Bcl-2 constitutively suppresses p53-dependent apoptosis in colorectal cancer cells

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To dissect apoptotic genes governing the survival of colorectal carcinoma cells, we employed RNAi to silence Bcl-2 and Bcl-x_L in isogenic clones of *p53*+/+ and *p53*–/– cells, and of *Bax*+/– and *Bax*–/– cells. We identify a novel proapoptotic function of p53 that does not require activation by genotoxic agents and that appears to be constitutively suppressed by Bcl-2. Silencing of *Bcl-2* induced massive p53-dependent apoptosis. The "Bcl-2/p53 axis" requires Bax and caspase 2 as essential apoptotic mediators. This newly discovered Bcl-2/p53 functional interface represents a key regulator of apoptosis which can be activated by targeting Bcl-2 in colorectal carcinoma cells.

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The pathways governing apoptosis in mammalian cells are complex, and the pro- and antiapoptotic permutations regulating cell viability vary according to species, cell type, and also between normal and cancer cells (for review, see Cory and Adams 2002; Johnstone et al. 2002; Reed 2002). Imbalance in favor of cell survival enables tumor progression and resistance to anticancer drugs. For example, the proapoptotic *Bax* gene is frequently mutated in DNA mismatch repair-deficient tumors, due to an unstable G8 tract at nucleotides 114–121 (Ionov et al. 1993; Rampino et al. 1997; Zhang et al. 2000). When both *Bax* alleles are mutated, the resultant Bax deficiency confers resistance to nonsteroidal anti-inflammatory drugs (NSAIDs) such as sulindac and indomethicin (He et al. 1999; Yamamoto et al. 1999; Zhang et al. 2000). Since predisposition to colorectal cancer is commonly associated with defective mismatch repair (Lynch 1999), mutation in *Bax* may explain acquired resistance to sulindac when administered as a chemopreventative agent. Indeed, sulindac enables clonal expansion of *Bax*-deficient cells in culture (Zhang et al. 2000) and may similarly favor clonal expansion of *Bax*-deficient cells in the colorectal epithelium of patients with inherited mismatch repair defects. However, *Bax*-deficient cells remain sensitive to 5-fluorouracil (5-FU), which activates p53-dependent apoptosis (Bunz et al. 1999; Zhang et al. 2000) and is the mainstream therapy for colorectal cancer.

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Other pathways may also influence the survival of colorectal cancer cells. Here we have combined RNA interference (RNAi) with gene knockout to investigate the putative apoptotic roles of p53, Bax, Bcl-2, and Bcl-x_L in cells cultured under normal growth conditions. Using isogenic clones of HCT116 human colorectal cancer cells, in which the parental cells contain normal p53 and Bax (Bunz et al. 1999; Zhang et al. 2000), our first experiments aimed to silence Bcl-2 expression by RNA interference. In mammalian cells, RNA interference is induced by short interfering RNA (siRNA) duplexes (Elbashir et al. 2001) which target homologous mRNA for degradation with exquisite selectivity and very high, sustained efficacy. Moreover, gene silencing by a single dose of siRNA is achieved within a few days (e.g., see Elbashir et al. 2001; Jiang and Milner 2002) and avoids the need for protracted long-term selection procedures such as those necessary to establish gene knockout cells. Thus RNA interference permits, for the first time, functional dissection of apoptotic pathways by silencing antiapoptotic genes in cells in which specific proapoptotic genes are already deleted.

We show that silencing of *Bcl-2* induces massive p53-dependent apoptosis, and that this occurs under normal cell growth conditions (i.e., without recourse to genotoxic drugs necessary to activate p53 as a transcription factor). Controls demonstrate that RNA interference per se is not sufficient to induce apoptosis in the parental HCT116 *p53*+/+ cells. The requirement for p53 is absolute, and other apoptotic genes such as *Bax* cannot substitute for *p53*. Moreover the requirement for p53 was also evident in other human colorectal carcinoma cell lines, indicating that *Bcl-2* silencing induces p53-dependent apoptosis in colorectal carcinoma cells in general. These observations place a novel proapoptotic function of p53 under Bcl-2 regulation, thus creating a constitutive Bcl-2/p53 axis regulating apoptosis in human colorectal epithelial cells. Further experiments using isogenic clones of *Bax*+/– and *Bax*–/– cells and caspase 2 siRNA clearly demonstrate that both Bax and caspase 2 are essential mediators of the Bcl-2/p53 apoptotic pathway.

Results and Discussion

Selective silencing of Bcl-2 expression

Initially we used paired isogenic clones of HCT116 *p53*+/+ and *p53*–/– cells (Bunz et al. 1999; Zhang et al. 2000), and to silence Bcl-2 expression we selected two Bcl-2 mRNA target sequences (Fig. 1A,B). Both are 100% conserved between human and murine *Bcl-2*. Silencing of Bcl-2 expression was monitored by immunoblotting the Bcl-2 protein. It should be noted that a previous, well controlled study failed to clearly detect Bcl-2 in immunoblots of HCT116 cell lysates (Zhang et al. 2000), and we confirm this observation when using the same antibody (N19; Fig. 1D). However, other antibodies clearly detect Bcl-2 in the HCT116 cells and, importantly, show that Bcl-2 protein levels are equivalent in the *p53*+/+ and *p53*–/– cells (Fig. 1E–H). The inevitable stress associated with the transfection process was not sufficient to activate p53 as a transcription factor in *p53*+/+ cells, as evi-

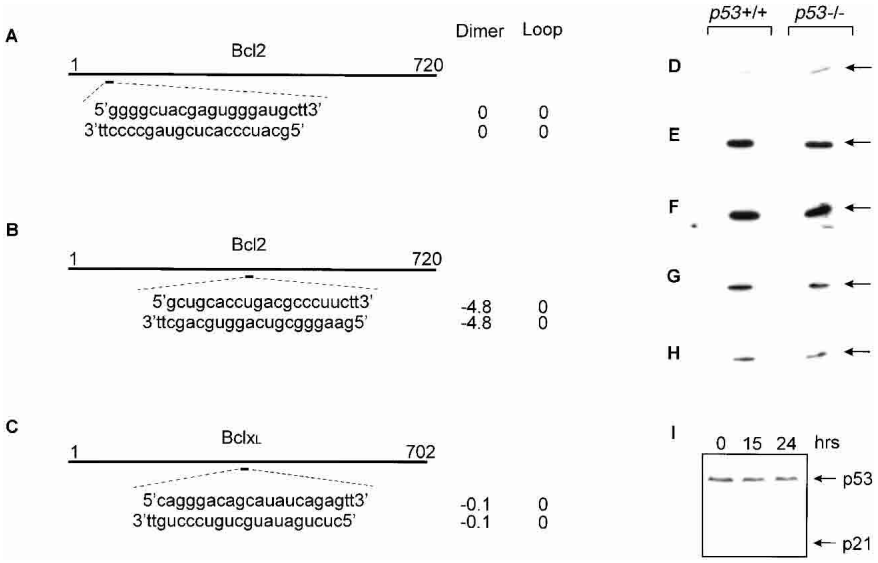

Figure 1. siRNA sequences used, and expression of Bcl-2 in HCT116 cells. (*A*,*B*) Bcl-2 siRNA sequences, equivalent to Bcl-2 mRNA nucleotides 77–95 (*A*) and 354–372 (*B*). (*C*) Bcl-x_L siRNA sequence, nucleotides 347–366. Predicted secondary structures with propensity for base-pairing out of register (dimers) or for forming stem-loop structures (loops) were derived using Vector NTI. Antisense RNA controls employed Bcl-2 antisense nucleotides 354–372, and Bcl-x_L antisense nucleotides 347–366. Control siRNA (Jiang and Milner 2002) and lamin A/C siRNA (Elbashir et al. 2001) were also used in this study. (*D–H*) Immunoblots of Bcl-2 protein (arrows) in HCT116 *p53*+/+ and *p53*–/– cell lysates using different anti-Bcl-2 antibodies: N19 (*D*), C-2 (*E*), Ab-1 (*F*; this antibody gave nonspecific cross-reactivity with multiple cellular proteins; data not shown), Ab-2 (*G*), and BD (*H*). The C-2 antibody was used in all subsequent experiments. (*I*) Immunoblots of p53 and p21 in HCT116 *p53*+/+ cells at different times after transfection with control siRNA as indicated.

dent from the absence of up-regulation of p21, a p53 target protein (Fig. 1I). The Bcl-2 protein fell to barely detectable levels within 24 h of transfection with Bcl-2 siRNA (Fig. 2A). Interestingly, only one of the two Bcl-2 siRNAs silenced Bcl-2 expression [Bcl-2(b); Fig. 2], indicating that the mRNA sequence homologous to the noneffective siRNA (nucleotides 77–95; Fig. 1A) must somehow be protected from recognition and/or degradation by RNA interference. Such protection may arise due to localized mRNA secondary structure or protein–mRNA interactions (Jiang and Milner 2002). Control transfections included a random siRNA sequence (control siRNA; Jiang and Milner 2002) and lamin A/C siRNA, previously shown to suppress lamin A/C protein expression without inducing apoptosis (Elbashir et al. 2001).

Bcl-2 silencing induces p53-dependent apoptosis

By 48 h, massive apoptosis was observed in the *p53*+/+ cells transfected with Bcl-2 siRNA [Bcl-2(b); Fig. 2B–D]. Apoptosis in cells transfected with Bcl-2 antisense RNA (antisense sequence 354–372) was negligible (Fig. 2D) and equivalent to that observed for control siRNA. This confirms that apoptosis induced by Bcl-2 siRNA in *p53*+/+ cells is due to RNA interference. siRNA silencing of lamin A/C failed to induce apoptosis in either the *p53*+/+ or *p53*–/– cells (Fig. 2B). This demonstrates that the process of RNA interference per se is not sufficient to activate apoptosis in HCT116 p53+/+ cells. Unexpectedly, the *p53*–/– cells failed to undergo apoptosis following silencing of Bcl-2 expression (Fig. 2B–D). Thus we conclude that selective silencing of Bcl-2 expression induces massive apoptosis of HCT116 colorectal cancer cells and that this effect is dependent upon p53.

It was recently demonstrated that Bcl-2 can regulate an apoptotic pathway that is activated independently of mitochondrial cytochrome c release and Apaf-1/caspase 9 activation (Marsden et al. 2002). This raises the possibility that p53 may enable this cytochrome c-independent pathway, thus accounting for the observed differences in apoptosis between p53+/+ and p53–/– cells following silencing of *Bcl-2* (Fig. 2). However, analysis of cytochrome c distribution clearly demonstrates release of cytochrome c into the cytosol in *p53*+/+ cells undergoing apoptosis following treatment with Bcl-2 siRNA (Fig. 2E). In long exposures of the immunoblots, mitochondrial cytochrome c release was also evident in adherent *p53*+/+ cells treated with Bcl-2 siRNA, whereas no cytosolic cytochrome c was detectable in parallel cultures of *p53*–/– cells treated with Bcl-2 siRNA (data not shown). These results indicate that *Bcl-2* silencing induces p53-dependent apoptosis via pathway(s) that involve the release of cytochrome c from the mitochondria.

Bcl-x_L silencing induces p53-independent apoptosis

The integrity of p53-independent apoptotic pathways was next confirmed by silencing the *Bcl-x_L* gene, again using RNA interference. *Bcl-x_L* is an antiapoptotic gene (Boise et al. 1993), and in colorectal epithelial cells, a decrease in the ratio of Bcl-x_L:Bax is sufficient to induce apoptosis (Zhang et al. 2000). Therefore we predicted that selective silencing of *Bcl-x_L* should induce apoptotic cell death in both *p53*+/+ and *p53*–/– cells. Indeed, this proved to be the case. First we ascertained that the selected Bcl-x_L siRNA sequence (Fig. 1C) effectively reduces Bcl-x_L protein expression (Fig. 3A), and then we demonstrated its capacity to induce apoptosis (Fig. 3B,C). Bcl-x_L protein levels declined between 24 and 48 h post-transfection with Bcl-x_L siRNA, and subsequently apoptosis was observed in both the *p53*+/+ and *p53*–/– cells. This demonstrates that p53 is not required for Bcl-x_L-regulated apoptotic pathway(s) in colorectal epithelial cells. Further verification of p53-independent apoptotic pathways was obtained by treating the cells with sulindac, which is known to activate Bax-dependent apoptosis (Zhang et al. 2001). Sulindac induced apoptosis in both *p53*+/+ and *p53*–/– cells (Fig. 3D; see also Zhang et al. 2000). On the basis of these overall results, we conclude that the observed lack of apoptosis in *p53*–/– cells treated with Bcl-2 siRNA (Fig. 2) cannot be attributed to either loss of Bax or another apoptotic pathway suppressed by Bcl-x_L. This is consistent with the isogenic nature of the two cell clones, and indicates that p53 is a selective requirement for apoptosis induced by *Bcl-2* silencing.

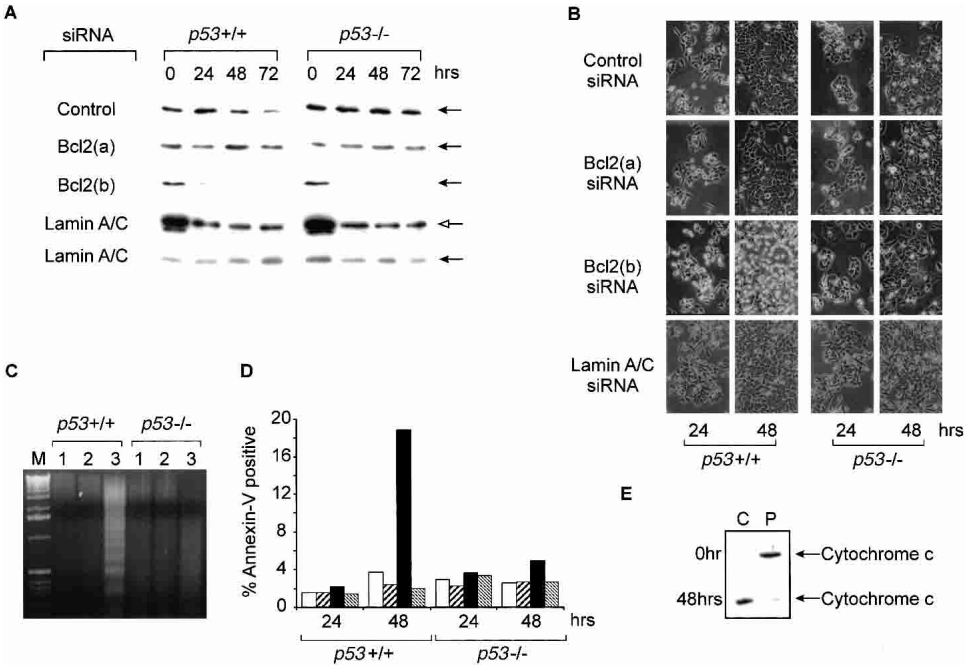

Figure 2. siRNA silencing of Bcl-2 induces p53-dependent apoptosis. Isogenic clones of *p53*+/+ and *p53*–/– HCT116 cells were cultured and transfected with siRNAs as described in the text. Transfection efficiency was 70%–80%. (*A*) Immunoblots of Bcl-2 (closed arrows), and lamin A/C (open arrow). Times posttransfection with control siRNA, Bcl-2 siRNAs, and lamin A/C siRNA are as indicated. (*B*) Phase contrast images of *p53*+/+ and *p53*–/– HCT116 cells at 24 and 48 h posttransfection with control siRNA, Bcl-2 siRNAs, or with lamin A/C siRNA. (*C*) Apoptotic cells confirmed by DNA laddering. M, marker; lane *1*, control siRNA; lane *2*, Bcl-2(a) siRNA; lane *3*, Bcl-2(b) si-RNA. Cells were harvested for analysis 48 h posttransfection. (*D*) Annexin V-positive apoptotic cells detected by FACS analysis. Cells were harvested at 24 and 48 h posttransfection as indicated. White bars indicate control siRNA; bars with thick hatch marks indicate Bcl-2(a) siRNA; black bars indicate Bcl-2(b) siRNA; bars with thin hatch marks indicate Bcl-2(b) antisense RNA. In all subsequent experiments, Bcl-2(b) siRNA was employed to silence Bcl-2 expression, and apoptosis was confirmed by the independent techniques of DNA laddering and annexin V labeling with FACS analysis. (*E*) Cytochrome c distribution in cells at the time of transfection (0 h) and release into the cytosol of nonadherent cells collected 48 h following transfection with Bcl-2 siRNA in HCT116 *p53*+/+ cells. P, pellet fraction; C, cytosolic fraction.

Bax and caspase 2 are required for apoptosis following silencing of Bcl-2 or Bcl-x_L

Thus far our results indicate that Bcl-2 constitutively suppresses apoptosis in colorectal cancer cells grown in culture and that, following silencing of Bcl-2 expression, the process of apoptosis requires p53. This is novel and places a proapoptotic function of p53 under Bcl-2 regulation. Moreover, this proapoptotic function of p53 does not require treatment of cells with cytotoxic agents such as 5-FU. (Note that the process of RNA interference by itself is not sufficient to activate p53-induced apoptosis, as demonstrated by the lack of apoptosis in p53+/+ cells treated with lamin A/C siRNA; see above). In addition, we show that silencing of *Bcl-x_L* induces apoptosis in a p53-independent manner (Fig. 3). This is consistent with previous work identifying Bax as a major player in the apoptotic response of colorectal cancer cells (Ionov et al. 2000; Zhang et al. 2001; LeBlanc et al. 2002) and Bcl-x_L as its antiapoptotic counterpart (when expressed exogenously; Zhang et al. 2001). These combined observations led us to reason that Bcl-2/p53 and Bcl-x_L/Bax might represent functional partners governing apoptosis in human colorectal epithelial cells. Within this scenario, at least two putative apoptotic pathways might be envisaged: (1) Bcl-2/p53 may define an apoptotic pathway that is essentially independent of Bcl-x_L/Bax; or (2) Bcl-2/p53 and Bcl-x_L/Bax may govern interrelated transitions in the apoptotic process. To discriminate between these two alternatives we silenced, individually, Bcl-2 and Bcl-x_L expression in isogenic clones of *Bax*+/– and *Bax*–/– HCT116 cells (note that the apoptotic response of *Bax*+/– cells is equivalent to that of *Bax*+/+ cells; Zhang et al. 2000). siRNA silencing of *Bcl-2* and of *Bcl-x_L* induced massive apoptosis in *Bax*+/– cells but failed to induce significant apoptosis in *Bax*–/– cells (Fig. 4C,D). This clearly demonstrates that Bax is required for apoptosis in both Bcl-2-regulated and Bcl-x_L-regulated pathways.

The above results demonstrate that in colorectal carcinoma cells, the Bcl-2 and Bcl-x_L cell death pathways share commonalities in their requirement for Bax, but differ in their requirements for p53. It is possible that p53 is required to prime a proapoptotic pathway that is selectively suppressed by Bcl-2, thus lowering the apoptotic threshold consequent to *Bcl-2* silencing. To further dissect the functional links between Bcl-2, Bcl-x_L, p53, and Bax, we next investigated whether caspase 2 is also involved. Apoptosis induced by *Bcl-2* or by *Bcl-x_L* silencing was blocked when caspase 2 siRNA was cotransfected with either Bcl-2 siRNA or Bcl-x_L siRNA, respectively (Fig. 4C; see Lassus et al. 2002 for caspase 2 siRNA sequence). siRNA silencing of *caspase 2* alone (Fig. 4C), or transfection with antisense caspase 2 RNA (data not shown), had no apparent effect on cell viability. Overall these results demonstrate that both Bax and caspase 2 are required for apoptosis following silencing of either *Bcl-2* or *Bcl-x_L* in *p53*+/+ colorectal cancer cells.

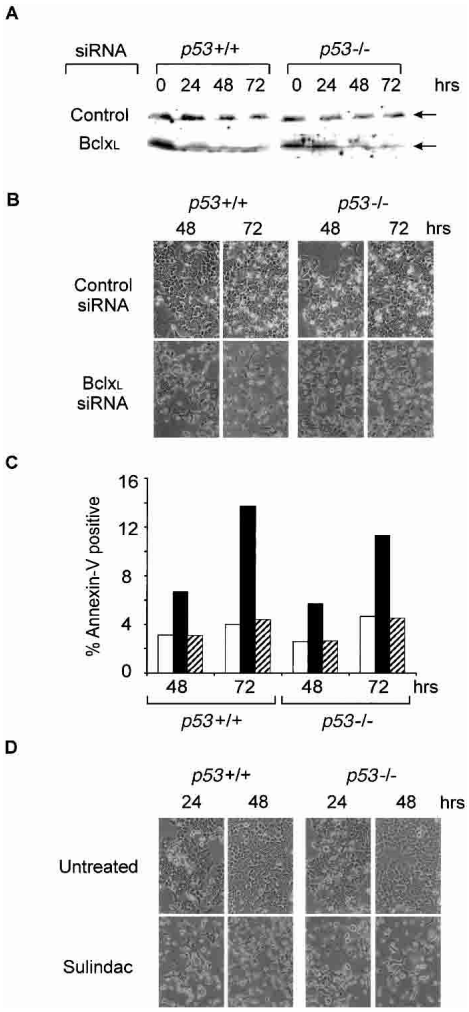

Figure 3. p53-independent apoptotic pathways in isogenic clones of HCT116 cells. (*A–C*) siRNA silencing of the antiapoptotic gene Bcl-x_L using the siRNA sequence shown in Figure 1C. (*A*) Immunoblot of Bcl-x_L protein at different times after transfection with control siRNA or Bcl-x_L siRNA. (*B*) Phase contrast images of cells at 48 and 72 h posttransfection with control siRNA or Bcl-x_L siRNA. (*C*) Early apoptotic cells detected by annexin V labeling and FACS analysis. Cells were harvested for analysis at 48 and 72 h as indicated. White bars indicate control siRNA; black bars indicate Bcl-x_L siRNA; bars with thick hatch marks indicate Bcl-x_L antisense RNA. (*D*) Apoptosis induced by treatment with sulindac. Phase contrast images of cells at 24 and 48 h posttreatment with sulindac, which activates Bax-dependent, p53-independent apoptosis (Zhang et al. 2000). Apoptosis was confirmed by DNA laddering and by FACS analysis of cells labeled with annexin V (not shown).

This is consistent with recent evidence that caspase 2 enables translocation of Bax into the mitochondria and subsequent mitochondrial membrane permeabilization marked by release of cytochrome c (Lassus et al. 2002).

Effects of Bcl-2 siRNA on individual colorectal carcinoma cell lines of varying p53 status

The above experiments involve isogenic clones of HCT116 cells and are thus tightly controlled for genetic variation. To test the generality of our observations, we silenced Bcl-2 in other human colorectal carcinoma cell lines, also defective for DNA mismatch repair and with defined p53 status (see Materials and Methods). In each case, the presence of wild-type p53 correlated with induction of apoptosis detectable 48 h posttransfection with Bcl-2 siRNA, whereas p53 deficiency correlated with background levels of apoptosis (Fig. 5). These results confirm our observations with isogenic clones of *p53+/+* and *p53–/–* HCT116 cells and are consistent with the concept that Bcl-2 constitutively suppresses p53-dependent apoptosis in colorectal cancer cells.

Conclusions

Given the complexity and diversity of mammalian apoptotic pathways, it is perhaps not surprising that the application of novel methodologies such as RNA interference can disclose unexpected regulatory interactions. For example, it has been widely accepted that caspase activation requires mitochondrial disruption. However, experiments using RNA interference now indicate that, for caspase 2, the converse is the case and that mitochondrial disruption requires caspase 2 gene expression (Lassus et al. 2002). Another unexpected discovery challenges the long held belief that Bcl-2 inhibits apoptosis by safeguarding mitochondrial integrity, thereby preventing cytochrome c release and activation of caspase 9: it now appears that the Bcl-2 apoptotic pathway can function independently of caspase 9 and its activator Apaf-1 (Marsden et al. 2002).

In the present study we used isogenic cell clones and siRNA to obtain defined combinations of pro- and antiapoptotic gene expression in cells that are otherwise genetically equivalent. Our observations indicate a new cell death pathway in which Bcl-2 constitutively suppresses p53-dependent apoptosis. Apoptosis can also be induced by treating the cells with agents such as 5-FU to activate p53 (Zhang et al. 2000; data not shown). This is consistent with established evidence that activated p53 functions upstream of Bcl-2 in response to genotoxic stress (Strasser et al. 1994; for reviews see Cory and Adams 2002; Johnstone et al. 2002). To accommodate our present observations within the context of previous studies, we suggest that Bcl-2 constitutively suppresses a novel proapoptotic function of p53 and that exposure to genotoxic stress overrides Bcl-2 suppression by inducing the transactivation potential of p53. Once activated as a transcription factor, p53 has the capacity to alter the expression ratios of Bcl-2 and Bcl-x_L (down-regulated) and Bax (up-regulated) in favor of apoptosis (Johnstone et al. 2002). From a clinical point of view this has proved very useful for anticancer therapy but carries the inherent risk of nonspecific cytotoxicity and genotoxicity caused by p53-activating agents. Our discovery that p53 possesses proapoptotic properties that appear to be constitutively active, albeit suppressed by Bcl-2, identifies Bcl-2 as a potential and promising target for anticancer therapy for colorectal cancer (see also Reed 2002), and demonstrates Bcl-2 accessibility for siRNA silencing. The survival of other epithelial tumors may be similarly susceptible to Bcl-2 silencing. With the development of RNA interference the selective silencing of specific genes is now a realistic possibility, and the continual inventive evolution of targeted delivery systems (e.g., see Hood et al. 2002) should enable application of RNA interference to prevent and to treat cancer.

This work also carries important implications for patients with inherited DNA mismatch repair deficiencies

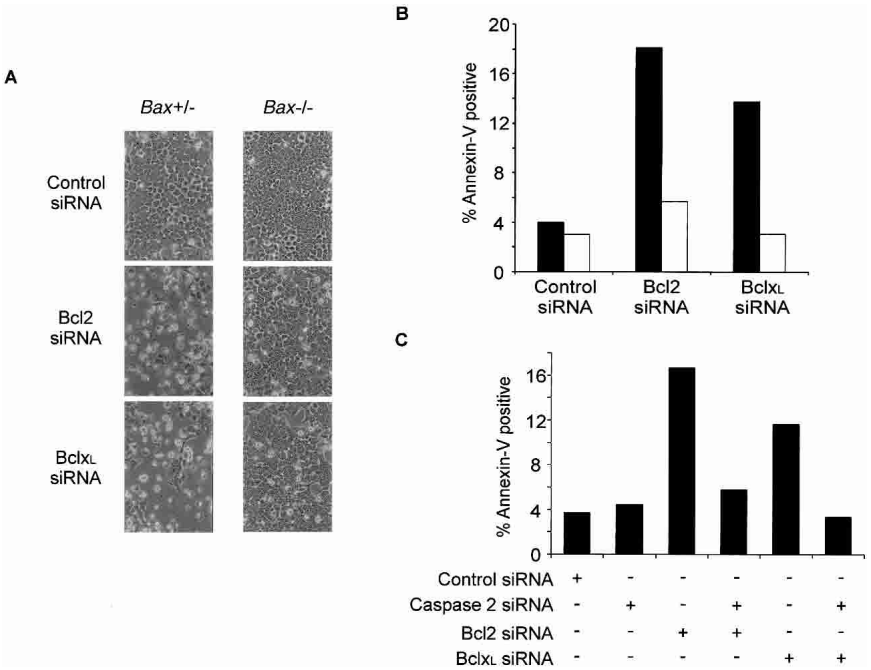

Figure 4. Apoptosis following silencing of Bcl-2 or Bcl-x_L depends upon Bax and caspase 2. (*A*) Phase contrast images of isogenic clones of *Bax*+/– and *Bax*–/– HCT116 cells at 72 h posttransfection with control siRNA, with Bcl-2 siRNA or with Bcl-x_L siRNA, as indicated. (*B*) Apoptotic cells in isogenic clones of HCT116 cells detected by labeling with annexin V and FACS analysis. The cells were harvested at 72 h posttransfection with control siRNA, Bcl-2 siRNA, or Bcl-x_L siRNA as indicated. Black bars indicate *Bax*+/– cells; white bars indicate *Bax*–/– cells. (*C*) Apoptotic cells in HCT116 *p53*+/+ cells (the same clone as used in Figs. 2, 3) 72 h following transfection with caspase 2 siRNA in combination with either Bcl-2 siRNA or Bcl-x_L siRNA as indicated.

and associated predisposition to colorectal cancer. In particular, it argues against the use of sulindac as a chemopreventative in such patients, because it is well established that defective mismatch repair renders the *Bax* gene susceptible to mutation and favors clonal expansion of *Bax*-deficient cells (see the introductory text above). In the present study we demonstrate that Bax is an essential mediator of apoptosis regulated by the newly discovered Bcl-2/p53 pathway (see above). It follows that, in patients with mismatch repair defects, any selective pressure for *Bax*-deficient cells may exacerbate tumorigenesis and should be avoided.

Future studies will investigate whether the newly identified constitutive proapoptotic function of p53 is linked with apical apoptosis in the normal colorectal epithelium. If so, failure of apoptosis in colorectal epithelial tumors might reflect inappropriate suppression of intrinsic p53-induced apoptosis. A strong candidate in this regard is Bcl-2, which constitutively blocks p53-induced apoptosis and enables the survival of colorectal cancer cells (the present study). Such a model is consistent with the late onset of p53 mutation in the malignant progression of colorectal cancer. It also reenforces Bcl-2 as a prime target for the development of novel anticancer agents.

Materials and Methods

Cell lines and transfections

HCT116 clones were cultured in DMEM with 10% FCS. All the cell clones were cultured with penicillin 100 units mL^{-1} and streptomycin 100 μg mL^{-1} at 37°C in 5% CO_2 in air. Other human colorectal cancer cells lines, also defective for DNA mismatch repair (Branch et al. 1995), were: LoVo and RKO (p53 wild type); DLD1, LS174T, SW48, and HT29 (all p53 mutant). Note that although LS174T cells are genotypically wild-type for p53, they are phenotypically p53-deficient. For transfection, the cells were trypsinized and subcultured into 6-well plates (10 cm^2) without antibiotics, 1.5×10^5 cells per well. Selected 21-nucleotide RNAs were synthesized and HPLC-purified (MWG) and annealed into siRNA duplexes according to the instructions supplied. Twenty-four hours after subculture, the cells were transfected with siRNA formulated into liposomes (Oligofectamine, Life Technologies) according to the manufacturer's instructions. The protocol includes a short incubation in serum-free medium, but controls demonstrated that this was not sufficient to activate a p53 response (see Results). The siRNA concentration was 0.58 μg per 1.5×10^5 cells per well. The final volume of culture medium was 1.5 mL per well. Cells were harvested for analysis at various times thereafter, as indicated in Results and Discussion. Each experiment with HCT116 cells was carried out four or more times. Transfection efficiencies were established by transfecting with liposomes containing FITC-dextran (Jiang and Milner 2002). Antisense RNA controls were included in each experiment, using the respective antisense sequences for Bcl-2(b), Bcl-x_L, and caspase 2 (see Fig. 1A and text).

Immunoblotting and mitochondrial cytochrome c release

For immunoblotting, the transfected cells were trypsinized and then washed in PBS, and an aliquot was removed for cell counting. The remaining cells were lysed in 50 μL lysis buffer (150 mM NaCl, 0.5% NP40, 50 mM Tris at pH 8.0) on ice for 30 min. Samples were diluted 1:1 in 4× strength Laemlli's buffer. Proteins were resolved by 15% SDS-PAGE and electroblotted onto nitrocellulose membrane for antibody detection. Molecular weight markers and purified recombinant human p53 were included as markers (data not shown). The following antibodies were employed: for Bcl-2 = N19 and C-2 (Santa Cruz Biotechnology); Ab-1 and Ab-2 (Oncogene; note that Ab-1 gave nonspecific background with multiple cellular proteins; data not shown); and BD (Fig. 1B; Pharmingen). The C-2 antibody gave the cleanest results and was subsequently used throughout this work. Lamin A/C = antibody 636

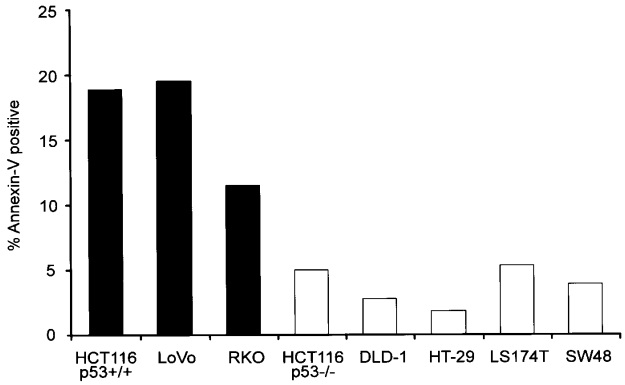

Figure 5. Apoptosis correlates with p53 status in individual human colorectal carcinoma cell lines following silencing of Bcl-2 expression. Cells were transfected with Bcl-2 siRNA, and apoptotic cells were determined after 48 h (as described in Fig. 2 legend and Materials and Methods). Black bars indicate cell lines expressing endogenous wild-type p53, white bars indicate p53-deficient cell lines.

(Santa Cruz Biotechnology); Bcl-x_L = Bcl-X antibody (Pharmingen; this antibody gave a relatively high nonspecific background); p53 = DO-1 antibody (Oncogene); and caspase 2 = caspase-2L antibody (F-7; Santa Cruz Biotechnology). Visualization of bound antibodies was by enhanced chemiluminescence (Roche). Cell fractionations and cytochrome c determinations were carried out as described (Marsden et al. 2002, and its supplementary information).

Cell growth, cell cycle analysis, and apoptosis

Cell growth curves were determined by cell counting. Induction of apoptosis by sulindac (Fig. 3) employed sulindac sulphide 120 µM (Calbiochem). For cell cycle analysis the cells were harvested, washed with PBS, and fixed in 90% ethanol overnight at –20°C. The fixed cells were pelleted, washed in PBS, and resuspended in PBS containing 0.1 µg/mL propidium iodide with 200 U/mL RNase A, and then analyzed by FACS. Apoptotic cells were identified using annexin-V-Fluos (Boehringer) following the manufacturer's protocol. Apoptosis was also verified by DNA laddering using the Suicide-track DNA ladder isolation kit (Oncogene) according to the manufacturer's instructions.

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References

Boise, L.H., Gonzalez-Garcia, M., Postema, C.E., Ding, L., Lindsten, T., Turka, L.A., Mao, X., Nunez, G., and Thompson, C.B. 1993. Bcl-x, a bcl-2-related gene that functions as a dominant regulator of apoptotic cell death. *Cell* **74:** 597–608.

Branch, P., Hampson, R., and Karran, P. 1995. DNA mismatch binding defects, DNA damage tolerance, and mutator phenotype in human colorectal carcinoma cell lines. *Cancer Res.* **55:** 2304–2309.

Bunz, F., Hwang, P.M., Torrance, C., Waldman, T., Zhang, Y., Dillehay, L., Williams, J., Lengauer, C., Kinzler, K.W., and Vogelstein, B. 1999. Disruption of p53 in human cancer cells alters the responses to chemotherapeutic agents. *J. Clin. Invest.* **104:** 263–269.

Cory, S. and Adams, J.M. 2002. The Bcl2 family: Regulators of the cell life-or-death switch. *Nat. Rev. Cancer* **2:** 647–656.

Elbashir, S.M., Harborth, J., Lendeckel, W., Yalcin, A., Weber, K., and Tuschl, T. 2001. Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. *Nature* **441:** 494–498.

He, T.C., Chan, T.A., Vogelstein, B., and Kinzler, K.W. 1999. PPARδ is an APC-regulated target of nonsteroidal anti-inflammatory drugs. *Cell* **99:** 335–345.

Hood, J.D., Bednarski, M., Frausto, R., Guccione, S., Reisfeld, R.A., Xiang, R., and Cheresh, D.A. 2000. Tumor regression by targeted gene delivery to the neovasculature. *Science* **296:** 2404–2407.

Ionov, Y., Peinado, M.A., Malkhosyan, S., Shibita, D., and Perucho, M. 1993. Ubiquitous somatic mutations in simple repeated sequences reveal a new mechanism for colon carcinogenesis. *Nature* **363:** 558–561.

Ionov, Y., Yamamoto, H., Krajewski, S., Reed, J.C., and Perucho, M. 2000. Mutational inactivation of the proapoptotic gene Bax confers selective advantage during tumour clonal evolution. *Proc. Natl. Acad. Sci.* **97:** 10872–10877.

Jiang, M. and Milner, J. 2002. Selective silencing of viral gene expression in HPV-positive human cervical carcinoma cells treated with siRNA, a primer of RNA interference. *Oncogene* **21:** 6041–6048.

Johnstone, R.W., Ruefli, A.A., and Lowe, S.W. 2002. Apoptosis: A link between cancer genetics and chemotherapy. *Cell* **108:** 153–164.

Lassus, P., Optiz-Araya, X., and Lazebnik, Y. 2002. Requirement for caspase 2 in stress-induced apoptosis before mitochondrial permeabilization. *Science* **297:** 1352–1354.

LeBlanc, H., Lawrence, D., Varfolomeev, E., Totpal, K., Morlan, J., Schow, P., Fong, S., Schwall, R., Sinicropi, D., and Ashkenazi, A. 2002. Tumor-cell resistance to death receptor-induced apoptosis through mutational inactivation of the proapoptotic Bcl-2 homologue Bax. *Nat. Med.* **8:** 274–281.

Lynch, H.T. 1999. Hereditary nonpolyposis colorectal cancer (HNPCRC). *Cytogenet. Cell Genet.* **86:** 130–135.

Marsden, V.S., O'Connor, L., O'Reilly, L.A., Silke, J., Metcalf, D., Ekert, P.G., Huang, D.C., Cecconi, F., Kuida, K., Tomaselli, K.J., et al. 2002. Apoptosis initiated by Bcl-2-regulated caspase activation independently of the cytochrome c/Apaf-1/caspase 9 apoptosome. *Nature* **419:** 634–637.

Rampino, N., Yamamoto, H., Ionov, Y., Li, Y., Sawai, H., Reed, J.C., and Perucho, M. 1997. Somatic frameshift mutations in the BAX gene in colon cancers of the microsatellite mutator phenotype. *Science* **275:** 967–969.

Reed, J.C. 2002. Apoptosis-based therapies. *Nat. Rev. Drug Discov.* **1:** 111–121.

Strasser, A., Harris, A.W., Jacks, T., and Cory, S. 1994. DNA damage can induce apoptosis in proliferating lymphoid cells via p53-independent mechanisms inhibitable by BCL-2. *Cell* **79:** 329–339.

Yamamoto, Y., Yin, M.J., Lin, K.M., and Gaynor, R.B. 1999. Sulindac inhibits activation of the NF-κB pathway. *J. Biol. Chem.* **274:**27307–27314.

Zhang, L., Yu, J., Park, B.H., Knizler, K.W., and Vogelstein, B. 2000. Role of BAX in the apoptotic response to anticancer agents. *Science* **290:** 989–992.