrations of ABT-737, but it was much lower than that induced in control cells (Fig. 4 B). A closer examination of the death pathways involved was performed by immunocytochemical analysis of cytochrome *c* localization and caspase 3 activation (using a conformation-specific antibody). The percentage of cells exhibiting cytochrome *c* release and caspase 3 activation after ABT-737 treatment was much lower in $Puma^{-/-}$ cells than in control cells (Fig. 4 C). ABT-737 at 10 μM had no detectable effect on cytochrome *c* release or caspase 3 activation in $Bax^{-/-}$ cells. Thus, Puma is implicated in induction of cytochrome *c* release, caspase 3 activation, and apoptotic cell death by ABT-737. As we could not detect any increase in Puma expression upon ABT-737 treatment (Fig. S3 A), we infer that endogenous levels of Puma are sufficient to mediate these effects. We checked that the knockdown of Puma expression in control HCT116 cells by RNA interference conferred resistance to ABT-737–induced caspase 3 activation and cell death (Fig. S3, B and C). Notably, the resistance of Puma knockdown cells was not as marked as that of Puma knockout cells, suggesting that the remaining levels of Puma in the knockdown cells could still mediate ABT-737–induced cell death. Microinjection of Hα1-Cter peptide into control HCT116 cells significantly inhibited ABT-737 induction of cell death (Fig. S3 D), further supporting the notion that Puma is involved in this process.

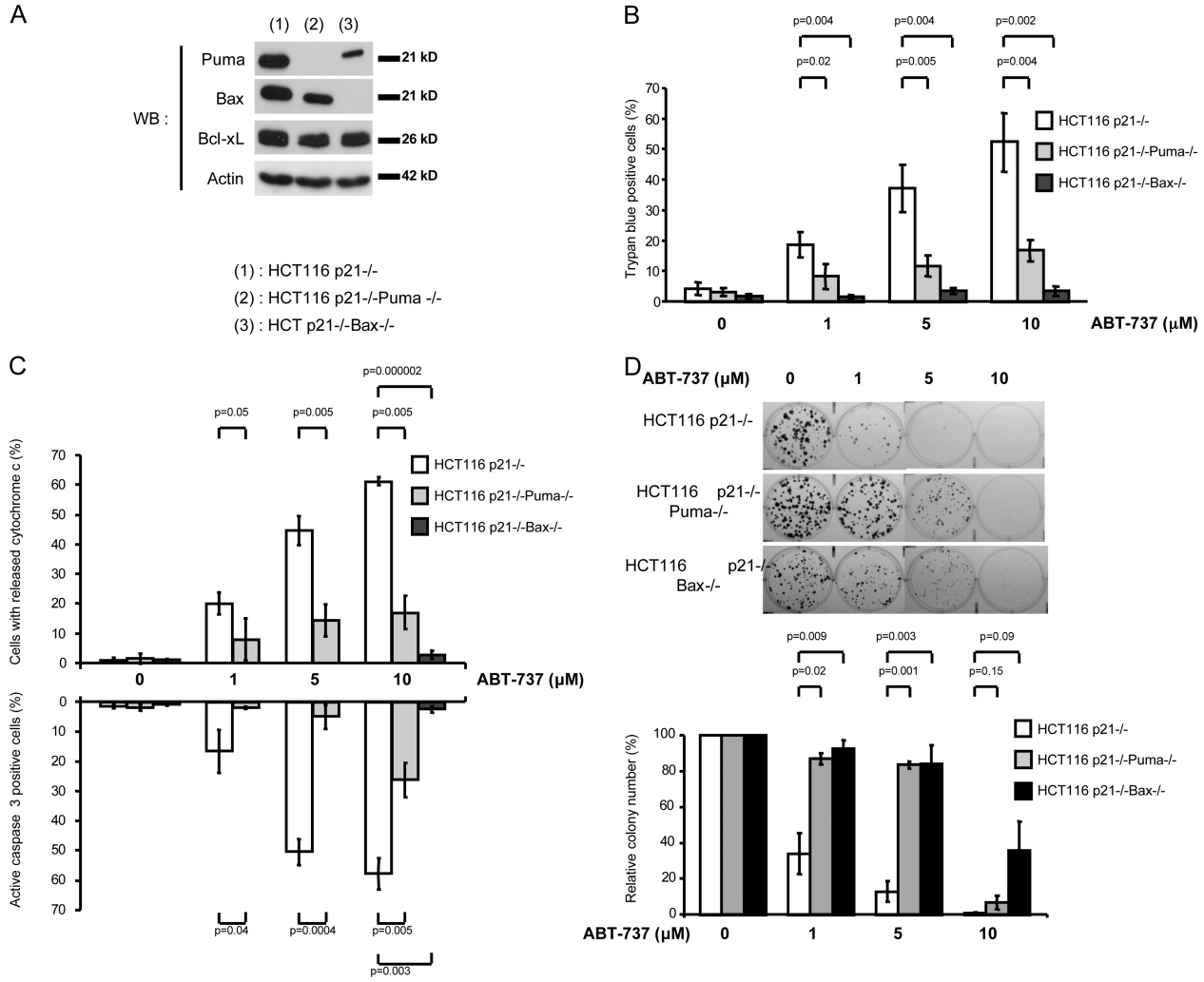

Figure 4. **Role of endogenous Puma in the biological activity of ABT-737.** (A) Expression of Bcl-2 family members in HCT116-derived cell lines. Expression of 50 µg Puma, Bax, and Bcl-xL in lysates from the indicated cells was analyzed by Western blotting. WB, Western blot. (B) Induction of cell death by ABT-737 in human colorectal cancer cells. The indicated cells were incubated with the indicated concentration of ABT-737 for 24 h, and induction of cell death was assayed by a trypan blue–staining procedure. Data are mean ± SEM of four independent experiments. (C) Induction of apoptosis by ABT-737 in human colorectal cancer cells. The indicated cells were incubated with the indicated concentration of ABT-737 for 24 h before immunocytochemical analysis of the percentage of cells exhibiting cytochrome *c* release and caspase 3 activation. Data are mean ± SEM of three independent experiments. (D) Long-term effects of ABT-737 on human colorectal cells. The indicated cells were treated for 2 wk with the indicated concentration of ABT-737 before analysis of viable clones by crystal violet staining. Data are mean ± SEM of at least three independent experiments and are expressed as a percentage of the number of colonies formed in untreated dishes. One experiment representative of three independent ones is shown in the top panel. (B–D) P-values were assessed using a Student's *t* test.

RNA interference experiments and microinjection assays using Hα1-Cter also showed that endogenous Puma contributes to the apoptotic response of primary GBM cells to ABT-737 (Fig. S3, E–G).

To analyze whether Puma was involved in long-term effects of ABT-737 on cell growth, control, $Puma^{-/-}$, and $Bax^{-/-}$ HCT116 cells were seeded at a low density and treated for 2 wk with 1–10 µM ABT-737 before visualization of viable clones. ABT-737 significantly affected the clonogenicity of control cells at all concentrations tested (Fig. 4 D). $Puma^{-/-}$ cells were strikingly more resistant to these long-term effects, as only 10 µM ABT-737 had a major effect on their growth. Because the growth of $Bax^{-/-}$ cells was also affected at this concentration, the implication of Bax-independent mechanisms, of nonapoptotic growth inhibitory mechanisms, and/or that of off-target effects of ABT-737 cannot be ruled out when high concentrations are used for long periods of treatment.

The aforementioned experiments suggest that some survival proteins targeted by ABT-737 constitutively maintain survival of the cells used in this study by counteracting Puma. We analyzed the acute effects of RNA interference–mediated Bcl-xL knockdown in control, $Puma^{-/-}$, and $Bax^{-/-}$ cells. We used a recombinant lentivirus that expresses GFP together with a short hairpin RNA targeting Bcl-xL (shBcl-xL) and infection conditions that lead to a profound loss of Bcl-xL expression (Fig. 5 A). Under these conditions, Bcl-xL knockdown induced the appearance of a significant number of cells with active caspase 3 2 d after infection and massive cell death 3 d after infection in control cells, whereas infection with a control (scr) lentivirus had no effect (Fig. 5, B and C). As shown in Fig. 5 (B and C),

Puma$^{-/-}$ and *Bax*$^{-/-}$ cells were refractory to caspase 3 activation and cell death induced by lentivirus-mediated down-regulation of Bcl-xL. Of note, a recombinant lentivirus targeting Bcl-2 had a strikingly weaker effect on cell viability than the shBcl-xL lentivirus, but its feeble effect was also Puma dependent (unpublished data). Interestingly, Fig. 5 A suggests that cell death induced by Bcl-xL knockdown in control cells may be slowed down by the unexpected ability of this knockdown to decrease Puma expression. The mechanism involved, which was also detected in Bax-deficient cells, remains to be characterized. It should also be noted that knockdown of Bcl-xL in control cells provoked the apparition of a truncated form of Bax of an apparent molecular mass of 18 kD. This may be a consequence of the induction of cell death, as this truncation, which has been reported in many cases of apoptosis induction and results in the removal of Bax Hα1 from the rest of the molecule (Cartron et al., 2004b), was not observed in Puma-deficient cells. In all cases, the view that emerges from these assays is that Bcl-xL acts in an ABT-737–sensitive manner to counteract a constitutive Puma-related death signal.

Puma mediates apoptosis induced by pharmacological inhibition of Bcl-xL combined with Mcl-1 down-regulation

ABT-737 does not inhibit Mcl-1 (Oltersdorf et al., 2005), and Puma does interact with this survival protein (Chen et al., 2005). Thus, the involvement of Puma in the aforementioned deleterious effects of ABT-737 may rely on the ability of this protein to counteract Mcl-1 rather than on its ability to activate Bax directly. We analyzed whether Puma was still involved in the induction of death by ABT-737 in cells devoid of Mcl-1. Because we were unable to produce a lentivirus construct that would significantly knock down Mcl-1 in HCT116 cells, we used a previously published procedure in which siRNAs targeting Mcl-1 were transiently transfected (Fig. 6 A; Maiuri et al., 2007). Mcl-1 knockdown by transfection of siRNAs induced only a modest increase in the amount of cells with released cytochrome *c* and active caspase 3 by itself (Fig. 6 B), but it allowed a more efficient induction of these apoptotic events by a 24-h treatment with 1 μM ABT-737 in control cells. In contrast, *Puma*$^{-/-}$ cells were not sensitized to ABT-737–induced cell death by Mcl-1 knockdown, and remained significantly more resistant to ABT-737–induced cell death under these conditions (Fig. 6 B). Thus, Puma still exerts proapoptotic activity when Mcl-1 is down-regulated and Bcl-xL is inhibited.

Conversely, we analyzed whether increased expression of Mcl-1 would protect cells from ABT-737–induced cell death and whether this would be Puma dependent. Flag-tagged Mcl-1 was transiently transfected in control and *Puma*$^{-/-}$ cells, cells were treated with a high concentration of ABT-737, and the percentage of Flag-positive cells exhibiting active caspase 3 was evaluated by immunocytochemistry. Although, in a Puma-proficient context, the percentage of cells displaying caspase 3 activation after ABT-737 treatment was lower in the Flag–Mcl-1–expressing population than in the mock-transfected one, this was not the case in a Puma-deficient background (Fig. 6 C). This puts forth the notion that Mcl-1 regulates ABT-737–induced cell death in great part in these cells by antagonizing Puma. It implies that Puma exerts proapoptotic activity that is efficient in the absence of Bcl-xL and Mcl-1 and that is restrained by these proteins.

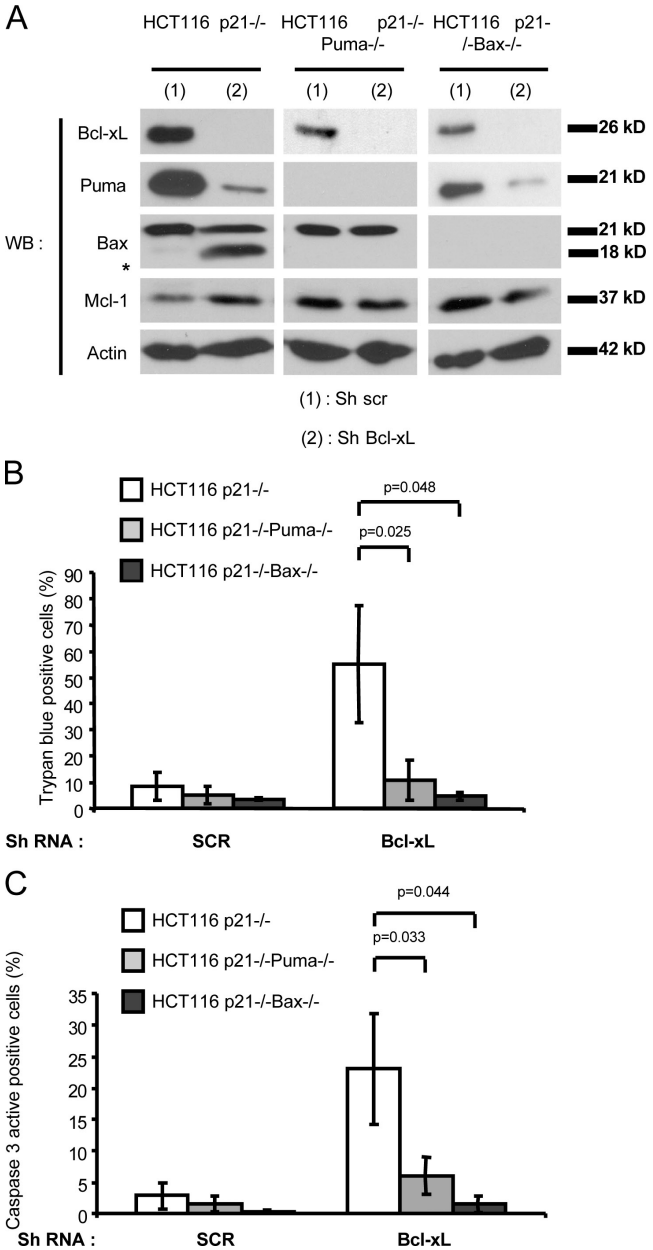

Figure 5. **Role of endogenous Puma in the induction of apoptotic cell death by down-regulation of Bcl-xL in human cancer colorectal cells.** (A) Expression of Bcl-2 family members in HCT116-derived cell lines after down-regulation of Bcl-xL. Western blot (WB) analysis of Puma, Bax, Bcl-xL, and Mcl-1 in lysates from the indicated cells was performed 3 d after infection with the indicated recombinant lentivirus. The asterisk indicates a truncated form of Bax of 18 kD. (B) Induction of cell death by down-regulation of Bcl-xL in human colorectal cancer cells. The indicated cells were infected with the indicated lentivirus, and induction of cell death was assayed by a trypan blue–staining procedure 3 d later. (C) Activation of caspase 3 by down-regulation of Bcl-xL in human colorectal cancer cells. The indicated cells were infected with the indicated recombinant lentivirus, and activation of caspase 3 was evaluated as described in Fig. 4 B 2 d later. (B and C) Data are mean ± SEM of three independent experiments. P-values were assessed using paired Student's *t* tests. ShRNA, short hairpin RNA.

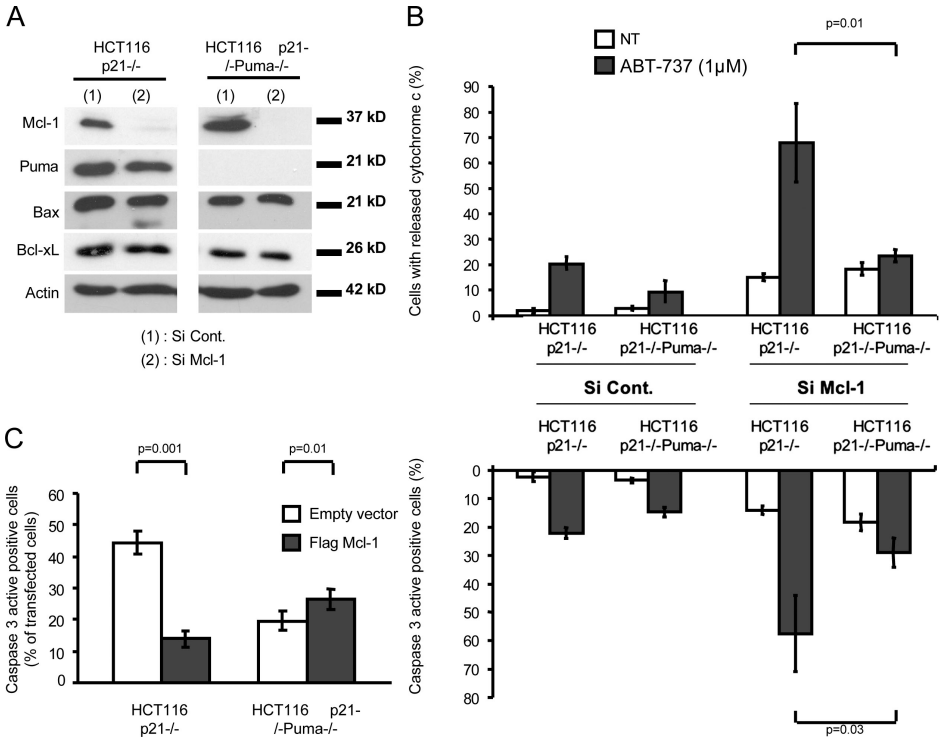

Figure 6. **Role of Mcl-1 in Puma-dependent apoptosis induced by ABT-737.** (A and B) Down-regulation of Mcl-1. The indicated cells were transfected with the indicated siRNA. 48 h later, Western blot analysis of Mcl-1, Puma, Bax, and Bcl-xL (A) and treatment with 1 μM ABT-737 (B) were performed. Induction of cytochrome *c* release and caspase 3 activation were analyzed as described for Fig. 4 B. NT, not treated. (C) Overexpression of Mcl-1. The indicated cells were transfected with the indicated plasmid. 24 h later, cells were treated with 10 μM ABT-737 for an additional 24 h. Immunocytochemical analysis of active caspase 3 and of Flag expression was then performed. For Flag–Mcl-1–transfected cells, the percentage of Flag-positive cells that also stained positive for active caspase 3 was evaluated. For mock-transfected cells, the percentage of cells that stained positive for active caspase 3 was evaluated in random populations. (B and C) Data are mean ± SEM of three independent experiments. P-values were assessed using a Student's *t* test.

Pharmacological inhibition of Bcl-xL enhances interactions between endogenous Puma and Bax

The aforementioned experiments show that the ability of Puma to mediate cell death induced by ABT-737–mediated Bcl-xL inhibition is independent from Mcl-1. They favor the notion that a direct interaction between Puma and Bax would be involved in this cell death process. We analyzed the effects of ABT-737 on intermolecular interactions between endogenous Bcl-xL, Bax, and Puma by coimmunoprecipitation experiments using lysates from cells treated with 1 μM ABT-737, which is an experimental condition that does not trigger major cell death (Fig. 4). As shown in Fig. 7 A, such treatment almost completely abolished the interactions between Bcl-xL and Bax in control cells. This was also the case in *Puma*$^{-/-}$ cells (Fig. 7 C). This indicates that inhibition of Bax–Bcl-xL interactions is not an indirect consequence of cell death induction. It implies that the role of Puma in ABT-737 activity is not to allow this compound to disrupt Bax–Bcl-xL complexes and, conversely, that disruption of Bax–Bcl-xL complexes that preexist in *Puma*$^{-/-}$ cells is insufficient to promote full-blown apoptosis. In control cells, ABT-737 also disrupted the interactions between Puma and Bcl-xL (Fig. 7 A). Most notably, coimmunoprecipitation showed that some interaction between Puma and Bax occurred in untreated cells and that it was enhanced by ABT-737 treatment (Fig. 7 B). Thus, ABT-737 treatment frees both Bax and Puma from Bcl-xL and allows these two proteins to interact substantially. Collectively, these assays suggest that the BH3-binding activity of Bcl-xL maintains survival by preventing Puma and Bax to interact with each other. Because some interaction was detected between Puma and Bax in viable cells, Bcl-xL may also protect cells from the consequence of these interactions.

Discussion

Although it has been proposed that Bax activation may be initiated by a subgroup of BH3 domains, so far, it has remained unclear whether the BH3 domain of Puma classifies as an activator. Analysis of Bax activation by truncated Bid in model assays has put forth the notion that Bax activation is a stepwise mechanism that relies on a series of reversible binding interactions with different rates initiated by transient events (Lovell et al., 2008). Thus, an initiating interaction between Bax and an activating BH3 domain may be elusive because of its fugacious nature and because of its potent inhibition by BH3-binding Bcl-2 homologues, which maintain cell survival. Thus, our analysis of a physical and functional interaction between Puma and Bax was focused on the definition of molecular motifs involved in this interaction and on the investigation of its consequences in cellular contexts in which Bcl-2 homologues are absent and/or inhibited.

We recently found that Puma could interact with the first α helix of Bax and then showed that Puma or its BH3 domain

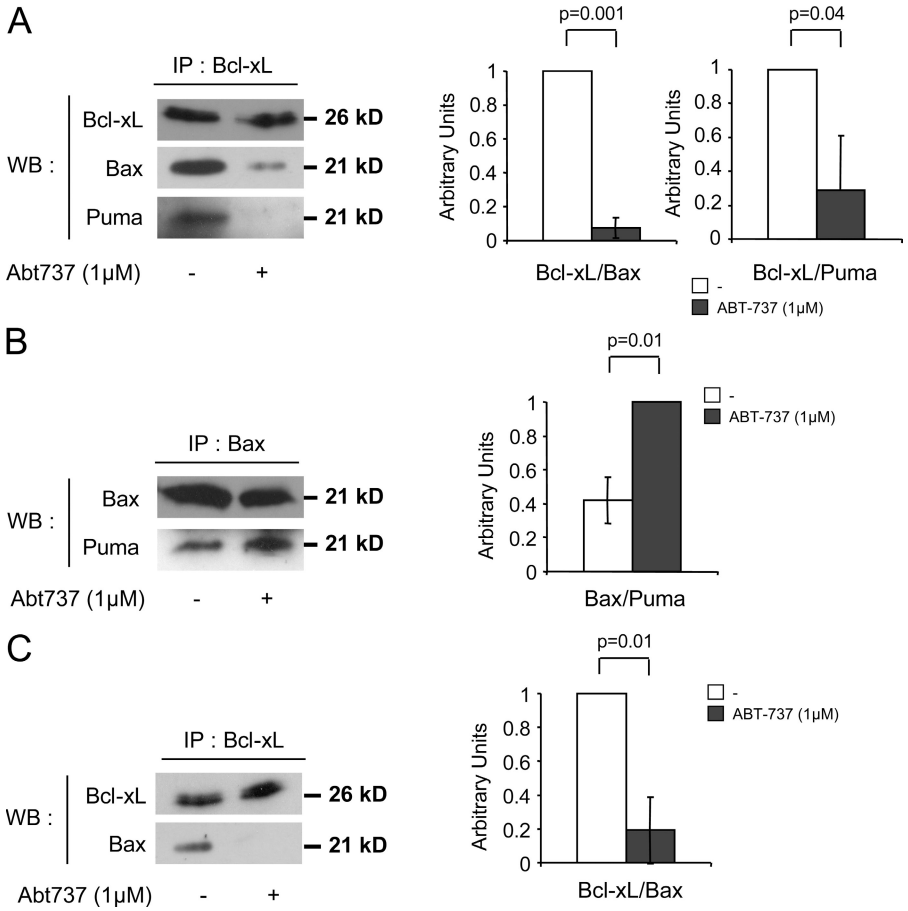

Figure 7. **Effect of ABT-737 on intermolecular interactions involving Bax, Bcl-xL, and Puma.** (A) Interactions between Bcl-xL and Bax or Puma. HCT116 $p21^{-/-}$ cells were treated or not treated (−) with 1 μM ABT-737 for 24 h. Cell lysates were immunoprecipitated (IP) with an anti–Bcl-xL antibody as described in Materials and methods, and the presence of Bcl-xL, Bax, and Puma in the immunoprecipitated fractions was analyzed by immunoblotting. The amount of Bax and Puma that were coimmunoprecipitated with Bcl-xL in each condition was evaluated by densitometric analysis and normalized to the amount of protein that coimmunoprecipitated with Bcl-xL in untreated cells. (B) Interactions between Puma and Bax. Experiments were performed as in A using an anti-Bax antibody for immunoprecipitation. The amount of Puma that was coimmunoprecipitated with Bax in each condition (evaluated by densitometric analysis) was normalized to the amount of protein that coimmunoprecipitated with Bax in treated cells. (C) Interactions between Bcl-xL and Bax in Puma knockout cells. Experiments were performed as in A using HCT116 $p21^{-/-}Puma^{-/-}$ cells. (A–C) Data are mean ± SEM of three independent experiments. P-values were assessed using a Student's *t* test. WB, Western blot.

could promote Bax interaction with mitochondria in cell-free assays and trigger its proapoptotic activity (Cartron et al., 2004a). The evidence provided in this study suggests that the latter effect involves, at least in part, a physical interaction between the BH3 domain of Puma and Bax, as the enhancement of Bax activity by $Puma^{BH3}$ is prevented by a minimal peptide that prevents this physical interaction. This peptide was designed from a Pepscan analysis, which further substantiated our previous description of an interaction between Bax Hα1 and Puma BH3 (Cartron et al., 2004a) and underscored the role of the C-terminal end of Bax Hα1 in this interaction. Our analysis also hinted to a possible interaction between $Puma^{BH3}$ and Bax Hα4, which is close to the Bax BH1 domain that was initially suggested to play a role in an interaction with Bid (Wang et al., 1996). However, a 7-mer peptide comprising residues 92–98 (within Bax Hα4) proved insufficient to prevent Puma BH3–Bax interactions in pull-down assays (unpublished data). The roles of other regions within Bax in its interaction with Puma also require further elucidation. In particular, a recent study published after submission of this manuscript shows that not only residues in Bax Hα1 but also residues in Bax Hα6 might be critically involved in ligand-induced activation of Bax by a stabilized Bim BH3 peptide (Gavathiotis et al., 2008). In our study, because Pepscan analysis using biotinylated Puma BH3 and peptides covering the sequence of Bax between residue 107 and residue 194 did not yield any significant result (unpublished data), we currently do not know whether, or which, additional regions in Bax might be involved in its interaction with Puma BH3. The involvement of regions other than the BH3 domain of Puma in the interaction between Puma and Bax has also been suggested by a recent study (Yee and Vousden, 2008).

In the native conformation of Bax, Bax Hα1 is in the vicinity of Bax Hα2, which contains the BH3 domain. Moreover, a salt bridge formed between D33 within the C-terminal end of Bax Hα1 and K64 within the BH3 domain participates in the maintenance of Bax in an inactive state (Cartron et al., 2005). Thus, it is tempting to speculate that an intermolecular interaction between Puma BH3 and Bax Hα1 might help to initiate an unfolding of the Bax molecule. This process, which could also play a role in Bax activation by Bim, as Bax Hα1 is also involved (Gavathiotis et al., 2008), might allow the complete activation of Bax to occur, possibly in combination with additional amplificatory mechanisms (Lakhani et al., 2006; Zhu et al., 2006). It should be noted that Bax Hα1 might be involved in other protein–protein interactions engaged by Bax such as that with TOM22 (Bellot et al., 2007). Thus, Puma and Bax may end up in distinct protein complexes during the course of cell death. This aspect is reminiscent of what was reported for the Bid–Bax interplay (Grinberg et al., 2005).

Yeast cells, which express no recognized Bcl-2 family proteins, tolerate the heterologous expression of wild-type, untagged Bax but undergo mitochondrial permeabilization and cell death when expressing constitutively active variants of Bax (Arokium et al., 2004). Thus, the fact that, in this paradigm,

Puma physically interacts with Bax and switches on its killing activity by a Bax Hα1–, Bcl-xL–sensitive process strongly underscores the potency of Puma to initiate Bax activation by itself. The ability of Puma to activate Bax in yeast is reminiscent of a similar effect recently reported for the short form of Bim (Weber et al., 2007). This work implies that the interaction of Bim with mitochondria is critical and that mechanisms other than protein–protein interactions could be at stake. Bax-activating signals can be provided by nonfamily proteins or nonproteinaceous factors (Kuwana et al., 2002; Lalier et al., 2007b). Therefore, it is possible that Puma may have an impact on such mechanisms, having an effect that, added to its ability to interact with Bax, will lead to efficient cell death. As we observed that, in yeast cells, Puma is essentially cytosolic (unpublished data), such additional signals would rely on different molecular mechanisms than those proposed for Bim (Weber et al., 2007). Because expression of cytosolic Puma leads to an accumulation of Bax at the mitochondria in yeast (unpublished data), the yeast system argues for a "touch and go" type of interaction between Puma and Bax.

A genuine activator BH3-only protein is expected to play an active role when mammalian cell death is induced by inhibition of Bcl-2 homologues. Along this line, it is important to note that endogenous Puma is involved in efficient induction of apoptosis in HCT116 cells by the small compound ABT-737. Although other death agonist BH3-only proteins might be mediators of ABT-737–induced cell death in certain cell systems (most notably Bim; Del Gaizo Moore et al., 2007), knockdown of Bim and Bid expression in HCT116 cells had no detectable impact on ABT-737–induced cell death (Fig. S4 A), implying that Puma's role in this case is not upstream of these proteins. Likewise, down-regulation of Bak had no detectable effect on ABT-737 induction of cell death in the same cells (Fig. S4 B), suggesting that this protein is also dispensable for this process and underlining the major role played by the Puma–Bax axis. Most importantly, Puma is involved in ABT-737–induced cell death even in conditions under which Mcl-1 expression is down-regulated, and, on a molecular level, ABT-737 treatment enhances Bax–Puma interactions at the same time as it prevents the interaction of each of these proteins with Bcl-xL. These observations support the notion that Puma is a death agonist BH3-only protein that, as such, provides a tonic death signal that the combined BH3-binding activities of Bcl-2 homologues will have to continuously counterbalance to maintain survival.

This notion is best exemplified by our observation that although Puma or Bax knockout cells tolerate Bcl-xL down-regulation, control HCT116 cells undergo massive cell death after such down-regulation. Compared with Bcl-xL down-regulation, Bcl-2 down-regulation (unpublished data) and Mcl-1 down-regulation (at the very least in the experimental conditions used) only had a modest effect on the viability of HCT116 cells. This indicates that Bcl-xL is more critically involved in maintaining the survival of these cells than its homologues. The reason for this apparent selectivity is unclear, but, strikingly, it recalls a previous study that showed that Bcl-xL much more potently prevents Puma function than Bcl-2 or Mcl-1 (Ming et al., 2006).

Importantly, low levels of Puma appear sufficient to prime cells to death and lock them in a Bcl-xL–dependent state. Thus, when Puma expression is increased, it is possible that its accumulation in complexes with Bcl-xL (and thus the ability of additional Puma molecules to interact with Bcl-xL) may play a significant role in Puma-dependent cell death (Chipuk et al., 2005; Ming et al., 2006; Yee and Vousden, 2008). Another implication is that Puma may exert a proapoptotic function that will be lost only when its expression is completely abrogated. This may explain differences found between experiments that, albeit performed similarly, used Puma knockout versus Puma knockdown cells (Kim et al., 2006; Willis et al., 2007). It is also noteworthy that Puma knockout HCT116 cells still show some response to ABT-737. Thus, Puma expression may not be an absolute requirement for induction of cell death by Bcl-xL inhibition. Along this line, it is important to note that we have shown that survival antagonist BH3 domains could promote the release of active Bax from Bcl-xL by themselves, implying that sensitizer molecules can launch a Bax-dependent apoptotic program without additional Bax-activating signals (Moreau et al., 2003; Cartron et al., 2005). The ability of ABT-737 to displace Bax from Bcl-xL in Puma-deficient cells may account for the small rates of Bax-dependent cell death we observed in these cells.

With increasing evidence of secondary effects and possible acquisition of resistance to ABT-737, predictive markers of sensitivity and resistance to this compound are crucial. Puma expression can be driven downstream of diverse tumor suppressor and/or oncogenic pathways because Puma is a direct transcription target of p53, p73, Foxo3A, and E2F-1 (Nakano and Vousden, 2001; Yu et al., 2001; Hershko and Ginsberg, 2004; Melino et al., 2004; You et al., 2006). Our results suggest that these pathways might install some sensitivity to wide-range or selective inhibitors of Bcl-2 homologues in cancer cells. Conversely, posttranslational mechanisms that negatively regulate Puma activity and allow cells to accumulate this protein without undergoing cell death are expected to promote resistance against such inhibitors.

Materials and methods

Cell culture and cell lines

BdGBM and BeGBM cells were described previously (Cartron et al., 2003). BdGBM(Bax-α), BdGBM(BaxD33A), and BdGBM(BaxK64A) cells were described previously (Cartron et al., 2004a). GBM shBax and GBM scr cells were also described previously (Manero et al., 2006). Human colorectal cancer cells lines derived from HCT116 ($p21^{-/-}$, $p21^{-/-}Puma^{-/-}$, and $p21^{-/-}Bax^{-/-}$) were provided by B. Vogelstein (The John Hopkins Kimmel Cancer Center, Baltimore, MD; Yu et al., 2003). GBM samples were provided by S. Martin (Hôpital G et R Laennec, Centre Hospitalier Universitaire, Nantes, France) and M. Campone (Centre René Gauducheau, Nantes-Atlantique, Nantes, France).

Antibodies

Anti–Bcl-xL (for immunoprecipitation [Epitomics] and for Western blotting [Transduction Laboratory]), anti–Bax 2D2 (for immunoprecipitation [Sigma-Aldrich] and for Western blotting [Dako]), anti–cytochrome *c* (R&D Systems), anti–active caspase 3 (BD), anti-Puma (Sigma-Aldrich), anti–Mcl-1 (Tebu-bio), antiactin (Millipore), and anti-Flag (Sigma-Aldrich) were used. Horseradish peroxidase–conjugated antibodies and enhanced chemiluminescence reagents were obtained from Santa Cruz Biotechnology, Inc. Fluorescent secondary antibodies were obtained from Invitrogen.

Peptides, recombinant proteins, and peptide scan
Peptides (Table S1) were obtained from Sigma-Aldrich and NeoMPS Polypeptide Laboratories. Small-scale preparation of recombinant His-Bax from bacterial lysates was performed as described previously (Cartron et al., 2002). Peptide scanning was performed as previously described using nitrocellulose membranes on which 12-mer peptides covering the indicated sequence of Bax were spotted (Bellot et al., 2007). 1 µg/ml biotinylated Puma^{BH3} was incubated with membranes overnight at 4°C and, after three washes with PBS and 1% BSA, was detected using extravidin-coupled horseradish peroxidase. Binding intensities were quantified using IP Laboratory Gel software (Signal Analytics).

Yeast assays
Yeast-expressing galactose-inducible Bax (or BaxD33A), Bcl-xL, and/or Puma was obtained by transforming the wild-type haploid strain W303-1B (Mata, ade2, his3, leu2, trp1, and ura3) with expression vectors expressing the corresponding cDNA under control of the *GAL1/10* promoter and expressing *URA3*, *HIS3*, and *TRP1* as yeast selection markers, respectively (Arokium et al., 2004). A chimeric Puma cDNA optimized for expression (Genescript) was used (Table S1). Survival after the addition of 0.5% galactose to cultures in early exponential growth phase was evaluated by plating 250 cells on complete medium and counting formed colonies 48 h later. Cytochrome *c* release was measured by redox spectrometry on isolated mitochondria as previously described using cytochrome *b* as an internal standard (Arokium et al., 2004). Immunoprecipitation experiments were performed with the IP-50 immunoprecipitation kit (Sigma-Aldrich) according to the manufacturer's instructions, using 2 µg of antibody and 2–5 mg of protein from zymolyase-treated whole cell lysates in 500 µl IP-50 buffer (Sigma-Aldrich).

Pull-down assays
1 µM biotinylated Puma^{BH3} was incubated with 500 µg of GBM cell extract in 500 µl of CHAPS buffer (10 mM Hepes, 150 mM NaCl, 1% CHAPS, pH 7.4, and EDTA-free protease inhibitors cocktail) with or without the competing peptide for 2 h under agitation at 4°C. 30 µl of avidin–agarose beads, previously washed twice with CHAPS buffer, were then added and incubated overnight under agitation at 4°C. After three washes in 500 µl of CHAPS buffer, beads were resuspended in 30 µl of 2× Laemmli buffer and boiled for 30 s at 95°C before Western blotting.

RNA interference and transfection assays
Lentivirus production and titration were performed as described previously (Qin et al., 2003) using HEK293-FT as packaging cells and FG12 lentivector in which an scr negative control (Applied Biosystems) or short hairpin RNA targeted to oligonucleotides 58–76 of human Bcl-xL under the control of the U6 promoter were subcloned. A multiplicity of infection of 4 was used. Mcl-1 and control siRNA were purchased from Applied Biosystems. Cells were transfected using the HiPerfect transfection reagent (QIAGEN) according to the manufacturer's instructions and were incubated with nucleotides for 48 h before subsequent experiments. Transfection with plasmids was performed using Lipofectamine 2000 reagent (Sigma-Aldrich) according to the manufacturer's instructions.

Cellular assays
Cytoplasmic microinjection and morphological analysis of cell death in fluorescent cells were described previously (Cartron et al., 2004a). For immunocytochemical assays, cells were washed with PBS and fixed with 4% paraformaldehyde and 0.15% picric acid in PBS for 30 min at RT. Cells were washed three times in PBS and permeabilized with 0.1% SDS in PBS for 10 min at RT. Cells were washed again and, after a 30-min saturation with 5% gelatin in PBS at 37°C, were incubated with primary antibodies diluted in 1% gelatin in PBS for 1.5 h at 37°C. After three washes in PBS, incubation with secondary antibodies (anti–mouse IgG coupled to Alexa Fluor 488 and anti–rabbit IgG coupled to Alexa Fluor 568) was performed in 1% gelatin in PBS for 1.5 h at 37°C. Cells were then washed and mounted in ProLong Gold antifade reagent (Invitrogen). Cells that had not been infected with lentivirus were inspected at RT with a fluorescent microscope (BX60; Olympus) equipped with a 40× NA 0.75 objective lens (UPlanFL) and a digital camera (CoolSNAP EZ; Photometrics). Representative images were acquired by MetaVue software (MDS Analytical Technologies). Lentivirus-infected cells were inspected at RT with a fluorescent microscope (DMLB; Leica) equipped with a 40× NA 0.75 objective lens (HCxPL FluoTar) and a color digital camera (DC300F; Leica). Representative images were acquired with the IM50 Image Manager software (Leica). Coimmunoprecipitation assays, using 2 µg of the indicated antibody and 500 µg of protein lysates in CHAPS buffer, were performed with the IP-50 immunoprecipitation kit according to manufacturer's recommendations. For long-term assays, 500 cells were seeded in each well of a 6-well plate 24 h before the start of a 2-wk continuous treatment, after which cells were stained with 0.5% crystal violet and 20% methanol.

Online supplemental material
Fig. S1 shows physical and functional interactions of Bax with BH3 peptides. Fig. S2 shows the impact of Hα1-Cter on Puma induction of apoptosis. Fig. S3 shows additional data regarding the role of Puma in ABT-737 induction of cell death. Fig. S4 shows the lack of contribution of Bid, Bim, and Bak in ABT-737 induction of cell death in HCT116 cells. Table S1 shows sequences of peptides and of Puma cDNA used in this study. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200809153/DC1.

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