Abstract
Aberrant nuclear factor-κB (NF-κB) signaling plays a role in cancer initiation and progression; thus, it represents a potential therapeutic target. We previously identified a mechanism of repression of NF-κB transcriptional activity and induction of apoptosis in colon cancer cells involving nuclear/nucleolar translocation of the RelA (p65) component of NF-κB. This response was stimulated by cellular stress-inducing agents, including aspirin, but not by tumor necrosis factor. Here, we investigate the upstream molecular mechanisms responsible for nucleolar targeting of RelA and show that aspirin activates the p38 mitogen-activated protein kinase (MAPK) pathway in colorectal cancer cells. We also show that aspirin causes rapid, ubiquitin-dependent degradation of cyclin D1, a known p38 target. Aspirin-induced p38 activation preceded cyclin D1 degradation, which was then followed by activation of the NF-κB pathway, suggesting a causative link. Indeed, chemical p38 inhibition (PD169316) and small interfering RNA directed against p38 blocked aspirin-induced cyclin D1 degradation, nucleolar translocation of RelA, and apoptosis. Furthermore, chemical inhibition of the cyclin D1/cyclin-dependent kinase 4 (CDK4) kinase complex, used as a surrogate for cyclin D1 degradation, caused nucleolar translocation of RelA, repression of κB-driven transcription, and apoptosis, thereby reproducing the effects of aspirin. In addition, we found that aspirin and the CDK4 inhibitor induced nucleolar translocation of RelA and apoptosis through a common mechanism involving the NH2-terminal nucleolar localization signal. Collectively, these data suggest that aspirin causes inhibition of cyclin D1/CDK4 through the p38 MAPK pathway. This inhibition stimulates the NF-κB pathway to induce nucleolar translocation of RelA and apoptosis. These novel findings have considerable relevance to the rational design of novel chemotherapeutic and chemopreventative strategies.

Introduction
Nuclear factor-κB (NF-κB) is a ubiquitously expressed, inducible transcription factor that regulates the expression of numerous genes controlling inflammation, proliferation, differentiation, and apoptosis (1). As appropriate regulation of these functions is critical for normal tissue homeostasis and for the suppression of tumor formation, NF-κB activity is generally tightly controlled. In resting cells, the NF-κB complex, most commonly a heterodimer of the p50/RelA (p65) polypeptides, is sequestered in the cytoplasm by the inhibitor protein IκB. Upon cellular stimulation by specific agents, the IκB protein is phosphorylated and then degraded by the proteasome, enabling NF-κB to translocate to the nucleus and regulate the transcription of target genes (2). Because NF-κB activates transcription of the IκB inhibitor, this acts as a negative feedback mechanism, ensuring that activation of the pathway is transient. However, in many human cancers, including colorectal cancer, NF-κB is aberrantly activated, and compelling evidence indicates that this constitutive NF-κB activity contributes to the carcinogenic process (3). For instance, suppression of NF-κB in cell lines and tumors showing aberrant activity causes decreased proliferation and apoptosis (4, 5). Oncogenes such as BCR-ABL and Ras require NF-κB activity for cell transformation (6), and genetic deletion of the NF-κB activator, IKK, can inhibit inflammation-associated cancer (7, 8). Hence, approaches that suppress the NF-κB pathway or restore the feedback mechanisms that limit its signaling response could have a significant therapeutic effect and are currently the focus of intense investigation.

Recent data from this laboratory (9) has revealed a novel mechanism for down-regulating NF-κB transcriptional activity and inducing apoptosis of transformed cancer cells. We found that activation of the NF-κB pathway by stress-inducing stimuli, such as serum deprivation, UV-C radiation, and aspirin, caused the RelA component of NF-κB to translocate from the cytoplasm to the nucleoplasm and then to the nuclear compartment, the nucleolus. In contrast, we found that RelA translocated to the nucleoplasm but was excluded from the nucleolus in response to the cytokines tumor necrosis factor-α (TNF-α) and TNF-related apoptosis-inducing ligand. We showed that nucleolar translocation of RelA was paralleled by a decrease in the basal levels of NF-κB transcriptional activity and apoptosis, whereas TNF-induced accumulation of RelA in the nucleoplasm was associated with activation of NF-κB transcription and inhibition of apoptosis (9). We identified a nucleolar localization signal at the NH2 terminus of RelA. Moreover, by using a dominant-negative mutant deleted for the nucleolar localization signal, we showed that nucleolar translocation of RelA was absolutely required for aspirin effects on NF-κB-driven transcription and apoptosis.

Our data showing differing NF-κB responses to aspirin/stress-inducing agents and TNF would suggest that these extracellular stimuli activate the NF-κB pathway through distinct upstream mechanisms. This notion is supported by our finding that aspirin and other agents that induce nucleolar translocation of RelA activate the NF-κB pathway with delayed kinetics, compared with the rapid activation observed in response to TNF (10, 11). The mode of TNF-induced NF-κB activation is well documented (1, 12). However, the mechanisms that trigger activation of NF-κB in
response to other agents, particularly stress inducers, are still poorly understood. Here, we investigate the upstream signaling pathways that stimulate cytoplasmic-nuclear/nucleolar translocation of NF-κB/RelA. Identification of these pathways may allow the design of a novel class of therapeutic agents that have the specific effect of targeting RelA to the nucleolus, thus down-regulating NF-κB transcriptional activity and inducing apoptosis in cancer cells.

One potential upstream candidate is the p38 mitogen-activated protein kinase (MAPK), which regulates inflammation, proliferation, and cell death. Activation of the p38 pathway is generally mediated by conditions of cell stress and culminates in phosphorylation of p38 on a conserved regulatory domain by the upstream kinases MKK3 and MKK6 (13). Once activated, p38 regulates multiple cellular processes, including transcription, translation, cell cycle progression, and apoptosis. Schwenger et al. (14) previously showed that sodium salicylate, the active component of aspirin, activates p38 to induce apoptosis, and the other stimuli we have shown to cause nucleolar translocation of RelA, namely UV-C radiation (15) and serum deprivation (16), are also known to stimulate this pathway. Furthermore, p38 is reported to lie upstream of NF-κB in response to proapoptotic stimuli such as UV radiation (17). Therefore, using aspirin as a model stimulus, we investigated the role of p38 in activation of the NF-κB pathway associated with nucleolar targeting of RelA.

Here, we show that aspirin activates the p38 MAPK pathway, leading to degradation of cyclin D1, nucleolar translocation of RelA, and apoptosis. We also show that chemical inhibition of the cyclin D1/CDK4 kinase complex mimics the effects of aspirin on NF-κB and programmed cell death. These findings contribute to our understanding of the complex intracellular signaling cascades that mediate activation of the NF-κB pathway and apoptosis, and reveal a novel mechanism of action of cyclin-dependent kinase (CDK) inhibitors.

Materials and Methods

Cell culture and reagents. Culture of SW480 and HT-29 cells has been previously described (18). The cells were plated at a density of 0.2 × 10^6/cm² and were treated in 0.5% serum when they were 60% to 80% confluent. Aspirin (Sigma, St. Louis, MO) was dissolved in water using 10 N NaOH and the pH was then adjusted to 7.0. SB203580, PD169316, MG132, and the CDK4 inhibitor 2-bromo-12,13-dihydro-5H-indole[2,3-a]pyrrolo[3,4-C]carbazole-5,7(6H)-dione (also known as “4d”), developed by Zhu et al. (19), were all obtained from Calbiochem, dissolved in DMSO, and added to the cells in 1:15 with 0.5% FCS.

Western blot analysis. Following treatment, whole-cell extracts were prepared using a commercially available lysis buffer according to the manufacturer’s instructions (Cell Signaling Technology, and Western blots were done as described previously (9). The membranes were probed with the following primary antibodies: phosphorylated p38 (Thr180/Tyr182), phosphorylated MKK3/MKK6 (Ser189/207), and MKK3 (Cell Signaling Technology); p38 (C-20) and I-βs-A (C-21; Santa Cruz Biotechnology); cyclin D1 (SP4; NeoMarkers, Inc.); and actin (Ab-1; Oncogene Research Products) as a control for protein loading. The horseradish peroxidase–conjugated secondary antibodies used were anti-rabbit IgG (Cell Signaling Technology) before the addition of the horseradish peroxidase–conjugated secondary antibodies (Jackson ImmunoResearch Laboratories) were used at a dilution of 1:2,000. The FITC- and Texas red–conjugated fluorescent microscopy and image capture were done as previously described (9). The primary antibodies used at 1:100 were as follows: NF-κB p65 (RelA; C-20) and p38 (C20; Santa Cruz Biotechnology); fibrillarin (AF801; Cytoskeleton, Inc.); cyclin D1 (SP4; NeoMarkers); and FLAG (Sigma), which was used at 1:2,000. The FITC- and Texas red–conjugated secondary antibodies (Jackson ImmunoResearch Laboratories) containing 1 μg/mL 4’,6-diamidino-2-phenylinole (DAPI).

Apoptosis assays. Staining for cell surface phosphatidylserine residues was conducted using an Annexin V-FITC apoptosis detection kit (Oncogene Research Products) according to the manufacturer’s instructions. The percentage of apoptotic cells was determined by fluorescent microscopy as described previously (9).

Transfection with short inhibitory RNA. SW480 cells were grown to 50% confluence, washed with PBS, and then transferred to L-15 medium containing 10% FCS without antibiotics. The cells were then transfected with either p38α (5’-UGAGAGACUGUAGGGCGAGAAG) or scrambled (5’-CAGUCGCGUUGGGUAGUGGAAG) small interfering RNA (siRNA) for 24 h with a fectin (FBE) transfection reagent (Promega) according to the manufacturer’s instructions. The transfection medium was removed 48 h later and the cells were treated with aspirin in reduced serum medium as described above.

Transfections and reporter assays. Cells were transfected with the 3henhancer CONA (3×RE ConA-Luc) NF-κB reporter (a kind gift from Professor R.T. Hay) and pCMV-β-galactosidase (Promega) plasmids as described previously (9). The relative luciferase activity was calculated as the units of luciferase activity per unit of β-galactosidase activity. FLAG-tagged vectors (a kind gift from Professor Jianghui Han; ref. 21) expressing either p38α wild-type (p38α WT) or a mutated form of p38α (dual TGY phosphorylation site converted to AGF-p38α AF) were transfected into SW480 cells using LipofectAMINE 2000 (Invitrogen) according to the manufacturer’s instructions. Immunochemistry was done on transfected cells as described above. The green fluorescent protein (GFP)–tagged vector expressing WT RelA was a kind gift from E. Qwarnström. The mutant vector, deleted for the nuclear localization signal (ΔΔ7–30), was generated in this laboratory and has been described previously (9). The cells were treated for 24 h after transfection with either 5 mmol/L aspirin or 2 μmol/L of the CDK4 inhibitor in reduced serum medium. The cellular distribution of GFP-tagged RelA (either WT or ΔΔ7–30) was examined using an Axiovert 100 inverted microscope with a ×40 objective lens and a Chroma 83000 filter set to capture fluorescent and phase-contrast images.

p38 kinase assay. A nonradioactive assay to determine p38 kinase activity was done on cells treated as outlined above using a commercially available kit according to the manufacturer’s instructions (Cell Signaling Technology). Briefly, 450 μg of whole-cell protein extract were immunopre-
Results

Activation of the p38 MAPK pathway by aspirin. To determine whether p38 signaling is involved in activation of the NF-κB pathway associated with nucleolar translocation of RelA, we initially examined the effects of aspirin on the p38 cascade in SW480 colon cancer cells. Using Western blot analysis with phosphospecific antibodies, we observed a rapid, dose- and time-dependent phosphorylation of p38 after aspirin treatment (Fig. 1A and B). In addition, phosphorylation of the upstream kinases, MKK3/MKK6, also occurred rapidly in response to this agent (Fig. 1C). Aspirin-induced phosphorylation of p38 was accompanied by an increase in the catalytic activity of the kinase, as evidenced by kinase assays using recombinant ATF-2 as a substrate for phosphorylated p38, which had been immunoprecipitated from aspirin-treated SW480 cells (Fig. 1C). Aspirin also induced the catalytic activity of p38 kinase in HT-29 colon cancer cells, showing that this effect is not exclusive to SW480 cells (Fig. 1D). Immunocytochemical analysis showed that these aspirin effects on p38 occurred before nuclear and nucleolar translocation of RelA, suggesting the possibility that a causative link might exist (Fig. 2A; refs. 9, 20). These data were confirmed using EMSAs, which revealed that aspirin stimulated the translocation of a p50/RelA complex to the nucleus and that this occurred after activation of p38 (Fig. 2B and C).

Aspirin induces cyclin D1 degradation and cell cycle arrest. p38 activity was observed minutes after aspirin treatment, whereas nuclear translocation of NF-κB did not occur for several hours (Figs. 1 and 2). Therefore, we hypothesized that p38 does not act directly on components of the NF-κB pathway, but that an intermediate signaling mechanism may be involved. One recognized target of the p38 kinase is the cell cycle protein, cyclin D1. p38 phosphorylates cyclin D1 on Thr328 in response to osmotic stress, and this phosphorylation targets the protein for degradation by the proteasome, thus mediating cell cycle arrest (22). This is of particular interest because salicylate and the stress-inducing agents we have shown to cause nuclear translocation of RelA have all previously been shown to reduce cellular levels of cyclin D1 and induce cell cycle arrest (23–25). Furthermore, perturbations in the cell cycle can activate the NF-κB pathway (26). Therefore, we next determined whether p38-mediated modulation of cyclin D1 was an intermediate pathway in the aspirin effects on NF-κB.

Using Western blot analysis, we found that aspirin caused a rapid decrease in the levels of cyclin D1 protein in both SW480 and HT-29 colon cancer cells (Fig. 3A and B). This decrease was blocked by the proteasome inhibitor, MG132 (Fig. 3C), suggesting that the reduction in protein levels was caused by phosphorylation and subsequent proteosomal degradation of cyclin D1. In addition, we found that decreased cyclin D1 levels were accompanied by cell cycle arrest (Fig. 3D), with a substantial increase in cells in S-phase and G2-M, shifted from G2-M, after aspirin exposure. Kinetic studies indicated that the proteasome-dependent degradation of cyclin D1 occurred after activation of the p38 cascade, but before nuclear/nucleolar translocation of RelA (Figs. 1A and 4A). This supports the notion that cyclin D1 is acting as an intermediate signal.

Blocking p38 activity attenuates aspirin effects on the cell cycle and NF-κB pathway. To investigate whether there is a causal relationship among aspirin-induced p38 activation, cell cycle arrest, and NF-κB responses, we used two specific p38 inhibitors, SB203580 and PD169316 (27, 28). Although both of these inhibitors have been reported to block phosphorylation of p38 in response to a variety of agents (29, 30), we found that only PD169316 inhibited aspirin-induced phosphorylation and kinase activity of p38 (Fig. 4A and data not shown). We then examined the effects of p38 inhibition on aspirin-induced cyclin D1 degradation. We found that PD169316 blocked the reduction in cyclin D1 protein levels, whereas SB203580 had no effect (Fig. 4A). This finding is in keeping with previous studies that have shown PD169316, but not SB203580, inhibits apoptosis induced by growth factor withdrawal and similar agents (31, 32). In view of these findings, we only used PD169316 in subsequent studies.
We examined the effects of PD169316 on aspirin-induced cell cycle arrest and discovered that chemical inhibition of p38 substantially abrogated the S-phase accumulation that occurred after aspirin treatment (Fig. 4B). We confirmed the specificity of PD169316 using sequence-specific siRNA directed against p38. Using Western blot analysis, we found that siRNA-mediated knockdown of p38 reduced aspirin-induced degradation of cyclin D1 (Fig. 4C). Immunocytochemistry also revealed that aspirin-mediated cyclin D1 degradation was inhibited in cultures transfected with p38 siRNA, compared with cultures transfected with scrambled siRNA (data not shown). To further show a critical role for p38 in aspirin-induced cyclin D1 degradation, we used FLAG-tagged vectors expressing either p38α WT protein or a mutated form of p38α in which the dual TGY phosphorylation site has been converted to AGF (p38α AF; ref. 21). Using immunocytochemistry, we found that cyclin D1 protein was highly expressed in nontransfected and p38α WT–transfected colon cancer cells before aspirin treatment (Fig. 4D). Following exposure to aspirin, the levels of cyclin D1 dramatically decreased in both p38α WT and nontransfected cells. However, cyclin D1 degradation by aspirin was attenuated in cancer cells transfected with the p38α AF vector (Fig. 4D, bottom). These data show a causal relationship between activation of the p38 signaling cascade and the reduction in cyclin D1 protein levels that occur after aspirin treatment.

Next, we investigated the role of p38 in aspirin-mediated activation of the NF-κB pathway and apoptosis. Using immunocytochemical analysis, we found that blocking p38 signaling using PD169316 inhibited aspirin-induced nucleolar translocation of RelA (Fig. 5A). We also found that transfection with p38 siRNA reduced the level of p38 protein expression in ~60% of cells (Fig. 5B, magenta arrows), which is consistent with the level of knockdown observed using Western blot analysis on whole-cell populations (Fig. 4C). In cells where p38 expression was knocked down, we found that RelA remained cytoplasmic in response to aspirin. In contrast, aspirin stimulated cytoplasmic to nuclear/nucleolar translocation of RelA in cells expressing normal levels of p38 (Fig. 5B, yellow arrows). Analysis of transfected cell cultures indicated that p38 siRNA reduced the proportion of cells containing nucleolar RelA after aspirin treatment from 16% to 7% (in keeping with the level of knockdown), whereas PD169316 decreased the number from 13% to <1% (Fig. 5C). Furthermore, RelA remained cytoplasmic after aspirin treatment in the presence of PD169316 and p38 siRNA, showing that p38 signaling is required to activate the NF-κB pathway, leading to cytoplasmic-nuclear/nucleolar translocation of RelA. In parallel with the effects on stimulation of the NF-κB pathway and nucleolar translocation of RelA, we found that p38 siRNA inhibited the apoptotic response to aspirin (Fig. 5D). The discrepancy between the proportion of cells with nucleolar RelA (16%) and the proportion undergoing apoptosis (40%) is due to differences in assay protocols because both adherent and floating cell populations are collected for apoptosis assays, whereas only adherent cells are analyzed by immunocytochemistry. In addition, the effects of aspirin on nucleolar RelA sequestration and apoptosis were attenuated by expression of the catalytically inactive p38α AF mutant (data not shown). Taken together, these data indicate that aspirin-induced activation of the
NF-κB pathway and apoptosis of colon cancer cells are also dependent on p38 activity.

Inhibition of CDK4 mimics the effects of aspirin. Because the data presented here show that aspirin rapidly activates the p38 MAPK cascade and that both cyclin D1 degradation/cell cycle arrest and activation of the NF-κB pathway are dependent on this effect, we wished to determine whether cyclin D1 degradation is causally associated with activation of the NF-κB pathway.

To stimulate cell cycle progression, cyclin D1 is required to interact with CDK4 and CDK6 to form an active kinase complex (33). To investigate the role of cyclin D1 degradation/cell cycle arrest in activation of the NF-κB pathway, we knocked out cyclin D1 function in colorectal cancer cells using a specific inhibitor of CDK4 (19). This CDK4 inhibitor blocks the activity of the cyclin D1/CDK4 kinase complex and should therefore replicate the effects of aspirin-mediated cyclin D1 degradation. Remarkably, we found that mimicking a reduction in cyclin D1 levels with the CDK4 inhibitor induced nucleolar translocation of RelA (Fig. 6A). This effect seemed to be specific to inactivation of the cyclin D1/CDK4 kinase complex, as roscovitine, which inhibits CDK1, CDK2, and CDK5, but not the cyclin D–type kinases CDK4 or CDK6, had no effect on the cellular distribution of RelA (Fig. 6A). In addition to its effects on nucleolar localization of RelA, inhibition of CDK4 also induced degradation of IκB and apoptosis in colon cancer cells in a similar manner to aspirin (Fig. 6B). Furthermore, by using NF-κB reporter assays, we found that the CDK4 inhibitor induced a decrease in NF-κB–driven transcription (Fig. 6B), a response that is associated with nucleolar translocation of RelA and apoptosis (9). These data suggest that inactivation of cyclin D1/CDK4 does indeed activate the NF-κB signaling cascade, resulting in nucleolar sequestration of RelA and down-regulation of κB-driven transcription, which ultimately leads to apoptosis.

We have previously shown that amino acids 27 to 30 in the NHR terminal of RelA are required for nucleolar targeting of this protein in response to aspirin and UV-C (9). To further investigate the mechanism by which inhibition of CDK4 induces nucleolar translocation of RelA, we used a mutant RelA protein that lacks this critical nucleolar localization sequence. In keeping with our immunocytochemistry data, we found that both aspirin and the CDK4 inhibitor induced nucleolar translocation of GFP-tagged RelA WT (Fig. 6C). However, when cells expressing the GFP-tagged RelA nucleolar localization signal deletion mutant (RelAΔ27–30) were exposed to either aspirin or the CDK4 inhibitor, RelA migrated to the nucleus but was excluded from the nucleolus (Fig. 6C, yellow arrows). Furthermore, apoptosis induced by both aspirin and the CDK4 inhibitor was attenuated in cells expressing RelAΔ27–30 (Fig. 6D). Collectively, these data suggest that aspirin and the CDK4 inhibitor function through a common mechanism to stimulate the NF-κB pathway to induce nucleolar sequestration of RelA. These findings also confirm that inactivation of cyclin D1/CDK4 is sufficient to drive the downstream responses, which, through nucleolar targeting of RelA, lead to apoptosis of colon cancer cells.

Discussion

The NF-κB transcription factor is one of the most important regulators of the cellular life/death balance and its aberrant activation is associated with cancer (4). Therefore, identifying mechanisms for switching off aberrant NF-κB activity could have a major therapeutic benefit. Here, we reveal a novel pathway for down-regulating NF-κB transcriptional activity and inducing apoptosis of colon cancer cells that involves activation of the p38 pathway, inhibition of the cyclin D1/CDK4 kinase complex, and consequent nucleolar targeting of RelA. These findings contribute to our knowledge of the complexities of NF-κB signaling. Furthermore, these findings have considerable relevance to understanding the mechanisms of chemoprevention and cancer therapeutics.

The data presented here provide evidence that p38-mediated inhibition of cyclin D1/CDK4 stimulates the NF-κB pathway to induce nucleolar sequestration of RelA. This conclusion is based on the following findings. First, p38 was rapidly activated in response to aspirin. Second, inhibiting p38 kinase activity by any of three approaches—chemical inhibition with PD169316, down-regulation of p38 protein with siRNA, or expression of a kinase-dead p38 mutant—all attenuated aspirin-induced cyclin D1 degradation and cell cycle arrest. This strongly suggests that these effects are mediated through the p38 pathway. Third, PD169316, siRNA, and kinase-dead p38 prevented aspirin-induced stimulation of cytoplasmic-nuclear/nucleolar translocation of RelA. These studies show that p38 is important for both the degradation of cyclin D1 and the NF-κB responses to aspirin, suggesting that these pathways may be linked. Fourth, we found that inhibiting cyclin D1/CDK4 kinase activity stimulated IκB degradation, nucleolar accumulation of RelA, repression of κB-driven transcription, and

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**Figure 3.** Aspirin induces cyclin D1 degradation and cell cycle arrest. A, SW480 cells were treated with 5 mmol/L aspirin for the times indicated in minutes (h), and the levels of cyclin D1 protein were determined by Western blot analysis. Actin was used as a loading control. B, HT-29 cells were exposed to 5 mmol/L aspirin for the times indicated in hours (h), and the levels of cyclin D1 were assessed by Western blotting. C, SW480 cells were pretreated with 25 μmol/L MG132 for 1 h and then exposed to 5 mmol/L aspirin for an additional hour. The levels of cyclin D1 protein were investigated by immunoblotting. D, SW480 cells were left untreated (open columns) or exposed to 5 mmol/L aspirin for 12 h (closed columns), and the proportion of cells in each phase of the cell cycle was analyzed. Columns, mean of three experiments; bars, SE.
Figure 4. Blocking p38 activity attenuates aspirin effects on the cell cycle. A, SW480 cells were pretreated with 5 μmol/L PD169316 or 10 μmol/L SB203580 for 1 h and then exposed to 5 mmol/L aspirin for a further 60 min. The levels of p38 kinase activity were then determined using kinase assays with ATF-2 as a substrate, as described above. The levels of cyclin D1 protein were also examined by Western blot analysis, with actin used to control for protein loading. B, SW480 cells were pretreated with 5 μmol/L PD169316 for 1 h before the addition of 5 mmol/L aspirin for 16 h. The proportion of cells in each phase of the cycle was determined, and the levels after aspirin treatment were expressed as a percentage of the levels without aspirin. Columns, mean of three experiments; bars, SE. Aspirin caused a significant increase in the level of cells in S-phase compared with control (P = 0.022), whereas there was no difference between control and aspirin-treated cells in the presence of PD169316. C, SW480 cells were transfected with p38 siRNA or a scrambled sequence for 48 h before the addition of 5 mmol/L aspirin for 1 h. The levels of p38 and cyclin D1 protein were then determined by Western blot analysis. D, SW480 cells were transfected with vectors encoding FLAG-tagged p38α WT protein or a catalytically inactive p38α mutant (p38α AF). The cells were then exposed to 5 mmol/L aspirin for 4 h, and the cellular distribution of cyclin D1 (FITC) and FLAG-p38 (Texas red) was assessed by immunocytochemical staining. The merged panel is a pseudocolor image generated by combining the FITC (green), Texas red (red), and DAPI (blue) channels. Bar, 10 μm. Yellow arrows, cells that are expressing FLAG-tagged p38α; magenta arrows, levels of cyclin D1 in cells exposed to aspirin.

apoptosis, supporting our hypothesis that p38 mediates its effects on the NF-κB pathway through inhibition of the cyclin D1/CDK4 complex. In further support of this notion, serum deprivation (16, 24) and UV-C (15, 25), which stimulate the NF-κB pathway, have also previously been shown to activate p38 signaling and induce cell cycle arrest. We have previously shown that, in addition to aspirin, each of these cellular insults cause nuclear targeting of RelA (9). Finally, our studies using the RelA nucleolar localization signal deletion mutant showed that inactivation of cyclin D1/CDK4 causes nuclear targeting of RelA and induces apoptosis through the same molecular mechanism as aspirin. The novel observations presented here are the first to show the MAPK cascade is involved in NF-κB signaling in response to nonsteroidal anti-inflammatory drugs and stimulation of the NF-κB pathway by a CDK4 inhibitor.

Although several studies have shown enhanced phosphorylation of p38 after salicylate treatment (34–36), this study is the first to show that aspirin activates MKK3/MKK6, the kinases upstream of p38. Our study also indicates that the aspirin-induced degradation of cyclin D1 is p38 dependent. Because p38 has been previously shown to directly phosphorylate cyclin D1 in response to osmotic stress (22), targeting the protein for degradation, we propose that p38 targets cyclin D1 directly in response to aspirin. Studies are ongoing in our laboratory to identify the targets upstream of MKK3/MKK6 that could be triggered by aspirin, for example, ASK1 (37), cdc42 (38), and cell surface integrins (39, 40), and to determine whether p38 directly phosphorylates cyclin D1.

Our finding that cyclin D1 degradation was associated with arrest in the S and G2-M phases of the cell cycle would seem to contradict the established understanding that cyclin D1 is a mediator of G1-S phase transition and that inhibition causes arrest in G1. However, more recently, cyclin D1 has been shown to be required at later stages of the cell cycle (41). Furthermore, other chemopreventative agents have been shown to induce S-phase arrest associated with degradation of cyclin D1 (42, 43). Interestingly, we were
unable to detect nucleolar sequestration of RelA in cells arrested using specific inhibitors of the G2-M phase (nocodazole or colcemid) or synchronized in S-phase after release from aphidicolin treatment (data not shown). This would suggest that specific inhibition of cyclin D1/CDK4 is required to induce nucleolar RelA translocation, as opposed to cell cycle arrest per se. This finding also implies that the cell cycle arrest we detect after aspirin treatment occurs as a consequence of down-regulation of cyclin D1/CDK4, but is not sufficient itself to drive the effects we observed on the NF-κB pathway.

Interestingly, we found that deletion of the nucleolar localization signal from RelA permitted nuclear translocation of the protein but prevented nucleolar accumulation of RelA after treatment with either aspirin or the CDK4 inhibitor. These data confirm that inhibition of cyclin D1/CDK4 stimulates the NF-κB pathway in the cytoplasm to induce cytoplasmic-nuclear/nucleolar translocation of RelA, but suggest that additional events requiring the nucleolar localization signal are needed for nucleolar targeting of the protein. It is possible that aspirin-induced inactivation of cyclin D1/CDK4 stimulates the NF-κB pathway in a manner that leads to the interaction of RelA with one or more cofactors, which either bind directly to the nucleolar localization signal or require an intact nucleolar localization signal to stabilize the protein-protein

Figure 5. Blocking p38 activity attenuates aspirin effects on the NF-κB pathway. A, SW480 cells were pretreated with 5 μmol/L PD169316 for 1 h before treatment with 5 mmol/L aspirin for 16 h. The subcellular localization of RelA (FITC) and of the nucleolar marker fibrillarin (Texas red) was then assessed by immunocytochemical staining. The merged panel is a pseudocolor image generated by combining the FITC (green), Texas red (red), and DAPI (blue) channels. Bar, 10 μm. B, SW480 cells were transfected with p38 siRNA or a scrambled sequence for 48 h before the addition of 5 mmol/L aspirin for 16 h. The cellular distribution of RelA (Texas red) and p38 (FITC) was then determined by immunocytochemistry, as described above. Yellow arrows, cells where RelA is sequestered in the nucleolus; magenta arrows, cells where p38 has been knocked down. C, cells were either pretreated with PD169316 (PD) or transfected with siRNA, and the number containing nucleolar RelA without aspirin treatment (open columns) or after exposure to 5 mmol/L aspirin for 16 h (closed columns) was quantified by counting a minimum of 200 cells per treatment per replicate. Columns, mean of three experiments; bars, SE. In untreated cells, aspirin caused a significant increase in nucleolar RelA (P = 0.034). However, the number of cells containing nucleolar RelA after treatment with PD16916 was not significantly different from control cells (P = 0.398). D, SW480 cells were transfected with p38 siRNA or a scrambled sequence for 48 h before the addition of 5 mmol/L aspirin for 16 h. The percentage of apoptosis in untreated cells (open columns), and in cells exposed to aspirin (closed columns), was then determined by fluorescence microscopy. Columns, mean of four experiments; bars, SE. Aspirin caused a significant increase in the levels of apoptosis in cells transfected with scrambled siRNA (P = 0.009), whereas there was no statistical difference between control and treated cells after transfection with p38 siRNA (P = 0.555). Inset, level of p38 knockdown by Western blot analysis.
Figure 6. Inhibition of CDK4 mimics the effects of aspirin. A, SW480 cells were exposed to 5 mmol/L aspirin, 2 μmol/L of a commercially available CDK4 inhibitor (CDK4i), or 10 μmol/L of the CDK1, CDK2, and CDK5 inhibitor, roscovitine, for 16 h. The cellular distribution of RelA (FITC) and the nucleolar marker fibrillarin (Texas red) was assessed by immunocytochemical staining. The merged panel is a pseudocolor image generated by combining the FITC (green), Texas red (red), and DAPI (blue) channels. Bar, 10 μm. B, cells were treated with 5 mmol/L aspirin or 2 μmol/L of the CDK4 inhibitor for 16 h. The levels of IκB protein were determined by Western blot analysis; actin was used to control for protein loading. The percentage of apoptosis (Annexin V–positive cells) was determined by fluorescence microscopy (closed columns). Columns, mean of three experiments; bars, SE. Both aspirin and the CDK4 inhibitor significantly increased the proportion of apoptotic cells, compared with the control (P = 0.046 and P = 0.015, respectively). Cells were transfected with a reporter plasmid containing a luciferase gene under the control of an NF-κB–driven promoter before treatment as described above. The extent of NF-κB–driven transcription was then assessed after treatment, and the levels of gene expression were calculated as a percentage of the control value (open columns). Columns, mean of four experiments; bars, SE. C, SW480 cells were transfected with a GFP-tagged construct encoding either RelA WT or a mutant RelA protein in which the nucleolar localization signal had been deleted (RelA Δ27–30). The cells were then exposed to either 5 mmol/L aspirin or 2 μmol/L of the CDK4 inhibitor for 16 h. The cellular distribution of the GFP-tagged proteins was then visualized using live cell imaging to obtain fluorescent and phase-contrast images of the cells with a ×40 objective lens. Bar, 10 μm. Each channel was recorded independently, and pseudocolor images were superimposed. Yellow arrows, where RelA is excluded from the nucleolus. D, cells were transfected and treated as described above, and the percentage of apoptosis was determined for cells expressing either RelA WT (open columns) or a mutant RelA protein in which the nucleolar localization signal had been deleted (RelA Δ27–30; closed columns). Columns, mean of three experiments; bars, SE. Both aspirin and the CDK4 inhibitor significantly increased the proportion of apoptotic RelA WT cells compared with the control (P = 0.024 and P = 0.025, respectively), whereas there was no significant difference between control and treated RelA Δ27–30 cells (P = 0.169 for aspirin and P = 0.193 for the CDK4 inhibitor).
interaction. We propose a model whereby the interaction between RelA and this cofactor(s) diverts the NF-κB complex away from its gene targets in the nucleoplasm and into the nucleolus, leading to repression of transcription and apoptosis. An alternative hypothesis, however, is that the interaction of RelA with the cofactor(s) forms a transcriptionally repressive complex, which down-regulates gene expression and then induces nucleolar sequestration of the proteins, possibly as a means to limit their effects. This latter theory is supported by previous studies (11) showing that RelA can interact with histone deacetylases after exposure to UV-C to form a transcriptionally repressive complex. Studies to identify this cofactor(s), which is activated/recruited after inactivation of cyclin D1/CDK4, are ongoing within our laboratory.

Our data showing that a CDK4 inhibitor mimics the effects of aspirin on the NF-κB pathway are particularly interesting given the current interest in cell cycle inhibitors as anticancer agents. There are several possible pathways by which inhibition of cyclin D1/ CDK4 could activate the NF-κB signaling cascade to bring about nucleolar sequestration of RelA. For example, previous studies have shown that inhibition of cyclin D1/CDK4 activity leads to hypophosphorylation of Rb protein and consequently down-regulation of E2F activity (33). As E2F is known to stabilize the NF-κB inhibitor IκB (44), attenuation of E2F activity would lead to destabilization of IκB and subsequent activation of NF-κB. Our current findings support this suggestion because chemical inhibition of CDK4 activity induced degradation of IκB (Fig. 6B). In addition, recent studies have shown that CDK4 can interact directly with IκB, suggesting another point of cross-talk between these pathways that could be involved in activation of NF-κB (45). Studies to investigate these hypotheses are currently under way in our laboratory.

The data presented here provide compelling evidence that aspirin induces apoptosis via effects on the p38 MAPK, cyclin D1/CDK4, and NF-κB pathways. Furthermore, the findings identify a novel effect of CDK4 inhibitors that is highly relevant to the antitumor effects of these agents. These studies contribute to understanding of the complex intracellular signaling cascades that control apoptosis through NF-κB and reveal novel pathways to down-regulate NF-κB transcriptional activity. Such understanding could be exploited to promote the development of chemotherapeutic agents.

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References

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