Previously, we showed that sulforaphane (SFN), a naturally occurring cancer chemopreventive agent, effectively inhibits proliferation of PC-3 human prostate cancer cells by causing caspase-9- and caspase-8-mediated apoptosis. Here, we demonstrate that SFN treatment causes an irreversible arrest in the G2/M phase of the cell cycle. Cell cycle arrest induced by SFN was associated with a significant decrease in protein levels of cyclin B1, cell division cycle (Cdc) 25B, and Cdc25C, leading to accumulation of Tyr-15-phosphorylated (inactive) cyclin-dependent kinase 1. The SFN-induced decline in Cdc25C protein level was blocked in the presence of proteasome inhibitor lactacystin, but lactacystin did not confer protection against cell cycle arrest. Interestingly, SFN treatment also resulted in a rapid and sustained phosphorylation of Cdc25C at Ser-216, leading to its translocation from the nucleus to the cytoplasm because of increased binding with 14-3-3β. Increased Ser-216 phosphorylation of Cdc25C upon treatment with SFN was the result of activation of checkpoint kinase 2 (Chk2), which was associated with Ser-1981 phosphorylation of ataxia telangiectasia-mutated, generation of reactive oxygen species, and Ser-139 phosphorylation of histone H2A.X, a sensitive marker for the presence of DNA double-strand breaks. Transient transfection of PC-3 cells with Chk2-specific small interfering RNA duplexes significantly attenuated SFN-induced G2/M arrest. HCT116 human colon cancer-derived Chk22− cells were significantly more resistant to G2/M arrest by SFN compared with the wild type HCT116 cells. These findings indicate that Chk2-mediated phosphorylation of Cdc25C plays a major role in irreversible G2/M arrest by SFN. Activation of Chk2 in response to DNA damage is well documented, but the present study is the first published report to link Chk2 activation to cell cycle arrest by an isothiocyanate.

Epidemiological studies have revealed an inverse correlation between the dietary intake of cruciferous vegetables and the risk for certain types of cancers, including prostate cancer (1–5). Laboratory studies indicate that the anticancer effect of cruciferous vegetables is caused by isothiocyanates that exist as thioglucoside conjugates (glucosinolates) in a variety of edible plants including broccoli, cabbage, watercress, and so forth (6–9). Cruciferous vegetable-derived organic isothiocyanates are generated by hydrolytic cleavage of corresponding glucosinolates through catalytic mediation of myrosinases, which are released when the plant cells are damaged because of cutting or chewing (6–9). Sulforaphane (SFN) is one such isothiocyanate analog that has received a great deal of attention not only because it is present in high concentrations in certain varieties of broccoli but also because of its potent anticancer activity (10–15). For example, oral administration of SFN (1-isothiocyanato-4-(methylsulfinyl)butane; CH3-SO-(CH2)4-N=C=S) caused a statistically significant reduction in 9,10-dimethyl-1,2-benzanthracene-induced mammary tumor incidence and multiplicity in rats (11). SFN was shown to offer impressive and prolonged protection of human retinal pigment epithelial cells, keratinocytes, and mouse leukemia cells against oxidative damage (13). The antioxidative effect of SFN was also observed in aortic smooth muscle cells from spontaneously hypertensive rats (14). Significantly, SFN exhibited bacterial activity against clinical isolates and antibiotic-resistant strains of Helicobacter pylori and inhibited benz[a]pyrene-induced forestomach cancer in mice (15). Moreover, SFN was effective in eradicating H. pylori in human gastric xenografts implanted in nude mice (16). The mutagenicity of food-derived heterocyclic amines was inhibited by a SFN analog in the Ames Salmonella/reversion assay (17). SFN as well as its N-acetyl-cysteine conjugate administered during the postinitiation period effectively reduced azoxymethane-induced colonic aberrant crypt foci formation in rats (18). Modulation of carcinogen metabolism resulting from inhibition of cytochrome P450-dependent monooxygenases and/or induction of phase II detoxification enzymes, such as glutathione transferases, is believed to be responsible for activity of SFN against chemically induced cancers (19–23).

Evidence is accumulating to indicate that SFN can inhibit proliferation of cancer cells in culture by causing apoptosis...
and/or cell cycle arrest (24–28). The growth-suppressive effect of SFN has been observed against HT29 and LS-174 human colon cancer cells (24, 25), PC-3 and LNCaP human prostate cancer cells (26, 28), and Jurkat T-leukemia cells (27). Apoptosis induction by SFN in Jurkat T-leukemia cells (27) and HT29 human colon cancer cells (24) correlated with overexpression of Bax and/or down-regulation of Bcl-2. Recent studies from our laboratory have indicated involvement of both caspase-8 and caspase-9 pathways in apoptosis induction by SFN in PC-3 cells (28). In addition, we showed that the growth of a PC-3 xenograft tumor is inhibited significantly upon oral administration of SFN at a concentration that may be generated through dietary intake of cruciferous vegetables (28). Although considerable progress has been made toward our understanding of the mechanism of SFN-induced apoptosis, the sequence of events leading to cell cycle arrest in SFN-treated cells is poorly defined. For example, previous studies have shown that SFN-treated HT29 colon cancer cells and Jurkat T-leukemia cells are arrested in the G2/M phase (24, 27), which may be of importance in the anticarcinogenic effect of SFN, but no attempts were made to define the mechanism of the cell cycle arrest.

In the present study, we demonstrate that SFN treatment causes an irreversible G2/M phase cell cycle arrest in PC-3 cells which is associated with a marked decrease in the expression of key G2/M-regulating proteins, including cyclin B1, cell division cycle 25B (Cdc25B) and Cdc25C. In addition, we provide evidence to indicate that cell cycle arrest in SFN-treated PC-3 cells is caused by the generation of reactive oxygen species (ROS) and ataxia telangiectasia-mutated (ATM)/checkpoint kinase 2 (Chk2)-mediated phosphorylation of Cdc25C at Ser-216. Phosphorylation of Cdc25C in SFN-treated cells leads to its retention in the cytosol through increased binding with 14-3-3. Phosphorylation of Cdc25C in SFN-treated cells leads to its retention in the cytosol through increased binding with 14-3-3. Phosphorylation of Cdc25C in SFN-treated cells leads to its retention in the cytosol through increased binding with 14-3-3. Activation of Chk2 in SFN-induced G2/M Arrest
with 0.5 μg of GST-Cdc25C in the presence of 50 μCi of [γ-32P]ATP and 10 μM cold ATP. Reaction mixtures were incubated at 30 °C for 25 min, and the reaction was terminated by the addition of an equal volume of sample buffer. The proteins were resolved by SDS-PAGE, transferred onto Immobilon-P membrane, and the radiolabeled proteins were visualized by autoradiography. After autoradiography, the membrane was probed with anti-Chk2 antibody to confirm equal protein loading. To determine whether SFN interacts with Chk2 to affect its kinase activity, 5 or 20 μM SFN was directly added to the kinase reaction mixture containing Chk2 immunoprecipitated from control or ionizing radiation-exposed cells.

siRNA Transfection—RNA interference of Chk2 was performed using 21-bp (including a 2-deoxynucleotide overhang) siRNA duplexes purchased from Dharmacon (Lafayette, CO). The coding strand for Chk2 siRNA was GAAACUGAGGACCAAGAACCAdTdT. For transfection, PC-3 cells were seeded in 6-well plates and transfected at 30% confluency with 200 μM siRNA duplexes using OligofectAMINE (Invitrogen) according to the manufacturer’s recommendations. Cells treated with OligofectAMINE (mock) or transfected with a control nonspecific siRNA duplex VIII (Dharmacon; ACUCUAUCUGCACGUGACUU) were used as controls for direct comparison. After 24 h of transfection, cells were treated with MeSO or SFN (20 μM) for 24 h. Both floating and adherent cells were collected, washed with PBS, and processed for analysis of cell cycle distribution or immunoblotting.

Measurement of ROS—Generation of intracellular ROS was examined by flow cytometry using H$_2$DCFDA (Molecular Probes). Briefly, PC-3 cells (0.5 × 10$^6$) were plated in T25 flasks and allowed to attach overnight. The cells were first exposed to 20 μM SFN for 0, 30 min, 1 h, or 3 h (37 °C) and then treated with 5 μM H$_2$DCFDA for 30 min at 37 °C. Subsequently, the cells were collected by trypsinization, washed twice with PBS, and analyzed for dichlorodihydrofluorescein fluorescence using a Coulter Epics XL Flow Cytometer.

Immunohistochemistry for Histone H2A.X Phosphorylation—PC-3 cells (10$^5$) were grown on coverslips and allowed to attach overnight. Cells were then exposed to MeSO (control) or 20 μM SFN for 1 and 2 h or 20 μM etoposide for 2 h (positive control) at 37 °C. Cells were then washed with PBS and fixed with 2% paraformaldehyde overnight at 4 °C. Subsequently, the cells were permeabilized with 0.1% Triton X-100 for 15 min at room temperature, washed with PBS, and incubated with normal goat serum (1:20 in PBS containing 0.5% (w/v) bovine serum albumin and 0.15% (w/v) glycine (BSA buffer)) for 45 min at room temperature. After washing with BSA buffer, cells were treated with antibodies against phospho-H2A.X (Ser-139) (2 μg/ml) for 1 h at room temperature and counterstained with 10 ng/ml DAPI. Slides were mounted and examined under a fluorescence microscope at ×40 (objective lens) magnification.

RESULTS

SFN Treatment Caused Irreversible G$_{2}$/M Phase Arrest in PC-3 Cells—We showed previously that SFN exhibits highly significant activity against proliferation of PC-3 cells in a concentration-dependent manner (28). In the present study, we used this cell line as a model to determine whether the inhibition of cell proliferation is caused by perturbations in the cell cycle progression. The effect of SFN on cell cycle distribution was determined using a flow cytometer after staining of the cells with propidium iodide, and the data are shown in Fig. 1. A 24-h exposure of PC-3 cells to 20 μM SFN resulted in a statistically significant increase in G$_{2}$/M phase cells which was accompanied by a decrease in G$_{0}$/G$_{1}$ phase cells (Fig. 1). At a 40 μM SFN concentration, the G$_{2}$/M phase arrest was abolished, but a significant increase in cells with sub-G$_{0}$/G$_{1}$ DNA content was evident, indicating apoptosis induction. The sub-G$_{0}$/G$_{1}$ fraction was minimal in MeSO-treated controls as well as in cells treated with 20 μM SFN (Fig. 1).

Cell cycle checkpoints are activated to ensure orderly and timely completion of critical events such as DNA replication and chromosome segregation. Activation of checkpoints in response to DNA damage, inhibition of DNA replication, or disruption of the mitotic spindles leads to cell cycle arrest to allow time for repair of damage; but in the case of severe damage, cell cycle arrest leads to apoptosis. To determine whether the cell cycle arrest induced by SFN was reversible, cells were first exposed to MeSO (control) or 20 μM SFN for 16 h and then either processed for analysis of cell cycle distribution (Fig. 2, A and B) or washed and cultured in drug-free fresh complete medium for an additional 24 h prior to cell cycle distribution analysis (Fig. 2, C and D). Consistent with the results shown in Fig. 1, SFN treatment caused a significant increase in the fraction of cells with G$_{2}$/M DNA content which was accompanied by a decrease in G$_{0}$/G$_{1}$ phase cells (compare A and B in Fig. 2). Cell cycle distribution was not altered when MeSO-treated cells were cultured in fresh complete medium for an additional 24 h (compare A and C in Fig. 2). Interestingly, culture of SFN-treated cells (16-h exposure to 20 μM SFN) in drug-free medium for 24 h resulted in a significant increase in sub-G$_{0}$/G$_{1}$ apoptotic cells (Fig. 2D). The fraction of cells with sub-G$_{0}$/G$_{1}$ DNA content was minimal in cells treated with MeSO or SFN for 16 h or in MeSO-treated cells that were cultured subsequently in fresh complete medium for 24 h. The increase in sub-G$_{0}$/G$_{1}$ fraction in SFN-treated cells cultured in drug-free medium was not accompanied by a decrease in G$_{2}$/M phase cells; instead it was associated with a decline in G$_{0}$/G$_{1}$ phase cells (Fig. 2D). Taken together, these results indicated that SFN-induced G$_{2}$/M cell cycle arrest was irreversible and not a secondary effect.

Effect of SFN on Levels of Proteins That Regulate G$_{2}$/M Transition—Eukaryotic cell cycle progression is regulated by sequential activation of Cdk5, whose activity is dependent upon their association with regulatory cyclins (32–34). A complex between cyclin-dependent kinase 1 (Cdk1; also known as p34cdc2) and cyclin B1 is important for entry into mitosis in most organisms (32–34). Although phosphorylation of Cdk1 at Thr-161 is essential for full activation of Cdk1-cyclin B1 kinase complex, reversible phosphorylations at Thr-14 and Tyr-15 of Cdk1 suppress its kinase activity (33, 34). Dephosphorylation
of Thr-14 and Tyr-15 of Cdk1, and hence activation of the Cdk1-cyclin B1 complex, is catalyzed by dual specificity phosphatases Cdc25B and Cdc25C, and this reaction is believed to be a rate-limiting step for entry into mitosis (32, 33, 35). To gain insights into the mechanism of cell cycle arrest upon treatment with SFN, levels of cyclin B1, Cdk1, Cdc25B, and Cdc25C proteins were compared by immunoblotting using lysates from control and SFN-treated cells, and representative blots are shown in Fig. 3A. In comparison with control, the level of cyclin B1 was reduced by about 56 and 90% in SFN-treated cells at the 4 and 16 h time points, respectively. Interestingly, the SFN-induced decline in cyclin B1 protein level was partially blocked at the 24 h time point (about 67% reduction compared with the control), indicating a biphasic response, which was observed in two independent experiments. Although the protein level of Cdk1 was not significantly altered by SFN treatment, the level of Cdc25C protein was reduced by about 41, 75, and 83% in cells treated with SFN for 4, 16, and 24 h, respectively, compared with control cells. A marked decrease in Cdc25B protein level (>60% reduction compared with control) was also evident in SFN-treated cells at the 16 and 24 h time points (Fig. 3A). These results indicated that the SFN-induced G2/M phase cell cycle arrest in PC-3 cells was associated with a marked decline in the protein levels of cyclin B1, Cdc25B, and Cdc25C, but not Cdk1.

SFN Treatment Enhanced Phosphorylation of Cdk1 (Tyr-15) and Cdc25C (Ser-216)—Because Cdc25B and Cdc25C, whose levels were reduced markedly in SFN-treated cells, play critical roles in dephosphorylation of Cdk1 (35), we hypothesized that SFN treatment might lead to accumulation of Tyr-15-phosphorylated (inactive) Cdk1. We examined this possibility by immunoblotting using an antibody specific for phospho-Cdk1 (Tyr-15). As shown in Fig. 3B, Tyr-15 phosphorylation of Cdk1 was increased by >200% in SFN-treated cells at the 16 and 24 h time points compared with the control.

The function of Cdc25C is negatively regulated by phosphorylation at Ser-216, which creates a binding site for 14-3-3 (36, 37), we examined the effect of SFN on Ser-216 phosphorylation of Cdc25C. As can be seen in Fig. 3B, the level of Ser-216-phosphorylated Cdc25C was significantly higher (between 150 and 300% increase over control) in SFN-treated cells compared with control. Increased Ser-216 phosphorylation of Cdc25C over control was evident as early as 1 h after SFN treatment and persisted for the duration of the experiment (24 h post-treatment; Fig. 3B).

Next, we determined whether SFN-induced decline in Cdc25C protein level (Fig. 3A) involved the ubiquitin-proteasome system because arsenic-induced G2/M phase cell cycle arrest was shown to be the result of ubiquitin/proteasome-mediated degradation of Cdc25C (38). We addressed this question by determining the effect of lactacystin, a specific inhibitor of proteasome, on the SFN-induced decline in Cdc25C protein as well as on cell cycle arrest. The decline in Cdc25C protein level upon treatment with SFN was nearly completely blocked in the presence of lactacystin (Fig. 3C). The blot was probed with anti-ubiquitin antibody to determine whether Cdc25C was ubiquitinated. Indeed, high molecular weight polyubiquitin conjugates were evident in the lane containing lysate from cells treated with SFN and lactacystin but not in control lysate (Fig. 3C). To our surprise, however, lactacystin treatment did not protect against SFN-induced G2/M arrest (Fig. 3D). These results indicated that, in our model, the Cdc25C protein level per se did not influence cell cycle arrest caused by SFN.

SFN Promoted Translocation of Cdc25C from the Nucleus to the Cytoplasm—Because Ser-216 phosphorylation of Cdc25C creates a binding site for 14-3-3 (36, 37), we examined the effect of SFN on the binding of Cdc25C with 14-3-3. The lysate proteins from control and SFN-treated cells (20 μM for 4 or 24 h) were immunoprecipitated using anti-14-3-3 antibody, and the immune complex was analyzed for the presence of Cdc25C by immunoblotting. As can be seen in Fig. 4A, SFN treatment resulted in increased binding of Cdc25C with 14-3-3β at the 4 and 24 h time points. These results suggested that SFN treatment might lead to translocation of Cdc25C from the nucleus to the cytoplasm because of increased binding with 14-3-3β. We examined this possibility by immunohistochemistry, and the data are shown in Fig. 4B. Cells were treated with Me2SO (control) or 20 μM SFN for 4 or 24 h and then stained with anti-Cdc25C antibody (red) or nucleic acid binding dye SYTOX® Green (green). In Me2SO-treated control cells, Cdc25C was localized in the cytoplasm (red staining surrounding SYTOX® Green-stained nuclei) as well as in the nucleus (brown-black staining in nucleus). In contrast, the nuclei of the cells treated with SFN for 24 h were brightly stained with SYTOX® Green, indicating translocation of Cdc25C from the nucleus to the cytoplasm. In agreement with the results of immunoblotting indicating a decline in Cdc25C protein level in SFN-treated cells (Fig. 3A), a marked decrease in Cdc25C immunostaining (red fluorescence around SYTOX® Green-stained nuclei) was observed in SFN-treated cells (Fig. 4B). SFN-induced decline in the Cdc25C protein level was more pronounced at 24 h than at the 4 h time point after treatment.

Cytoplasmic accumulation of Cdc25C upon treatment with
SFN was confirmed by biochemical fractionation of cytoplasmic and nuclear fractions from control (Me$_2$SO-treated) and SFN-treated (20 µM for 4 h) cells followed by immunoblotting using anti-Cdc25C antibody, and the results are shown in Fig. 4C. A 4 h time point was selected to minimize influence of SFN-induced decline in Cdc25C protein level. In Me$_2$SO-treated control, the intensity of Cdc25C immunoreactive band was significantly higher in the lane corresponding to nuclear fraction than in the cytoplasmic fraction. Treatment of cells with SFN resulted in a decrease in nuclear Cdc25C signal intensity with a concomitant increase in cytoplasmic Cdc25C signal intensity (Fig. 4C). The blot was stripped and reprobed with anti-α-tubulin (middle panel) and anti-α-Ran (bottom panel) antibodies to normalize for equal protein loading as well as to rule out cross-contamination of the nuclear and cytoplasmic fractions.

SFN Treatment Increased Thr-68 Phosphorylation of Checkpoint Kinase 2 (Chk2)—Several kinases including Chk1 and...
Chk2 have been implicated in Ser-216 phosphorylation of Cdc25C (36, 39–41). Chk1 and Chk2 are intermediaries of DNA damage checkpoints and activated by phosphorylation on Ser-345/Ser-317 and Thr-68, respectively (42–46). We therefore examined whether SFN treatment affects phosphorylation of Chk1 or Chk2. Representative immunoblots for total and phospho-Chk2 (Thr-68) showed increased Thr-68 phosphorylation of Chk2 over control which was evident as early as 1 h after SFN treatment and persisted for the duration of the experiment (Fig. 5A). The level of Chk2 protein was not affected by SFN treatment. SFN treatment did not affect either the Chk1 protein level or its phosphorylation (data not shown). The kinase activity of Chk2 was determined in the lysates prepared from control (MeSO4-treatment) and SFN-treated cells (20 μM for 4 h) using GST-Cdc25C as a substrate. As can be seen in Fig. 5B, the Chk2 kinase activity was significantly higher in SFN-treated cells than in control cells. ATM is an upstream kinase implicated in phosphorylation and hence activation of Chk2 (46). Immunoblotting using an antibody specific for phospho-ATM (Ser-1981) showed increased Thr-68 phosphorylation of Chk2 was evident for up to 24 h after SFN removal.

To determine whether activation of Chk2 persisted even after drug removal, the cells were first treated with MeSO4 or 20 μM SFN for 4 h, washed, and cultured in drug-free fresh complete medium for 0, 2, 8, or 24 h prior to harvesting. Lysates were prepared and subjected to immunoblotting using phospho-Chk2 (Thr-68) antibody. The blot was stripped and reprobed with anti-actin antibody to ensure equal protein loading. F, effect of SFN on phosphorylation of Chk2 (Thr-68), Chk1 (Ser-345), Cdc25C (Ser-216), and Cdk1 (Tyr-15) in 293 cells. SFN was then added to the kinase reaction mixture containing immunoprecipitated Chk2, and the incubation was carried out for 25 min. Chk2 kinase activity was determined using GST-Cdc25C as a substrate. The membrane was probed with anti-Chk2 antibody to ensure equal protein loading. G, effect of SFN on Chk1 and Chk2. Representative immunoblots for total and phospho-Chk1 and Chk2 protein to affect its kinase activity, we exposed 293 cells to 10-Gy ionizing radiation (to activate Chk2) prior to preparation of lysates and immunoprecipitation of Chk2 protein. 5 or 20 μM SFN was then added directly to the kinase reaction mixture containing immunoprecipitated Chk2, and the incubation was carried out for 25 min. Chk2 kinase activity was determined using GST-Cdc25C as a substrate and reprobed with anti-Chk2 antibody to ensure equal protein loading. G, effect of SFN on Chk1 and Chk2. Representative immunoblots for total and phospho-Chk1 and Chk2 protein to affect its kinase activity, we exposed 293 cells to 10-Gy ionizing radiation (to activate Chk2) prior to preparation of lysates and immunoprecipitation of Chk2 protein. 5 or 20 μM SFN was then added directly to the kinase reaction mixture containing immunoprecipitated Chk2, and the kinase activity was determined using GST-Cdc25C as a substrate after a 25-min incubation in the presence of SFN. As shown in Fig. 5D, the addition of SFN to the kinase reaction mixture had no appreciable effect on Chk2 kinase activity.

SFN-induced Phosphorylation of Chk2 Is Not Unique to PC-3 Cells—To rule out a possibility that activation of Chk2 in SFN-treated PC-3 cells was a cell line-specific effect, we examined 293 cells for SFN-induced Chk2 activation. Similar to the
results obtained in PC-3 cells, SFN caused activation of Chk2 at the 1, 16, and 24 h time points (Fig. 5F). Additionally, SFN-induced phosphorylation of Cdc25C (Ser-216) and accumulation of Tyr-15-phosphorylated Cdk1 was also observed in 293 cells. Although IR and UV light induced phosphorylation of Chk1, SFN failed to activate Chk1 (Fig. 5F). These results clearly indicated that SFN-induced activation of Chk2 was not unique to the PC-3 cell line.

siRNA-mediated Down-regulation of Chk2 Partially Blocked SFN-induced G2/M Arrest—To experimentally verify the role of Chk2 in SFN-induced cell cycle arrest, we used siRNA technology to suppress Chk2 protein expression. As can be seen in Fig. 6A, transfection with a siRNA targeted to Chk2 suppressed Chk2 protein expression by >70% compared with the mock control. The expression of Chk2 was not affected in cells transfected with a nonspecific control siRNA (Fig. 6A). Chk2 siRNA-transfected cells and control cells (mock and control siRNA-transfected cells) were then treated with SFN, and their cell cycle distribution was assessed after 24 h (Fig. 6B). Treatment of control siRNA-transfected cells with SFN (20 μM for 24 h) resulted in a 2.2-fold increase in G2/M phase cells (Fig. 6B). A similar effect of SFN on cell cycle distribution was observed in mock-transfected cells. The SFN-induced G2/M block was partially but statistically significantly attenuated in cells transfected with Chk2 siRNA (Fig. 6B).

The effect of Chk2 down-regulation on SFN-induced phosphorylation of Chk2 and Cdc25C was also examined, and the results are shown in Fig. 7. The SFN-induced Thr-68 phosphorylation of Chk2 was relatively more pronounced in the mock control (about a 4-fold increase over Me2SO control) than in Chk2 siRNA-transfected cells (about a 1.9-fold increase over Me2SO control; Fig. 7, top panel). Likewise, SFN-induced Ser-216 phosphorylation of Cdc25C was relatively more pronounced in mock-transfected cells (about a 4-fold increase) than in the cells transfected with Chk2 siRNA (about 2-fold). Consistent with the results in Fig. 3, treatment of mock-transfected cells with SFN resulted in about a 90% decrease in the Cdc25C protein level. In contrast, a decrease of only about 50% in the Cdc25C protein level was observed when Chk2 siRNA-transfected cells were treated with SFN (Fig. 7, bottom panel).

Two important conclusions can be drawn from data in Fig. 7. First, it is clear that down-regulation of Chk2 protein level reduces SFN-induced phosphorylation of both Chk2 and Cdc25C. Furthermore, inhibition of Chk2 and Cdc25C phosphorylation seems to stabilize Cdc25C protein, suggesting that Ser-216 phosphorylation of Cdc25C may regulate its degradation.

Immunoblotting for phospho-Cdc25C (Fig. 7, middle panel) revealed the presence of additional bands with reduced electrophoretic mobility in the lane containing lysate protein from SFN-treated cells but not control cells. The phospho-Cdc25C immunoreactive bands with reduced electrophoretic mobility were not observed if the lysate was treated with λ-protein phosphatase prior to immunoblotting (data not shown). These results indicated that the slower migrating bands were phosphorylated forms of Cdc25C.

HCT116-derived Chk2−/− Cells Were Significantly More Resistant to SFN-induced G2/M Arrest than Wild Type Cells—The role of Chk2 in cell cycle arrest by SFN was investigated further using HCT116-derived Chk2−/− and Chk2+/+ human colon cancer cells. As can be seen in Fig. 8A, the G2/M blockade induced by SFN was relatively more pronounced in Chk2+/+ HCT116 cells than in the HCT116-derived Chk2−/− cells. Consistent with the results in PC-3 and 293 cells, SFN treatment (20 μM for 24 h) caused phosphorylation of Chk2 (Thr-68) and accumulation of Tyr-15-phosphorylated Cdk1 in Chk2+/+ cells (Fig. 8B). These observations provided additional support to the conclusion that Chk2 plays an important role in SFN-induced cell cycle arrest. Because SFN caused a significant increase in fraction of cells with G2/M DNA content in both Chk2+/+ and Chk2−/− HCT116 cells (Fig. 8A), compensatory Chk2-independent mechanisms are likely to contribute to the cell cycle arrest in Chk2−/− HCT116 cells.
 Activation of Chk2 in SFN-induced G2/M Arrest

The data shown in Fig. 7 suggested that inhibition of SFN-induced Cdc25C phosphorylation by siRNA-based knockdown of Chk2 could stabilize Cdc25C protein. We explored this possibility further by determining the effect of SFN on level and Ser-216 phosphorylation of Cdc25C in Chk2−/− and Chk2+/+ cells; and between SFN-treated Chk2−/− and Chk2+/+ cells by one-way analysis of variance (p < 0.05) followed by Bonferroni’s Multiple Comparison Test. B, effect of SFN on phosphorylation of Chk2 (Thr-68) and Cdk1 (Tyr-15) in Chk2−/− and Chk2+/+ cells. Lysates from control (Me2SO-treated) and SFN-treated Chk2−/− and Chk2+/+ cells (solid bars) cells were treated with Me2SO (control) or 20 μM SFN for 48 h prior to analysis of cell cycle distribution. Data are the mean ± S.E. (n = 3). Asterisks (*) indicate that the fraction of G2/M phase cells was statistically significantly different between control and SFN treatment groups for both Chk2−/− and Chk2+/+ cells, and between SFN-treated Chk2−/− and Chk2+/+ cells by one-way analysis of variance (p < 0.05) followed by Bonferroni’s Multiple Comparison Test. B, effect of SFN on phosphorylation of Chk2 (Thr-68) and Cdk1 (Tyr-15) in Chk2−/− and Chk2+/+ cells. Lysates from control (Me2SO-treated) and SFN-treated Chk2−/− and Chk2+/+ cells (solid bars) were subjected to immunoblotting using antibodies specific for phospho-Chk2 (top panel) or phospho-Cdk1 (middle panel). The blot was probed with anti-Cdk1 antibody (bottom panel) to confirm equal protein loading. Lane 1, lysate from Me2SO-treated Chk2−/− cells; lane 2, lysate from SFN-treated Chk2−/− cells; lane 3, lysate from SFN-treated Chk2−/− cells; and lane 4, lysate from SFN-treated Chk2−/− cells. C, effect of SFN on Ser-216 phosphorylation and protein level of Cdc25C in Chk2−/− and Chk2+/+ cells. Lysates from control (Me2SO-treated) and SFN-treated Chk2−/− and Chk2+/+ cells (solid bars) were subjected to immunoblotting using antibodies against phospho-Cdc25C (Ser-216) and total Cdc25C. The blots were probed with anti-actin antibody to ensure equal protein loading. Lane 1, lysate from Chk2−/− cells; lane 2, lysate from SFN-treated Chk2−/− cells; lane 3, lysate from SFN-treated Chk2−/− cells; and lane 4, lysate from SFN-treated Chk2−/− cells.

DISCUSSION

We have shown previously that SFN effectively inhibits proliferation of PC-3 human prostate cancer cells by causing caspase-8- and caspase-9-mediated apoptosis and that the growth of PC-3 xenografts in nude mice is retarded significantly upon oral administration of SFN (28). Moreover, inhibition of PC-3 xenograft growth was observed at a dose of SFN which can be generated through dietary intake of cruciferous vegetables. These results prompted us to examine further the mechanism by which SFN inhibits proliferation of cancer cells. Data presented herein indicate that SFN-treated PC-3 cells are arrested irreversibly in G2/M phase of the cell cycle. Cell cycle arrest in SFN-treated cells was accompanied by a marked decline in the levels of cyclin B1, Cdc25B, and Cdc25C. It is reasonable to postulate that SFN treatment may affect activity of Cdk1/cyclin B1 kinase not only by reducing complex formation because of a reduction in the level of cyclin B1 protein but also by causing accumulation of Thr-14/Tyr-15 phosphorylated (inactive) Cdk1 because of a decline in the level of Cdc25B and Cdc25C proteins. Indeed, Western blotting using anti-phospho-Cdk1 antibody revealed a significant increase in the level of Tyr-15-phosphorylated Cdk1 in SFN-treated cells. The SFN-induced decline in the Cdc25C protein level in PC-3 cells was nearly fully blocked in the presence of proteasome inhibitor lactacystin. Interestingly, lactacystin-mediated restoration of Cdc25C protein level did not significantly affect SFN-induced cell cycle arrest. These results suggested that in our model, the level of Cdc25C protein does not influence G2/M arrest by SFN.

Cell cycle arrest in the presence of SFN has been demonstrated previously in other cellular models, but the results are inconsistent. For example, a net increase in the percentage of G2/M phase cells upon treatment with SFN was observed in HT29 human colon cancer cells as well as in Jurkat T-leukemia...
The mechanism of G₂/M arrest was not investigated thoroughly in any of the above studies, but an increase in the level of cyclin A and cyclin B protein was reported in SFN-treated HT29 cells (24). In contrast, Chiao et al. (26) showed that treatment of LNCaP human prostate cancer cells with 3-50 μM SFN resulted in an enrichment of G₁ phase cells. Although the reasons for this discrepancy are not yet clear, the inconsistency could be caused by differences in the genetic background of the cells. For example, PC-3 cells do not require androgen for growth and lack functional p53, whereas LNCaP cells are androgen-responsive and contain wild type p53. It would be of interest to determine whether p53 status influences SFN-induced cell cycle arrest.

The activity of Cdc25C is negatively regulated by phosphorylation at Ser-216, which creates a binding site for 14-3-3 (36, 37). The binding with 14-3-3 hinders nuclear accumulation of Cdc25C, which is required for activation of the Cdk1-cyclin B kinase complex in the nucleus (36, 37). Therefore, phosphorylation of Cdc25C on Ser-216 represents an important regulatory mechanism by which cells delay or block mitotic entry under normal conditions as well as in response to DNA damage (36, 37). In our model, SFN treatment caused an increase in Ser-216 phosphorylation of Cdc25C which was evident as early as 1 h after treatment and persisted for the duration of the experiment (24 h after the SFN treatment). We also observed an increase in binding of Cdc25C with 14-3-3β in SFN-treated cells compared with control (Fig. 4A).

Activation of Chk2 in SFN-induced G₂/M Arrest

Chk1 and Chk2, which are important intermediaries of DNA damage checkpoint pathways, are implicated in Ser-216 phosphorylation of Cdc25C (39, 40). Chk1 and Chk2 are activated in response to DNA damage by IR and/or UV light and by interference with DNA replication (29, 39-46). Chk1 is activated by ATR (ATM and Rad3-related protein kinase), whereas ATM-dependent phosphorylation at Thr-68 leads to activation of Chk2 (42-46). The results of the present study indicate that increased Ser-216 phosphorylation of Cdc25C in SFN-treated cells is associated with ATM-dependent activation of Chk2. Phosphorylation of ATM (Ser-1981) and Chk2 (Thr-68) was very low in Me2SO-treated control PC-3 cells but increased rapidly and dramatically upon treatment with SFN (Fig. 5). The time course for phosphorylation of Cdc25C upon treatment with SFN mirrored that of Chk2 phosphorylation (Figs. 3B and 5A). The kinase assays using immunoprecipitated Chk2 clearly indicated an increase in Chk2 kinase activity in SFN-treated cells compared with the control. Moreover, transfection of PC-3 cells with Chk2-specific siRNA not only reduced expression of Chk2 protein but also caused a marked decrease in SFN-in-
duced phosphorylation of both Chk2 (Thr-68) and Cdc25C (Ser-216) (Fig. 7). SFN-induced G2/M arrest was partially but statistically significantly attenuated in Chk2 siRNA-transfected cells (Fig. 6B). SFN-induced cell cycle arrest in Chk2 siRNA-transfected cells was only partially blocked probably because Chk2 siRNA did not fully eliminate Chk2 protein expression. Such a finding raises another possibility that Chk2-independent mechanisms may also contribute to SFN-induced cell cycle arrest. Consistent with this possibility, SFN-induced G2/M phase arrest was observed in Chk2-null HCT116 cells. Nonetheless, the results of the present study clearly indicate that the Chk2 protein level affects sensitivity of cells to SFN-induced G2/M arrest.

It is widely accepted that activation of checkpoints in response to DNA damage leads to cell cycle arrest; but in the case of severe damage, the cell cycle arrest leads to apoptotic cell death. The effects of SFN are compatible with this model. SFN treatment caused generation of ROS which was associated with increased phosphorylation of H2A.X at Ser-139, suggesting the treatment caused generation of ROS which was associated with the absence of cell death clearly shows that cell cycle arrest is not a secondary event; rather it leads to apoptosis because culture of SFN-treated (G0/M arrested) cells in drug-free medium led to a >5-fold increase in sub-G1/G0 fraction (Fig. 2D).

The SFN-induced decline in Cdc25C protein level was blocked by about 50% in Chk2 siRNA-transfected cells (Fig. 7, bottom panel) compared with mock-transfected cells. Because Chk2 depletion also led to a reduction in SFN-induced phosphorylation of Cdc25C, it is possible that Ser-216 phosphorylation of Cdc25C regulates its degradation. Results in HCT116-derived Chk2−/− cells also support this possibility because the SFN-induced decline in the Cdc25C protein level was relatively more severe in Chk2−/− than in HCT116-derived Chk2−/− cells (Fig. 8C). It is important to mention that Chk1-dependent phosphorylation of Cdc25A has been shown to regulate its stability (52). Specifically, these investigators showed that loss of Chk1 resulted in the accumulation of a hypophosphorylated form of Cdc25A protein, and Chk1-deficient cells failed to degrade Cdc25A after ionizing radiation treatment (52).

A fundamental question, which remains unanswered, is how SFN treatment causes DNA damage to activate ATM/Chk2. One possibility is that SFN reacts directly with the nucleophile sites in DNA to cause damage, which is probable because SFN is a highly electrophilic molecule capable of reacting with nucleophiles such as GSH (47). Alternatively, SFN treatment may cause transient oxidative stress and subsequent DNA damage because of its reaction with cellular antioxidant GSH. Even though further studies are needed to explore the above mentioned possibilities, data presented in this paper demonstrate an increase in ROS upon treatment of PC-3 cells with SFN.

In conclusion, the results of the present study indicate that SFN-treated PC-3 cells are irreversibly arrested in G2/M phase because of ROS-mediated activation of ATM/Chk2 leading to Ser-216 phosphorylation and cytoplasmic sequestration of Cdc25C.

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