Bcl2 and c-Myc are two major oncogenic proteins that can functionally promote DNA damage, genetic instability, and tumorigenesis. However, the mechanism(s) remains unclear. Nitrosamine 4-(methylnitrosamo)-1-(3-pyridyl)-1-butanone (NNK) is the most potent carcinogen contained in cigarette smoke that induces cellular DNA damage. Here we found that Bcl2 potently suppresses the repair of NNK-induced abasic sites of DNA lesions in association with increased c-Myc transcriptional activity. The Bcl2 BH4 domain (amino acids 6–31) was found to bind directly to c-Myc MBI domain (amino acids 106–143), and this interaction is required for Bcl2 to enhance c-Myc transcriptional activity and inhibit DNA repair. In addition to mitochondria, Bcl2 is also expressed in the nucleus, where it co-localizes with c-Myc. Expression of nuclear-targeted Bcl2 enhances c-Myc transcriptional activity with suppression of DNA repair but fails to prolong cell survival. Depletion of c-Myc expression from cells overexpressing Bcl2 significantly accelerates the repair of NNK-induced DNA damage, indicating that c-Myc may be essential for the Bcl2 effect on DNA repair. It is known that apurinic/apyrimidinic endonuclease (APE1) plays a crucial role in the repair of abasic sites of DNA lesions. That overexpression of Bcl2 results in up-regulation of c-Myc and down-regulation of APE1 suggests APE1 may function as the downstream target of Bcl2/c-Myc in the DNA repair machinery. Thus, Bcl2, in addition to its survival function, may also suppress DNA repair in a novel mechanism involving c-Myc and APE1, which may lead to an accumulation of DNA damage in living cells, genetic instability, and tumorigenesis.

Bcl2 was discovered by cloning the (14,18)(q32;q21) chromosomal breakpoint characteristic of human follicular B-cell lymphomas (1–3). Bcl2 has the unique role of extending cell survival rather than promoting cell proliferation (4). B- and T-cell lineage Bcl2 transgenic mice models demonstrate an oncogenic phenotype (5–7). For example, Eμ-Bcl2 transgenic mice show an increased incidence of B-cell lymphomas (7), whereas lck promoter-driven Bcl2 in T-cells can develop peripheral T-cell lymphomas (5). Thus, Bcl2 not only functions as a potent anti-apoptotic molecule but also as a cellular proto-oncogene that can promote tumor development. Several reports indicate that Bcl2 can enhance DNA damage and attenuate DNA repair in association with genetic instability (8–11). However, the mechanism(s) by which Bcl2 regulates DNA damage and repair remains enigmatic. c-Myc is a proto-oncogene that normally regulates cellular growth and proliferation and in some contexts induces apoptosis (12–13). Overexpression of c-Myc is thought to cause tumorigenesis by promoting unrestrained cellular proliferation and blocking differentiation. c-Myc also contributes to tumorigenesis by inducing genomic destabilization (14–16). The genomic damage induced by c-Myc can be broadly grouped into two classes of abnormalities. First, overexpression of c-Myc induces loss of chromosomal integrity associated with chromosomal aberrations such as gene amplifications, double minutes, and fusions. Second, c-Myc overexpression can cause inappropriate DNA replication, resulting in endoreduplication. The former type of genomic abnormalities can be caused by defects in the repair of double-strand DNA breaks (DSBs) since c-Myc overexpression disrupts the repair of DSBs (17). c-Myc is one of those most frequently implicated in carcinogenesis (13, 18–19). Bcl2 and c-Myc are two major oncogenic proteins that can functionally cooperate in cell proliferation, transformation, apoptosis, and tumorigenesis (20). To avoid c-Myc-induced cell death and ensure its oncogenic property, Bcl2 functions as one of the most potent Myc-cooperating oncoproteins, which is a global inhibitor of apoptosis, likely through multiple mechanisms (13, 18, 21). Because Bcl2, in addition to its antiapoptotic function, is able to regulate DNA damage and repair (8–11), we hypothesize that Bcl2 may regulate c-Myc to inhibit DNA repair and retain such damage in surviving cells leading to genomic instability and tumorigenesis.

Nicotine and nitrosamine 4-(methylnitrosamo)-1-(3-pyridyl)-1-butanone (NNK) are two important components in cigarette smoke (22). Recent reports indicate that nicotine promotes survival of both normal human lung epithelial and lung cancer cells (23–24). NNK is formed from nicotine by opening of the pyrrolidine ring and nitrosation. This occurs during the processing of tobacco leaves and in the body of smoker (25–29). In contrast to nicotine, NNK is a more potent carcinogen that not only stimulates survival and proliferation of normal lung epithelial and lung cancer cells but also induces single-strand DNA breaks (SSBs) and oxidative DNA damage in culture cells as well as whole animals (24, 30–35). The outcome of DNA damage is generally adverse, contributing to degenerative processes such as aging and cancer (36). Apurinic/apyrimidinic (AP) sites are expected to be one of the most frequent lesions in DNA. AP sites can be formed by spontaneous hydrolysis of the N-glycosyl bond or as a consequence of the removal of damaged or inappropriate bases by DNA N-glycosylases. The vast majority of damaged and inappropriate bases in DNA are removed by specific DNA N-glycosylases yielding AP sites. Cleavage of AP sites by AP endonucleases or by DNA N-glycosylases/AP lyases results in the
formation of SSBs with 3’- or 5’-blocked ends that cannot be used as substrates by DNA polymerases or DNA ligases. The 3’- or 5’-blocked SSBs can be converted into DSBs after DNA replication (36). The specific DSBs may lead to chromosomal translocations that contribute to genomic instability and/or tumorigenesis (37). AP sites are the central intermediate in DNA base excision repair (BER) and are processed by AP endonucleases (38). Apurinic/apyrimidinic endonuclease (APE1) is a multifunctional protein possessing both DNA repair and redox regulatory activities and is involved in the maintenance of genomic integrity (39 – 40). In BER, APE1 can recognize and bind to abasic DNA and is responsible for processing spontaneous, chemical, or monofunctional DNA glycosylase-initiated AP sites via its 5’-endonuclease activity and 3’-end-trimming activity (41). Thus, APE1 plays an essential role in the repair of AP sites of DNA damage.

We recently discovered that NNK promotes survival and proliferation of human lung cancer cells through phosphorylation of Bcl2 and c-Myc (42). However, it is not clear whether NNK can induce AP sites in cellular genomic DNA and whether Bcl2 and c-Myc can regulate the repair of NNK-induced DNA damage including AP sites to promote genomic instability and/or tumorigenesis (37). AP sites are the central substrates by DNA polymerases or DNA ligases. The 3’-o r 5’-o r 5’-o r 3’-blocked ends that cannot be used as substrates to activate the enzyme. The conditions for PCR amplification were denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, and extension at 68 °C for 45 s. The samples were run for 30 cycles. PCR products were ligated directly into PCR 2.1-TOPO vector, amplified using TOP10 cells (Invitrogen), and screened by PCR for positive clones. Positive clones in PCR 2.1 TOPO vector were digested with the restriction enzymes NcoI/NotI and subcloned into pCMV/Myc/Nuc vector. Each mutant was verified by DNA sequencing.

**Stable Transfection**—The pCIneo or pCMV/Myc/Nuc plasmid bearing the WT or ΔBH4 Bcl2 mutant was transfected into H1299 cells using Lipofectamine™ 2000 (Invitrogen). Clones stably expressing WT or the ΔBH4 Bcl2 mutant were selected in a medium containing G418 (0.6 mg/ml). The expression levels of exogenous Bcl2 were analyzed by Western blot analysis using a Bcl2 antibody. Three separate clones for each mutant expressing similar levels of exogenous Bcl2 were selected for further analysis.

**Preparation of Total Cell Lysate**—Cells were washed with 1 × PBS and resuspended in ice-cold EBC buffer (0.5% Nonidet P-40, 50 mM Tris, pH 7.6, 120 mM NaCl, 1 mM EDTA, 1 mM β-mercaptoethanol, 50 mM sodium fluoride, and 1 mM sodium orthovanadate) with a mixture of protease inhibitors (Calbiochem). The cells were lysed by sonication and centrifuged at 14,000 × g for 10 min at 4 °C. The resulting supernatant was collected as the total cell lysate as described (42).

**Generation of GST-tagged c-Myc Fusion Proteins**—pGEX-2T/GST-Myc (N262) and pGEX-2T/GST-/Myc(C176) were kind gifts from Dr. Bernhard Lüscher (43). These plasmids contain the amino acids of human c-Myc, indicated in parentheses in the name of each plasmid. Various c-Myc mutants in pGEX vector were transformed into BL21 Star™ (DE3) cells (Invitrogen). GST-c-Myc fusion proteins were produced and purified using BugBuster™ protein extraction reagent (Novagen) and glutathione-Sepharose® 4B (Amersham Biosciences) according to the manufacturer’s instruction.

**Immunofluorescence Staining**—H460 cells expressing endogenous c-Myc and Bcl2 or H1299 cells expressing nuclear targeted WT Bcl-2 were seeded and cultured on Lab-Tek® chamber slide (Nalge Nunc International) overnight at 37 °C with 5% CO2. Cell culture medium was removed, and cells were incubated with prewarmed (37 °C) growth medium containing 200 nM MitoTracker Red CMXRos (Molecular Probes). The cells were incubated for 30 min under normal growth conditions. After staining, the cells were washed with prewarmed growth medium and fixed with freshly prepared, prewarmed growth medium containing 3.7% formaldehyde and incubated at 37 °C for 15 min. Cells were washed with PBS and incubated with ice-cold acetone for 5 min. Cells were blocked with PBS containing 10% normal rabbit serum for 20 min at room temperature. Then cells were incubated with a rabbit Bcl2 primary antibody at room temperature for 90 min. After washing, cells were incubated with fluorescein isothiocyanate-conjugated anti-rabbit secondary antibody for 60 min. Cells were washed with PBS and stained with 4’,6-diamidino-2-phenylindole. Samples were observed under a fluorescence microscope (Zeiss) or a confocal fluorescent microscope (Leica, TCS SP2). Pictures were taken and colored with the same exposure setting for each experiment. To determine subcellular regions of protein co-localization, individual green-, red-, and blue-stained images derived from the same field were merged using Openlab 3.1.5 software from Improvision, Inc. (Lexington, MA).
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**c-Myc Transcriptional Activity Assay**—c-Myc transcriptional activity was assessed using a dual luciferase reporter assay system (Promega) because this system allows for a more reliable interpretation of the experimental data by reducing extraneous influence. The term “dual reporter” refers to the simultaneous expression and measurement of two individual reporter enzymes within a single system. The “experimental” reporter (i.e. pMyc-TA-Luc luciferase reporter) is correlated with the effect of specific experimental conditions, whereas the activity of the co-transfected “control” reporter (i.e. pTK Renilla luciferase reporter) provides an internal control that serves as a base-line response. Normalizing the activity of the experimental reporter to the activity of the internal control will minimize experimental variability caused by differences in cell viability, transfection efficiency, or other extraneous influences. A 20:1 mixture of the pMyc-TA-Luc luciferase and pTK Renilla control luciferase reporter vectors were cotransfected with Bcl-2/pClneo or Bcl2/pCMV/Myc/Nuc constructs in various human lung cancer cells using Lipofectamine 2000 (Invitrogen). The pTA-Luc vector was used as a negative control. After 48 h cells were lysed in passive lysis buffer. Firefly luciferase activity (pMyc-TA-Luc reporter) to Renilla luciferase activity (internal control) was assessed according to the Promega technical manual and analyzed by a Monolight 3010 luminometer (BD Biosciences). Each experiment was repeated three times, and data represent the mean \pm S.D. of three determinations.

**Comet Assay**—A single-cell gel electrophoresis (Comet Assay) kit was employed for evaluating DNA damage following the manufacturer’s instructions (Trevigen, Inc., Gaithersburg, MD). Comet Assay is an effective method for detection of DNA damage in cells. The principle of the assay is based upon the ability of denatured, cleaved DNA fragments to migrate out of the cell under the influence of an electric field, whereas undamaged DNA migrates slower and remains within the confines of the nucleus when a current is applied. After treatment of cells with DNA damage agents (i.e. NNK, methylmethane sulphonate, or H2O2), cells in 1× PBS (Ca2+ and Mg2+-free) at 1× 10^6/ml were combined with molten LMAgarose (at 37 °C) at a ratio of 1:10 (v/v) and immediately pipetted 75 \( \mu l \) onto CometSlide™. After a gentle cell lysis, samples were treated with alkali to unwind and denature the DNA and hydrolyze sites of damage. The samples were submitted to electrophoresis, stained with a fluorescent DNA intercalating dye (i.e. SYBR Green 1 nucleic acid), and visualized by a fluorescence microscope (Zeiss Axioplan-2 Imaging, Thornwood, NY).

**AP Site Counting in Genomic DNA**—One of the most prevalent lesions in DNA is the apurinic/apyrimidinic (AP) site. After treatment of cells with NNK, genomic DNA was purified using a DNA isolation kit (Dojindo Molecular Technologies, Inc., Gaithersburg, MD). The number of AP sites was assessed using a DNA damage quantification (AP Site Counting) kit according to the manufacturer’s instructions (Dojindo Molecular Technologies). Aldehyde-reactive probe (ARP) reagent (N’-aminooxymethylcarbonylhydrazino-d-biotin) can react specifically with an aldehyde group which is the open ring form of the AP site. After treating DNA-containing AP sites with ARP reagent, AP sites are tagged with biotin residues. By using an excess amount of ARP, all AP sites can be converted to biotin-tagged AP sites. Standard ARP DNA and purified ARP-labeled sample genomic DNA were fixed on a 96-well plate with DNA binding solution. Then the number of AP sites in the sample DNA was determined by the biotin-avidin-peroxidase assay. The absorbance of the samples was analyzed using a microplate reader with a 650-nm filter. Each experiment was repeated three times, and data represent the mean \pm S.D. of three determinations.

**RNA Interference (RNAi)**—This is a technique for down-regulating the expression of a specific gene in living cells by introducing a homologous double-stranded RNA. H460 cells or H1299 cells expressing WT Bcl2 were transfected with Bcl2 siRNA or c-Myc siRNA, respectively, according to the siPORT lipid siRNA transfection protocol from the manufacturer (Ambion, Inc). A control siRNA (non-homologous to any known gene sequence) was used as a negative control. The levels of Bcl2 or c-Myc expression were analyzed using Western blotting using a Bcl2 or c-Myc antibody, respectively. DNA damage was assessed by AP Site Counting or Comet Assay after various treatments. Specific silencing of the targeted Bcl2 or c-Myc gene was confirmed by at least three independent experiments.

**Cell Viability Assay**—Apoptotic and viable cells were detected using an ApoAlert annexin-V kit (Clontech) according to the manufacturer’s instructions. The percentage of annexin-Vlow (i.e. viable) or annexin-Vhigh (i.e. apoptotic) cells was determined using the data obtained by fluorescence-activated cell sorter analysis as described (42). Cell viability was confirmed using the trypan blue dye exclusion method.

**RESULTS**

**Treatment of Cells with NNK Enhances c-Myc Transcriptional Activity in Association with Increased DNA Damage**—NNK has been demonstrated to induce DNA damage and promote proliferation of human lung cancer cells (22), which contribute to tumor development. NNK-induced SSBs could result from hydrolysis of alkali-labile sites, N7-Gua methylation, and enzymatic incision during excision repair (44–45). Because c-Myc has been reported to enhance DNA damage and suppress DNA repair (17) and is ubiquitously expressed in both small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC) cells (Fig. 1A), NNK may stimulate c-Myc to further enhance DNA damage. To test this, the pMyc-TA-Luc luciferase reporter construct was used to monitor the transcriptional activity of c-Myc in the NNK-mediated signal transduction pathway. First, H460 cells expressing endogenous c-Myc and Bcl2 were transfected with a 20:1 mix of the pMyc-TA-Luc luciferase reporter and the pTK Renilla control luciferase reporter constructs. After 48 h cells were treated with NNK (1 \( \mu M \)) for various times and then lysed in passive lysis buffer. The firefly (pMyc-TA-Luc reporter) activity relative to the Renilla (control) luciferase activity was assessed using the dual luciferase reporter assay kit and analyzed using the Monolight™ 3010 Luminometer. Intriguingly, exposure of cells to NNK significantly enhances c-Myc transcriptional activity (Fig. 1B). To evaluate NNK-induced DNA damage, H460 cells were treated with increasing concentrations of NNK (i.e. 1–5 \( \mu M \)) for 60 min. DNA damage was analyzed by Comet Assay as described under “Experimental Procedures.” Results reveal that NNK induces DNA damage in a dose-dependent manner (Fig. 1C). Cells that have accumulated DNA damage appear as fluorescent “comets” with tails of DNA fragmentation or unwinding, whereas normal undamaged DNA does not migrate far from the origin (Fig. 1C). The Comet Assay is able to detect SSBs, DSBs, and apurinic sites as well as apyrimidinic sites. Several previous reports indicated that NNK induces SSBs in both culture cells and whole animals, which were determined by a classic alkaline elution assay (32, 45–46). Therefore, we considered that NNK-induced DNA damage observed in the Comet Assay is likely SSBs. Thus, in addition to hydrolysis of alkali-labile sites and N7-Gua methylation, NNK-induced DNA damage may also result from increased c-Myc activity.

**Expression of Bcl2 Enhances c-Myc Transcriptional Activity and Suppresses the Repair of NNK-induced DNA Damage**—Bcl2 and c-Myc are two major oncogenic proteins that can cooperatively promote tumorigenesis (20). However, the mechanism is not fully understood. To test whether Bcl2 affects c-Myc transcriptional activity, H82, H23, H157, and H1299 cells expressing low or undetectable levels of endogenous Bcl2 were co-trans-
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Could result in base substitution mutations and genetic instability (47). Bcl2 may inhibit AP site repair induced by NNK and retain such damage in surviving cells, leading to genomic instability and tumorigenesis. These findings reveal that Bcl2 suppression of DNA repair may occur in a novel mechanism at least in part through enhancing c-Myc transcriptional activity. Similar results were also obtained by using H$_2$O$_2$, a known DNA damage agent, for induction of AP sites (data not shown), suggesting a role for Bcl2 in regulating the repair of AP sites of DNA damage induced by various agents.

**Specific Depletion of Endogenous Bcl2 by RNAi Down-regulates c-Myc Transcriptional Activity and Accelerates the Repair of NNK-induced DNA Damage**—To further demonstrate the physiological role of Bcl2 in regulating c-Myc transcriptional activity and DNA repair, a gene silencing approach for specific knockdown of endogenous Bcl2 was employed. H460 cells that express high levels of endogenous Bcl2 were transfected with Bcl2 siRNA or control siRNA as described under “Experimental Procedures.” Results indicate that transfection of Bcl2 siRNA significantly reduces expression levels of endogenous Bcl2 by more than 95% in H460 cells (Fig. 3A). This effect of Bcl2 siRNA on Bcl2 expression is highly specific since control siRNA has no effect (Fig. 3A). Importantly, specific depletion of endogenous Bcl2 results in down-regulation of c-Myc transcriptional activity and acceleration of DNA repair (Fig. 3, B–D). These findings provide further evidence that physiological Bcl2 may negatively regulate DNA repair by a mechanism involving c-Myc.

**c-Myc Co-localizes and Interacts with Both Nuclear and Mitochondrial Bcl2**—It has traditionally been viewed that Bcl2 is primarily localized in mitochondria with some minor expression in nuclear and endoplasmic reticulum membrane systems (48–49). Recent reports indicate that Bcl2 also resides in the nucleoplasm and may function within the nuclear compartment (47, 50). In contrast, the majority of c-Myc is localized in the nucleus of normal cells, and cytoplasmic c-Myc has also been frequently found in various tumor cells (51). In support of this, subcellular fractionation experiments indicate that c-Myc co-localizes with endogenous Bcl2 in both the nucleus and the mitochondrial membranes (Fig. 4A). Densitometry analysis reveals that approximately 85% of endogenous Bcl2 distributes in mitochondrial membranes, and 15% is observed in the nucleus. By contrast, about 84% of endogenous c-Myc is distributed in the nucleus, and 16% is localized in mitochondria in NSCLC H460 cells (Fig. 4A). This implies that the physiological cooperation between Bcl2 and c-Myc could occur at either the mitochondrial or nuclear level. To verify the purity of the subcellular fractions obtained, fraction-specific proteins were assessed by probing the same filters. Prohibitin, a protein exclusive to the mitochondria (52), was detected only in the mitochondrial fraction, whereas proliferating cell nuclear antigen, which is a nuclear marker (53–54), was detected exclusively in the nuclear fraction (Fig. 4A). These data reveal that the mitochondrial and nuclear fractions are pure without cross-contamination. To further confirm these findings, an immunofluorescent staining was performed using a rabbit c-Myc primary antibody and a fluorescein isothiocyanate-conjugated anti-rabbit secondary antibody. Results show that the majority of c-Myc is localized in the nucleus (Fig. 4B). Intriguingly, a certain amount of c-Myc can also be observed in mitochondria under a confocal fluorescent microscope. This is characterized by observing punctuate cytoplasmic c-Myc staining (Fig. 4B). Importantly, co-immunoprecipitation studies using c-Myc antibody indicate that nuclear Bcl2 can directly interact with c-Myc in vivo in H460 cells expressing endogenous Bcl2 or H1299 cells expressing exogenous Bcl2 (Fig. 4C, lanes 2 and 4). A rabbit preimmune serum was used as a control and failed to precipitate either c-Myc or Bcl2 (Fig. 4C, lane 1).
Bcl2 BH4 Domain Functionally Associates with c-Myc at Its MB II (Amino Acids 106–143) Domain—Bcl2 family members share homology in the Bcl2 homology (BH) domains including BH1, BH2, BH3, and BH4 (Ref. 55; Fig. 5A). To directly assess whether c-Myc binds to Bcl2 at these BH domains, purified c-Myc protein was incubated with purified recombinant WT, H9004 BH1, H9004 BH2, H9004 BH3, or H9004 BH4 Bcl2 deletion mutants in EBC lysis buffer at 4 °C for 2 h. The c-Myc-associated Bcl2 was co-immunoprecipitated with an agarose-conjugated c-Myc antibody and analyzed by Western blot using a Bcl2 antibody. Results demonstrate that c-Myc is able to associate with WT, H9004 BH1, H9004 BH2, H9004 BH3, or H9004 BH4 Bcl2 deletion mutants in EBC lysis buffer at 4 °C for 2 h. The c-Myc-associated Bcl2 was co-immunoprecipitated with an agarose-conjugated c-Myc antibody and analyzed by Western blot using a Bcl2 antibody. Results demonstrate that c-Myc is able to associate with WT, H9004 BH1, H9004 BH2, and H9004 BH3 but not with the H9004 BH4 Bcl2 mutant (Fig. 5B), indicating that the BH4 domain is the c-Myc binding site on Bcl2. That WT Bcl2 could not be immunoprecipitated in the absence of c-Myc (Fig. 5B, lane 1 versus lane 2) suggests Bcl2/c-Myc binding is specific in this assay.

The N-terminal transactivation domain of c-Myc (amino acids 1–144) has both transcriptional activation and repression activities. The C terminus of the c-Myc protein contains a basic-helix-loop-helix-leucine zipper (b-HLH-LZ) domain that is a characteristic of many known transcription factors (56). To determine the Bcl2 binding site on c-Myc, GST-tagged N-terminal 262 amino acids (i.e. N262) or the C-terminal 176 amino acids (i.e. C176) of c-Myc was incubated with purified recombinant WT Bcl2 in EBC lysis buffer. GST-beads were used to pull down the GST-c-Myc Bcl2 complex. The c-Myc-associated Bcl2 or GST-tagged c-Myc mutants were analyzed by Western blot using a Bcl2 or a GST antibody, respectively. Results indicate that the N-terminal N-262 but not the C-terminal C176 c-Myc mutant directly interacts with Bcl2 (Fig. 6A), indicating that Bcl2 binds c-Myc at its N-terminal domain, which has both transcriptional activation and repression activities. Within this domain are two evolutionarily conserved regions termed Myc Box (MB) I (amino acids 47–62) and MB II (amino acids 106–143). Deletion of MB I has been shown to diminish Myc-mediated transactivation, whereas deletion of MB II (amino acids 106–143) completely abrogates transformation and cell cycle progression (56). To further test the Bcl2 binding site in the c-Myc N terminus,
WT or c-Myc deletion mutants (i.e. ΔMBI (41–53) or ΔMBII (106–143)) and WT Bcl2 were co-transfected in c-Myc null HO15.19 cells using Lipofectamine™ 2000. Co-immunoprecipitation using agarose-conjugated c-Myc antibody indicates that WT and ΔMBI c-Myc mutant but not the ΔMBII mutant interact with Bcl2 (Fig. 6B). Reciprocally, WT Bcl2 associates with both WT and the ΔMBI c-Myc mutant but fails to interact with the ΔMBII c-Myc mutant (Fig. 6C). These results reveal that Bcl2 associates with c-Myc at the MBII domain in its N terminus. To test the role of the Bcl2/c-Myc binding via the MBII domain in regulating DNA repair, Bcl2 was co-transfected with WT or ΔMBII c-Myc mutant into c-Myc null HO15.19 cells. After 48 h, cells were washed and incubated in regular cell culture medium for 24 h. AP sites (C) and DNA damage (D) were assessed using an AP Site Counting Kit or Comet Assay kit, respectively. The data from AP Site Counting represent the mean ± S.D. of three separate determinations.

**FIGURE 3.** Depletion of endogenous Bcl2 expression by RNAi results in down-regulation of c-Myc transcriptional activity and acceleration of DNA repair. A, H460 cells expressing high levels of endogenous Bcl2 were transfected with Bcl2 siRNA or control siRNA as described under “Experimental Procedures.” Expression levels of Bcl2 were analyzed by Western blotting using a Bcl2 antibody. B, H460 cells were transfected with Bcl2 siRNA or control siRNA. After 48 h, c-Myc transcriptional activity was analyzed as described in the legend of Fig. 18. C and D, H460 cells were transfected with Bcl2 siRNA or control siRNA for 48 h. Then cells were treated with NMO (5 μM) for 60 min. Cells were washed and incubated in regular culture medium for 24 h. AP sites (C) and DNA damage (D) were assessed using an AP Site Counting Kit or Comet Assay Kit, respectively. The data from AP Site Counting represent the mean ± S.D. of three separate determinations.

**FIGURE 4.** c-Myc co-localizes and interacts with Bcl2 in nucleus and mitochondria. A, subcellular fractionation was performed in H460 cells expressing endogenous Bcl2 to isolate nuclear (Nuc), mitochondrial (Mito), and cytosolic (Cyt) fractions as described under “Experimental Procedures.” Western blot analysis of subcellular fractions was performed to detect Bcl2 and c-Myc. Prohibitin or proliferating cell nuclear antigen (PCNA) was used as a mitochondrial marker (52) or a nuclear marker (53), respectively, to verify the purity of each fraction. B, H460 cells were fixed with 3.7% formaldehyde and ice-cold acetone and blocked with PBS containing 10% normal rabbit serum. Then cells were incubated with a rabbit against human c-Myc antibody. Fluorescein isothiocyanate-conjugated anti-rabbit secondary antibody was used to visualize c-Myc localization under a confocal fluorescent microscope (Leica). C, nuclear proteins were isolated from H460 cells expressing endogenous Bcl2, H1299 cells expressing exogenous WT Bcl2, or vector control cells as described under “Experimental Procedures.” Co-immunoprecipitation (IP) experiments were performed using an agaro-conjugated rabbit anti-human c-Myc antibody. The c-Myc-associated Bcl2 (i.e. bound nuclear Bcl2) or c-Myc was analyzed by Western blotting using Bcl2 or c-Myc antibody, respectively. Rabbit preimmune serum was used as a control.

**FIGURE 5.** c-Myc directly interacts with Bcl2 at its BH4 domain. A, schematic representation of the BH in Bcl2 protein. B, purified recombinant c-Myc (0.1 μg) was incubated with purified WT, ΔBH1, ΔBH2, ΔBH3, or ΔBH4 Bcl2 deletion mutants (0.1 μg each) in EBC lysis buffer at 4 °C for 2 h. The c-Myc-associated Bcl2 was co-immunoprecipitated (IP) with an agaro-conjugated c-Myc antibody and analyzed by Western blot using a Bcl2 antibody.
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**FIGURE 6.** Bcl2 directly associates with c-Myc at its MBII domain. A, GST-tagged N-terminal 262 amino acids (i.e. N262) and the C-terminal 176 amino acids (i.e. C176) of c-Myc fusion proteins were incubated with purified recombinant Bcl2 in EBC lysis buffer. GST beads were used to pull down the GST-c-Myc-Bcl2 complex. The c-Myc-associated Bcl2 or GST-tagged c-Myc mutants were analyzed by Western blot using a Bcl2 or a GST antibody, respectively. IP, immunoprecipitates. B and C, WT or c-Myc deletion mutants (i.e. ΔMBI or ΔMBII) and WT Bcl2 were co-transfected into c-Myc-null H105.19 cells using Lipofectamine™ 2000. After 48 h, cells were lysed in EBC buffer. Co-immunoprecipitation experiments were carried out using agarose-conjugated Bcl2 or c-Myc antibody, respectively. c-Myc, Bcl2, bound c-Myc, and bound Bcl2 were analyzed by Western blotting. D, MBII mutant were transfected into c-Myc null H105.19 cells using Lipofectamine™ 2000. After 48 h cells were treated with H2O2 (50 μM) for 60 min. Cells were then washed and incubated in normal cell culture medium for 24 h. DNA damage was assessed using an AP Site Counting Kit. Data represent the mean ± S.D. of three separate determinations.

The Bcl2 BH4 Domain Is Required to Enhance c-Myc Transcriptional Activity and Suppress the Repair of NNK-induced DNA Damage—Our findings reveal that c-Myc directly interacts with Bcl2 at its BH4 domain (Fig. 5), suggesting that the BH4 domain may be important for the Bcl2 effect on regulation of c-Myc transcriptional activity and/or DNA repair. To test this, a BH4 deletion Bcl2 mutant ΔBH4 (Δ6–31) was created and constructed in the pClneo vector as described under “Experimental Procedures.” WT, the ΔBH4 Bcl2 mutant, and pClneo vector control were stably transfected into H1299 cells. Clones expressing quantitatively similar levels of Bcl2 were selected and tested (Fig. 7A). Each study was performed on at least three separate occasions with at least three independently derived clones expressing similar levels of exogenous Bcl2, and data for one representative clone are shown throughout. Results indicate that expression of WT but not the ΔBH4 Bcl2 mutant prolongs cell survival after H2O2 treatment (Fig. 7, A and B). This supports the notion that the BH4 domain is essential for the Bcl2 antiapoptotic function (57–59). Intriguingly, c-Myc transcriptional activity is enhanced in cells expressing WT but not the ΔBH4 Bcl2 mutant compared with vector control (Fig. 7C), and expression of the BH4 domain-deficient Bcl2 mutant fails to suppress DNA repair after treatment of cells with NNK (Fig. 7, D and E). These findings indicate that the BH4 domain is required for Bcl2 multiple functions including enhancement of c-Myc transcriptional activity, inhibition of DNA repair, and cell survival.

Expression of Bcl2 in the Nucleus Enhances c-Myc Transcriptional Activity in Association with Suppression of DNA Repair, and Nuclear Expressed Bcl2 Fails to Support Cell Survival—Both our results and those of others have demonstrated that, in addition to mitochondria, Bcl2 also localizes in the nucleus (Fig. 4A; Refs. 47 and 50). However, the functional role of nuclear Bcl2 is not clear. To test this, nuclear-targeted WT and ΔBH4 Bcl2 mutant (Bcl2/pCMV/Myc/Nuc) constructs were created and transfected into H1299 cells. Expression of the nuclear-targeted Bcl2 was analyzed by immunostaining or by Western blot. Results reveal that nuclear-targeted Bcl2 is exclusively expressed in the nucleus but not in mitochondria, which is characterized by co-localization with 4',6-diamidino-2-phenylindole but not Mitotraker (Fig. 8A). To test the effect of nuclear Bcl2 on cell survival, H1299 cells expressing nuclear-targeted WT or ΔBH4 mutant were treated with cisplatin (40 μM) for 48 h. Cell viability shows that expression of WT nuclear Bcl2 displays less viable cells compared with vector control or the ΔBH4 Bcl2 mutant after cisplatin treatment (Fig. 8B). However, expression of nuclear-targeted WT but not the ΔBH4 Bcl2 mutant potently enhances c-Myc transcriptional activity with suppression of DNA repair (Fig. 8, D–F). These results reveal that the BH4 domain is also essential for nuclear Bcl2 to activate c-Myc as well as attenuate DNA repair capacity.

Depletion of c-Myc Expression from Cells Overexpressing Bcl2 Accelerates the Repair of NNK-induced DNA Damage—Our findings suggest that Bcl2-induced suppression of DNA repair may occur by a mechanism involving transcriptional activation of c-Myc, which requires its MBII domain binding to the Bcl2 BH4 domain. To test whether c-Myc is essential for Bcl2 attenuation of DNA repair, an RNAi approach was employed to specifically deplete c-Myc expression. H1299 cells that overexpress WT Bcl2 were transfected with c-Myc siRNA as described under “Experimental Procedures.” Results show that the c-Myc siRNA efficiently and specifically silences c-Myc expression (more than 90%) in H1299 cells, whereas control siRNA has no effect (Fig. 9A). The c-Myc siRNA does not influence expression levels of Bcl2 (Fig. 9A). Cells were treated with NNK for 60 min, and then NNK was removed from the culture medium. Results indicate that AP sites and the DNA damage observed in Comet Assay are repaired within 24 h after depletion of c-Myc expression. By contrast, the majority of DNA damage in control cells expressing WT Bcl2 remains unrepaired (Fig. 9). These findings suggest that c-Myc may be essential for Bcl2 suppression of DNA repair.

Expression of Bcl2 Up-regulates c-Myc and Down-regulates APE1—Our data indicate that Bcl2 suppression of the repair of NNK- or H2O2-induced DNA lesions (i.e. AP sites) may occur through direct interaction with c-Myc and enhancement of c-Myc transcriptional activity. However, the downstream target(s) in the DNA repair machinery remains unclear. It is known that AP endonuclease (APE1) plays a central role in repairing AP sites of DNA damage through the BER pathway (39–40). It is possible that Bcl2 inhibits the repair of AP sites of DNA lesions in a mechanism involving regulation of APE1. Studies were designed to test whether expression of Bcl2 affects expression of c-Myc and APE1. Intriguingly, overexpression of Bcl2 results in increased levels of c-Myc and decreased levels of APE1 (Fig. 10). These data suggest that Bcl2 may attenuate DNA repair in a mechanism that involves, at least in part, up-regulation of c-Myc and down-regulation of APE1.
Thus, APE1 may function as a downstream target of Bcl2/c-Myc in the DNA repair machinery.

**DISCUSSION**

DNA repair mechanisms are essential for the maintenance of genomic integrity. Improperly repaired chromosomal damage can result in tumorigenesis. Bcl2 overexpression, which results from the translocation of t(14;18), leads to the dysregulation of apoptotic cell death during carcinogenic process in follicular B-cell lymphoma and lymphomagenesis in transgenic mice (3, 6). This indicates that Bcl2 is important in carcinogenesis. Currently, it is believed that Bcl2 may contribute to the development of carcinogenesis by inhibiting apoptosis because increasing the resistance to apoptosis in cells appears to make them susceptible to genetic alterations that may otherwise lead to cell death (5–7). Several studies have demonstrated that Bcl2 not only functions as a survival molecule but also can attenuate DNA repair capacity to promote genomic instability (8–11, 60), indicating that Bcl2 may enhance mutagenesis and carcinogenesis by both attenuating DNA repair and inhibiting apoptosis. However, the precise mechanism(s) by which Bcl2 inhibits DNA repair is not clear. Recent reports indicate that c-Myc, a known cooperative oncoprotein of Bcl2, can induce DNA damage and interfere with the ability of cells to repair DSBs (17). Therefore, the capacity of c-Myc to impair DNA repair and induce genomic instability plays a key role in tumorigenesis. In addition to cell survival, it is possible that Bcl2 and c-Myc may be involved in regulating DNA repair after exposure of cells to DNA-damaging agents (i.e. NNK or \( \text{H}_2\text{O}_2 \)).

NNK is formed by nitrosation of nicotine and has been identified as the most potent carcinogen in cigarette smoke (22, 27). Treatment of cells with increasing concentrations of NNK (1–5 \( \mu \text{M} \)) clearly induces DNA damage as determined by the Comet Assay, which is associated with increased c-Myc transcriptional activity (Fig. 1). AP Site Counting indicates that NNK significantly enhances abasic sites (AP sites; Fig. 2D), which are one of the most frequent type of lesions and promutagenic events in genomic DNA (61). It is known that NNK can induce SSBs (31–32, 45–46). Because cleavage of AP sites by AP endonucleases can generate SSBs (36), NNK-induced SSBs likely result, at least in part, from cleavage of AP sites. Intriguingly, the majority of NNK-induced AP sites and visible DNA damage observed in the Comet Assay are repaired within 24 h after removal of NNK from the culture medium in H1299 vector control cells expressing no detectable endogenous levels of Bcl2 (Fig. 2, C and D). In contrast, overexpression of Bcl2 in H1299 cells results in delayed DNA repair compared with vector control (i.e. from 24 to 96 h) in association with increased c-Myc transcriptional activity (Fig. 2). Importantly, specific knockdown of Bcl2 expression from H460 cells that express high levels of endogenous Bcl2 down-regulates c-Myc transcriptional activity and accelerates DNA repair (Fig. 3). Because c-Myc has been reported to inhibit DNA repair with enhanced DNA damage (3, 17), these findings suggest that Bcl2 suppression of DNA repair may occur by a novel mechanism involving enhancing c-Myc transcriptional activity. A recent report indicates that Bcl2 suppresses mismatch repair activity through inhibition of E2F1 transcriptional activity in GM00637 cells (60). E2F1 transcriptional activity was also tested in our system. Unexpectedly, no difference was observed in Bcl2 expressing and vector control H1299 cells (data not shown), which may be due to cell type difference or other
unknown reason. Because Bcl2 can enhance c-Myc transcriptional activity in multiple lung cancer cell lines (Fig. 2), our finding strongly suggests that activation of c-Myc by Bcl2 may play a key role in suppressing DNA repair in human lung cancer cells exposed to NNK.

The endogenous Bcl2 physically co-localizes and directly interacts with c-Myc in both mitochondria and nucleus (Fig. 4; Ref. 42). It is likely that Bcl2 may potentially function as a direct, physiological regulator of c-Myc. Bcl2 family members share homology in one to four regions designated the BH domains including BH1, BH2, BH3, and BH4 (55). All four homology regions are present in Bcl2. The BH4 domain (amino acids 6–31) of Bcl2, which encompasses an amphipathic α-helix, has been suggested to play a crucial role in the prevention of apoptosis. This is substantiated by studies revealing that deletion of this region renders Bcl2 defective for suppression of apoptosis (57–59). The transactivation domain and the C-terminal basic helix-loop-helix zipper domains are important for c-Myc functions (43). To potentially uncover the molecular mechanism(s) by which Bcl2 can enhance c-Myc transcriptional activity, structure-function studies with Bcl2 and c-Myc deletion mutants were performed. Results reveal that the BH4 domain of Bcl2 directly interacts with c-Myc at its MBII domain (Figs. 5 and 6), indicating that the BH4 domain is the c-Myc binding site on Bcl2 that may be critical for regulation of c-Myc activity. That the BH4 domain-deficient Bcl2 mutant not only fails to enhance c-Myc transcriptional activity but also loses the ability of Bcl2 to suppress DNA repair (Fig. 7) suggests the BH4 domain is required for Bcl2 attenuation of DNA repair function. Mechanistically, Bcl2/c-Myc binding can enhance the c-Myc half-life and allow c-Myc to accumulate with enhanced total activity, as described pre-

FIGURE 8. Expression of nuclear-targeted Bcl2 fails to prolong cell survival but enhances c-Myc transcriptional activity with suppression of DNA repair. A, WT Bcl2/pCMV/Myc/Nuc construct was transfected into H1299 cells and stained with Mitotracker (red) in the culture medium. Then cells were fixed with 3.7% formaldehyde and ice-cold acetone and incubated with a rabbit antibody against Bcl2, fluorescein isothiocyanate-conjugated anti-rabbit antibody (green), and 4',6-diamidino-2-phenylindole (DAPI; blue) as described under “Experimental Procedures.” Samples were observed under a fluorescent microscope (Zeiss). Green-, red- and blue-stained images were merged using Openlab 3.1.5 software. B, WT or the ΔBH4 Bcl2 mutant in pCMV/Myc/Nuc vector was transfected into H1299 cells. Expression levels of nuclear targeted Bcl2 were analyzed by Western blotting using a Bcl2 antibody. C, H1299 cells expressing nuclear targeted WT, the ΔBH4 Bcl2 mutant, or vector control were treated with cisplatin (40 μM) for 48 h. Cell viability was analyzed using an ApoAlert annexin-V kit. D, H1299 cells expressing nuclear targeted WT, the ΔBH4 Bcl2 mutant, or pCMV/Myc/Nuc vector control were transfected with a 20:1 mix of the pMyc-TA-Luc luciferase reporter and the pTK Renilla control luciferase report constructs using Lipofectamine™ 2000. After 48 h, c-Myc transcriptional activity was assessed using the dual luciferase reporter assay kit. E and F, H1299 cells expressing nuclear targeted WT, the ΔBH4 Bcl2 mutant, or vector control were treated with NNK (5 μM) for 60 min. Cells were washed and incubated in regular culture medium for 24 h. AP sites (E) and DNA damage (F) were assessed using an AP Site Counting kit or Comet Assay kit, respectively. The data from AP Site Counting represent the mean ± S.D. of three separate determinations.
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AP sites of DNA lesions, and expression of Bcl2 potently inhibits the repair of such type of DNA damage in a mechanism involving enhancement of c-Myc transcriptional activity (Fig. 2). APE1 is a major AP endonuclease in mammalian cells and plays a pivotal role in repairing AP sites of DNA lesions (39–40). That expression of Bcl2 not only up-regulates c-Myc but also down-regulates expression of APE1 (Fig. 10) suggests APE1 may act as a potential downstream target of Bcl2/c-Myc pathway in the DNA repair machinery. It is possible that Bcl2-activated c-Myc may repress APE1 expression at the transcriptional level through an unknown mechanism. Additional studies will be required to demonstrate this possibility.

In summary, results reported here have identified a novel signaling pathway by which Bcl2 suppresses the repair of NNK-induced AP sites and damage (i.e. SSBs) in genomic DNA through a mechanism involving enhanced c-Myc transcriptional activity and decreased APE1 expression. The binding between the Bcl2 BH4 domain and the c-Myc MBII domain may be required for the Bcl2 effect on c-Myc and DNA repair. Thus, Bcl2 may activate c-Myc through direct interaction in regulating DNA damage and repair, which may facilitate carcinogens (i.e. NNK)-induced genetic instability and tumorigenesis. Results from these studies may have potential clinical relevance for prevention and treatment of tobacco-related cancer, specifically lung or other Bcl2- and c-Myc-expressing malignancies and may contribute significantly to the development of novel strategies specifically aimed at functionally disrupting the interaction of Bcl2 and c-Myc.

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