Bcl2 Impedes DNA Mismatch Repair by Directly Regulating the hMSH2-hMSH6 Heterodimeric Complex*

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Bcl2 has been reported to suppress DNA mismatch repair (MMR) with promotion of mutagenesis, but the mechanism(s) is not fully understood. MutSα is the hMSH2-hMSH6 heterodimer that primarily functions to correct mutations that escape the proofreading activity of DNA polymerase. Here we have discovered that Bcl2 potently suppresses MMR in association with decreased MutSα activity and increased mutagenesis. Exposure of cells to nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone results in accumulation of Bcl2 in the nucleus, which interacts with hMSH6 but not hMSH2 via its BH4 domain. Deletion of the BH4 domain from Bcl2 abrogates the ability of Bcl2 to interact with hMSH6 and is associated with enhanced MMR efficiency and decreased mutation frequency. Overexpression of Bcl2 reduces formation of the hMSH2-hMSH6 complex in cells, and purified Bcl2 protein directly disrupts the hMSH2-hMSH6 complex and suppresses MMR in vitro. Importantly, depletion of endogenous Bcl2 by RNA interference enhances formation of the hMSH2-hMSH6 complex in association with increased MMR and decreased mutagenesis. Therefore, Bcl2 suppression of MMR may occur in a novel mechanism by directly regulating the heterodimeric hMSH2-hMSH6 complex, which potentially contributes to genetic instability and carcinogenesis.

Bcl2 is a proto-oncogene that was first identified at the chromosomal breakpoint of t(14;18) found in non-Hodgkin lymphoma, which is involved in dysregulated apoptotic cell death during the carcinogenic process (1). Bcl2 is widely expressed in various malignancies, including hematopoietic lung, breast, prostate, and nasopharyngeal cancers (2). However, the mechanism(s) by which Bcl2 facilitates oncogenesis is not fully understood. Because overexpression of Bcl2 results in lymphomagenesis in transgenic mice, this suggests that Bcl2, in addition to its survival activity, may also potentially have an oncogenic property (3). It has been reported that Bcl2 can enhance the benzene metabolite-induced DNA damage and mutagenesis in human promyelocytic HL60 cells (4). Overexpression of Bcl2 not only attenuates the nucleotide excision repair capacity and DNA replication in UV-irradiated HL60 cells (5) but also inhibits gamma ray-induced homologous recombination repair pathways, which result in elevated frequencies of mutagenesis (6). A recent report has proposed that Bcl2 can suppress DNA mismatch repair (MMR) by inhibiting E2F transcriptional activity (7). Thus, the oncogenic activity of Bcl2 may occur through multiple regulatory pathways.

MMR is a critical mechanism for maintaining genetic integrity by correcting base substitution and insertion/deletion loop mismatches that occur because of errors in DNA replication and recombination as well as DNA lesions resulting from a variety of internal and external stresses (8). The MMR system in human cells is composed of at least eight genes, including hMSH2, hMSH3, hMSH4, hMSH5, hMSH6 (GTBP), hMLH1, hPMS1, and hPMS2 (9, 10). The mismatch is detected by two major mismatch recognition complexes consisting of the hMSH2-hMSH6 heterodimer, which acts on single base mispairs and loops of one or two bases, and the hMSH2-hMSH3 heterodimer, which preferentially binds loops of three and four bases (11). Genetic studies have suggested that the MSH2-MSH6 complex is required for repair of single base substitution mispairs (12, 13), and the MSH6-defective mice have been found to develop cancer (14), suggesting that MSH2 and MSH6 are critical components in the MMR system.

Nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) is the most potent carcinogen contained in cigarette smoke that can induce cellular DNA damage (15–17). The NNK-induced DNA damage may have the potential to undergo mutagenesis during DNA replication and recombination. We previously discovered that Bcl2 potently suppresses the repair of NNK-induced abasic sites of DNA lesions by regulating c-Myc and APE1 (16). It is currently unclear whether Bcl2, in addition to its negative role in repairing NNK-induced DNA damage, may also affect NNK-induced mutagenesis by regulating the MMR machinery. Here we have discovered that Bcl2 enhances both spontaneous and NNK-induced mutagenic frequencies in a novel mechanism by down-regulating MMR efficiency via disrupting the hMSH2-hMSH6 complex.

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3 The abbreviations used are: MMR, mismatch repair; MutSα, MSH2/MSH6 heterodimer; GTBP, G/T binding protein; SAEC, small airway epithelial cell; NNK, nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; BH, Bcl2 homology; siRNA, small interfering RNA; WT, wild type; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid.

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which may potentially contribute to genetic instability and carcinogenesis.

EXPERIMENTAL PROCEDURES

Materials—Bcl2, hMSH2, hMSH3, hMSH6, and prohibitin antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). NNK was purchased from Toronto Research Chemicals (Toronto, Canada). The GTBP DNA repair kit was obtained from Active Motif (Carlsbad, CA). Purified recombinant wild type (WT), ΔBH1, ΔBH2, ΔBH3, and ΔBH4 Bcl2 mutant proteins were obtained from ProteinX Lab (San Diego, CA). Synthetic Bcl2 short interfering RNA (siRNA) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The M13mp2 heteroduplex DNA-containing mismatch was obtained from Dr. Thomas A. Kunkel (National Institute of Environmental Health Sciences). All of the reagents used were obtained from commercial sources unless otherwise stated.

Generation of Various Bcl2 Deletion Mutants—To create ΔBH1, ΔBH2, ΔBH3, and ΔBH4 Bcl2 deletion, the 5'-phosphorylated mutagenic primers for various precise deletion mutants were synthesized as follows: ΔBH1, 5'-GAG CGC TTT GCC AGC GTG GTG GAG GTG AGC GTG AAC AGG GAG ATG-3'; ΔBH2, 5'-GAG TAC CTG AAC CGG CAT CTG CAC CGA CCT CTG TTT GAT TTC TCC TGG-3'; ΔBH3, 5'-GCC TCT AGC CCT GTG CCA CCT GTG GAC TTC GCA GAG ATG TCC AGT CAG-3'; and ΔBH4, 5'-GGA AGT AGG CAC GAA GCC GGG AGA GCT GGA GAT GCG GAC GCC GCG CCC CTG-3'. The WT-Bcl2/pUC19 construct was used as the target plasmid that contains a unique Ndel restriction site for selection against the unmutated plasmid. The Ndel selection primer is 5'-GAG TGC ACC ATG GCC GTG GTG AAA-3'. Various Bcl2 BH deletion mutants were created by using a mutagenesis kit (Clontech) according to the manufacturer’s instructions and confirmed by sequencing of the cDNA. The WT and various Bcl2 deletion mutants were then cloned into the pcIneo (Promega) mammalian expression vector.

Cell Lines, Plasmids, and Transfections—Various human lung cancer cells were maintained as previously described (16). DLD1 (MSH6-/-) cells were obtained from the American Type Culture Collection (Manassas, VA) and cultured in Dulbecco’s minimum essential medium containing 10% serum. Human normal small airway epithelial cells (SAECs) were obtained from Cambrex Bio Science, Inc. and were maintained following the manufacturer’s protocol. The pCIneo plasmid containing each Bcl2 mutant cDNA was transfected into H1299 cells, DLD1 cells, or SAECs using Lipofectamine 2000™ (Invitrogen). Clones stably expressing WT and Bcl2 deletion mutants were selected in a medium containing G418 cells (0.6 mg/ml). The expression levels of exogenous Bcl2 were compared by Western blot analysis. Three separate clones for each mutant expressing similar amounts of exogenous Bcl2 were selected for further analysis.

Preparation of Cell Lysates—Cells were washed with 1x phosphate-buffered saline and resuspended in ice-cold 1% CHAPS lysis buffer (1% CHAPS, 50 mM Tris, pH 7.6, 120 mM NaCl, 1 mM EDTA, 1 mM NaVO₄, 50 mM NaF, and 1 mM β-mercaptoethanol) with a mixture of protease inhibitors (Calbiochem). Cells were lysed by sonication and centrifuged at 14,000 x g for 10 min at 4 °C. The resulting supernatant was collected as the total cell lysate and used for protein analysis or co-immunoprecipitation as described (18).

Subcellular Fractionation—Subcellular fractionation was performed as described previously (16). Briefly, cells (2 x 10⁶) were washed once with cold 1x phosphate-buffered saline and resuspended in isotonic mitochondrial buffer (210 mM mannitol, 70 mM sucrose, 1 mM EGTA, 10 mM Hepes, pH 7.5) containing the protease inhibitor mixture set I (Calbiochem). The resuspended cells were homogenized with a polytron homogenizer operating for four bursts of 10 s each at a setting of 5 and then centrifuged at 2000 x g for 3 min to pellet the nuclei and unbroken cells. The supernatant was centrifuged at 13,000 x g for 10 min to pellet mitochondria. The mitochondria was washed with mitochondrial buffer, resuspended with 1% Nonidet P-40 lysis buffer, rocked for 60 min, and then centrifuged at 17,530 x g for 10 min at 4 °C. The supernatant containing mitochondrial proteins was collected. For nuclear fractionation, the cells were washed with 1x phosphate-buffered saline and suspended in 2 ml of Buffer A (10 mM Tris-HCl, pH 7.4, 10 mM NaCl, 3 mM MgCl₂, and 0.03% Nonidet P-40 with fresh protease inhibitor mixture set I). The samples were incubated on ice until >95% of the cells could be stained by trypan blue. The samples were then centrifuged at 500 x g for 5 min. The resulting nuclear pellet was washed with Buffer B (50 mM NaCl, 10 mM Hepes, pH 8.0, 25% glycerol, 0.1 mM EDTA, 0.5 mM spermidine, 0.15 mM spermine) and then resuspended in 150 μl of Buffer C (350 mM NaCl, 10 mM Hepes, pH 8.0, 25% glycerol, 0.1 mM EDTA, 0.5 mM spermidine, 0.15 mM spermine) and rocked at 4 °C for 30 min. After centrifugation (14,000 x g) at 4 °C, the supernatant (nuclear fraction) was collected. Protein (100 μg) from each fraction was subjected to SDS-PAGE and analyzed by Western blotting using a Bcl2 antibody.

Measurement of MutSα Activity—MutSα DNA binding activity in nuclear extract isolated from various human lung cancer cells was analyzed using GTBP DNA repair kits according to the manufacturer’s instructions (Active Motif). GTBP DNA repair kits contain 96-well plates on which has been immobilized linear oligonucleotides containing G/T mismatches. The MSH2-MSH6 heterodimer contained in nuclear extract can specifically bind the G/T mismatch base pairs in the oligonucleotide. The primary antibody used in the GTBP DNA repair kit recognizes an epitope on the GTBP protein that is accessible upon DNA binding. After binding of a secondary horseradish peroxidase-conjugated antibody, developing solution was added to each well and incubated for 15 min. After the addition of stop solution, samples were read on a spectrophotometer within 5 min at 450 nm. Raji nuclear extract provided in the kit or the boiled recombinant Bcl2 protein were used as a positive or negative control, respectively. Each experiment was repeated three times, and data represent the mean ± S.D. of three separate determinations.

Purification of hMutSα—Human MutSα was purified from the nuclear extracts of H1299 cells as described previously (19). In brief, the samples used for hMutSα purification were precipitated from H1299 nuclear extracts between an ammonium sulfate saturation of 30 and 65%. The extract was diluted three
times before being loaded onto a single strand DNA cellulose column (1 x 1.5 cm; United States Biochemical). The proteins were eluted by a 10-ml (of 0.2–1 M NaCl) gradient in buffer A. The fractions containing the MSH2-MSH6 complex were diluted to bring the conductivity of the pool to an equivalent to 100 mm NaCl before being loaded onto Mono S (HR5/5). The bound proteins were eluted with a 20-ml (of 0.1 to 1 M NaCl) gradient in Buffer A. The purified hMutSα was immediately supplemented with 1 mg/ml bovine serum albumin and 10% sucrose, frozen in liquid N₂, and stored at −80 °C. Purified hMutSα were confirmed by Western blot using hMSH2 and hMSH6 antibodies.

**MMR Assay**—MMR activity was analyzed using an M13mp2 lacZ α-complementation method as described previously (20). The double-stranded M13mp2 heteroduplex DNA contains a nick in the (−) strand and a A:C, G:G or G:T mismatch in the lacZ α-complementation gene. The (+) strand encodes a colorless plaque phenotype, whereas the (−) strand encodes a blue plaque phenotype. The repair was directed to the (−) strand of M13mp2 by the presence of a nick. Introduction of this heteroduplex DNA into an *Escherichia coli* strain defective in mismatch repair yields M13 plaques on host indicator plates that are blue, colorless, or a mixture of the two. The repair reactions (25 μl) contained 30 mM Hepes (at pH 7.8), 7 mM MgCl₂, 4 mM ATP, 200 μM each of CTP, GTP, and UTP, 100 μM each of dATP, dGTP, dTTP, and dCTP, 40 mM creatine phosphate, 100 mg ml⁻¹ creatine phosphokinase, 15 mM sodium phosphate, 5 ng of purified heteroduplex DNA, and 50 μg of the nuclear extract protein isolated from human lung cancer cells or SAECs. After incubation for 30 min at 37 °C, the DNA was introduced into a *mutS* strain by electroporation. The cells were plated to score the plaque colors. As the nick directs repair to the (−) strand, the (+) phenotype increases and the (−) phenotype decreases, that is, producing more colorless plaques in this instance. The repair efficiency was expressed in percentage, as 100 × (ratio of mixed colonies in the control group − the ratio of mixed colonies in repaired group)/(ratio of mixed colonies in the control group). Each experiment was repeated three times, and data represent the mean ± S.D. of three separate determinations.

**Mutagenic Frequency Assay**—The shuttle vector pS189 and host *E. coli* strain MBM7070 were obtained from Dr. No-Hee Park (University of California Los Angeles School of Dentistry, Los Angeles, CA). The mutagenic frequency assay was performed as described previously (21). Briefly, cells were transiently transfected with pS189 plasmids using Lipofectamine 2000™ reagent (Invitrogen). After 24 h, the cells were exposed to NNK (1 μM) for 2 h and cultured in fresh medium without NNK for an additional 24 h. The repair efficiency was expressed in percentage, as 100 × (ratio of mixed colonies in the control group − the ratio of mixed colonies in repaired group)/(ratio of mixed colonies in the control group). Each experiment was repeated three times, and data represent the mean ± S.D. of three separate determinations.

**Expression of Endogenous Bcl2 Is Associated with Increased Mutagenic Frequency, Decreased MMR Efficiency, and Lower Levels of MutSα Activity in Human Lung Cancer Cells**—MMR is responsible for correcting base substitution and insertion or deletion loop mismatches generated during DNA replication (8). hMSH2 and hMSH6 are the major mismatch repair proteins that are required for MMR and are widely expressed in both small cell lung cancer and non-small cell lung cancer cells (Fig. 1A). Intriguingly, Bcl2, a major anti-apoptotic and/or oncogenic protein, is co-expressed with hMSH2 and hMSH6 in H69 and H460 but not in other lung cancer cells tested (Fig. 1A).

To test whether endogenous expression of Bcl2 may potentially regulate mutagenesis, various lung cancer cells were transfected with pS189 plasmids. After 24 h, the cells were exposed to NNK (1 μM) for 2 h and cultured in fresh medium without NNK for an additional 24 h. The mutagenic frequency was analyzed using the host *E. coli* strain MBM7070 as described under “Experimental Procedures.” Results indicate that H69 and H460 cells expressing high levels of endogenous Bcl2 represent a higher rate of either spontaneous or NNK-induced mutagenesis compared with other cell lines that express low or undetectable levels of Bcl2 (Fig. 1B). Because MMR is a key mechanism in maintaining genetic stability (23), an increased mutagenic frequency may result from decreased MMR. To test this possibility, nuclear extracts were prepared from various human lung cancer cells, and MMR efficiency was analyzed using three heteroduplexes containing A:C, G:G, or G:T mismatches. The percentage of MMR (i.e. A:C, G:G, and G:T) is significantly lower in the Bcl2-expressing H69 and H460 cells than that of other lung cancer cell lines that express undetectable levels of Bcl2 (Fig. 1C).

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MutSα is involved in recognizing and repairing mismatched bases and insertion or deletion loop mismatches in double-stranded DNA (24). To assess whether expression of Bcl2 affects MutSα activity, the mismatch binding capacity of MutSα was assessed using nuclear extracts isolated from various lung cancer cells and a DNA repair kit that contains a G:T
mismatched double-stranded linear DNA following the manufacturer's instructions. Because lower levels of MutS\textsubscript{a} activity were observed in H69 and H460 cells, this indicates that expression of endogenous Bcl2 is associated with the decreased mismatch binding capacity of MutS\textsubscript{a} (Fig. 1D), which may contribute to decreased MMR and increased mutagenesis.

**Overexpression of Exogenous Bcl2 Suppresses MMR in Association with Decreased MutS\textsubscript{a} Activity and Increased Mutagenesis**—To directly test the effect of Bcl2 on MMR, MutS\textsubscript{a} activity, and mutagenesis, Bcl2 was overexpressed in H1299 cells that do not express detectable levels of endogenous Bcl2, primary normal lung small airway epithelial cells (SAECs) that express low levels of endogenous Bcl2. Results indicate that overexpression of exogenous Bcl2 significantly inhibits both MMR efficiency and MutS\textsubscript{a} activity in association with enhanced mutagenic frequency in both H1299 and SAECs (Fig. 2). An MMR-deficient DLD1 (MSH6\textsuperscript{-/-}) cell line was used as a negative control (25). As expected, a low level of MutS\textsubscript{a} activity, low MMR efficiency, and high mutation frequency were observed in DLD1 cells. Overexpression of Bcl2 does not affect MutS\textsubscript{a} activity, MMR, and mutation frequency in DLD1 (MSH6\textsuperscript{-/-}) cells (Fig. 2), and this suggests that MSH6 is necessary for the effect of Bcl2 on either MMR or mutation frequency. Purified MutS\textsubscript{a} was added back to the extracts from
MMR-deficient DLD1 cells as an MMR-positive control. Results indicate that MutSα can restore MMR from DLD1 vector control cells (Fig. 2B). However, overexpression of Bcl2 in DLD1 cells inhibits MutSα-mediated MMR (Fig. 2B). Each study was performed on at least three separate occasions with at least three independently derived clones expressing similar levels of exogenous Bcl2, and results for one representative clone are shown throughout. These findings strongly suggest that the oncogenic property of Bcl2 may result from the inhibition of MMR.

**Treatment of Cells with the DNA-damaging Agent NNK Promotes Bcl2 Accumulation, Co-localization, and Association with hMSH6 in the Nucleus**—Bcl2 is primarily localized in the outer mitochondrial membranes with minor expression in nuclear and endoplasmic reticulum membrane systems (26, 27). Recent reports indicate that Bcl2 also resides in the nucleus and functions within the nucleus (16, 28, 29). We have previously demonstrated that the nuclear localized Bcl2 has no anti-apoptotic activity but is able to suppress DNA repair (16). By contrast, hMSH2 and hMSH6, the major components in the MMR machinery, primarily localize and function in the nucleus (30). To test how Bcl2 regulates hMSH6 following DNA damage, H460 cells expressing high levels of endogenous Bcl2 and hMSH6 were exposed to NNK (5 μM) for 60 min. Subcellular distribution of Bcl2 and hMSH6 was then examined by co-immunofluorescent staining. Consistent with our previous findings (16), the majority of Bcl2 is localized in cytoplasm, and only a small proportion is located in the nucleus in untreated cells. Intriguingly, Bcl2 is accumulated and co-localized with hMSH6 in nucleus after exposure of cells to NNK for 60 min, because the merged images become more yellow in the NNK-treated cells than untreated cells (Fig. 3A). However, our previous report indicates that treatment of H82 cells with NNK for 24 h did not affect Bcl2 nuclear localization (31). These different results may be due to the time difference of cell treatment (60 min versus 24 h). To test this possibility, a time course experiment was performed. H460 cells were treated with NNK (5 μM) for various times up to 24 h. Subcellular fractionation was carried out to isolate mitochondrial and nuclear fractions. Results reveal that nuclear Bcl2 expression is enhanced within 60 min (Fig. 3B). After 3 h, the increased levels of nuclear Bcl2 are gradually reduced to the same level as the no treatment control (i.e. at a 12 or 24 h time point; Fig. 3B), indicating that NNK-enhanced nuclear Bcl2 expression occurs in a time-dependent manner. These findings can explain why treatment of cells with NNK for 24 h did not affect nuclear Bcl2 expression in our earlier report (31). By contrast, the levels of mitochondrial Bcl2 showed no significant change in the time course experiment (Fig. 3B, lower panel). Thus, increased nuclear Bcl2 may not result from a movement from mitochondria into nucleus. This effect on Bcl2 following exposure of cells to NNK may occur through a transcriptional or other unknown mechanism(s). Further study is required to demonstrate this possibility.

To test for a direct interaction between Bcl2 and hMSH2 or hMSH6 in the nucleus, a co-immunoprecipitation was performed using isolated nuclear extracts and an agarose-conjugated Bcl2 or hMSH6 antibody. Results reveal that

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![FIGURE 3. DNA-damaging agent NNK promotes Bcl2 nuclear accumulation, co-localization, and association with hMSH6.](image)

A. H460 cells expressing high levels of endogenous Bcl2 were treated with NNK (5 μM) for 60 min. Subcellular distribution of Bcl2 and hMSH6 was examined by immunofluorescent staining using a mouse antibody against human hMSH6, rabbit Bcl2 antibody, and fluorescein isothiocyanate-conjugated anti-mouse (green) or Alexa 594 (red)-conjugated anti-rabbit secondary antibodies. Red- and green-stained images were merged using OpenLab version 3.1.5 software. Areas of co-localization appear yellow. B. H460 cells were treated with NNK (5 μM) for various times. Subcellular fractionation was performed to isolate nuclear and mitochondrial fractions. Levels of Bcl2 in the nuclear or mitochondrial fraction were analyzed by Western blotting using a Bcl2 antibody. C. H460 cells were treated with increasing concentrations of NNK for 60 min. A co-immunoprecipitation (IP) experiment was performed in isolated nuclear extracts using Bcl2, hMSH6, or hMSH2 antibody, respectively. Bcl2, hMSH2, hMSH6, and hMSH3 were analyzed by Western blot.

NNK-induced DNA damage promotes Bcl2 to associate with hMSH6 (but not hMSH2) in a dose-dependent manner (Fig. 3C, upper panel). NNK enhanced Bcl2-hMSH6 binding results in a decreased hMSH2-hMSH6 association (Fig. 3C, middle and lower panels), suggesting that Bcl2 may replace hMSH2 from the hMSH2-hMSH6 heterodimer and form a new Bcl2-hMSH6 complex upon DNA damage. Thus, Bcl2 suppression of MMR may occur in a novel mechanism by disrupting the hMSH2-hMSH6 heterodimerization. Intriguingly, NNK-induced Bcl2-hMSH6 binding promotes hMSH2-hMSH3 interaction (Fig. 3C, lower panel). It is possible that Bcl2 replaces hMSH2 from the hMSH2-hMSH6 complex to liberate hMSH2, which may benefit the formation of the hMSH2-hMSH3 complex.
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Bcl2 Directly Interacts with hMSH6 at the BH4 Domain, and Deletion of the BH4 Domain from Bcl2 Results in Loss of the Ability of Bcl2 to Suppress MMR and MutSα Activity, Which Leads to Reduced Mutagenesis—Bcl2 family members share homology in the Bcl2 homology (BH) domains including BH1, BH2, BH3, and BH4 (32). To directly assess whether Bcl2 directly binds to hMSH6 via its BH domains, purified recombinant hMSH6 protein was incubated with purified recombinant WT, ΔBH1, ΔBH2, ΔBH3, or ΔBH4 Bcl2 deletion mutants in 1% CHAPS lysis buffer at 4 °C for 2h. The hMSH6-associated Bcl2 was co-immunoprecipitated with an agarose-conjugated hMSH6 antibody. Results demonstrate that hMSH6 is able to directly bind to hMSH6 via its BH domains, purified recombinant hMSH6 (Fig. 4B, upper panel, lane 1 versus lane 2) and purified recombinant hMSH6 (Fig. 4B, lower panel, lane 1 versus lane 2) suggests that the binding of Bcl2 to hMSH6 is specific in this assay. Functionally, expression of WT, ΔBH1, ΔBH2, and ΔBH3 but not with the ΔBH4 Bcl2 mutants (Fig. 4B), indicating that the BH4 domain is the hMSH6 binding site on Bcl2. Because WT Bcl2 could not be immunoprecipitated by the hMSH6 antibody in the absence of hMSH6 (Fig. 4B, low panel, lane 1 versus lane 2) suggests that the binding of Bcl2 to hMSH6 is specific in this assay. Functionally, expression of WT, ΔBH1, ΔBH2, and ΔBH3 but not the ΔBH4 Bcl2 mutants significantly suppresses MMR and MutSα activity in H1299 cells (Fig. 4, C–E). Intriguingly, deletion of the BH4 domain (i.e. the hMSH6 binding site) from Bcl2 results in failure of Bcl2 to enhance mutagenic frequency (Fig. 4F). This supports the notion that the binding of Bcl2 to hMSH6 is critical for their activities in MMR. Because Bcl2 not only directly interacts with hMSH6 but also suppresses hMSH6 activity in association with decreased MMR efficiency (Figs. 2 and 3), Bcl2 may affect the functional heterodimerization of hMSH2-hMSH6 via binding to hMSH6. To test this, association of hMSH2-hMSH6 was compared in H1299 cells expressing WT Bcl2 and vector-only control cells in the absence and presence of NNK. Expression levels of Bcl2 in H1299 cells does not affect the expression levels of either hMSH2 or hMSH6 (Fig. 5A). However, lower levels of the hMSH2-hMSH6 complex were observed in the Bcl2-overexpressing cells compared with vector control cells, suggesting that expression of Bcl2 inhibits hMSH2-hMSH6 heterodimerization in cells (Fig. 5B). To further test whether Bcl2 can directly dissociate the hMSH2-hMSH6 complex in vitro, the hMSH2-hMSH6 complex was co-immunoprecipitated from H1299 parental cells expressing undetectable levels of endogenous Bcl2 using an agarose-conjugated hMSH6 antibody. The immune complex was incubated with increasing concentrations of purified, recombinant Bcl2 at 4 °C for up to 2 h, and proteins released from the complex were identified in the supernatant following centrifugation at 14,000 × g for 5 min.

FIGURE 4. Bcl2 directly interacts with hMSH6 at the BH4 domain and deletion of the BH4 domain from Bcl2 results in loss of the ability of Bcl2 to suppress mismatch repair and hMSH6 activity, which leads to reduced mutagenesis. A, schematic representation of the Bcl2 homology domains (BH) in Bcl2 protein. B, purified recombinant hMSH6 (0.1 μg) was incubated with purified WT, ΔBH1, ΔBH2, ΔBH3, or ΔBH4 Bcl2 deletion mutants (0.1 μg each) in 1% CHAPS lysis buffer at 4 °C for 2 h. The hMSH6-associated Bcl2 was co-immunoprecipitated with hMSH6 antibody. The hMSH6-associated Bcl2 was analyzed by Western blot. C, WT, ΔBH1, ΔBH2, ΔBH3, and ΔBH4 Bcl2 mutants were stably transfected into H1299 cells. Expression levels of Bcl2 were analyzed as described in the legend to Fig. 1. Data represent the mean ± S.D. of three separate determinations.
Surprisingly, Bcl2 directly disrupts the hMSH2-hMSH6 complex in vitro, because the addition of purified Bcl2 results in decreased levels of bound hMSH2 on beads and increased levels of non-bound hMSH2 in the supernatant (Fig. 5C). Functionally, the addition of recombinant Bcl2 to the extracts from H1299 parental cells (but not DLD1 cells) results in a dose-dependent reduction in MMR in vitro (Fig. 5D). These findings suggest that Bcl2-mediated attenuation of MMR may occur, at least in part, through disrupting a functional hMSH2-hMSH6 heterodimerization.

Depletion of Bcl2 by RNA Interference Enhances Formation of the hMSH2-hMSH6 Complex in Association with Increased MMR Efficiency and Decreased Mutagenesis—To test a physiological role for Bcl2 in regulating MMR and mutagenesis, a gene silencing approach was employed to specifically knock down the endogenous Bcl2. H460 cells that express 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 blot. B, H460 cells expressing Bcl2 siRNA or control siRNA were treated with NNK (5 μM) for 60 min. Cells were then disrupted in 1% CHAPS lysis buffer as described under “Experimental Procedures.” Co-immunoprecipitation (IP) was performed using an agarose-conjugated hMSH2 antibody. The hMSH6-associated hMSH2, the hMSH6-associated hMSH2, total hMSH2, and total hMSH6 were then analyzed by Western blot. C–E, MMR efficiency, MutSα activity, and mutagenesis in H1299 cells expressing Bcl2 siRNA or control siRNA were analyzed as described in the legend for Fig. 1. Data represent the mean ± S.D. of three separate determinations.

**DISCUSSION**

Bcl2 functions as a potent anti-apoptotic molecule as well as a cellular proto-oncogene that facilitates tumor development (3, 18, 33). Several reports indicate that Bcl2 can enhance DNA
damage and suppress DNA repair, including MMR in association with genetic instability (4–7, 34). However, the mechanisms by which Bcl2 regulates DNA damage and MMR are not fully understood. The major role of the MMR machinery, including hMSH2-hMSH6, is to recognize and correct mutations that can occur as a result of replication errors, recombination events, or chemically induced or spontaneous modification of DNA bases (24). Because mice lacking MSH6 have been shown to be prone to cancer (14), this indicates that the MSH6 involved in MMR plays an essential role in maintaining genetic stability and/or preventing carcinogenesis. Here, we have discovered that either endogenous or exogenous expression of Bcl2 significantly reduces MutSα activity and MMR efficiency in association with enhanced mutagenesis (Figs. 1 and 2). This helps explain why Bcl2 possesses oncogenic activity in the development of various cancers. Because Bcl2 has no effects on MMR and the mutation frequency in DLD1 (MSH6−/−) cells (Fig. 2), this indicates that the effects of Bcl2 are MMR-dependent.

In addition to mitochondrial localization, Bcl2 has also been found in the nucleoplasm and may function within the nucleus (28, 29). Approximately 85% of endogenous Bcl2 distributes in mitochondrial membranes, and 15% is observed in the nucleus in H460 human lung cancer cells (16). Intriguingly, the nuclear Bcl2 has no survival activity but still has ability to regulate DNA repair (16). Here we have found that NNK-induced DNA damage can stimulate Bcl2 accumulation in the nucleus, which subsequently interacts with hMSH6 (Fig. 3). This uncovers a potential mechanism by which Bcl2 down-regulates MutSα activity with suppression of MMR.

Bcl2 family members share homology in regions designated as the Bcl2 homology (BH) domains BH1, BH2, BH3, and BH4, in which only anti-apoptotic proteins, such as Bcl2, Bcl-XL, and MCL1, bear the NH2-terminal BH4 domain (32). Structure-function studies with Bcl2 deletion mutants reveal that hMSH6 directly interacts with Bcl2 at its BH4 domain (Fig. 4), indicating that the BH4 domain is the hMSH6 binding site on Bcl2. Because the BH4 domain-deficient Bcl2 mutant failed to suppress either MutSα activity or MMR (Fig. 4C, D and E), this suggests that the BH4 domain may function as the hMSH6 docking site, which is required for the effect of Bcl2 on MMR. It is known that caspase-mediated cleavage or mutagenic removal of the Bcl2 BH4 domain not only loses its survival activity but also converts Bcl2 into a pro-apoptotic molecule (35). Our data indicate that deletion of the BH4 domain abrogates the capacity of Bcl2 to enhance mutagenesis (Fig. 4F), suggesting that the BH4 domain is critical for Bcl2 survival and oncogenic functions. Thus, inhibition of the Bcl2/hMSH6 binding, via either pharmacologically or structurally based molecular targeting of the BH4 region, may provide a double whammy to the anti-apoptotic and oncogenic properties of Bcl2, which potentially represents a novel clinical strategy for prevention and/or treatment of a variety of cancers.

Our previous report indicates that the BH4 domain of Bcl2 (amino acids 6–31) was found to bind directly to c-Myc, and this interaction is required for Bcl2 to enhance c-Myc transcriptional activity and inhibit the repair of NNK-induced abasic sites of DNA lesions (16). It is known that APE1 plays a crucial role in the repair of abasic sites of DNA lesions (36). We have recently demonstrated that APE1 functions as the downstream target of Bcl2/c-Myc in regulating the repair of abasic sites of DNA lesions (16). Interestingly, our present data show that the BH4 domain of Bcl2 can also interact with MSH6 to impede MMR (Fig. 4). Thus, these two pathways may be functionally linked, because they share the binding site (i.e. BH4 domain) on Bcl2. Our preliminary data reveal that overexpression of c-Myc not only reduces the Bcl2-MSH6 binding but also inhibits the MSH2-MSH6 interaction (data not shown), suggesting that c-Myc may also play a role in regulating MMR. Further study is required to test this possibility.

hMSH2 functionally interacts with hMSH6 to form a complex (hMutSα complex) that can recognize mispaired bases in DNA, which may be essential for MMR (11). Our findings indicate that Bcl2 can directly interact with hMSH6 (but not hMSH2) both in vivo and in vitro (Figs. 3 and 4). Importantly, overexpression of Bcl2 in cells or the addition of purified Bcl2 to the hMSH2-hMSH6 complex significantly disrupts hMSH2-hMSH6 binding (Fig. 5), which leads to suppression of MMR in vivo and in vitro (Fig. 2B and Fig. 5D). A recent report indicates that expression of Bcl2 down-regulates hMSH2 expression through the inhibition of E2F1 transcriptional activity in GM00637 cells (7). However, either endogenous or exogenous expression of Bcl2 does not affect the expression levels of hMSH2 or hMSH6 in human lung cancer cells (Figs. 1A and 5A), which may be due to cell type difference or other unknown reason. By contrast, depletion of endogenous Bcl2 by RNA interference from H460 cells enhances the hMSH2-hMSH6 heterodimerization with elevated hMSH6 activity and MMR efficiency, which results in a reduced mutation frequency (Fig. 6). These findings uncover a novel mechanism by which Bcl2 promotes mutagenesis.

In summary, our findings have identified a novel signaling pathway by which Bcl2 enhances mutagenesis through a mechanism involving the suppression of MutSα activity and MMR. An NNK-induced DNA damage signal facilitates Bcl2 accumulation in the nucleus and interaction with hMSH6 via its BH4 domain leading to decreased MutSα activity and attenuation of MMR. Because MMR is the major mechanism for maintaining genetic integrity, Bcl2-mediated suppression of MMR may cooperate with carcinogens (i.e. NNK) to facilitate genetic instability and/or cancer development. Results from these studies may have potential clinical relevance for the prevention and treatment of tobacco-related cancer, especially lung or other Bcl2-expressing malignancies, by functionally disrupting the Bcl2-hMSH6 interaction.

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