Tobacco-specific Nitrosamine 4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone Promotes Functional Cooperation of Bcl2 and c-Myc through Phosphorylation in Regulating Cell Survival and Proliferation*

Zhaohui Jin, Fengqin Gao, Tammy Flagg, and Xingming Deng‡

From the University of Florida Shands Cancer Center, Department of Medicine and Department of Anatomy & Cell Biology, University of Florida, Gainesville, Florida 32610-0232

Nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) is formed by nitrosation of nicotine and has been identified as the most potent carcinogen contained in cigarette smoke. NNK significantly contributes to smoking-related lung cancer, but the molecular mechanism remains enigmatic. Bcl2 and c-Myc are two major oncogenic proteins that cooperatively promote tumor development. We report here that NNK simultaneously stimulates Bcl2 phosphorylation exclusively at Ser70 and c-Myc at Thr58 and Ser62 through activation of both ERK1/2 and PKCα, which is required for NNK-induced survival and proliferation of human lung cancer cells. Treatment of cells with staurosporine or PD98059 blocks both Bcl2 and c-Myc phosphorylation and results in suppression of NNK-induced proliferation. Specific depletion of c-Myc expression by RNA interference retards G1/S cell cycle transition and blocks NNK-induced cell proliferation. Phosphorylation of Bcl2 at Ser70 promotes a direct interaction between Bcl2 and c-Myc in the nucleus and on the outer mitochondrial membrane that significantly enhances the half-life of the c-Myc protein. Thus, NNK-induced functional cooperation of Bcl2 and c-Myc in promoting cell survival and proliferation may occur in a novel mechanism involving their phosphorylation, which may lead to development of human lung cancer and/or chemoresistance.

Lung cancer is the predominant cause of cancer deaths worldwide, with a high mortality rate and a five year survival rate of 15% (1). Cigarette smoking is the most important risk factor in the development of lung cancer (2). About 90% of male and 75–80% of female lung cancer deaths are caused by smoking each year in the United States (3). Cigarette smoke contains about 4,000 chemicals, 55 of which have been evaluated as carcinogens (3). Nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), a nicotine-nitrosated derivative, has been identified as the most potent lung carcinogen (4, 5). Cigarette smoke contains a substantial amount of NNK, and the total dose experienced by a smoker in a lifetime of smoking is remarkably close to the lowest dose shown to induce lung cancer in rats (5). Therefore, NNK is considered to be a major contributor to lung carcinogenesis in smokers (6, 7).

It has been demonstrated that NNK induces DNA damage, including single-strand DNA breaks, alkylphosphotriester formation (8), DNA adduct formation, increased oxidative stress (9), as well as induction of p53 and Ras mutations (6, 8). Traditional views have emphasized the formation of promutagenic DNA adducts from reactive metabolites of NNK (10) and resulting point mutations in the k-Ras gene (11) as important mediators of smoking-associated lung carcinogenesis. However, k-Ras mutations are absent in small cell lung cancer (SCLC; Ref. 12). The lack of these mutations strongly suggests that the development of SCLC may be caused by cellular events other than Ras mutations. In the search for potential mechanisms of SCLC development, our studies focus on the role of NNK-associated signal transduction pathways.

In addition to DNA damage, the mechanisms that promote cellular survival and proliferation may be the critical steps in NNK-initiated carcinogenesis. NNK, a nicotine-nitrosated carcinogenic derivative, retains some structural similarities with nicotine and binds to the α-nicotinic acetylcholine receptor (αnAChR), resulting in the influx of Ca2+, release of 5-hydroxytryptamine, and activation of a protein kinase cascade (i.e. PKC/Raf-1/MEK/ERK1/2; Refs. 6 and 7). However, the downstream survival and proliferative substrates of NNK-activated protein kinases have not been identified.

Among the known proto-oncogenes, the cellular Myc gene (c-Myc) is one of those most frequently implicated in carcinogenesis (13–15). Deregulated expression of the structurally unaltered Myc protein is sufficient to drive continuous cell proliferation and apoptosis in response to growth-promoting and growth-inhibitory signals, respectively (14). Expression of c-Myc can initiate proliferation and increase sensitivity to apoptosis under low serum conditions when antiapoptotic mechanisms are not activated (16). Bcl2 and c-Myc are two major oncogenic proteins that can functionally cooperate in cell proliferation, transformation, apoptosis, and tumorigenesis (17). To avoid c-Myc-induced cell death and ensure continuous cell proliferation, Bcl2 functions as one of the most potent Myc-cooperating oncogenes (13, 18), which is a global inhibitor of apoptosis, likely through multiple mechanisms (14, 19). Bcl2

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† To whom correspondence should be addressed: University of Florida Shands Cancer Center, 1600 SW Archer Rd., Academic Research Bldg., R4-216, P.O. Box 100232, Gainesville, FL 32610-0232. Tel.: 352-392-9232; Fax: 352-392-5802; E-mail: xdeng@ufscf.ufl.edu.

‡ The abbreviations used are: NNK, nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; SCLC, small-cell lung cancer; αnAChR, α-nicotinic acetylcholine receptor; PKC, protein kinase c; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; e-BTX, α-bungarotoxin; siRNA, small interfering RNA; MEK, MAPK/ERK kinase; JNK, c-Jun N-terminal kinase; WT, wild type; PBS, phosphate-buffered saline; AA, T88A/S62A.

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Assay of PKCs Activity and Phosphorylation of c-Myc and Bcl2 in Vitro—PKCs was immunoprecipitated from cell lysate with PKCa antibody following treatment with NNK. Immunoprecipitated PKCs was suspended in 50 μl of kinase assay buffer containing 20 mM Hesper, pH 7.4, 100 mM CaCl2, 10 mM MgCl2, 200 μg/ml histone-1, 100 μM ATP, 100 μg/ml phosphatidylserine, 2 μg/ml of γ-32P]ATP, and 0.03% Triton X-100. The mixture was incubated for 30 min at 30 °C. The reaction was stopped by the addition of 2× SDS sample buffer and boiling the sample for 5 min. The samples were separated by SDS-PAGE. The activities of PKCs were determined by autoradiography. To assess phosphorylation of Bcl2 and c-Myc in vitro, Bcl2 or c-Myc was immunoprecipitated from cell lysates using Bcl2 or c-Myc antibody, respectively. The immune complex was then incubated with purified, activated PKCa (Panviva, CA), ERK1, or ERK2 (Calbiochem) in assay buffer containing γ-32P]ATP as described previously (19). Phosphorylation of Bcl2 or c-Myc was analyzed by autoradiography.

**Subcellular Fractionation**—The cells (2 × 10^7) were washed once with cold 1× PBS and resuspended in isotonic mitochondrial buffer (210 mM mannitol, 70 mM sucrose, 1 mM EGTA, 10 mM Hesper, pH 7.5) containing protease inhibitor mixture set I. 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 × g for 3 min to pellet the nuclei and unbroken cells. The supernatant was centrifuged at 13,000 × g for 10 min to pellet mitochondria as described (25). The supernatant was further centrifuged at 150,000 × g to pellet light mitochondrial contaminants. The resulting pellets containing mitochondrial fractions. The mitochondria was washed with mitochondrial buffer twice, resuspended with 1% Nonidet P-40 lysis buffer, rocked for 60 min, and then centrifuged at 17,530 × g for 10 min at 4 °C. The supernatant containing mitochondrial proteins was collected. For nuclear fractionation, the cells were washed with 1× PBS and suspended in 2 ml of Buffer A (10 mM Tris-HCl, pH 7.4, 10 mM NaCl, 3 mM MgCl2, 0.01% Nonidet P-40 with fresh protease inhibitor mixture set I). The samples were incubated on ice until more than 95% of cells could be stained by trypan blue. The samples were then centrifuged at 500 × g at 4 °C for 5 min. The resulting nuclear pellet was washed with Buffer B (50 mM NaCl, 10 mM Hesper, 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 Hesper, 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 × g) at 4 °C, the supernatant (nuclear fraction) was collected (28). Protein (100 μg) from each fraction was subjected to SDS-PAGE and analyzed by Western blotting using Bcl2 or c-Myc antibody, respectively. The purity of the fractions was confirmed by assessing localization of fraction-specific proteins including prohibitin (a mitochondrial marker; Ref. 27) and proliferating cell nuclear antigen (a nuclear marker; Refs. 28 and 29).

**Treatment of Mitochondria with Proteinase K in Vitro**—Purified mitochondria fractions were collected as described above. Purified mitochondria were treated with various concentrations of proteinase K (0.5, 2.5, 5, 25, 50, and 100 μM) for 25 min on ice. Phenylmethanesulfonyl fluoride was then added to a final concentration of 2 mM, and the samples were incubated for another 10 min on ice as described (30). The mitochondria were pelleted by centrifugation at 13,000 × g for 10 min and then washed twice with isotonic mitochondria buffer. The pellets were suspended in 1% Nonidet P-40 lysis buffer and rocked for 60 min and then centrifuged at 17,530 × g for 10 min at 4 °C. Protein (100 μg) from the supernatant was subjected to SDS-PAGE and analyzed by Western blotting using Bcl2 or c-Myc antibody, respectively.

**Cell Viability Assay**—The apoptotic and viable cells were detected using an ApoAlert Annexin-V kit (Clontech) according to the manufacturer's instructions. This is a colorimetric assay for the quantification of cell proliferation, based on the cleavage of the tetrazolium salt WST-1 by mitochondrial dehydrogenases in viable cells (percentage of viable cells) or annexin-V<sup>−</sup> cells (percentage of apoptotic cells) is determined using the data obtained by fluorescence-activated cell sorter analysis as described (31). Cell viability was also confirmed using the trypan blue dye exclusion method.

**Cell Proliferation Assay**—H922 or NCi-H69 cells were seeded at a concentration of 2 × 10<sup>4</sup> cells/well in 100 μl of culture medium and treated with NNK in the absence or presence of various inhibitors. Cell proliferation was assessed using a WTS-1 kit according to the manufacturer's instructions (Roche Applied Science). This is a colorimetric assay for the quantification of cell proliferation, based on the cleavage of the tetrazolium salt WST-1 by mitochondrial dehydrogenases in metabolically active cells. The absorbance of each sample was measured using a microplate (enzyme-linked immunosorbent assay) reader.
performed to confirm and quantify Bcl2 protein (lower panels). For present the means Bcl2 and c-Myc in various lung cancer cell lines were analyzed by

Labeled with [32P]orthophosphoric acid and treated with NNK (100 pM) H69 cells expressing high levels of endogenous Bcl2 were metabolically

Growth curves of NCI-H69 cells expressing endogenous Bcl2 and c-Myc reprorbed using c-Myc antibody to quantify the total c-Myc protein.

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Bcl2 functions as a potent antiapoptotic molecule, and phosphorylation of Bcl2 in the flexible loop domain can positively regulate its survival function (19, 23, 33). In contrast, the

c-Myc protein, encoded by the c-Myc proto-oncogene, is a potent inducer of both cell proliferation and apoptosis (34). Phosphorylation of c-Myc has been reported to enhance both its proliferation and apoptotic functions (35–37). NNK may functionally facilitate a cooperation of Bcl2 and c-Myc in a mechanism involving their phosphorylation. To experimentally test this, SCLC H69 cells expressing endogenous Bcl2 and c-Myc were metabolically labeled with [32P]orthophosphoric acid and treated with NNK (100 pM) for various times as indicated. Bcl2 phosphorylation was analyzed by autoradiography. Phosphorylation of c-Myc was assessed by Western blot using a phospho-specific c-Myc antibody. Results indicate that NNK induces Bcl2 phosphorylation with a peak at 15 min (Fig. 1B). Simultaneously, NNK induces c-Myc phosphorylation at Thr58 and Ser62 as determined by using a Thr58/Ser62 dual phosphospecific c-Myc antibody that only recognizes the dual phosphorylated c-Myc protein (Fig. 1B and Ref. 32). Because growth curves show that NNK significantly stimulates proliferation (Fig. 1C), this suggests that NNK-induced proliferation of lung cancer cells may occur through phosphorylation of Bcl2 and c-Myc to facilitate their cooperation. We chose H69 cells for testing the effect of NNK on Bcl2 and c-Myc phosphorylation because high levels of endogenous Bcl2 and c-Myc are expressed in H69 cells (Fig. 1A). Thus, the results obtained from this cell line may have more physiological relevance.

Knockdown of c-Myc Expression by RNA Interference Blocks NNK-induced Proliferation of Human Lung Cancer Cells—Previous studies reveal that expression of endogenous c-Myc in human lung cancer cells is associated with high proliferative activity (21). To determine whether c-Myc is required for NNK-stimulated lung cancer cell proliferation, an RNA interference approach was employed. It has recently been demonstrated that 21-base double-stranded siRNA is a potent mediator of the RNA interference effect in mammalian cells (38). H69 cells were transfected with c-Myc siRNA as described under “Experimental Procedures.” Results show that the c-Myc siRNA efficiently and specifically reduces c-Myc expression in SCLC H69 cells, whereas control siRNA has no effect (Fig. 2A). In contrast to the control, knockdown of c-Myc expression reduces the percentage of cells in the S phase (i.e. 18.41% versus 47.53%) with a greater percentage of cells in the G0-G1 phase (66.14% versus 35.99%; Fig. 2B). Importantly, depletion of c-Myc expression by RNA interference significantly inhibits NNK-induced cell proliferation (Fig. 2C). These findings suggest that expression of c-Myc may be essential for G0/S cell cycle transition as well as NNK-stimulated phosphorylation of SCLC H69 cells.

NNK-induced Bcl2 Phosphorylation at Ser70 Inhibits Apoptotic Cell Death Following Treatment with the Chemotherapeutic Drug Cisplatin—We have previously found that Ser70 is the major phosphorylation site of Bcl2 and that phosphorylation at Ser70 may be required for the antiapoptotic function of Bcl2 (19, 23). To identify the NNK-induced Bcl2 phosphorylation site, serine → alanine/glutamate mutants at Ser70 including S70A and S70E were created to abrogate or mimic, respectively, phosphorylation at this site. WT, S70A, and S70E Bcl2 mutants were stably transfected into SCLC NCI-H82 cells. We chose this cell line because H82 cells express very low levels of endogenous Bcl2 (Fig. 1A). Thus, the survival function of individual Bcl2 mutants could be more accurately evaluated because any possible effect from endogenous Bcl2 could be minimized. H82 cells expressing similar levels of exogenous WT, S70A, or S70E Bcl2 were selected and tested (Fig. 3A). Because NNK induces WT but not S70A or S70E Bcl2 phosphorylation (Fig. 3B), this indicates that NNK-induced Bcl2 phosphorylation occurs at the Ser70 site. To test whether NNK-induced

### RESULTS

**NNK Simultaneously Induces Phosphorylation of Both Bcl2 and c-Myc, Which Is Associated with Increased Proliferation of SCLC Cells**—The tobacco-specific lung carcinogen NNK functions as a site-selective and high affinity agonist for the α7 nAChR (7, 32). Interaction of NNK with the α7 nAChR results in activation of PKC/RAF/MAPK/ERK1/2 protein kinase cascade that may promote lung cancer development and proliferation of tumor cells (7). Bcl2 and c-Myc have been demonstrated to be two functional cooperative oncoproteins that are expressed in both SCLC and non-SCLC cells (Fig. 1A and Refs. 18, 20, and 21). Bcl2 functions as a potent antiapoptotic molecule, and phosphorylation of Bcl2 in the flexible loop domain can positively regulate its survival function (19, 23, 33). In contrast, the

**Fig. 1.** NNK induces Bcl2 and c-Myc phosphorylation and facilitates proliferation of human SCLC cells. A, expression levels of Bcl2 and c-Myc in various lung cancer cell lines were analyzed by Western blotting using Bcl2 or c-Myc antibody, respectively. B, NCI-H69 cells expressing high levels of endogenous Bcl2 were metabolically labeled with [32P]orthophosphoric acid and treated with NNK (100 pM) for various times as indicated. Bcl2 was immunoprecipitated by using Bcl2 antibody. Phosphorylation of Bcl2 was determined by autoradiography (upper panels). Western blot analysis using Bcl2 antibody was performed to confirm and quantify Bcl2 protein (lower panels). For c-Myc phosphorylation, NCI-H69 cells were treated with NNK (100 pM) for various times. The cells were harvested and lysed at each time point. Phosphorylation of c-Myc was assessed by Western blotting using a Thr58/Ser62 dual phosphospecific c-Myc antibody. The same filter was reprobed using c-Myc antibody to quantify the total c-Myc protein. C, growth curves of NCI-H69 cells expressing endogenous Bcl2 and c-Myc in the presence or absence of NNK (100 pM) were assessed using Coulter Counter. The experiment was repeated three times, and the data represent the means ± S.D. of three determinations. NSCLC, non-SCLC.
Ser70 site phosphorylation or mimicking the charge conferred by phosphorylation at this site inhibits chemotherapeutic agent-induced apoptosis, H82 cells expressing WT, S70A, or vector control were treated with cisplatin (10 μM) in the absence or presence of NNK. Cell viability was determined by analyzing annexin-V binding with a fluorescence-activated cell sorter as described under “Experimental Procedures.” The results indicate that NNK potently enhances survival of cells expressing WT but not S70A Bcl2 (Fig. 3C). These results indicate that NNK-induced Bcl2 phosphorylation at Ser70 can inhibit cisplatin-induced apoptosis. To further demonstrate a requirement of Ser70 site phosphorylation for inhibition of che-
motherapeutic agent-induced apoptosis, S70E Bcl2 mutant that mimics continuous Bcl2 phosphorylation at Ser70 was also tested. Interestingly, expression of the phosphomimetic S70E Bcl2 mutant represents more antiapoptotic activity in the absence of NNK compared with WT (Fig. 3C). NNK has no additional survival effect on cells expressing S70E Bcl2. These comparative results provide strong evidence that phosphorylation of Bcl2 at Ser70 may be required for NNK-enhanced survival of human SCLC cells.

**Phosphorylation of c-Myc Is Essential for NNK-induced Proliferation of Human Lung Cancer Cells**—We have identified that NNK stimulates c-Myc phosphorylation at Thr58 and Ser62 sites using a Thr58/Ser62 dual phosphospecific c-Myc antibody (Fig. 1B). To test the role of c-Myc phosphorylation in NNK-induced cell proliferation, WT and the nonphosphorylatable AA c-Myc mutant were transfected into NCI-H1299 lung cancer cells expressing WT Bcl2. We used this cell line because H1299 cells express very low levels of endogenous c-Myc (Fig. 4A).

Thus, the proliferative function of exogenous WT and AA c-Myc mutant could be more accurately evaluated because any possible effect from endogenous c-Myc could be minimized. H1299 cells expressing WT or AA c-Myc mutant or vector control were treated with NNK (100 pM) for 72h. Cell proliferation was assessed using the cell proliferation assay kit (WST-1) as described under “Experimental Procedures.” The results reveal that NNK significantly enhances proliferation of cells expressing WT but not the nonphosphorylatable AA mutant c-Myc (Fig. 4), suggesting that phosphorylation of c-Myc may be required for NNK-stimulated cell proliferation.

**NNK Activates PKCa and MAPK ERK1/2 in Human Lung Cancer Cells, and ERK1 and ERK2 Directly Induce c-Myc Phosphorylation in Vitro**—Our previous studies have demonstrated that PKCa and MAPKs (ERK1/2) are physiological Bcl2 kinases that directly induce Bcl2 phosphorylation in *vitro* and *in vivo* (19, 39). ERK1 and ERK2 also function as physiological c-Myc kinases to phosphorylate c-Myc (Fig. 5A and Refs. 32 and 36). This indicates that c-Myc and Bcl2 may share the same upstream protein kinases in certain signal transduction pathways. Because NNK can induce both Bcl2 and c-Myc phosphorylation (Fig. 1), we tested whether NNK activates the physiological kinases of Bcl2 and c-Myc in human SCLC cells. Time course experiments indicate that NNK induces activation of PKCa and ERK1/2 within 60 min (Fig. 5, B and C). By contrast, NNK has no effect on either JNK1 or p38 (Fig. 5, D and E). These findings suggest that NNK-induced Bcl2 and c-Myc phosphorylation may occur through activation of PKCa and/or ERK1/2.

Staurosporine is a potent PKC inhibitor, whereas PD98059 can specifically inhibit MEK1-induced ERK1 or ERK2 activation (19, 40). Because staurosporine can also inhibit NNK-induced ERK1/2 activation (Fig. 5G), this indicates that PKC may function upstream of ERK1/2 in the NNK-activated protein kinase cascade. These pharmacological data strongly suggest that NNK-induced ERK1/2 phosphorylation may be dependent on PKC activity.

**Staurosporine and PD98059 Block NNK-induced Bcl2 and c-Myc Phosphorylation as Well as Cell Proliferation**—To further test whether PKCa and MAPK/ERK1/2 are involved in NNK-induced Bcl2 and c-Myc phosphorylation, H82 cells expressing WT Bcl2 were metabolically labeled with [32P]orthophosphoric acid and treated with NNK in the absence or presence of staurosporine or PD98059 or in combination. The results indicate that staurosporine and/or PD98059 potently inhibits NNK-induced phosphorylation of both Bcl2 and c-Myc in a dose-dependent manner (Fig. 6, A and B). This suggests that NNK-induced phosphorylation of Bcl2 and c-Myc occurs through activation of PKC and ERK1/2. To test the functional effect of PKC and ERK1/2 in NNK-induced phosphorylation of Bcl2 and c-Myc, cells expressing WT or S70E Bcl2 mutant were treated with NNK in the presence or absence of various concentrations of staurosporine or PD98059. Cell proliferation was assessed using the cell proliferation assay kit (WST-1) as described under “Experimental Procedures.” As expected, both staurosporine and PD98059 potently block NNK-induced proliferation of SCLC cells (Fig. 6C). Importantly, cells expressing the S70E Bcl2 mutant are less sensitive to inhibition of cell proliferation by staurosporine or PD98059 compared with WT (Fig. 6C), suggesting that expression of the gain-of-function S70E mutant partially restores NNK-induced cell proliferation in the presence of various concentrations of staurosporine or PD98059.

**The α2 nAChR-specific Inhibitor a-Bungarotoxin (a-BTX) Blocks NNK-induced Bcl2 and c-Myc Phosphorylation in Association with Decreased Cell Proliferation**—a-BTX has been identified as the site-selective antagonist for α2 nAChR (32, 41, 42). Because α2 nAChR plays an important role in lung cancer cell signaling (32) and NNK is a site-selective, high affinity agonist for the α2 nAChR (7, 32), we tested whether a-BTX affects NNK-induced Bcl2 and c-Myc phosphorylation in human SCLC cells. The results indicate that a-BTX potently

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Fig. 4. NNK enhances proliferation of cells expressing WT but not AA c-Myc mutant. A, WT and AA c-Myc mutant cDNAs were overexpressed in H1299 lung cancer cells. Expression levels of c-Myc protein were determined by Western blotting using a c-Myc antibody. B, H1299 cells expressing WT or AA mutant c-Myc or vector control were treated with NNK (100 pM) for 96 h. Cell proliferation was assessed using WTS-1 kit as described under “Experimental Procedures.” The data represent the means ± S.D. of three determinations.
blocks both Bcl2 and c-Myc phosphorylation in a mechanism involving inhibition of NNK-induced activation of ERK1 and ERK2 that are physiological Bcl2 and c-Myc kinases (Fig. 7, A–C). Correlative, α-BTX significantly inhibits NNK-stimulated cell proliferation (Fig. 7D). These findings suggest that α2 nAChR functions as the upstream receptor in NNK-induced Bcl2 and c-Myc phosphorylation.

Bcl2 Co-localizes with c-Myc in Nucleus as Well as on Outer Mitochondrial Membranes—To determine where Bcl2/c-Myc interaction may occur in SCLC cells, co-immunoprecipitation studies were performed using c-Myc antibody and isolated mitochondrial or nuclear protein from H82 cells expressing the gain-of-function mutant S70E or nonphosphorylatable S70A Bcl2. Results reveal that S70E but not S70A Bcl2 can directly interact with c-Myc in both nucleus and mitochondria (Fig. 10A). These findings suggest that functional cooperation between Bcl2 and c-Myc may occur at both mitochondrial and nuclear levels. To further analyze the subcellular localization of Bcl2/c-Myc association, an experiment for proteinase K-dependent degradation of outer mitochondrial membrane proteins was performed using H82 cells expressing S70E Bcl2 mutant as described (30). Notably, proteinase K treatment has been used extensively to selectively remove proteins from the surface of mitochondrial outer membranes (30, 48). Because the majority of Bcl2 is removed from the outer mitochondrial...
membrane after treatment with proteinase K (Fig. 10B), this is consistent with previous findings that Bcl2 is integrally associated with the outer mitochondrial membrane (49, 50). Because mitochondrial c-Myc is very sensitive to proteinase K treatment (Fig. 10B), this suggests that c-Myc is also associated with the outer mitochondrial membrane. It is known that prohibitin is associated with the inner mitochondrial membrane (27). This helps to explain why prohibitin is not affected by this treatment (Fig. 10B). These results reveal that the association of c-Myc and Bcl2 on mitochondria may occur at the outer mitochondrial membrane.

Interaction between Bcl2 and c-Myc Increases c-Myc Protein Stability—Activation of the Ras/Raf/ERK pathway has been reported to induce c-Myc phosphorylation resulting in an increase of the half-life ($t_{1/2}$) of the c-Myc protein and thus enhances the accumulation of c-Myc activity (36, 37). Our findings show that phosphorylation of Bcl2 at Ser70 enhances its ability to associate with c-Myc (Fig. 9). However, the functional role of this association is not clear. It is possible that this direct interaction between Bcl2 and c-Myc may stabilize the c-Myc protein to enhance its multiple functions. To test this, $t_{1/2}$ studies were performed in H82 cells expressing the phosphomimetic S70E Bcl2 mutant with the classical [35S]methionine pulse-chase method. We chose H82 cells expressing S70E because S70E Bcl2 more potently associates with c-Myc (Fig. 9B). Results indicate that Bcl2-associated c-Myc (i.e. bound c-Myc) has a longer half-life compared with the unbound c-Myc in cells expressing S70E Bcl2. The half-life of the bound c-Myc is more than 60 min, whereas the half-life of the unbound c-Myc is about 30 min (Fig. 11), suggesting that Bcl2/c-Myc association enhances stability of the c-Myc protein. Thus, the functional cooperation between Bcl2 and c-Myc may occur, at least in part, through their interaction to stabilize c-Myc protein and enhance the accumulation of c-Myc activity.

**DISCUSSION**

Both active and passive smoking have been implicated in lung cancer development (2). SCLC accounts for about 25% of human lung cancer and demonstrates a strong etiologic association with smoking (51). Cigarette smoking has also been reported to promote the development of non-SCLC (32, 52). NNK is the most potent carcinogenic agent contained in cigarette smoke and is formed by nitrosation of nicotine (6). Recent reports indicate that NNK is not only an important etiological factor in lung tumor development but also potently stimulates proliferation of SCLC cells (4, 32). Because Bcl2 and c-Myc are two major oncoproteins that cooperatively regulate cell survival and proliferation (17–18), NNK may functionally target these two oncoproteins to promote proliferation of lung cancer cells. To inhibit apoptotic potential and ensure the oncogenic and proliferative functions of c-Myc, cooperation with a survival gene like Bcl2 is required (17). Our results indicate that
NNK can induce phosphorylation of both Bcl2 and c-Myc in association with increased proliferation of human SCLC cells (Fig. 1). This suggests that NNK may facilitate a functional cooperation between Bcl2 and c-Myc in a mechanism involving phosphorylation of both regulators.

Deregulation of c-Myc occurs in a broad range of human tumors, including lung cancer (21). c-Myc not only promotes G₁ to S cell cycle progression in a mechanism involving activation of cyclin-E/CDK2 (53, 54) but also sensitizes cells to apoptosis (13, 18). To test whether the cooperation between Bcl2 and c-Myc is required for NNK-induced lung cancer cell proliferation, expression of c-Myc is specifically depleted by RNA interference. This maneuver significantly reduces NNK-stimulated proliferation of H69 cells (Fig. 2), indicating that c-Myc may be required for NNK-induced proliferation of lung cancer cells.

Growth factor (i.e., interleukin-3)-induced Bcl2 phosphorylation at Ser70 in the flexible loop domain has been reported to enhance the survival function of Bcl2 (19, 23). NNK not only induces Bcl2 phosphorylation at Ser70 to positively regulate cell survival but also simultaneously stimulates c-Myc phosphorylation at Thr58 and Ser62 to enhance the multiple functions of c-Myc including proliferation and apoptotic potential (Figs. 1 and 3; Refs. 14 and 55). Because Bcl2 is known to block c-Myc-mediated apoptosis in a cooperative mechanism (13, 18), NNK-induced phosphorylation of Bcl2 at Ser70 may specifically inhibit c-Myc-induced apoptosis to ensure the proliferative function of c-Myc. To test whether NNK-induced Bcl2 phosphorylation at Ser70 enhances the antiapoptotic function of Bcl2, H82 cells expressing WT, S70A, or S70E were treated with cisplatin. NNK was found to significantly prolong survival of cells expressing WT but not S70A Bcl2 following treatment with the chemotherapeutic agent, cisplatin (Fig. 3). This reveals that phosphorylation of Bcl2 at Ser70 is required for NNK-induced survival. Thus, Bcl2 phosphorylation at Ser70 may cooperatively inhibit the apoptotic effect of c-Myc to promote the oncogenic and proliferative functions of c-Myc.

NNK binds to and activates the α7 nAChR with a high affinity in SCLC (7, 32). Cellular membrane depolarization

**Fig. 7.** The α7 nAChR specific inhibitor α-BTX inhibits NNK-induced Bcl2 and c-Myc phosphorylation and reduces cell proliferation. A, NCI-H82 cells were treated with NNK (100 pM) in the absence or presence of various concentrations of α-BTX for 30 min. ERK1/2 phosphorylation was analyzed as in Fig. 5C. B, NCI-H82 cells expressing WT Bcl2 were metabolically labeled with [32P]orthophosphoric acid and treated with NNK (100 pM) in the absence or presence of various concentrations of α-BTX for 30 min. Bcl2 phosphorylation was analyzed as in Fig. 1B. C, NCI-H82 cells expressing WT Bcl2 were treated with NNK (100 pM) in the absence or presence of various concentrations of α-BTX for 30 min. Phosphorylation of c-Myc was assessed by Western blotting using a Thr58/Ser62 dual phosphospecific c-Myc antibody. D, NCI-H82 cells expressing WT Bcl2 were treated with NNK (100 pM) in the absence or presence of various concentrations of α-BTX for 72 h. Cell proliferation was assessed using WTS-1 kit as described under “Experimental Procedures.”

**Fig. 8.** Bcl2 co-localizes with c-Myc in nucleus and on mitochondrial membranes of SCLC cells. Subcellular fractionation was performed in NCI-H82 cells expressing WT 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 (27) or a nuclear marker (28), respectively, to verify the purity of each fraction.
resulting from the binding of NNK to the \( \alpha_7 \) nAChR causes the opening of voltage-gated ion channels, resulting in the influx of \( \text{Ca}^{2+} \) and activation of PKC that can trigger the Raf/MEK/ERKs protein kinase cascade (7, 32). Our results indicate that NNK potently activates PKC and ERK1/2, the physiological kinases for both Bcl2 and c-Myc, but has no effect on either p38 or JNK1 activity (Fig. 5). The potent PKC inhibitor, staurosporine, and the MEK specific inhibitor, PD98059, not only inhibit NNK-induced phosphorylation of both Bcl2 and c-Myc but also reduce NNK-induced cell proliferation in a dose-response manner (Fig. 6). These data suggest that NNK-induced Bcl2 and c-Myc phosphorylation occurs as the result of a signal transduction pathway involving the activation of PKC and/or the ERK1/2 kinases. Because inhibition of Bcl2 and c-Myc phosphorylation correlates with decreased cell proliferation (Fig. 6), this supports a functionally inextricable relationship between Bcl2/c-Myc phosphorylation and cell proliferation. Therefore, we propose that NNK-induced phosphorylation of both Bcl2 and c-Myc may facilitate the ability of Bcl2 to cooperate with c-Myc and enhance cell proliferation. This could also explain the role of NNK in tumorigenesis and tumor development, at least in those tumors expressing both Bcl2 and c-Myc.

Nicotinic acetylcholine receptors are cationic channels whose opening is controlled by acetylcholine and nicotinic receptor agonists. The NNK binds to and
activates α2 nAChR, which results in the influx of Ca2+ and triggers a PKC/RAF/MEK/ERK protein kinase cascade (7). The α2 nAChR-specific inhibitor α-BTX potently blocks NNK-induced activation of ERK1/2 as well as phosphorylation of Bcl2 and c-Myc in association with decreased cell proliferation (Fig. 7). Thus, NNK-induced cell proliferation may occur through activation of the α2 nAChR signal transduction pathway involving α2 nAChR/PKC/ERK/Bcl2/c-Myc in SCLC cells. These findings suggest that α-BTX may abolish Bcl2/c-Myc cooperation in a mechanism involving inhibition of their phosphorylation that dampens NNK-induced cell proliferation. Therefore, α-BTX may have potential clinical relevance in strategies designed to restrain cell proliferation through this novel mechanism in patients with lung cancer.

Previous reports indicate that Bcl2 can specifically block the apoptotic function of c-Myc while leaving its proliferative activity unaffected (18). Bcl2 suppression of c-Myc-induced apoptosis exposes multiple oncogenic properties of c-Myc and triggers carcinogenic progression (17, 55, 57). However, the molecular mechanism(s) by which Bcl2 may functionally synergize with c-Myc remains unclear. Our data reveal that Bcl2 and c-Myc co-localize in mitochondria as well as the nucleus and NNK potently enhances a direct interaction between Bcl2 and c-Myc in human SCLC cells (Figs. 8 and 9). This may uncover a novel mechanism by which NNK promotes a functional cooperation between these two oncoproteins in both the mitochondria and nucleus when they are both expressed. Because the phosphomimetic S70E mutant Bcl2 more efficiently associates with c-Myc than WT Bcl2, whereas the nonphosphorylatable S70A Bcl2 demonstrates little if any binding to c-Myc (Fig. 9), this indicates that Bcl2 phosphorylation at Ser70 is necessary for maximal association between Bcl2 and c-Myc. Importantly, the phosphomimetic S70E mutant Bcl2 directly binds to c-Myc in nucleus and on mitochondrial membranes (Fig. 10A). This association significantly enhances the half-life of the c-Myc protein (Fig. 11), which may enhance the accumulation of c-Myc activity during the initial stage of cell proliferation. Because Bcl2 is an outer mitochondrial membrane-integral protein (49–50), its N terminus may be localized to the cytoplasmic face of the outer mitochondrial membrane, and its C terminus, bearing a hydrophobic region (49), may be inserted in the outer mitochondrial membrane. Our findings indicate that both Bcl2 and c-Myc could be removed from the outer mitochondrial membrane after treatment with proteasine K (Fig. 10B), suggesting that Bcl2 may function as a mitochondrial “receptor” associating with c-Myc through its N terminus on the surface of the outer mitochondrial membrane. Recent studies have suggested that the MBIID domain (amino acids 106–143) of c-Myc is required for its apoptotic and cell cycle progression functions (35). It is possible that Bcl2 may associate with c-Myc at MBIID domain to specifically abolish the apoptotic potential of c-Myc. However, further studies will be required to test this hypothesis.

In summary, results reported here have identified a novel NNK-induced proliferation signal transduction pathway that depends on Bcl2 and c-Myc phosphorylation in SCLC cells (Fig. 12). Thus, in addition to DNA damage and p53 or Ras gene mutation, NNK also potently activates a PKC/Raf/MEK/ERK1/2 protein kinase cascade through the α2 nAChR, which triggers phosphorylation of Bcl2 and c-Myc. NNK-induced phosphorylation of Bcl2 at Ser70 facilitates a cooperative interaction between Bcl2 and c-Myc that stabilizes the c-Myc protein and enhances the accumulation of c-Myc activity leading to the promotion of lung cancer development and tumor cell growth.

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Tobacco-specific Nitrosamine 4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone Promotes Functional Cooperation of Bcl2 and c-Myc through Phosphorylation in Regulating Cell Survival and Proliferation

Zhaohui Jin, Fengqin Gao, Tammy Flagg and Xingming Deng

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