Transcriptional Activation of p21<sub>Waf1/Cip1</sub> by Alkylphospholipids: Role of the Mitogen-Activated Protein Kinase Pathway in the Transactivation of the Human p21<sub>Waf1/Cip1</sub> Promoter by Sp1

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ABSTRACT

Alkylphospholipids (ALKs) are a novel class of antitumor agents with an unknown mechanism of action. The first ALK tested in the clinic, miltefosine, has been approved recently in Europe for the local treatment of patients with cutaneous metastasis. Perifosine, the only available oral ALK, is being studied currently in human cancer clinical trials. We have shown previously that perifosine induces p21<sup>Waf1/Cip1</sup> in a p53-independent fashion and that induction of p21<sup>Waf1/Cip1</sup> is required for the perifosine-induced cell cycle arrest because cell lines lacking p21<sup>Waf1/Cip1</sup> are refractory to perifosine. In this report, we investigated the mechanism by which perifosine induces p21<sup>Waf1/Cip1</sup> protein expression. We observed that perifosine induces the accumulation of p21<sup>Waf1/Cip1</sup> mRNA without affecting p21<sup>Waf1/Cip1</sup> mRNA stability. Using several p21<sup>Waf1/Cip1</sup> promoter-driven luciferase reporter plasmids, we observed that perifosine activates the 2.4-kb full-length p21<sup>Waf1/Cip1</sup> promoter as well as a p21 promoter construct lacking p53-binding sites, suggesting that perifosine activates the p21<sup>Waf1/Cip1</sup> promoter independent of p53. The minimal p21 promoter region required for perifosine-induced p21 promoter activation contains four consensus Sp1-binding sites. Mutations in each particular Sp1 site block perifosine-induced p21<sup>Waf1/Cip1</sup> expression. Moreover, we showed that perifosine activates the mitogen-activated protein/extracellular signal-regulated kinase pathway, and this activation promotes the phosphorylation of Sp1 in known mitogen-activated protein kinase residues (threonine 453 and 739), thereby leading to increased Sp1 binding and enhanced p21<sup>Waf1/Cip1</sup> transcription. These results represent a novel mechanism by which alkylphospholipids modulate transcription, and may contribute to the discovery of new signal transduction pathways crucial for normal and neoplastic cell cycle control.

INTRODUCTION

Alkylphospholipids (ALKs) belong to a novel class of antineoplastic compounds that display potent antiproliferative activity against several in vitro and in vivo human tumor models. ALKs include ET-18-OCH<sub>3</sub> (eldeposine), hexadecylphosphocholine (miltefosine), and octadecylphosphiridine (perifosine; Refs. 1, 2). In addition to its striking curative effects in leishmaniasis (3), miltefosine has been approved recently in Europe for the local treatment of patients with cutaneous metastasis (4). Perifosine is an analogue of miltefosine with similar antiproliferative effects but with better oral tolerability in preclinical models (1, 2). On the basis of these unique features, several clinical trials with oral perifosine are being conducted worldwide (5, 6). Although ALKs have demonstrated antitumoral effects in clinical trials with oral perifosine are being conducted worldwide (5, 7, 8), the exact mechanism of action remains elusive (2, 9, 10).

Cell cycle progression is controlled by the cyclic activation of a family of serine-threonine kinases, the cyclin-dependent kinases (cdks; Refs. 11–13). Regulation of cdk activity occurs mainly through the complex with cyclin cofactors and through the stoichiometric binding of endogenous cdk inhibitors to the cdk/cyclin complex (11–13). The cdk inhibitor p21<sup>Waf1/Cip1</sup> is the prototype of an endogenous cdk inhibitor. This general cdk inhibitor is a downstream effector of the tumor suppressor gene p53 (14–17). Several important physiologic functions are ascribed to p21<sup>Waf1/Cip1</sup>, including cell cycle arrest, differentiation, DNA repair, apoptosis, and senescence, among other functions (18–20). The regulation of p21<sup>Waf1/Cip1</sup> occurs primarily at the transcriptional and post-transcriptional levels (21–24). The tumor suppressor gene p53 is the bona fide transcriptional activator of p21<sup>Waf1/Cip1</sup> because this promoter displays p53 binding site in its sequence (15). However, p21<sup>Waf1/Cip1</sup> can also be transcriptionally regulated by p53-independent mechanisms (18, 23–26).

Initial efforts in our laboratory demonstrated that ALKs (particularly perifosine) promote cell cycle arrest at the G<sub>1</sub>-S and G<sub>2</sub>/M by up-regulation of p21<sup>Waf1/Cip1</sup> protein levels, independent of p53 function (9). Increased levels of p21<sup>Waf1/Cip1</sup> induced by perifosine are associated with cdkks, thereby inhibiting cdk activity. The cell cycle arrest induced by perifosine requires p21<sup>Waf1/Cip1</sup> because HCT 116 cell lines lacking p21 (HCT 116 p21<sup>−/−</sup>) are refractory to perifosine. Thus, the induction of p21<sup>Waf1/Cip1</sup> by ALKs is p53 independent and is required for the cell cycle effects of ALKs (9).

In this study, we investigated the mechanism by which ALKs induce p21<sup>Waf1/Cip1</sup> protein accumulation. We showed that the induction of p21<sup>Waf1/Cip1</sup> by ALKs is due to a p53-independent transcriptional activation of the p21<sup>Waf1/Cip1</sup> promoter. We also showed that the minimal promoter region required by perifosine is similar to the minimal region required by Ras to induce p21<sup>Waf1/Cip1</sup>. Furthermore, we demonstrated that perifosine activates the mitogen-activated protein/extracellular signal-regulated kinase (ERK) kinase (MEK)/ERK mitogen-activated protein kinase (MAPK) pathways, and this activation leads to increased Sp1 phosphorylation, leading to enhanced Sp1 DNA-binding activity. Altogether, our results illustrate a novel mechanistic aspect by which ALKs regulate p21<sup>Waf1/Cip1</sup> transcription.

MATERIALS AND METHODS

Cell Culture. Exponentially growing HaCaT human keratinocyte cells were grown in DMEM containing 10% fetal bovine serum (Invitrogen, Carlsbad, CA) in a 5% CO<sub>2</sub> humidified atmosphere at 37°C as shown previously (9).

Reagents. Zentaris (Frankfurt, Germany) provided perifosine and other ALKs (miltefosine and edelfosine). For in vitro studies, perifosine was reconstituted as a 100 mM stock solution in PBS. PD98059 (Calbiochem, Darmstadt, Germany) and actinomycin D (Sigma Chemical Co., St. Louis, MO) were diluted in DMSO, stock concentrations of 25 μM and 5 μg/ml, respectively. PP2A was obtained from Upstate Cell Signaling (Lake Placid, NY).

Immunoblot Analysis. To study the role of MEK/ERK pathways, HaCaT cells were cultured under serum deprivation conditions for 16 h and exposed to increasing concentrations of perifosine for 30 min, or to 10 μM of perifosine for increasing time periods. Cell lysates were obtained as described previously (9, 27). Briefly, 25 μg of protein were electrophoretically resolved in 4–20% Tris Glycine SDS-PAGE gel (Invitrogen) and transferred to a polyvinylidene fluoride membrane.
concentrations of perifosine for 30 min. After treatment, cells were lysed and serum starved for 16 h and exposed to vehicle (PBS) or to increasing concentrations of perifosine for 30 min. After treatment, cells were lysed as described previously (28). Briefly, 200 µg of total cellular lysate was immunoprecipitated with ERK-2 antibody (Santa Cruz Biotechnology, Inc.) for 1 h at 4°C. Then, Gammabind G Sepharose (Amersham Pharmacia Biotech, Piscataway, NJ) was used to capture the immune complexes. After three washes, kinase reactions were performed in kinase assay buffer (12.5 mM 4-morpholinepropanesulfonic acid (pH 7.5), 12.5 mM β-glycerophosphate, 7.5 mM MgCl₂, 0.5 mM EGTA (pH 8.0), 0.5 mM NaF, and 0.5 mM Na₃VO₄) containing γ-[32P]ATP (3000 Ci/mmol; NEN, Boston, MA). Twenty µM cold ATP, and 50 µg of myelin basic protein as substrate. Reactions were incubated at 37°C for 30 min and terminated by the addition of SDS-gel loading buffer. The resolved and dried gels were subjected to autoradiography and quantified by Phospho-Imager (Molecular Dynamics).

**Northern Blot Analysis.** Total RNA was extracted using TRIZOL (Invitrogen) following the manufacturer’s recommendations. Twenty-five µg of total RNA were separated on 1% formaldehyde-agarose gels and transferred to nylon membranes (Hybond-XL; Amersham Pharmacia Biotech) by capillary blotting. A p21<sup> wt/lqpl </sup> probe was generated by digestion of pCMV p21<sup> wt/lqpl </sup> (obtained from Michael R. Karin, New York University, NY) with XhoI and HindIII, and then gel purified using a GeneClean spin kit (Qiogene, Inc., Carlsbad, CA). Glyceraldehyde-3-phosphate dehydrogenase was obtained from Ambion, Inc. (Austin, TX). Probes were labeled with [α-32P]dATP by random priming using StripEZ DNA kit (Ambion, Inc.,). Northern blot hybridization to [α-32P]labeled probes was performed using HybriS blot solution (Intergen Company, Purchase, NY) following the manufacturer’s instructions. Signals were detected by exposure to BioMax MR film (Kodak, Rochester, NY), and quantitation of signals was performed by densitometry.

**Real-Time Quantitative PCR.** HaCaT cells were plated and grown overnight to ~70% confluence and treated with increasing concentrations of perifosine (1, 3, 5, and 10 µM) for 12 h or with 10 µM perifosine for several time periods (1, 3, 6, 9, and 12 h). Total RNA was extracted using TRIZOL (Invitrogen) following the manufacturer’s recommendations. Two µg of RNA were incubated in a final volume of 20 µl containing 2 µl of real-time buffer (10×), 2 µl of deoxycytidine triphosphate mix (5 mM), 2 µl of oligodeoxy- thymidylic acid primer (10 µM) and 1 µl of real-time Omniscript enzyme (Qiagen, Valencia, CA). After 1 h at 37°C, quantitative PCR was performed with 1 µl of cDNA, 1 µl of primer mix (10 µM), and 12.5 µl of SYBR Green (Quantitect SYBR Green PCR kit; Qiagen) in a final volume of 25 µl. The primers used were 5′CTG GAG ACT CTC AGG AGC GTA AAT′ and 5′GAA TTA GGG CCT CTT GGA G′ for p21<sup> wt/lqpl </sup> and 5′ GAA GGT GAA GTG CGT AC C′ and 5′ GAA GAT GAT GAT GAG ATT TC′ for p21<sup>∞/∞</sup>. Quantitative PCR was performed in an ABI Prism 7700 Sequence Detector cycler (Perkin-Elmer Applied Biosystems, Foster City, CA) as follows, 94°C for 5 min, then 40 cycles at 95°C for 30 s and at 60°C for 30 s. To determine whether the accumulation of p21<sup>∞/∞</sup> induction by perifosine is due to post-transcriptional control (i.e., increase in p21<sup>∞/∞</sup> mRNA half-life), HaCaT cells were treated for 12 h with 10 µM of perifosine, washed with PBS, and exposed to actinomycin D (1 µg/ml) or actinomycin D and perifosine for increasing time periods (0.5, 1, 2, 4, 6, or 8 h). Cells were harvested, RNA was isolated using TRIzol, and real-time and quantitative PCR were performed as described above.

**Luciferase Promoter Analysis.** HaCaT cells were transfected with different expression plasmids using Fugene (Roche, Indianapolis, IN) according to the manufacturer’s protocol, 1 µg of each of the reporter plasmids, and 0.01 µg of pRL-null (a plasmid expressing the enzyme Renilla luciferase from Renilla reniformis) as an internal control, adjusting the total amount of plasmid DNA with empty vector (pcDNAIII-β-gal, a plasmid expressing the enzyme β-galactosidase). Twelve h after transfection, cells were exposed to 10 µM perifosine for 18 h. Firefly and Renilla luciferase activities present in cellular lysates were assayed using the Dual-Luciferase Reporter System (Promega, Madison, WI), and light emission was quantitated using the MLX Microplate Luminometer as specified by the manufacturer (DyneX Technologies, Chantilly, VA). Luciferase activities were normalized based on total protein concentrations and Renilla luciferase activity. All of the p21<sup> wt/lqpl </sup> promoter constructs used herein were reported previously (26). Briefly, p12PSma1-luc was created by the digestion of the 2.4-kb full-length promoter (p21Luc-wt) with Smal and religation. p12PSma-ura promoter (minimal promoter region for Rac activity) was created by cloning the 50-bp Smal fragment of the p21<sup> wt/lqpl </sup> promoter into pGL-2 basic (26). Mutagenesis of p21P93-S-luc was performed to generate p21P93-Smt#2 to 5 and p21P93-Smt#2. Sp1–1 site was mutated from the wild-type CCCCCTCCTT to TATCTAGAC (p21P 93-S mut#2-luc); Sp1–2 was mutated from TGGAGCCGGG to CCTCTAGAAT (p21P 93-S mut#3-luc); Sp1–3 was mutated from CCGGGCGGGG to ATCTAGA CAT (p21P 93-S mut#4-luc); and Sp1–4 was mutated from CGGGTGATAT to TCTAGACGTT (p21P 93-S mut#5-luc; Ref. 26). Plasmids encoding vectors for the activating mutant for Ras (Ras V12) and dominant-negative MEK (MEKAA) were provided by Silvio Gukind (NIH, Bethesda, MD) and have been described previously (28).

**Electrophoretic Mobility Shift Assays.** Oligonucleotides corresponding to the wild-type consensus Sp1 site (5′ ATT CGA TCG CGG GGC GGC GAC C′) were obtained from Promega, or an oligonucleotide corresponding with the mutant Sp1 sequence in the p21<sup> wt/lqpl </sup> promoter (p21P 93-S mut#2; CCC GCC TCA AGG ATC CGG GAC CCG C′) was end-labeled with [γ-32P]dATP using T4 polynucleotide kinase (Invitrogen). The resulting end-labeled oligo was purified using a G25 column (Amersham Pharmacia Biotech). Approximately 10,000 cpm of labeled probe was used in each gel shift analysis reaction. To prepare nuclear extracts, HaCaT cells were plated in 150-mm plates and grown to 70% confluency. Cells were then incubated with vehicle (PBS) or 10 µM perifosine for 12 h. As a positive control, HaCaT cells were transfected with 500 ng of activated Ras (Ras V12). Cells were washed in cold PBS and lysed in 400 µl of lysis buffer [0.1 mM HEPES (pH 7.9) 1 mM KCl, 0.02 µM EDTA, 0.04 µM EGTA, 1 mM DTT, 1 µg/ml aprotinin, 1 µg/ml leupeptin, and 0.5 mM phenylmethylsulfonyl fluoride]. After 15 min, 25 µl of 10% of NP40 was added and vigorously vortexed for 10 s. After centrifugation (13,000 × g), nuclear pellets were resuspended in 50 µl of ice-cold hypotonic lysis buffer [20 mM HEPES (pH 7.9), 0.4 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 µg/ml aprotinin, 1 µg/ml leupeptin, and 0.5 mM phenylmethylsulfonyl fluoride]. Six µg of nuclear protein extract were incubated at 4°C with 1 µg of poly(deoxyinosinic-deoxytidylic acid) in 10 µl of buffer reaction [24 mM HEPES (pH 7.8), 120 mM KCl, 4 mM MgCl₂, 0.24 mM EDTA, 0.6 mM DTT, 0.6 mM phenylmethylsulfonyl fluoride, and 24% glycerol] and 0.1 µg of salmon sperm DNA for 15 min. Wild-type or mutant probes were then added, and after 20 min of incubation at room temperature, the reaction was stopped with loading buffer and resolved on a 5% acrylamide gel at 100 V for 4 h at 4°C. Gels were subsequently dried and exposed to autoradiograph film. For Sp1 supershift assays, 0.4 µg of Sp1 (PEP-2)-specific antibody (Santa Cruz Biotechnology, Inc.) was added to the binding reaction before the addition of radiolabeled probe for 15 min.

**Immunofluorescence Studies.** HaCaT cells were seeded on glass coverslips and serum starved for 16 h. HaCaT cells were exposed to vehicle alone or to 10 µM perifosine for 1 h. Also, HaCaT cells were preincubated with 50 µM PD98059 for 15 min before perifosine exposure (for a total of 75 min). Cells were washed twice with 1× PBS, fixed, and permeabilized with 4% formaldehyde and 0.5% Triton X-100 in 1× PBS for 10 min. After washing with PBS, cells were blocked with 1% BSA and incubated with the indicated primary antibodies for 1 h, rabbit polyclonal anti-phospho-Thr453 (1:120), rabbit polyclonal anti-phospho-Thr739 (1:120; Ref. 29), or Sp1 (PEP-2; 1:100; Santa Cruz Biotechnology). After an incubation period of 1 h, cells were washed three times with 1× PBS, then incubated additionally for 1 h with the corresponding secondary antibodies (Cy-3-conjugated donkey antirabbit; Jackson Immunoresearch Laboratories, Inc., West Grove, PA; 1:500). Coverslips were washed three times, mounted in Vectashield mounting medium with 4',6-diamidino-2-phenylindole (Vector Laboratories, Inc., Burlingame, CA), and viewed using a Leica TCS-SP2 confocal system (Heidelberg, Germany).

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Perifosine Increases p21waf1/cip1 mRNA and Protein Levels in HaCaT Cells. We demonstrated previously that perifosine promotes cell cycle arrest by inducing p21waf1/cip1 protein in a p53-independent fashion (9). To further study the mechanism by which perifosine induces p21waf1/cip1 accumulation, we used HaCaT cell lines, an immortalized human keratinocyte cell line with deficient p53 function, as a model (30). Initial dose response analysis performed in HaCaT cells demonstrated that maximal activation of p21waf1/cip1 protein was reached after a 12-h treatment with 10 μM perifosine (Fig. 1A). Time response analysis in the same cell line showed that 10 μM perifosine promotes a significant accumulation of p21waf1/cip1 protein after 6 h of treatment (Fig. 1B). To determine whether perifosine-induced accumulation of p21waf1/cip1 protein occurred at the mRNA level, we performed Northern blot analyses in parallel samples. As shown in Fig. 1C, p21waf1/cip1 mRNA was induced significantly by perifosine at concentrations ≥3 μM at 6 h of treatment, reaching ~2.5-fold induction with 10 μM at 12 h of exposure, suggesting that perifosine accumulates p21waf1/cip1 protein by increasing p21waf1/cip1 mRNA levels. To determine whether perifosine-induced accumulation of p21waf1/cip1 was due to post-transcriptional control, we conducted pulse-chase analyses in the presence of actinomycin D and measured p21 mRNA by reverse transcription-PCR. We observed that p21 mRNA expression decreased to a similar extent in both vehicle and perifosine up to 8 h after actinomycin D exposure (17% ±2 and by 12% ±1, respectively). These experiments demonstrated that the p21waf1/cip1 induction by perifosine cannot be explained by p21 mRNA stabilization, suggesting that the accumulation of p21waf1/cip1 mRNA by perifosine is mainly due to transcriptional effects.

Perifosine Activates the p21waf1/cip1 Promoter by a p53-Independent Mechanism. To investigate whether the increase in p21waf1/cip1 mRNA by perifosine is due to transcriptional activation of the p21waf1/cip1 promoter, we transiently transfected HaCaT cells with wild-type and several p21waf1/cip1 promoter-driven luciferase mutant reporter plasmids (26, 31). Twelve h after transfection, HaCaT cells were exposed to perifosine for 18 h, and luciferase activity was measured as described in “Materials and Methods” (26). As shown in Fig. 2, perifosine significantly enhanced the transcriptional activity of the full-length (2.4 kb) p21 promoter (p21P-luc) by 6-fold; similar activation (~10-fold) was observed when cotransfected Ras V12 (the activated form of Ras) was used as a positive control (26). To investigate whether the transcriptional activation of p21waf1/cip1 by perifosine is related to p53 function, we transfected the p53-mutant HaCaT cell lines with the p21PAP53-luc reporter, a p21waf1/cip1 reporter construct lacking 250 bp from the 5’ end corresponding to a p53 consensus DNA-binding site. Perifosine activates this construct (~9-fold) despite the lack of the p53 consensus site, indicating that neither the presence of p53 binding sites in the p21waf1/cip1 promoter nor the presence of p53 protein was required for perifosine to activate the p21waf1/cip1 promoter.

Sp1 Sites in the Minimal p21 Promoter Are Required for Perifosine Effects. To determine the minimal promoter region required for the transcriptional activation of p21waf1/cip1 by perifosine, additional promoter deletion constructs were tested. We initially used the p21PSma-luc construct (26, 31), which contains the p21waf1/cip1 promoter sequence from base −111 through the transcriptional initiation site. Of note, this construct contains four consensus Sp1-binding sites, and this is the minimal promoter region activated by Ras (26). Sp1 is a member of a multigene family that binds DNA through COOH-terminal zinc-finger motifs and is important for the transcription of multiple cell cycle genes, including p21waf1/cip1 (32, 33). As shown in Fig. 2, perifosine significantly activated this minimal promoter (~20-fold), similar to Ras V12. Moreover, when HaCaT cells were transfected with full-length p21waf1/cip1 construct lacking the Ras minimal promoter region (p21PSmaA1-luc), the induction was lost for both Ras V12 and perifosine. Of note, induction of p21PSMA-luc was also observed when edelfosine and miltefosine were used as controls (data not shown). Thus, the minimal p21waf1/cip1 promoter region for ALK5 is similar to that of Ras and seems to be represented by a region in the p21waf1/cip1 promoter proximal to the Smad site at −111 (p21PSma-luc).

To additionally define the cis-acting elements in the p21waf1/cip1 minimal promoter required for perifosine induction, we tested a series of mutant Sp1 constructs (p21 93-S-luc) as described in “Materials and Methods.” The “wild-type” p21P93-S-luc reporter plasmid contains the promoter sequence between −93 and −34, and spans four Sp1-binding sites: Sp1 site 1 (−84 to −79) and Sp1 site 2 (−70 to −65) are independent binding sites, whereas Sp1 site 3 (−60 to −55)
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Fig. 3. Sp1 DNA-binding sites in the p21WAF1/CIP1 promoter are required for the activation by perifosine. HaCaT cells were transfected with the minimal promoter region (p21P93-S-luc) and deletion/mutant p21WAF1/CIP1 minimal promoter constructs (p21P93-SM2-luc, p21P93-SM2.2-luc, p21P93-SM3-luc, p21P93-SM4-luc, p21P93-SM5-luc; see “Materials and Methods”), exposed to 10 μM perifosine for 18 h, then harvested and assayed for luciferase activity. As a positive control, cells were cotransfected with expression vector for the activated forms of Ras (Ras V12), as indicated. Histograms represent the mean luciferase activity in each sample, normalized to the corresponding efficiency of transfection and protein expression, and expressed as fold induction relative to controls for each reporter, of which the values were taken as 1. All of the values are the average of triplicate samples from a typical experiment, bars, ±SE. In each case, similar results were obtained in three additional experiments. ◊, scrambled Sp1 site; □, point mutation in site #2; ◯, vehicle treatment; ■, Ras; □, perifosine; Luc, luciferase; Sp1, Sp1 DNA consensus sites.

Fig. 4. Activation of p21WAF1/CIP1 promoter and p21WAF1/CIP1 protein expression induced by perifosine is dependent on the activation of mitogen-activated protein kinase (MAPK) pathway. Activation of MAPK activity by perifosine in HaCaT cells. A, endogenous extracellular signal-regulated kinase (ERK) 1/2 activity was analyzed by in vitro kinase assay (“kinase”) or using specific anti-phospho MAPK antibodies, as indicated (B). Serum-starved HaCaT cells were exposed to increasing concentrations of perifosine for 30 min (A) and/or with 10 μM perifosine for increasing time periods (B). Equal expression of ERK1/2 in the different samples was also confirmed. C, stimulation of p21WAF1/CIP1 promoter transcriptional activity in HaCaT cells transfected with the p21WAF1/CIP1 promoter reporter plasmid (pSMA-Luc) and treated with perifosine (Lanes 2–4), with PD98059 (Lanes 9), or pretreated with PD98059 (50 μM) 20 min before perifosine exposure (Lanes 4). Moreover, HaCaT cells were cotransfected with an expression vector for the dominant-negative form of mitogen-activated protein/ERK kinase (MEKAA), as indicated. As a positive control, cells were cotransfected with expression vector for the activated forms of Ras (Ras V12). Histograms represent firefly luciferase activity in each sample, normalized for the corresponding efficiency of transfection and protein expression, and expressed as fold induction relative to controls for each reporter, of which the values were taken as 1. All values are the average of triplicate samples from a typical experiment; bars, ±SE. In each case, similar results were obtained in three additional experiments. Luc, luciferase; MBP, myelin basic protein; S, serum stimulation. Ab, no antibody. D, induction of p21 protein expression is blunted by MEK inhibition. Serum-starved HaCaT cells were exposed to perifosine in the presence (or absence of) PD98059 and protein lysates were obtained for Western blots against the indicated antibodies. Equal loading expression in the different samples was confirmed by Western blots against HSP90.
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Fig. 5. Perifosine promotes increased Sp1 DNA binding by increased Sp1 phosphorylation. A, nuclear extracts prepared from perifosine-treated (Lanes 3, 4, and 6), transfected with Ras (Lanes 4, 5, and 9) or untreated HaCaT cells (Lanes 1, 2, and 8) were incubated with an end-labeled Sp1-binding sequence oligonucleotides wild-type or mutant probes as described in “Materials and Methods.” In Lanes 2, 4, and 6, nuclear extracts were preincubated with Sp1 antibody and supershift bands were observed. B, nuclear extracts prepared from untreated HaCaT cells (Lanes 1 and 4), treated with perifosine (Lanes 3 and 6), and with an expression vector for the activated forms of Ras (Ras V12; Lanes 2 and 5) were incubated with an end-labeled Sp1-binding sequence oligonucleotides wild-type. In Lanes 4 through 6, phosphatase treatment (PP2A) of nuclear extracts occurred before incubation with end-labeled Sp1 oligonucleotide. Retention signals due to binding of Sp1 and Sp3 signals are indicated.

exposed to perifosine with a radiolabeled Sp1 response element containing oligonucleotide. As shown in Fig. 5, we were able to detect several protein-DNA binding complexes, which were undetected when a mutant Sp1 oligonucleotide was used in the binding reaction, indicating that these complexes contained specific proteins binding to the Sp1 consensus site. Supershift assays using specific Sp1 antibody confirmed that the slowest migrating complex represents Sp1 (Fig. 5, Lane 2). Of note, this band was not visualized when an irrelevant IgG was used (data not shown). Moreover, treatment with perifosine or overexpression of Ras V12 increased the specific DNA-binding activity of Sp1, as demonstrated by the supershift of the Sp1 complexes (compare Lanes 2, 4, and 6 in Fig. 5A). Of note, several bands in both perifosine- and Ras-treated cells remained unaltered when incubated with specific Sp1 antibodies. Moreover, they were not present when nuclear extracts were incubated with mutant Sp1 oligonucleotides. These bands, as previously reported elsewhere in the case of Ras, represent increased binding to Sp3, a transcriptional factor that binds to DNA using the same DNA-binding consensus sequence as Sp1 (26). Taken together, these results suggest that perifosine, similar to Ras, activates \( p21^{\text{waf1/cip1}} \) transcription by increasing the binding of the Sp1 transcription factor to the minimal promoter region of the \( p21^{\text{waf1/cip1}} \) promoter.

To additionally investigate whether perifosine-induced Sp1 DNA-binding activity was due to Sp1 phosphorylation, we incubated the nuclear extract with the serine-threonine phosphatase PP2A before the binding reaction. As observed in Fig. 5B, the increased Sp1 DNA-binding activity provoked by perifosine was blunted in the samples treated with PP2A. These data suggest that perifosine activates \( p21^{\text{waf1/cip1}} \) transcription by increasing Sp1 phosphorylation and consequent Sp1 DNA-binding activity.

Perifosine-Induced Sp1 DNA Activity Requires the Phosphorylation of Sp1 by MEK/ERK Pathway. Previous studies using Ras and/or activators of the MEK/ERK pathway have demonstrated that phosphorylation of Sp1 increased its transactivation capacity (39, 40). A recent study demonstrated that Ras induces the phosphorylation of Sp1 at two specific sites, threonine 453 and 739, and this phosphorylation increases Sp1 transactivation activity (29). To determine whether perifosine can promote the phosphorylation of Sp1 in these MAPK-specific sites, nuclear extracts obtained from HaCaT cells exposed to perifosine were immunoblotted with Sp1 phosphospecific antibodies. As clearly shown in Fig. 6A, increased Sp1 phosphorylation at residue threonine 453 (a known MAPK site) was observed within 60 min of perifosine incubation. Moreover, preincubation of perifosine-treated cells with PD98059 (a known MEK inhibitor) demonstrated loss in Sp1 phosphorylation, suggesting that the increased phosphorylation induced by perifosine in this particular site is due to MEK activation. As a loading control, we used lamin C, an intermediate filament protein localized in the nucleus (41).

To determine whether the other known MAPK site, Sp1 threonine 739, was phosphorylated by perifosine, HaCaT cells were exposed to perifosine, and Sp1 phosphorylation was monitored by immunofluorescence with phosphospecific Sp1 antibodies. Again, a specific increase in Sp1 phosphorylation was evident in threonine 739 (Fig. 6B, left panels) with perifosine treatment. Similar effects were observed with antibodies against Sp1-T453 (Fig. 6A). In contrast, total Sp1 levels were not significantly changed with perifosine treatment as evaluated by immunofluorescence (Fig. 6B, right panels). As a positive control, cells were transfected with an expression vector for Ras V12. Increased phosphorylation at both sites was observed, as has been reported previously (data not shown; Ref. 29). Preincubation of HaCaT cells with PD98059 followed by perifosine treatment revealed a decrease in Sp1 phosphorylation at both sites (data not shown), indicating that the Sp1 phosphorylation induced by perifosine was dependent on ERK activation.

We then examined whether the increased Sp1 DNA-binding activity by perifosine is mediated by the MEK/ERK pathway. To this end, nuclear extracts obtained from HaCaT cells exposed to perifosine in the presence of MEK blockers (PD98059 or MEKAA) were used in

Fig. 6. MAPK activation by perifosine is required for Sp1 phosphorylation and increased Sp1 DNA binding to the \( p21^{\text{waf1/cip1}} \) promoter. A, Western blotting from serum-starved HaCaT cells untreated (Lane 1) or treated with perifosine (Lanes 2, 3, and 5), treated with 50 \( \mu \text{M} \) PD98059 (Lane 4), pretreated with 50 \( \mu \text{M} \) PD98059 20 min before perifosine (Lane 3), or lysates treated with PP2A (Lanes 5 and 6) were performed using specific anti-phospho Sp1 threonine 453 site (P-453) and total Sp1. Equal loading expression in the different samples was confirmed by Western blots against laminin C. B, immunofluorescence studies of untreated HaCaT cells (left panel) or HaCaT cells treated with perifosine. Sp1 threonine 453 site (P-453), anti specific anti-phospho Sp1 threonine 739 site (P-739), and total Sp1 were used (data not shown; Ref. 29). Preincubation of HaCaT cells with PD98059 followed by perifosine treatment revealed a decrease in Sp1 phosphorylation at both sites (data not shown), indicating that the Sp1 phosphorylation induced by perifosine was dependent on ERK activation.

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the binding reactions. As demonstrated in Fig. 6C, the increase in Sp1 binding activity induced by perifosine was blunted by the presence of either PD98059 or MEKAA, indicating that the increased Sp1 DNA-binding activity promoted by perifosine occurs as a result of activation of MEK/ERK leading to Sp1 phosphorylation.

In summary, the ALK perifosine promotes cell cycle arrest as a result of transcriptional activation of p21 waf1/cip1. This effect occurs because of activation of the MEK/ERK pathway, and this enhanced activity promotes the specific phosphorylation of Sp1 protein, thereby increasing Sp1 DNA binding to the p21 waf1/cip1 promoter.

**DISCUSSION**

We demonstrated that ALKs, a novel class of small-molecule cell cycle modulators, promote cell cycle arrest by transcriptional activation of p21 waf1/cip1. Detailed studies of the p21 waf1/cip1 promoter revealed that the minimal promoter region required by ALKs is similar to the region modulated by Ras. Furthermore, the activation of the minimal promoter region requires MAPK activation, which, in turn, phosphorylates the transcription factor Sp1, thereby promoting increased Sp1 DNA-binding activity to the p21 waf1/cip1 promoter.

Initial efforts from our laboratory demonstrated that ALKs promote G1 and G2/M arrest in tumor cell lines because of up-regulation of p21 waf1/cip1. The endogenous cdki inhibitor p21 waf1/cip1 is a known universal cdki inhibitor of which the induction may lead to several phenotypes, including cell cycle arrest, cellular senescence, apoptosis, and differentiation (14, 15, 16, 42–50). Perifosine induced accumulation of p21 protein at the transcriptional level by an increase in mRNA message, not by an increase in p21 mRNA stability. Studies using full-length and deletion mutant p21 waf1/cip1 luciferase reporters demonstrated a significant activation of the full-length 2.4-kb (p2IP-luc) and the construct lacking p53-binding sites, suggesting that the transcriptional activation of p21 waf1/cip1 by ALKs was independent of p53 function. Suprisingly, the minimal p21 promoter region is the same minimal region (pSMA-luc) required by the proto-oncogene Ras to induce p21 waf1/cip1 promoter (26, 31). To test whether the p21 waf1/cip1 transcriptional activation by perifosine occurred in other cell types, we transfected pSMA-luc into HCT116 isogenic cell lines (wild-type or p53 null cells). Again, perifosine activated pSMA-luc in both cell lines (data not shown), demonstrating that the induction of the p21 minimal promoter by perifosine occurred in other cell types and was also independent of p53 function. Interestingly, the induction of pSMA-luc by Ras or perifosine appeared to be higher than the full-length construct (p21P-luc). This higher activation could be explained by the presence of a distal negative regulatory region in the p21 promoter for both Ras and ALKs. We are currently studying the cause of this event.

The pSMA-luc construct contains several Sp1-binding sites that play a major role in the transcriptional regulation of p21 waf1/cip1 by Ras (26, 31). Mutational analysis of this minimal promoter demonstrated that mutations in each Sp1 site had a profound effect on p21 transcription by perifosine, similar to Ras. Taken together, these results suggest a critical role of Sp1 in p21 waf1/cip1 activation by perifosine, as has been observed previously with Ras (26).

The proto-oncogene Ras is a known activator of prototypical MAPK pathways, ERK1 and ERK2 (51, 52). Activated ERK, in turn, phosphorylates multiple transcription factors, thereby enhancing its transcriptional activation (53). On the basis of the similar effects of ALKs and Ras on the p21 waf1/cip1 promoter, we determined the effects of perifosine on MEK activation. A very potent activation of the MEK/ERK pathway was observed within minutes of perifosine exposure. Moreover, this activation preceded the p21 waf1/cip1 mRNA accumulation by perifosine. The role of MEK activation in p21 waf1/cip1 induction by perifosine was analyzed using dominant-negative alleles and/or chemical inhibitors of MEK (PD98059). Blockade of MEK clearly blunt the p21 waf1/cip1 promoter induction. Thus, perifosine mediated the transcriptional activation of the p21 waf1/cip1 promoter by activation of MEK, similar to Ras. Moreover, MEK blockade blunted the induction of p21 waf1/cip1 at the protein level, indicating that MAPK activation induced by perifosine was required for p21 accumulation. Interestingly, addition of the chemical MEK inhibitor PD98059 to HaCaT cells somewhat increased p21 waf1/cip1 protein expression. This phenomenon may be explained by the lack of specificity of chemical inhibitors in general (54). Thus, it is possible that PD98059 may inhibit, in addition to MEK, a kinase relevant for p21 waf1/cip1 expression. To explore how MAPK activation leads to an increase in p21 waf1/cip1 expression, gel-shift studies demonstrated that perifosine increased Sp1 DNA-binding activity. Moreover, MEK ablation by PD98059 and/or dominant-negative MEK prevented increased Sp1 DNA-binding activity by perifosine. Taken together, the transcriptional transactivation of the p21 waf1/cip1 promoter by perifosine required MEK activation.

Sp1 is a member of a multigene family that binds DNA through COOH-terminal zinc-finger motifs (32, 33). Sp1 is a ubiquitous transcription factor that has been implicated in the activation of many genes (32, 55, 56). Although Sp1 activity was initially thought to be constitutive, it has been shown that it can be regulated at different levels (20, 57–60). Sp1 can be phosphorylated (a modification that affects its binding to the DNA; Refs. 29, 39, 57, 58) and O-glycosylated (a modification that confers resistance to proteosome-dependent degradation; Ref. 61). Milanini-Mongiat et al. (29) demonstrated recently that Ras modulates the transcription of the vascular endothelial growth factor promoter by the phosphorylation of Sp1 at two specific sites (threonines 453 and 739) by activation of the MEK/ERK pathways. On the basis of the potent activation of MEK/ERK by perifosine, we asked whether perifosine modulates Sp1 phosphorylation. As expected, increased phosphorylation was demonstrated at both phosphorylation sites by Western blotting and/or immunofluorescence. Moreover, the increased specific phosphorylation and enhanced Sp1 DNA-binding activity induced by perifosine was blunted with pretreatment with the MEK inhibitor PD98059 and/or incubation of lysates with the serine-threonine phosphatase PP2A. Thus, it appears that activation of the MAPK pathway not only regulates the phosphorylation of Sp1 but also mediates the enhanced DNA-binding activity and increases transcriptional p21 waf1/cip1 promoter activation induced by perifosine. Experiments in our laboratory are being undertaken to elucidate this novel mechanism.

Notably, it appears that the increased Sp1 phosphorylation coincides with an increase in Sp1 protein, and this increase appears to be partially mediated by activation of MEK because PD98059 can, to some extent, blunt the induction of Sp1 by perifosine. Noe et al. (39) demonstrated that acute exposure (~15 min) of Chinese hamster ovary cells to 12-O-tetradecanoylphorbol-13-acetate, a known activator of MEK/ERK pathway (62), promotes increase in Sp1 mRNA and protein levels, leading to increased Sp1 DNA-binding activity to the dihydrofurole reductase promoter. Thus, our observations confirm separate observations by other investigators (29, 39, 40) that the activation of MEK/ERK pathways by different stimuli promote the increase in Sp1 expression, phosphorylation, and DNA-binding activity, thereby enhancing Sp1 transcriptional activation. In our study, MEK blockade prevented p21 transactivation/expression, decreased Sp1 DNA binding, and decreased Sp1 phosphorylation with perifosine exposure. Thus, these data indicate that the activation of MAPK by ALKs is required for all of the events necessary for p21 transactivation.

Although Sp1 is a transcriptional factor involved in the transcrip-
tion of several genes, we demonstrated the specificity and requirement of Sp1 in p21 transcription by ALKs using several strategies: first, we showed that the minimal promoter region of the p21 promoter (~60 bp) is composed of several Sp1 sites that, when mutated, render the promoter inactive; second, perifosine promoted a specific increase in Sp1 DNA-binding activity at concentrations and times required for p21 up-regulation; third, phosphorylation of Sp1 at specific MAPK-dependent sites was required for p21 transcription; and fourth, MEK blockade promoted loss in p21 transcription that is associated with loss in Sp1 phosphorylation and loss in Sp1 DNA binding.

One intriguing detail to note is the ability of ALKs to activate the MEK/ERK MAPK pathway. It is counterintuitive that a small molecule that belongs to a known class of antiproliferative agents, ALKs, may have the capacity to activate a proliferative pathway such as the MEK/ERK MAPK. However, other antiproliferative agents, including paclitaxel and cisplatin, do activate MEK/ERK MAPK pathways (63, 64). Furthermore, Ras (a known activator of MEK/ERK) itself can modulate several signal transduction pathways, including proto-oncogenes or drugs can, in some circumstances, due to activation of the MEK/ERK pathways (65, 64). Ras (a known activator of MEK/ERK) itself can activate several signal transduction pathways. Ras induces p21Cip1/Waf1 cyclin kinase inhibitor transcriptionally through Sp1 binding sites. Oncogene, 12: 2805–2809, 1999.

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Transcriptional Activation of $p21^{\text{waf1/cip1}}$ by Alkylphospholipids: Role of the Mitogen-Activated Protein Kinase Pathway in the Transactivation of the Human $p21^{\text{waf1/cip1}}$ Promoter by Sp1

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