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Indole-3-carbinol (I3C) induced cell growth inhibition, G1 cell cycle arrest and apoptosis in prostate cancer cells

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Prostate cancer is one of the most common cancers in men and it is the second leading cause of cancer related death in men in the United States. Recent dietary and epidemiological studies have suggested the benefit of dietary intake of fruits and vegetables in lowering the incidence of prostate cancer. A diet rich in fruits and vegetables provides phytochemicals, particularly indole-3-carbinol (I3C), which may be responsible for the prevention of many types of cancer, including hormone-related cancers such as prostate. Studies to elucidate the role and the molecular mechanism(s) of action of I3C in prostate cancer, however, have not been conducted. In the current study, we investigated whether I3C had any effect against prostate cancer cells and, if so, attempts were made to identify the potential molecular mechanism(s) by which I3C elicits its biological effects on prostate cancer cells. Here we report for the first time that I3C inhibits the growth of PC-3 prostate cancer cells. Induction of G1 cell cycle arrest was also observed in PC-3 cells treated with I3C, which may be due to the observed effects of I3C in the up-regulation of $p21^{WAF1}$ and $p27^{Kip1}$ CDK inhibitors, followed by their association with cyclin D1 and E and down-regulation of CDK6 protein kinase levels and activity. The induction of $p21^{WAF1}$ appears to be transcriptionally upregulated and independent of the p53 responsive element. In addition, I3C inhibited the hyperpohosphorylation of the Retinoblastoma (Rb) protein in PC-3 cells. Induction of apoptosis was also observed in this cell line when treated with I3C, as measured by DNA laddering and poly (ADP-ribose) polymersae (PARP) cleavage. We also found an up-regulation of Bax, and down-regulation of Bcl-2 in I3C-treated cells. These effects may also be mediated by the down-regulation of NF-κB observed in I3C treated PC-3 cells. From these results, we conclude that I3C inhibits the growth of PC-3 prostate cancer cells by inducing G1 cell cycle arrest leading to apoptosis, and regulates the expression of apoptosis-related genes. These findings suggest that I3C may be an effective chemopreventive or therapeutic agent against prostate cancer. *Oncogene* (2001) **20,** 2927–2936.

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Introduction

Prostate cancer is one of the most common cancers in men and is the second leading cause of male cancer death in the United States (ACS, 2000). The mortality of prostate cancer has increased, with increased incidence in recent years (Krongrad *et al*., 1998). Recent studies involving detailed histological analysis of prostate glands from autopsy specimens have shown that foci of prostate cancer can be detected in much younger age groups without clinical manifestation, indicating that there is a long incubation period for the development of clinically manifested disease (Sakr, 1995). Moreover, this phenomenon also suggests that the majority of these cancer foci will remain indolent and will not manifest into clinically relevant disease, indicating the importance of the role of prostate cancer prevention apart from its treatment. Furthermore, since the disease-free survival of patients treated with radical prostatectomy has not been significantly improved despite many recent advances, further studies are warranted to improve the outcome of this disease. Recent dietary and epidemiological studies have suggested the benefit of dietary intake of fruits and vegetables, which provide phytochemicals, particularly indole-3-carbinol (I3C), that may play an important role in the prevention of many types of cancer, including hormone-related cancer (Dashwood, 1998; Kelloff *et al*., 1996; Safe, 1995; Steinmetz and Potter, 1996).

Indole-3-carbinol (I3C) is produced endogenously from naturally occurring glucosinolates contained in a wide variety of plant food substances including members of the family Cruciferae, and the genus Brassica, when they are crushed or cooked (Beier, 1990). I3C possesses anti-carcinogenic effects in experimental animals depending on the time of I3C exposure (Bailey *et al*., 1987; Dashwood, 1998; Dashwood *et al*., 1989; Grubbs *et al*., 1995). Exposure of I3C prior to carcinogenic insult had clearly shown to have protective effect (Guo *et al*., 1995; Xu *et al*., 1996), and that I3C has been shown to inhibit the growth of human cancer cells (Katdare *et al*., 1998; Tiwari *et al*., 1994).

Although there are some conflicting reports in the literature, such as the exposure of I3C during post-initiation phase shown to have carcinogenic effects (Dashwood *et al*., 1989; Kim *et al*., 1997), numerous reports favoring the anti-carcinogenic effects of I3C is overwhelming. Because of these findings, the interest in I3C, as a cancer chemopreventive agent, has increased significantly in the past several years (Bradfield and Bjeldanes, 1991; Broadbent and Broadbent, 1998). Although dietary and epidemiological studies have shown the benefit of consuming cruciferous vegetables in the prevention of cancer (Cohen *et al*., 2000; Kolonel *et al*., 2000), and most studies report inhibitory or protective effects of I3C *in vivo* (Dashwood, 1998), the molecular mechanism(s) by which I3C exerts its tumor suppressive effect on prostate cancer are unknown.

Eukaryotic cellular proliferation is regulated by expression and sequential activation of cell cycle dependent cyclins, cyclin dependent kinases (CDKs) and CDK inhibitors (CDKIs). The transition from G1 to S phase or commitment of cell proliferation is controlled by the activation of G1 CDKs and phosphorylation of Retinoblastoma (Rb) protein (Sherr, 1994). Cyclin molecules regulate the progression of each phase of cell cycle by associating with corresponding phase specific CDKs. Cyclin D isoforms associate with CDK4 and CDK6 and this association leads to activation of CDK4 and CDK6, which help maintaining and progressing through the early G1 phase of the cell cycle (Lukas *et al*., 1995a,b). Cyclin E and CDK2 proteins play a role in the transition from the G1 to S phase of the cell cycle (Ohtsubo *et al*., 1995). The G1 phase specific cyclin and CDK complexes, phosphorylates the Retinoblastoma protein and represses its inhibitory activity by associating with the E2F transcription factor, which mediates the expression of S phase specific genes (Sherr, 1994). The activity of CDKs is regulated by the cell cycle phase specific expression of cyclins and inhibited by cyclin dependent kinase inhibitors. $p21^{WAF1}$ and $p27^{Kip1}$ belong to the family of CDKIs (el-Deiry *et al*., 1993; Polyak *et al*., 1994b), whose expression regulates the G1 phase CDKs (Sherr and Roberts, 1995). The loss of cell cycle control in G1 has been implicated in tumor development and proliferation, and I3C has been shown to induce G1 cell cycle arrest in human breast cancer cells (Cover *et al*., 1998). However such studies have not been reported in prostate cancer. Our data showed that I3C is an effective agent in the induction of G1 cell cycle arrest in PC-3 prostate cancer cells, and that $p21^{WAF1}$, $p27^{Kip1}$ and CDK6 may play an important role in mediating the effects of I3C in prostate cancer cells.

The anti-tumor effect of I3C could be due to either cell growth inhibition, induction of apoptosis or both. Apoptosis is one of the important pathways through which chemopreventive and chemotherapeutic agents inhibit the growth of cancer cells. Thus, we investigated whether I3C may be involved in the induction of apoptosis apart from cell growth inhibition as suggested in the literature. The molecular analysis of cell growth inhibition and apoptosis may provide information delineating the molecular mechanisms by which I3C elicits its biological effects on prostate cancer (PC-3) cells. Our data showed that I3C is an effective agent in the induction of cell growth inhibition of PC-3 prostate cancer cells. Moreover, I3C induced apoptosis in PC-3 cells when treated for 24–72 h. The induction of cell growth inhibition was associated with the up-regulation of $p21^{WAF1}$ and $p27^{Kip1}$ and subsequent association with Cyclin D1 and E. G1 cell cycle arrest was associated with the down-regulation of CDK6 protein kinase levels and activity and Rb phosphorylation. Finally, I3C induction of programmed cell death was associated with up-regulation of Bax and down-regulation of Bcl-2 and inhibition of NF-κB activity.

Results

I3C induces inhibition of cell proliferation

To test the effects of I3C on cell growth, we treated PC-3 cells with 0.1% DMSO as a control, or 30, 60 and 100 μM I3C dissolved in DMSO (final concentration 0.1%) for 24–72 h. In Figure 1, we show I3C's dose-dependent inhibition of cell proliferation. Inhibition of cell proliferation could be the result of the induction of apoptosis or cell cycle growth arrest. We hypothesized that I3C's inhibition of cell proliferation was due to alterations in cell cycle control and programmed cell death.

I3C-induced G1 cell cycle arrest in PC-3 cells is mediated by alterations in G1 cell cycle proteins

Previous studies have shown that I3C induces G1 cell cycle arrest in breast cancer cells (Cover *et al*., 1998),

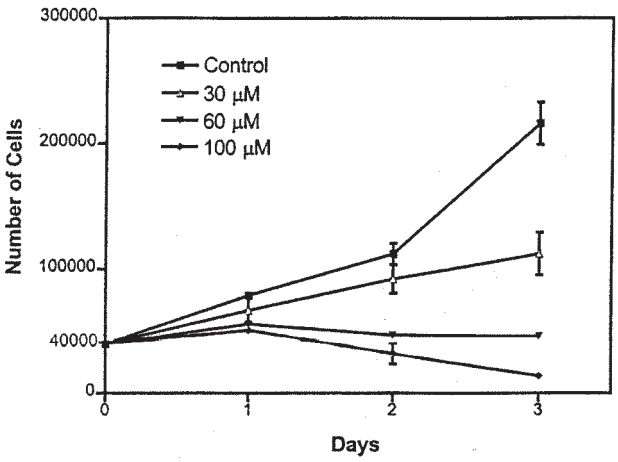

Figure 1 Cell growth inhibition by I3C. PC3 cells treated with 30, 60 and 100 μM I3C, and control cells with 0.1% DMSO were harvested by trypsinization and cell numbers were determined. The number of living cells was plotted versus the days of I3C exposure

hence we investigated whether G1 cell cycle regulatory molecules are altered in I3C treated PC-3 cells. The expression of $p21^{WAF1}$ and $p27^{Kip1}$ in PC-3 cells was investigated in I3C-treated and untreated cells by Western blot analysis. We demonstrated a significant induction of both CDKI protein levels with as low as 30 μM I3C treatment for 48 h (Figure 2a). In order to obtain a quantitative value for the protein expression of $p21^{WAF1}$ and $p27^{Kip1}$, we measured the relative optical density of the CDKI and actin bands (see Materials and methods). The ratios of $p21^{WAF1}$ and $p27^{Kip1}$ to β-actin protein expression revealed that cells treated with 100 μM I3C for 72 h showed at least a 10–20-fold increase in both CDKIs compared to the untreated control (Figure 2b). The induction of both $p21^{WAF1}$ and $p27^{Kip1}$ protein expression were generally associated with the inhibition of cell growth.

I3C-induced $p21^{WAF1}$ is independent of p53 status

To further identify the proximal promoter elements regulating the I3C induced expression of $p21^{WAF1}$ gene, three different $p21^{WAF1}$ promoter constructs fused with luciferase gene were transfected to PC-3 cells and luciferase activity was measured in cell extracts prepared from I3C treated or untreated control cells (Figure 3). The $p21^{WAF1}$ promoter construct devoid of the p53 responsive element (construct-2) induced $p21^{WAF1}$ expression comparable with full-length construct (construct-1) indicating that the I3C induced $p21^{WAF1}$ expression is independent of p53 function. The third construct devoid of one SP1 element but retains majority of GC rich region, reduced I3C induced $p21^{WAF1}$ promoter activity by 30% compared to full length construct (construct-1), indicating that SP1 elements and upstream GC rich region are important in I3C-mediated induction of $p21^{WAF1}$ gene expression. These data indicate that in PC-3 cells, I3C can circumvent the loss of p53 wild type function and induce $p21^{WAF1}$ expression through activation of SP1 components involved in the proximal promoter activity of the $p21^{WAF1}$ gene.

I3C-mediated alteration in cyclin D1 and E complexes in PC-3 cells

To further elucidate the role of CDK inhibitors, we investigated the association of CDKIs with cyclin D and E in PC-3 cells treated with 60 and 100 μM of I3C for 48 h (Figure 4). Both CDKIs were recruited into cyclin D and E complexes which, in turn, lead to the inhibition of cyclin associated kinase activities.

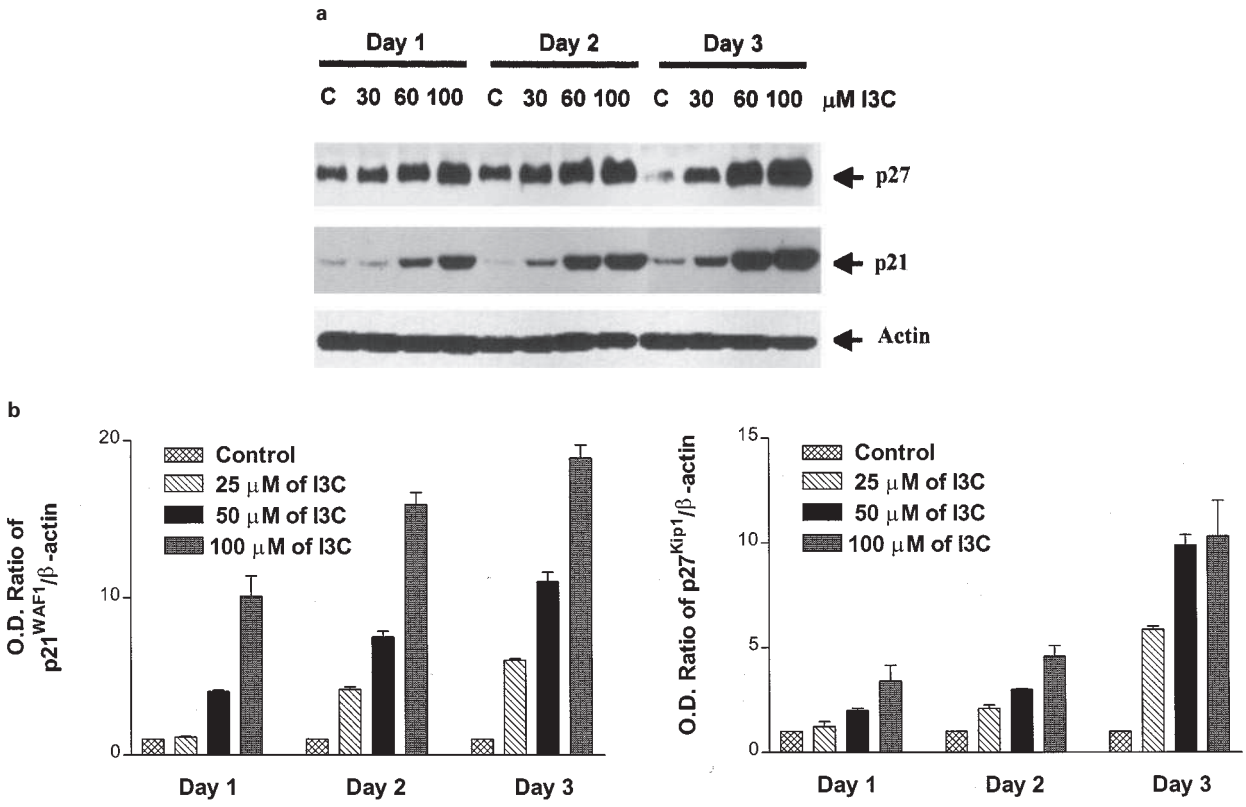

Figure 2 I3C induces expression of $p21^{WAF1}$ and $p27^{Kip1}$ in PC-3 cells. PC-3 cells were treated with either DMSO alone or with 30, 60 and 100 μM I3C for 24, 48 and 72 h. Cell lysates were prepared and Western blot analysis was performed with indicated antibodies. (**a**) Western blot analysis of PC-3 cell lysates for $p21^{WAF1}$, $p27^{Kip1}$ and β-actin protein levels (data from a representative experiment is shown from a total of three independent experiments). (**b**) Densitometric quantification (means $\pm$ s.e.m of three independent experiments)

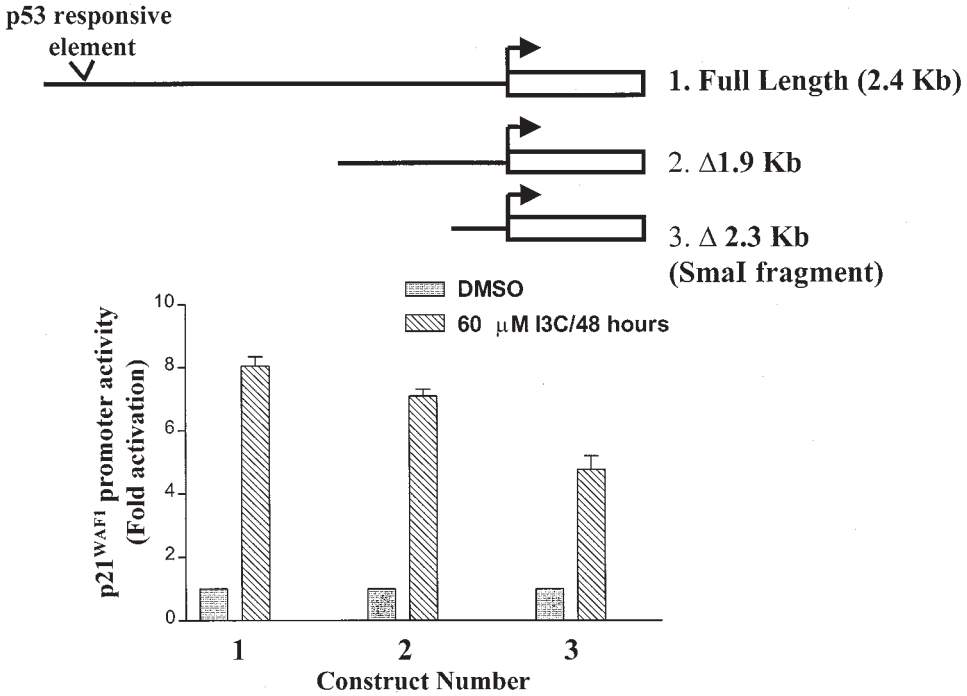

Figure 3 I3C induces $p21^{WAF1}$ gene expression independent of p53 responsive element and partly dependent on GC rich SP1 binding elements. PC-3 cells transfected with indicated $p21^{WAF1}$ promoter constructs and cells were untreated or treated with 60 μM of I3C for 48 h. Cell lysates were assayed for luciferase activity. The values are means $\pm$ s.e.m of three independent experiments

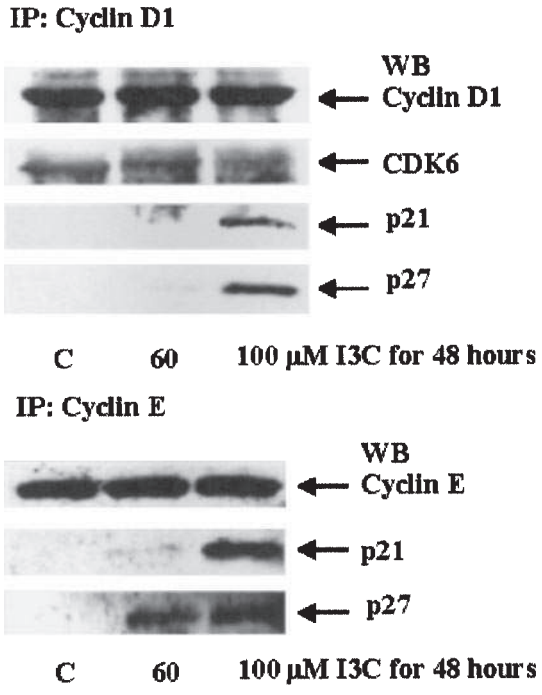

Figure 4 I3C induces association of $p21^{WAF1}$ and $p27^{Kip1}$ with cyclin D1 and E. Sixty μM and 100 μM I3C treated or DMSO treated PC-3 cell lysates were immunoprecipitated with anti-cyclin D1 or cyclin E antibodies and Western immunoblotted with corresponding antibody shown in the panel. Autoradiographs of a representative experiment from three independent experiments are shown

Furthermore, we investigated the protein expression level of CDK6 (Figure 5a), which interacts with D type cylcins and facilitates the progression of cells through G1 cell cycle phase. I3C treated PC-3 cells showed a dose dependent decrease in the total levels of CDK6 protein and at 100 μM concentration a time dependent decrease was observed. This decrease was observed at higher concentrations starting from 24 h treatment of the cells, and the expression was significantly reduced at 100 μM concentrations at 72 h (Figure 5b). We also investigated the CDK6 kinase activity in PC-3 cells treated with 60 and 100 μM concentrations of I3C by immunoprecipitation followed by kinase assay using GST-Rb protein as a substrate. The CDK6 kinase activity was decreased up to 70% of control upon treatment with 100 μM I3C for 48 h (Figure 6). The decrease in protein expression closely correlated with the concomitant decrease in the kinase activity of CDK6 in I3C treated PC-3 cells.

Collectively, these results indicate a novel mechanism of I3C-induced G1 cell cycle arrest in PC-3 cells. We further sought to investigate whether additional molecular mechanism(s) exists by which I3C inhibits G1 cell cycle arrest and studied the phosphorylation status of Retinoblastoma (Rb) protein levels, which is a central molecule in G1 cell cycle control. During the progression of G1 phase and entering into S phase, the Rb protein is hyperphosphorylated by early and late G1 phase specific CDKs and releases E2F transcription factors which, in turn, activate S phase specific genes

and help progress cells enter into S phase. I3C treatment showed progressive inhibition of Rb phosphorylation, and that this effect was more pronounced at 60–100 μM concentration within 48 h of treatment (Figure 7). In addition to cell cycle arrest, the growth inhibition induced by I3C treatment could also be due to programmed cell death, hence, we investigated whether I3C induces apoptosis.

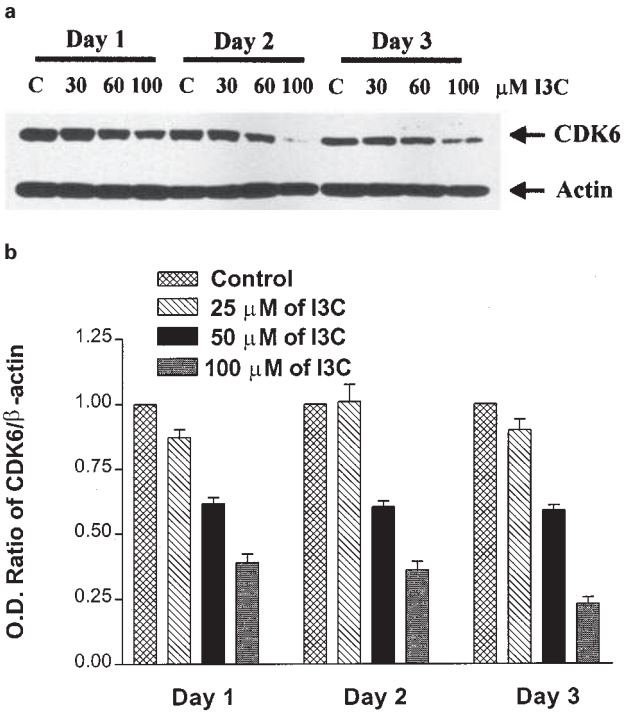

Figure 5 I3C down-regulates CDK6 protein levels. PC-3 cells treated or untreated with indicated concentrations of I3C for 24, 48 and 72 h, and the cell lysates were subjected to Western immunoblot analysis with anti CDK6 antibody. (**a**) Western blot analysis of PC-3 cell lysates for CDK6 and β-actin protein levels (data from a representative experiment is shown from a total of three independent experiments). (**b**) Densitometric quantification (means $\pm$ s.e.m of three independent experiments)

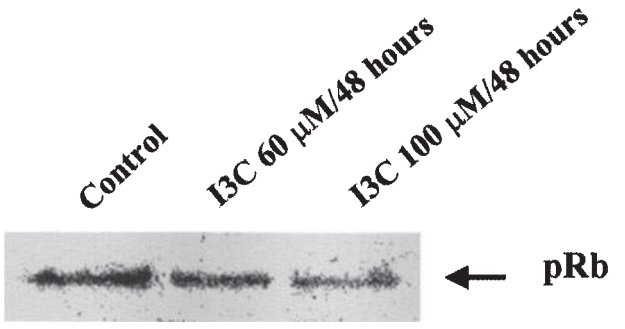

Figure 6 I3C inhibits CDK6 activity in PC-3 cells. CDK6 protein was immunoprecipitated with anti-CDK6 antibody from 60 and 100 μM I3C treated or untreated PC-3 cells and *in vitro* kinase reaction was performed using recombinant GST-Rb as substrate. A representative autoradiograph of [γ-^{32}P] incorporation into GST-Rb is shown, indicating kinase activity

I3C induces apoptosis in PC-3 cells

Apoptosis was observed in PC-3 cells treated with 100 μM of I3C as measured by the DNA ladder (Figure 8a). The induction of apoptosis was pronounced at 48 h of treatment, and appears to be correlated with cell growth inhibition. In addition, PARP cleavage analysis showed that the full size PARP (116 KD) protein was cleaved to yield an 85 kDa fragment after treatment with I3C for 48 h (Figure 8b) in PC-3 cells providing further evidence for the induction of apoptosis in I3C treated PC-3 cells. These two independent methods of measuring apoptosis provided strong evidence that apoptosis was induced in PC-3 cells treated with I3C. In order to explore the mechanism(s) by which I3C induces apoptosis, we investigated the alterations in the expression of selected genes that are involved in the complex processes of apoptosis.

Bax and Bcl-2 protein expression was altered in I3C treated PC-3 cells

The constitutive levels of Bax or Bcl-2 and the time course for the effect of I3C on Bax or Bcl-2 expression in PC-3 cells were studied by Western blot analysis.

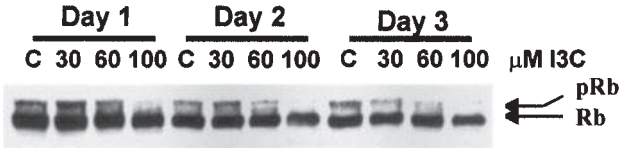

Figure 7 I3C inhibits hyperphosphorylation of Retinoblastoma protein. PC-3 cells were treated with DMSO alone or with 30, 60 and 100 μM I3C for 24, 48 and 72 h. Cell lysates were prepared and Western immunoblotted with anti-Rb antibody. A representative autoradiograph from three independent experiments is shown

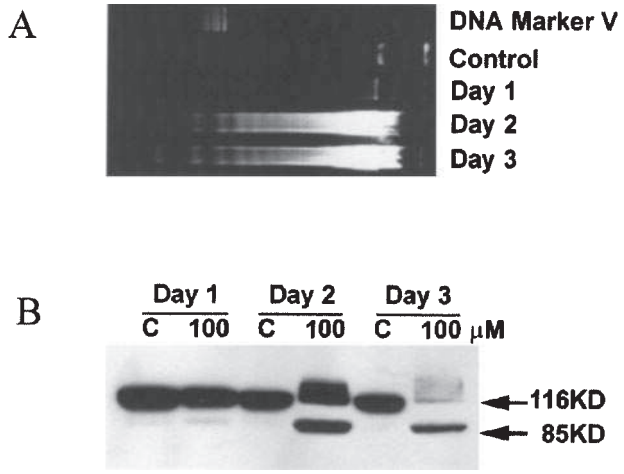

Figure 8 Activation of apoptosis by I3C in PC-3 cells. (**a**) DNA ladder formation in PC-3 cells treated with 100 μM I3C for 24, 48 and 72 h. (**b**) Western blot analysis of PARP cleavage in PC-3 cells treated with 100 μM I3C for 24, 48 and 72 h

The levels of Bcl-2 expression in PC-3 cells were down-regulated in I3C treated cells exposed for 24–72 h (Figure 9a). In contrast, the expression of Bax was up-regulated after 24 and 48 h with 60 and 100 μM of I3C treatment (Figure 9a). Optical density measurement was also conducted to obtain a quantitative value for the protein expression of Bax and Bcl-2. The ratios of Bcl-2 to β-actin protein expression showed at least an 80% decrease after 72 h of I3C treatment (Figure 9b). The ratios of Bax to β-actin revealed that cells treated with 100 μM of I3C showed a 1.9-fold increase in Bax at 24 h of treatment, which may contribute to the induction of apoptotic processes. In order to further delineate the molecular mechanism by which I3C elicits its effects on prostate cancer cells, we focused our investigation on a central transcription factor, NF-κB which is known to play important roles in cell growth and apoptotic processes.

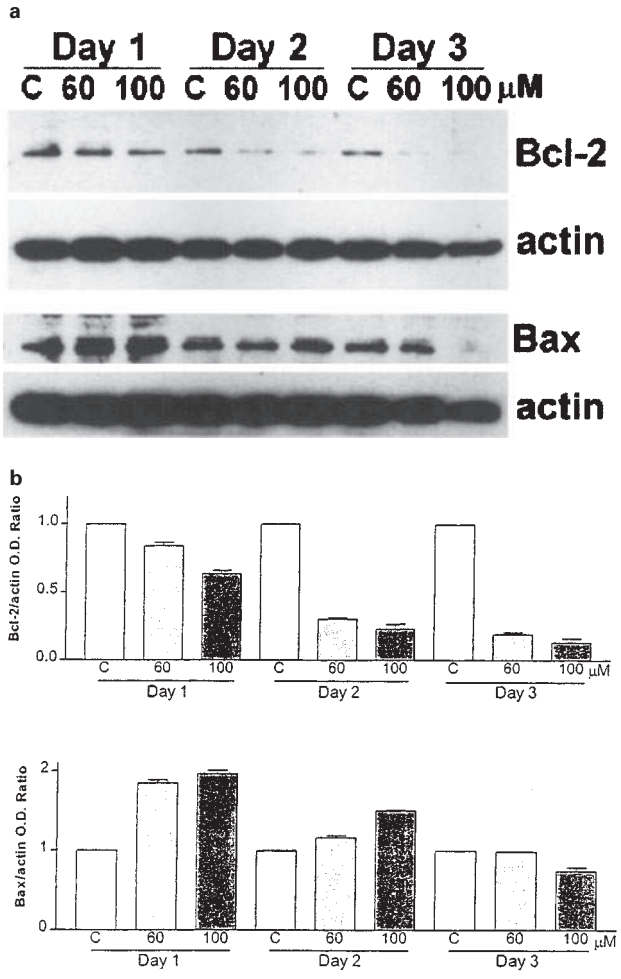

Figure 9 I3C induces apoptosis in PC-3 cells by down-regulation of Bcl-2 and up-regulation of Bax. PC-3 cells were treated with DMSO alone or 60 and 100 μM of I3C for 24, 48 and 72 h. Cell lysates were prepared and Western immunoblotted with indicated antibodies. (**a**) Western blot analysis of PC-3 cell lysates for Bcl-2, Bax and β-actin protein levels (data from a representative experiment is shown from a total of three independent experiments). (**b**) Densitometric quantification (means $\pm$ s.e.m of three independent experiments)

I3C inhibits NF-κB activation in PC-3 cells

We investigated the effect of I3C on NF-κB activation in PC-3 cells. Several lines of evidence suggests the role of NF-κB for protection against apoptosis (Van Antwerp *et al*., 1996; Wang *et al*., 1996), and a chemokine TNF-α and a reactive oxygen species H_2O_2 shown to induce the NF-κB transcription factor activity. Nuclear extracts were prepared from PC-3 cells treated with I3C for 48 h or TNF-α stimulation alone. NF-κB transcription activity was significantly decreased in cells treated with I3C (Figure 10). Furthermore, TNF-α stimulated NF-κB transcription factor activity in PC-3 cells. The observed gel-shift band could be super-shifted with p65 or p50 specific antibodies in addition to the inhibition of the complex formation in the presence of unlabeled NF-κB specific oligonucleotides but not by mutant oligonucleotides (data not shown). The above results indicate that the induction of apoptosis induced by I3C may be potentially mediated by the down-regulation of NF-κB transcription factor activity which, in turn, induces both cell growth inhibition and induction of apoptotic processes. However, further in-depth investigation is warranted in order to delineate the signaling pathway by which NF-κB is down-regulated in I3C treated PC-3 cells.

Discussion

Indole-3-carbinol (I3C), a naturally occurring component of Brassica vegetables such as cabbage, broccoli, and Brussels sprouts, has been shown to reduce the incidence of spontaneous and carcinogen-induced mammary and endometrial tumors in rats, and to inhibit the growth of human mammary cancer cells *in*

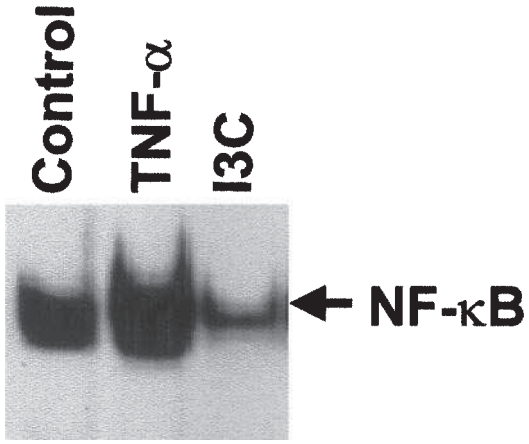

Figure 10 I3C inhibits NF-κB binding activity in PC-3 cells. PC-3 cells treated with DMSO alone or with 60 μM of I3C for 48 h $\pm$ TNF-α treatment were analysed for NF-κB transcription factor activity by gel shift assay

vitro (Cover *et al*., 1998; Grubbs *et al*., 1995; Huber *et al*., 1997; Kojima *et al*., 1994; Niwa *et al*., 1994). Treatment of cultured human MCF-7 breast cancer cells with I3C inhibited the growth of these cells (Cover *et al*., 1998). The data from *in vivo* studies suggest that I3C, at a minimum effective dose schedule of 300 mg per day, is a promising chemopreventive agent for breast cancer prevention (Michnovicz *et al*., 1997; Telang *et al*., 1997). In our study, we found that I3C also inhibited the growth of PC-3 prostate cancer cells in a time and dose dependent manner at a concentration that is similar to those reported earlier (Cover *et al*., 1998), suggesting that I3C induced cell growth inhibition and apoptosis are new and novel observation in the PC-3 model system. The inhibition of cell growth observed in I3C-treated cells may be due to induction of G1 cell cycle arrest as a result of multiple gene activities. We found that two important CDKIs, $p21^{WAF1}$ and $p27^{Kip1}$, were up-regulated in PC-3 cells treated with I3C. The $p21^{WAF1}$ has been known to play a major role in regulating early and late cell cycle phase specific cyclin dependent kinases. Its over-expression and association with CDKs is implicated in the induction of blockade at a specific stage of the cell cycle. PC-3 cells harbor a mutation in the p53 gene and the majority of $p21^{WAF1}$ gene expression appears to be independent of p53 regulation (Figure 3). Similar p53 independent regulation of $p21^{WAF1}$ expression has been described earlier (Biggs *et al*., 1996; Datto *et al*., 1995a; Liu *et al*., 1996a). The $p21^{WAF1}$ gene expression in cultured cells is induced by diverse agents, including growth factors (Gartel *et al*., 1996; Liu *et al*., 1996a; Missero *et al*., 1995; Parker *et al*., 1995), TGF-β (Datto *et al*., 1995b), phorbol esters (Biggs and Kraft, 1999), retinoids (Liu *et al*., 1996a) and Vitamin D3 (Liu *et al*., 1996b), indicating convergence of diverse signaling pathways at the level of transcription. P53 independent transcriptional up-regulation of $p21^{WAF1}$ by TGF-β is mediated by several GC rich regions in the $p21^{WAF1}$ promoter region, which spans between -62 to 124 bp relative to the transcription start site contains SP1 transcription factor binding sites (Datto *et al*., 1995b). Our results also provide evidence that deletion construct lacking part of this SP1 element (construct-3) loses approximately 30% of I3C inducibility of $p21^{WAF1}$ in PC-3 cells and retains majority of I3C induced $p21^{WAF1}$ expression (Figure 3), indicating that I3C regulates some unknown signaling mechanism culminating in transcriptional activation of SP1, leading to the up-regulation of the $p21^{WAF1}$ gene. The $p27^{Kip1}$ is another member of CDKI, which could bind and inhibit a broader range of CDKs. The expression of $p27^{Kip1}$ has been shown to be up-regulated during TGF-β mediated antiproliferative response (Polyak *et al*., 1994a; Slingerland *et al*., 1994), serum starvation or density arrested cells (Nourse *et al*., 1994). Cells failing to progress to mitosis are, thus, destined to apoptosis by I3C treatment. Therefore, the up-regulation of $p21^{WAF1}$ and $p27^{Kip1}$ may be an important molecular mechanism through which I3C inhibits cancer cell growth and induces apoptosis.

The growth inhibitory effect of I3C could also be directly related to its ability to down-regulate CDK6, which is one of the critical molecules required for early progression of the G1 cell cycle (Cover *et al*., 1998). Alterations in the expression levels of CDK6 have been implicated in the transformation processes during tumorigenesis. In addition, it has been shown to be amplified in human gliomas (Costello *et al*., 1997), and its activity was also found to be elevated in certain human squamous cell carcinoma cell lines (Timmermann *et al*., 1997). The expression of CDK6 has been shown to be inhibited in two breast cancer cells exposed to I3C in a dose dependent manner, and it was found to be independent of ER status of the cell lines, indicating that CDK6 down-regulation is a specific effect of I3C. Similarly CDK2 gene expression has been shown to be down regulated by retinoic acid in MCF-7 cancer cells (Teixeira and Pratt, 1997). A recent study of cloning and analysing CDK6 promoter revealed that I3C responsiveness was largely confined to the 300 bp fragment upstream of transcription start site (Cram *et al*., 2000). This fragment contains a NF-κB consensus-binding site, indicating that I3C mediated down-regulation of CDK6 could be attributed to the function of NF-κB activity. In our study, we found that I3C down-regulates basal activity of NF-κB in PC-3 cells (Figure 10), suggesting that the down-regulation of NF-κB induced by I3C could be important in the subsequent down-regulation of CDK6. We postulate that I3C mediated down-regulation of NF-κB activity could lead to the inhibition of cell growth regulation and induction of the apoptotic processes, and that these effects are mediated by selective alterations in G1 cell cycle regulatory proteins and the induction of apoptotic processes. However, a clear association between NF-κB activity and CDK6 gene expression require further in-depth investigation to establish molecular pathways by which CDK6 promoter activity is modulated. These investigations could be focused by promoter deletion studies and by investigating the effect of I3C on dominant negative mutant of IκB expressing cells, which will inhibit NF-κB translocation into the nucleus and, in turn, may down-regulate CDK6 activity.

In our study, we found that I3C inhibits the growth of PC-3 prostate cancer cells. The inhibition of cell growth observed in I3C treated cells may also be due to apoptotic cell death in addition to cell cycle arrest. Thus, we employed two methods to detect apoptosis in our system. The nucleosomal DNA ladder has been widely used as a biochemical marker of apoptosis for several years (Huang *et al*., 1997). In addition, the cleavage of PARP has also been used as an early marker of apoptosis (Darmon *et al*., 1995). Using these various techniques, we found that there was an induction of apoptosis in PC-3 cells treated with I3C. DNA ladder formation and PARP cleavage were also observed in I3C-treated prostate cancer cells. Collectively, these results clearly suggest that I3C inhibits the growth of prostate cancer cells and induces apoptosis, and that these results corroborate the findings of many

published studies in other cancer cell lines (Ge *et al*., 1996; Katdare *et al*., 1998; Wong *et al*., 1997; Cover *et al*., 1998).

Bcl-2 and Bax play a major role in determining whether cells will undergo apoptosis under experimental conditions that promote cell death (Salomons *et al*., 1997; Sedlak *et al*., 1995). Bcl-2 protects cells from apoptosis (Sedlak *et al*., 1995), while increased expression of Bax can induce apoptosis (Salomons *et al*., 1997). The ratio of Bax:Bcl-2, rather than Bcl-2 alone, is important for the survival of drug-induced apoptosis in leukemia cell lines (Sedlak *et al*., 1995). In our study, a decrease in Bcl-2 expression was observed in PC-3 prostate cancer cells after treatment with I3C. The expression of Bax, however, was up-regulated in PC-3 prostate cancer cells after treatment for 24 and 48 h, hence, the ratio of Bax to Bcl-2 was altered in favor of apoptosis. Our results suggest that up-regulation of Bax and down-regulation of Bcl-2 may be another molecular mechanisms through which I3C induces apoptosis. However, further studies are needed to firmly establish the role of Bcl-2, Bax and $p21^{WAF1}$ in I3C induced apoptosis in prostate cancer cells. Furthermore, the association between up-regulation of $p21^{WAF1}$, $p27^{Kip1}$ and Bax, and down-regulation of Bcl-2, CDK6 kinase activity and Rb dephosphorylation with down regulation of NF-κB in I3C treated PC-3 cells is not fully understood and require additional studies.

In conclusion, the results of our studies provide experimental evidence, for the first time, that I3C inhibits cell growth of PC-3 prostate cancer cells and induces apoptosis with alterations in Bcl-2, Bax, $p21^{WAF1}$, $p27^{Kip1}$ and CDK6 protein expression. The nature of I3C in mediating the above mentioned responses in PC-3 cells, in conjunction with its non-toxic nature, could make it a potentially effective chemopreventive or therapeutic agent against prostate cancer. However, further *in vivo* studies are needed to establish the role of I3C as a chemopreventive or therapeutic agent against prostate cancer.

Materials and methods

Cell culture and growth inhibition studies

The human prostate cancer cell line, PC-3, was obtained from ATCC and cultured in RPMI 1640 medium (Gibco, Rockville, MD, USA), supplemented with 10% FBS, 1% penicillin/streptomycin in a 5% CO_2 atmosphere at 37°C. The cells were seeded at a density of 4×10^5 cells well in a six well culture dish. After 24 h, the cells were treated with 30, 60 and 100 μM I3C dissolved in DMSO (final concentration 0.1%) and control cells were treated with 0.1% DMSO. Cells treated with I3C or DMSO for 24, 48 and 72 h were harvested by trypsinization, stained with 0.4% Trypan blue and counted using a hemocytometer.

Protein extraction and Western blot analysis

The prostate cancer cells were plated and cultured in complete medium and allowed to attach for 24 h followed by the addition of 30, 60 and 100 μM I3C. Incubation was carried out for 24, 48 and 72 h. Control cells were incubated in the medium with 0.1% DMSO for the same time period. After the indicated incubation period, the cells were harvested by scraping the cells from culture dishes and collected by centrifugation. Cells were re-suspended in Tris-HCl (pH 6.8) buffer, sonicated for two times for 10 s and lysed using an equal volume of 4% SDS. Protein concentration was then measured using protein assay reagents (Pierce, Rockford, IL, USA). Cell extracts were boiled for 10 min and chilled on ice (for 5 min), subjected to 10% SDS–PAGE (unless otherwise indicated), and electrophoretically transferred to a nitrocellulose membrane. The following primary antibodies were used: $p21^{WAF1}$ (1 : 2000, Upstate, Lake Placid, NY, USA), $p27^{Kip1}$ (1 : 250, Novocastra Laboratories, Newcastle-upon-Tyne, UK), CDK6 (1 : 1000, Santa Cruz Biotechnology, Santa Cruz, CA, USA), Rb (Neomarker, Fremont, CA, USA), Bcl-2 (1 : 250, Oncogene, Cambridge, MA, USA), Bax (1 : 500, Oncogene, Cambridge, MA, USA), β-actin (1 : 2000, Sigma, St. Louis, MO, USA). Each membrane was incubated with primary antibodies, washed with TTBS (20 mM Tris pH 7.6, 150 mM NaCl and 0.05% Tween 20), incubated with secondary antibodies conjugated with peroxidase, and visualized by a chemiluminescent detection system (Pierce, Rockford, IL, USA).

Autoradiograms of different exposures of the Western blots were scanned with the Gel Doc 1000 image scanner (Bio-Rad, Hercules, CA, USA) and band intensities were quantified and analysed with the Molecular Analyst software program (Bio-Rad, Hercules, CA, USA).

Immunoprecipitation of cyclin D1 and E complexes

1×10^6 PC-3 cells were lysed in NP-40 lysis buffer containing 50 mM Tris (pH 8.0), 0.5% NP-40, 0.5% sodium deoxycholate, 150 mM NaCl, 5 μg/ml Pepstatin, 5 μg/ml leupeptine, 5 μg/ml aprotinin, 1 mM PMSF, 1 mM benzamidine, 100 mM sodium floride, 2 mM sodium orthovanadate and 10 mM sodium pyrophosphate. Protein concentration was determined using Protein A assay kit from Pierce (Rockford, IL, USA) and equal amount of proteins from I3C treated and untreated cell lysates were incubated with either cyclin D1 or cyclin E (Santa Cruz Biotechnology, Santa Cruz, CA, USA) primary antibody for overnight at 4°C, followed by the addition of Protein-A agarose and incubating at 4°C for 1 h. Agarose beads were washed for five times with NP-40 lysis buffer and re-suspended in 2 × SDS sample buffer and subjected to Western-immunoblot analysis with appropriate antibody.

CDK6 kinase assay

For CDK6 kinase assay, immunoprecipitated samples using CDK6 antibodies were washed twice with NP-40 lysis buffer and re-suspended in kinase buffer containing 50 mM HEPES (pH 8.0), 10 mM $MgCl_2$, 2.5 mM EGTA, 1 mM DTT, 20 μM ATP, 2 μg of GST-Rb, 10 μCi [γ-^{32}P]ATP and supplemented with protease and phosphatase inhibitors. The kinase assay was performed by incubating complexes at 30°C for 15 min. The reaction was terminated by the addition of 2 × SDS sample buffer and analysed on SDS–PAGE followed by autoradiography.

Analysis of cleavage of PARP

Cells treated with 100 μM I3C or 0.1% DMSO for 48 h were lysed in lysis buffer (10 mM Tris-HCl, pH 7.1, 50 mM sodium

chloride, 30 mM sodium pyrophosphate, 50 mM sodium fluoride, 2 mM sodium orthovanadate, 2 mM iodoacetic acid, 5 mM $ZnCl_2$, 1 mM phenylmethylsulfonyl fluoride and 0.5% Triton X-100). The lysates were kept on ice for 1 h and vigorously vortexed before centrifugation at 12 500 *g* for 20 min. Fifty μg of total proteins were resolved on 10% SDS–PAGE and then transferred to membrane. The membrane was incubated with primary monoclonal anti-human PARP antibody (1 : 5000, Biomol Research, Plymouth, PA, USA), washed with TTBS and incubated with secondary antibody conjugated with peroxidase. The signal was then detected using the chemiluminescent detection system (Pierce, Rockford, IL, USA).

DNA ladder formation

Nuclear DNA from cells treated with 100 μM I3C for 24, 48 and 72 h or with 0.1% DMSO for 48 h, was extracted using 10 mM Tris (pH 8.0), 1 mM EDTA and 0.2% Triton X-100. The lysate was centrifuged for 15 min at 13 000 *g* to separate the fragment DNA (soluble) from intact chromatin (nuclear pellet). The supernatant from the lysate was treated with RNase, followed by SDS-Proteinase K digestion and isopropanol precipitation. DNA was separated through a 1.5% agarose gel. After electrophoresis, gels were stained with ethidium bromide and the DNA was visualized under UV light.

Transient transfection of cells with $p21^{WAF1}$ promoter reporter plasmids

Transfections were done using Fugene 6 (Boehringer Mannheim, Indianapolis, IN, USA) reagent. The following three different $p21^{WAF1}$ promoter constructs were used (gift from Dr Wang, Duke University Medical Center) for transfecting PC-3 cells. Construct-1 is 2.4 kb genomic fragment of $p21^{WAF1}$ promoter cloned into pGL2-basic (Promega, Madison, WI, USA) luciferase vector, construct-2 (Δ1.9) is lacking 1.9 kb fragment at 5′ region and the construct-3 (*Sma*I) contains a $p21^{WAF1}$ promoter from -113 to transcription start site (Datto *et al*., 1995b). PC-3 cells were grown in 35 mm plates at a density of 5×10^5 cells per dish. The following day, cells were washed with PBS and overlaid with serum free RPMI media. Three μl of Fugene 6, 1 μg of DNA and 100 μl of RPMI medium were incubated at room temperature for 20 min and overlaid on cells, and the plates were transferred to incubator containing 5% CO_2 atmosphere at 37°C. The cells were allowed to transfect with lipid DNA complexes for 4–5 h and washed with RPMI media, and transferred to incubator. The following day, cells were treated with indicated concentrations of I3C or DMSO.

For luciferase assay cells were lysed in reporter lysis buffer (Promega, Madison, WI, USA) and lysates were assayed for luciferase activity using luminometer. Lysates were also assayed for β-galactosidase activity to normalize for transfection efficiency using cells co-transfected with a control vector.

Preparation of nuclear extracts and gel mobility shift assay/ electrophoretic mobility shift assay (GMSA/EMSA)

PC-3 cells were plated at a density of 1×10^6 cells in 100 mm dishes and on the following day treated with 60 μM I3C for 48 h and stimulated with or without 20 ng/ml of TNF-α for 10 min. The cells were scrapped and cell pellet was resuspended in 0.5 ml of 10 mM Tris (pH 7.5), 5 mM $MgCl_2$, 0.05% Triton X-100 and lysed with 20 strokes in a 1 ml Dounce homogenizer. Nuclear proteins were extracted from cell pellet with the addition of two pellet volumes of 10 mM Tris (pH 7.5), 5 mM $MgCl_2$ and 500 mM NaCl and by incubating on ice for 30 min followed by centrifugation and collecting the supernatant fraction. The protein concentrations of each sample were estimated using BCA protein assay reagent (Pierce, Rockford, IL, USA).

NK-κB consensus oligo nucleotide (Promega, Madison, WI, USA) was end labeled with T4 polynucleotide kinase according to manufacturers instructions (GIBCO–BRL, Rockville, MD, USA). For gel mobility shift assay, 5 μg of nuclear extracts were incubated in gel shift binding buffer containing 4% glycerol, 1 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM dithiothreitol, 50 mM NaCl, 10 mM Tris-HCl (pH 7.5) with 0.25 mg/ml poly(dI):poly(dC) for 10 min at room temperature followed by the addition of 20 000 c.p.m. of ^{32}P-labeled NF-κB oligonucleotide to the reaction mixture and incubated for an additional 10 min at room temperature. The binding reaction was terminated with the addition of 1 μl of 10× sample loading dye (250 mM Tris-HCl, pH 7.4, 40% glycerol, 0.2% bromophenol blue). The samples were loaded on a prerun 8% polyacrylamide gel and electrophoresed at 30 mA constant current for 45 min. The gel was dried, exposed to X-ray film for overnight at -70°C, and then developed.

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