Treatment of a Human Papillomavirus Type 31b-Positive Cell Line with Benzo[a]Pyrene Increases Viral Titer through Activation of the Erk1/2 Signaling Pathway[▽]

Brian S. Bowser, Samina Alam, and Craig Meyers*

*Department of Microbiology and Immunology, Pennsylvania State University College of Medicine,
500 University Drive, Hershey, Pennsylvania 17033*

Received 19 January 2011/Accepted 18 February 2011

Numerous epidemiological studies have implicated cigarette smoking as a cofactor in the progression to cervical cancer. Tobacco-associated hydrocarbons have been found in cervical mucus, suggesting a possible interaction with human papillomavirus (HPV)-infected cells. The polycyclic aromatic hydrocarbon benzo[a]pyrene (BaP) is a major component of cigarette smoke condensate that has received significant attention due to its ability to induce carcinogenesis. We have previously demonstrated by conventional methods for determining viral titer that high concentrations of BaP increase HPV31b titers within the context of organotypic raft cultures compared with the level for vehicle controls. However, a definitive mechanism for explaining this increase in viral titer was lacking. Here, we show that BaP treatment activates the Ras-Raf-Mek1/2-Erk1/2 signaling pathway. The importance of Erk1/2 pathway activation to the BaP-mediated increase in viral titer was determined by Erk pathway inhibition with multiple Erk1/2 pathway inhibitors. Finally, BaP treatment activated p90RSK and its downstream target CDK1. These data indicate that the Erk1/2 signaling pathway plays an important role in mediating the response to BaP treatment that ultimately leads to increased viral titers.

Cervical cancer is the third most common female cancer and is the second most common cause of cancer-related deaths among women (67). More than 90% of all cervical cancer cases are associated with human papillomavirus (HPV) infection (76, 88–90). Papillomavirus infections are established in basal epithelial cells, where the viral genome is maintained as an episome, tethered to the host genome (22, 80, 86). HPV genome replication, gene expression, and virion synthesis are tightly linked to the differentiation state of the infected cell (51, 57, 61). The progression to cervical cancer is associated with the integration of high-risk HPV genomes into host chromosomes (18, 30). However, only a small percentage of women infected with high-risk HPV types (such as HPV16, HPV18, and HPV31) actually progress to cervical cancer (7, 21), suggesting a role for cofactors in the progression to cervical cancer.

Ample epidemiological evidence exists to implicate cigarette smoking as a cofactor with HPV infection in cervical cancer progression (4, 24, 43, 55, 70, 82, 85). Polycyclic aromatic hydrocarbons (PAHs) are among the numerous carcinogens associated with cigarette smoking. Benzo[a]pyrene (BaP) is a PAH that is present at roughly 8 to 25 nanograms per cigarette (27) and is found at elevated levels in the cervical mucus of women who smoke (48). BaP is metabolized via cytochrome P450 enzymes to intermediates or metabolites, some of which have the potential to bind to DNA, forming DNA adducts (16,

72). Unrepaired DNA adducts represent an important initiator of carcinogenesis (27, 73). PAHs like BaP have also been shown to increase cell proliferation, likely through the activation of specific signaling pathways (8, 31, 62, 74, 75). BaP has been shown to activate both mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3 kinase (PI3K)/Akt signaling pathways (8, 9, 42, 60).

MAPK signaling is involved in the regulation of cell proliferation, apoptosis, and differentiation. MAPK signals are propagated through the sequential phosphorylation and activation of a MAPKKK, MAPKK, and MAPK. Activated MAPKs subsequently phosphorylate downstream substrates to affect numerous cellular functions. Four major MAPK signaling cascades exist to respond to various stimuli. Of these MAPK signaling cascades, Erk1/2 has been studied the most extensively as a target of BaP. Several studies have demonstrated activation of the Erk1/2 pathway by BaP in multiple cell types over a range of concentrations from 0.36 to 3 μ M (8, 42, 60). Given this information, we studied the significance of Erk1/2 pathway activation by BaP to the HPV viral life cycle.

Erk1/2 pathway activation leads to the activation of a large number of transcription factors, p90RSK, and antiapoptotic factors (19, 26, 41, 79). Once activated, p90RSK regulates transcription through the activation of a panel of transcription factors (12, 14, 20, 53, 81), regulates translation through the activation of the mammalian target of rapamycin (mTOR) pathway (10, 65), and regulates the cell cycle (23, 59). Cell cycle regulation by p90RSK occurs through the modulating activity of factors such as CDK1 and p27. Activation of p90RSK leads to the downregulation of Myt1 and subsequent activation of CDK1-cyclin B complexes (59), while p90RSK-mediated phosphorylation of the CDK inhibitor p27KIP1 in-

* Corresponding author. Mailing address: Department of Microbiology and Immunology, H107, The Pennsylvania State College of Medicine, 500 University Drive, Hershey, PA 17033. Phone: (717) 531-6240. Fax: (717) 531-4600. E-mail: cmm10@psu.edu.

[▽] Published ahead of print on 2 March 2011.

hibits its translocation to the nucleus and thereby inhibits its cell cycle-inhibitory effects (23).

Despite extensive epidemiological evidence supporting the role of cigarette smoking as a cofactor in cervical carcinogenesis, a mechanistic explanation is still lacking. To understand this correlation, we have explored the effects of cigarette smoke carcinogens on the HPV life cycle. Specifically, we have focused our analyses on abundant cigarette smoke carcinogens with known tumorigenic potential. Given that BaP is abundant in cigarette smoke and is found concentrated in the cervical mucus of women who smoke, we targeted our early studies on BaP. Detailed studies for measuring the amount of BaP in cervical mucus have not been reported; however, the concentration of other cigarette smoke carcinogens in the cervical mucus has been determined and may be used to estimate an amount of BaP that is physiologically relevant. The carcinogen NNK has been measured as high as 0.56 μM , nicotine has been measured at 3 μM , and cotinine has been measured at 0.36 μM (63, 66). Based on this information, the HPV-positive cell line CIN-612 9E was treated with BaP over a range of concentrations from 0.001 to 10 μM . This study demonstrated that BaP affected multiple steps of the HPV life cycle (2). Treatment of CIN-612 9E cells with low concentrations of BaP (0.001 μM) promoted a 2-fold increase in genome amplification, while treatment with higher concentrations of BaP (1.0 μM) increased viral titers roughly 10-fold and increased the abundance of the major capsid protein L1 (2). We also found that treatment with 1.0 μM BaP treatment increased CDK1 kinase activity (1). Inhibition of CDK1 kinase activity with purvalanol A dramatically decreased HPV infectivity in BaP-treated raft cultures but had no effect on viral genome equivalents (1). These data implicated CDK1 activity in infectivity. As a whole, the data indicated that treatment of an HPV-positive cell line with physiologically relevant concentrations of BaP could affect both the virus life cycle and the cellular environment.

Here, we explored the signaling pathways that give BaP the ability to increase HPV31b titers. We demonstrated Erk1/2 signaling pathway activation by BaP in our organotypic raft culture system. We also demonstrated that inhibition of the Erk1/2 signaling pathway significantly reduced HPV31b infectivity, indicating that the biological endpoint of Erk1/2 pathway activation by BaP is increased infectious viral titers. However, the activation of the Erk1/2 pathway by BaP did not directly affect viral gene expression but instead altered the infectivity of virus particles through CDK1 activation.

MATERIALS AND METHODS

Cell lines. Monolayer cultures of J2 3T3 cells were maintained in Dulbecco's modified Eagle's medium with 10% newborn calf serum and gentamicin. The cell line CIN-612 9E was generated from a cervical intraepithelial neoplasia type I (CINI) biopsy specimen and maintains approximately 50 episomal copies of the HPV31b genome per cell (5). CIN-612 9E cells were maintained in E medium with 5% fetal bovine serum in the presence of mitomycin C-treated J2 3T3 feeder cells, as described previously (50). Organotypic raft cultures of CIN-612 9E cells were grown as described previously (50). Briefly, 2×10^5 CIN-612 9E cells were plated on J2 3T3 collagen matrices then lifted to the air-liquid interface. Raft cultures were fed with E medium with 5% fetal bovine serum and 10 μM 1,2-dioctanoyl-sn-glycerol (C8) through the dermal equivalent once every 2 days.

Measurement of HPV31b viral titer. HPV titers were determined by quantitative reverse transcription-PCR (RT-PCR), as we have previously described (17). Briefly, CIN-612 9E organotypic raft cultures were grown for 12 days and treated with 1.0 μM BaP or an equivalent concentration of dimethyl sulfoxide

TABLE 1. Densitometric analysis of Western blot data^a

Protein	Fold increase	SE
L1	2.2	0.6
pErk1/2	3.2	0.9
pMek1/2	2.4	0.5
Total Mek1/2	1.8	0.5

^a Data represent fold increases in protein abundance with BaP treatment compared with the levels for the DMSO controls.

(DMSO) as a vehicle control. Virus was harvested and used to infect HaCaT cells for 48 h. Total RNA was isolated from the infected HaCaT cells by use of a SurePrep TrueTotal RNA purification kit (Fisher BioReagents). Reverse transcription and cDNA quantitation were performed in the same reaction using a Quantitect probe RT-PCR kit (Qiagen) with the primer set consisting of 31E15'qPCR (5'-TGG CTG ATC CAG CAA GTG AC-3') and 31E43'qPCR (5'-AGG CGC AGG TTT TGG AAT TC-3'). Detection of HPV31b E1'E4 cDNAs was performed using the probe 31E1'E4 (5'-CAA AGC TAC CAA CAG CCA ACA ACA-3'). TATA binding protein (TBP) transcripts were measured as described previously (17) and used to normalize E1'E4 transcript levels from sample to sample. The amplification of each sample was performed in duplicate, and each titer-determining experiment was performed at least three times.

Western blot analysis. Total protein extracts were prepared as follows. For each sample, one frozen organotypic raft tissue was thawed on ice and then Dounce homogenized in 400 μl of buffer A (50 mM NaPO₄ [pH 7.2], 5 mM EDTA, 50 mM NaF, 0.5 $\mu\text{g/ml}$ leupeptin, 0.5 $\mu\text{g/ml}$ pepstatin, 20 $\mu\text{g/ml}$ aprotinin, 0.2 mM Na₃VO₄, 1 mM dithiothreitol [DTT], 1 mM phenylmethylsulfonyl fluoride). To this was added 400 μl of buffer B (50 mM Na₃PO₄ [pH 7.2], 5 mM EDTA, 2% [wt/vol] sodium dodecyl sulfate [SDS], 50 mM NaF, 0.5 $\mu\text{g/ml}$ leupeptin, 0.5 $\mu\text{g/ml}$ pepstatin, 20 $\mu\text{g/ml}$ aprotinin, 0.2 mM Na₃VO₄, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride), and this was boiled in a water bath for 8 min. Supernatants were collected by centrifugation at $13,000 \times g$ for 5 min at room temperature. A 500- μl aliquot of each sample was added to 125 μl of 5 $\times$ sample loading buffer (100 mM Tris, pH 8.0, 5 mM EDTA, 10% [wt/vol] sucrose, 2% [wt/vol] SDS, 33 mM DTT, and 0.1 mg/ml pyronin Y). Protein concentrations were determined by the Peterson protein assay as described previously (49). Western blot analyses were performed as described previously (3). The primary antibodies used were as follows: HPV16 L1 D9 primary antibody (ND Christensen), mouse monoclonal anti-HPV16 E7 (Santa Cruz), rabbit monoclonal anti-Ras (27H5) (Cell Signaling), rabbit polyclonal anti-Raf-1 (C-12) (Santa Cruz), mouse monoclonal anti-MEK1/2 (L38C12) (Cell Signaling), rabbit monoclonal anti-phospho MEK1/2 (Ser217/221) (41G9) (Cell Signaling), rabbit monoclonal anti-p44/42 MAP kinase (137F5) (Cell Signaling), rabbit monoclonal anti-phospho-p44/42 MAPK (Thr 202/Tyr204) (197G2) (Cell Signaling), rabbit polyclonal anti-phospho-p90RSK (Thr573) (Cell Signaling), rabbit monoclonal anti-RSK1/RSK2/RSK3 (32D7) (Cell Signaling), rabbit polyclonal anti-glyceraldehyde-3-phosphate dehydrogenase (anti-GAPDH) (Santa Cruz), and mouse monoclonal antiactin (MP Biomedicals). Each Western blot analysis was performed at least three times. Densitometric analysis was performed on each Western blot using a Bio-Rad G8-800 calibrated densitometer, and the data were analyzed using the Quantity One (version 4.5.0) software package. Fold changes in protein abundance were calculated by dividing the band intensities of BaP-treated samples by the levels for the DMSO controls. The results represent the averages for three independent experiments $\pm$ standard errors of the means (Table 1).

Histochemical analysis of raft tissue. CIN-612 9E organotypic raft cultures were grown for 12 days and were treated with 1.0 μM BaP or an equivalent concentration of DMSO with or without 20 μM U0126 or 50 μM PD98059. The inhibitors were added on days 7 and 8, 9 and 10, or 11 and 12. Raft tissue was harvested, and histochemical analysis was performed as previously described (50).

Transfections and luciferase assays. Luciferase assays were performed to assess the effects of BaP treatment on HPV promoter activity. Luciferase reporter plasmids containing either the late (p742) (pGL2B-630) or the early (p99) and late (pGL2B-1706) HPV31b promoters in the context of the pGL2-Basic luciferase vector were generated previously (6). CIN-612 9E cells were transfected with either HPV31b promoter plasmid or empty pGL2-Basic vector using the FuGENE HD transfection reagent (Roche Applied Science) according to the manufacturer's instructions. The cells were incubated at 37°C for 3 h and then

suspended in 1.6% methylcellulose with 1.0 μ M BaP or an equivalent concentration of DMSO as a vehicle control. The methylcellulose suspensions were incubated at 37°C for 48 h and then harvested for luciferase assays. Cell pellets were analyzed for luciferase activity using the Luciferase Assay system (Promega) according to the manufacturer's instructions. Each experiment was performed in triplicate.

Measurement of L1/L2 transcript abundance. The abundance of HPV 31b L1 and L2 transcripts was determined by quantitative RT-PCR analysis with or without 1.0 μ M BaP treatment. CIN-612 9E cells were grown in the context of organotypic raft cultures for 12 days and treated with 1.0 μ M BaP or an equivalent concentration of DMSO as a vehicle control every 48 h. Thirty milligrams of each raft was Dounce homogenized in 300 μ l of buffer RLT (Qiagen), and RNA was purified using an RNeasy fibrous tissue minikit (Qiagen) according to the manufacturer's instructions. A Roche 1st-strand cDNA synthesis kit was used to convert 100 ng of each total RNA sample into cDNA. Each RNA sample was converted to cDNA in 20 μ l reaction mixtures containing 100 ng total RNA, 1 $\times$ reaction buffer, 5 mM MgCl₂, 4 μ g random primer p(dN)₆, >20 units AMV reverse transcriptase, 0.2 μ g/ μ l gelatin, and 10 mM each deoxynucleoside triphosphate (dNTP). Reverse transcription was performed 25°C for 10 min, 42°C for 60 min, 99°C for 5 min, and 4°C for 5 min. The relative quantities of L1 and L2 cDNAs were determined by quantitative real-time PCR analysis. L1 cDNAs were amplified using the primer set consisting of 31bSYBRL1(5') (5'-CAC TGA AAA CTC TAA TAG ATA TGC CGG TGG-3') and 31bSYBRL1(3') (5'-CTA CAA GGA CTA CCT TTA CCC CAA TGC-3'). L2 cDNAs were amplified using the primer set consisting of 31bSYBRL2(5') (5'-GAC CAC CAG TTA GCA TTG ACC CTG-3') and 31bSYBRL2(3') (5'-CAG TTG TAG CAA TGT CAA ACC CAG ATG TTG-3'). TBP cDNAs were amplified using the primer set consisting of 5'-CAC GGC ACT GAT TTT CAG TTC T-3' and 5'-TTC TTG CTG CCA GTC TGG ACT-3'. Amplification of 1 μ l of each cDNA using each of the above-mentioned primer sets was detected using IQ SYBR green Supermix (Bio-Rad). Thermocycling was performed using a Bio-Rad CFX96 real-time system. The thermocycling profile was as follows: 15 min hot start at 95°C followed by 40 cycles of 15 s at 94°C, 30 s at 52°C, and 30 s at 72°C. Data collection was performed during the extension phase.

Kinase assay. Protein extracts were prepared from raft tissues as described previously (1). Protein concentrations were determined by the Bradford assay. Immunoprecipitations were performed using 200 μ g of each protein extract and 5 μ l anti-cdc2 antibody (Oncogene Research Products), as described previously (3). CDK1 kinase activity was assessed by a kinase assay with histone H1 as a substrate, as described previously (3).

RESULTS

Treatment of HPV-positive organotypic raft cultures with 1.0 μ M BaP increases viral titer. Our previous data for titer determination using endpoint RT-PCR analysis demonstrated that treatment of HPV-positive organotypic raft cultures with 1.0 μ M BaP promotes an increase in viral titer (2, 11). To confirm these data and to quantitate this increase, we performed quantitative RT-PCR analysis, as described previously (17). CIN-612 9E cells were grown for 12 days in organotypic raft cultures treated with 1.0 μ M BaP or an equivalent concentration of DMSO as a vehicle control. Virus particles were isolated from the raft tissue and were used to infect HaCaT cells for 48 h. RNA isolated from the infected HaCaT cells was reverse transcribed into cDNA, and the abundance of E1⁺E4 transcripts was measured by quantitative RT-PCR. This measurement demonstrates the infectivity of the viruses generated in the raft cultures rather than a direct effect of BaP upon E1⁺E4 transcription. E1⁺E4 transcript abundance was normalized to TATA binding protein transcript levels and is represented as fold increase over the level for the DMSO control (Fig. 1A). These data indicate that treatment with 1.0 μ M BaP leads to a 6-fold increase in HPV titer (Fig. 1A).

We have previously demonstrated that BaP treatment increases the expression of the HPV31b major capsid protein L1 (2). We have extended this analysis to include the early protein

FIG. 1. (A) Quantitative RT-PCR infectivity assay. CIN-612 9E organotypic raft cultures were grown for 12 days and were treated with 1.0 μ M BaP or an equivalent concentration of DMSO, and virus titers were measured by a quantitative RT-PCR infectivity assay. Data are presented as fold increases in E1⁺E4 level compared with the level for the DMSO control. (B) HPV31b gene expression. The virus lysates from panel A were used in Western blot analysis with antibodies specific for HPV L1, HPV E7, and actin.

E7 since Maher et al. have recently shown that BaP treatment of Caski cells in monolayer cultures promotes an increase in the expression of E7 protein (45). The virus preparations whose titers are shown in Fig. 1A were processed for Western blot analysis. As shown in Fig. 1B, organotypic raft cultures treated with BaP demonstrated a 2.2-fold increase in L1 expression; however, E7 expression was unchanged. These data indicated that BaP treatment increased L1 expression without a general increase in HPV31b gene expression. The fact that E7 expression was unchanged here but increased in the study by Maher et al. may reflect differences in cell type (Caski versus CIN-612 9E), BaP treatment schedule, and/or the natures of the cell cultures (monolayer versus organotypic rafts). Further analysis will be necessary to understand these differences.

BaP treatment activates the Erk1/2 signaling pathway. To understand how BaP affects viral titer, we focused on known activities associated with BaP. Previous studies demonstrated that BaP activates MAPK signaling. The MAPKs affect many cellular functions, including gene expression, proliferation, and apoptosis. Given its role in regulating gene expression through activation of both transcription and translation factors (19, 36, 47) and considering that BaP has been shown to activate Erk1/2 (8, 60), we focused on the Erk1/2 pathway. Total protein extracts were prepared from 12-day organotypic raft cultures of CIN-612 9E cells grown with medium only or treated with 1.0 μ M BaP or an equivalent concentration of DMSO as a vehicle control. Protein expression and activation of several Erk1/2 pathway factors were assessed by Western blot analysis, and densitometry was performed to quantitate changes in protein abundance. As shown in Fig. 2, treatment of CIN-612 9E raft cultures with BaP increased the abundance of phosphorylated Erk1/2 3.2-fold compared with the level for the DMSO controls despite no change in total Erk1/2 levels, indicating

FIG. 2. Erk1/2 pathway activity. CIN-612 9E organotypic raft cultures were grown for 12 days and were treated with 1.0 μ M BaP, an equivalent concentration of DMSO, or medium only. Cell lysates were generated and used in Western blot analysis with antibodies specific for total Ras, total Raf, total Mek1/2, phospho-Mek1/2, total Erk1/2, phospho-Erk1/2, or actin.

activation of the Erk signaling pathway. Erk1/2 activation occurs in response to the Ras-Raf-Mek-Erk pathway or through cross talk with PKC signaling via PKC α phosphorylation of Raf-1 (35, 46). To determine whether Erk1/2 activation occurs downstream of the Ras-Raf-Mek1/2 pathway, we analyzed the activation of Ras, Raf, and Mek1/2 by Western blotting. As shown in Fig. 2, treatment of CIN-612 9E raft cultures with 1.0 μ M BaP promoted the phosphorylation of Mek1/2 2.4-fold above the level for the DMSO controls and also led to a 1.8-fold increase in the abundance of total Mek1/2. Previous studies have demonstrated stimuli that resulted in changes to the abundance of total Mek1/2 (34, 44, 77); however, Mek1/2 phosphorylation is necessary for the activation of Erk1/2 as seen here and is likely the more significant response to BaP treatment (25). In addition to Mek1/2 activation, BaP treatment also led to increases in the abundances of both Raf-1 and Ras, indicating that the Ras-Raf-Mek1/2-Erk1/2 pathway was activated in response to BaP treatment. These experiments have been repeated multiple times, with reproducible results.

Inhibition of the Erk1/2 signaling pathway inhibits the BaP-mediated increase in viral titer. To measure the significance of Erk1/2 pathway activation in response to BaP treatment, we treated CIN-612 9E organotypic raft cultures with 1.0 μ M BaP as before, while inhibiting Erk1/2 signaling with the pharmacological inhibitor U0126. The concentration of U0126 and the duration of treatment were titrated to achieve the strongest inhibition of Erk1/2 activation without adversely affecting organotypic raft growth. These data revealed that treatment of organotypic raft cultures with 20 μ M U0126 for 48 h significantly inhibited Erk1/2 activity while maintaining the integrity of the raft tissue. Microscopic examination of hematoxylin and eosin (H&E)-stained sections of U0126-treated rafts and controls is presented in Fig. 3A. As demonstrated previously (2),

treatment of CIN-612 9E raft cultures with 1.0 μ M BaP permitted normal growth of raft tissue compared with the level for the DMSO controls (Fig. 3A). Similarly, treatment with 1.0 μ M BaP and 20 μ M U0126 together also permitted normal growth of raft culture tissue, indicating that the treatment regimen was not toxic to the raft cultures (Fig. 3A).

To confirm that the U0126 treatment regimen used was sufficient to inhibit the Erk1/2 pathway, Western blot analyses were performed using an antibody specific to the phosphorylated form of Erk1/2. Organotypic raft cultures of CIN-612 9E cells were grown and treated with 1.0 μ M BaP or an equivalent concentration of DMSO for the duration of raft growth. Rafts were also treated with 20 μ M U0126 on days 7 and 8, 9 and 10, or 11 and 12 or were treated with an equivalent concentration of DMSO. Cell lysates were prepared on days 8, 10, and 12. The Western analysis was repeated three times, and a representative blot is shown in Fig. 3B. As before (Fig. 2), BaP treatment increased the phosphorylation of Erk1/2 compared with the level for the DMSO controls (Fig. 3B). Treatment of the rafts with U0126 reduced Erk1/2 phosphorylation to levels below those for the DMSO controls at each time point, demonstrating the effectiveness of the Erk pathway inhibitor in our system (Fig. 3B). Interestingly, the phosphorylation of Erk1/2 was strongest on days 9 and 10. This result may indicate that the phosphorylation of Erk1/2 by BaP treatment is a temporally regulated process. We have seen a similar temporal regulation of gene expression in organotypic raft cultures previously (58). Additional analysis will be necessary to determine how this precise timing occurs.

The effects of Erk1/2 signaling pathway inhibition on HPV titers were determined by quantitative RT-PCR analysis. Organotypic raft cultures of CIN-612 9E cells were grown for 12 days and treated with 1.0 μ M BaP with or without 20 μ M U0126. U0126 was added to the raft culture medium on days 7 and 8, 9 and 10, or 11 and 12. Virus isolated from the rafts was used to infect HaCaT keratinocytes, and relative E1⁺E4 expression was determined as before (Fig. 1) (17). E1⁺E4 transcript levels were normalized to TATA binding protein transcript abundance, and the data are represented as a fold change in E1⁺E4 expression compared with the level for the DMSO control, which was set to 1. Treatment of the cells with BaP and U0126 on days 7 and 8 resulted in a 0.21-fold increase in E1⁺E4 expression compared to the level for the DMSO controls and 28.6-fold inhibition compared to the level for BaP alone ($P < 0.01$) (Fig. 3C). Treatment on days 9 and 10 with BaP and U0126 also resulted in a 0.21-fold increase in E1⁺E4 expression compared with the level for the DMSO controls and 28.6-fold inhibition compared with the level for BaP alone ($P < 0.01$). Finally, treatment of the cells with BaP and U0126 on days 11 and 12 resulted in a 0.74-fold increase in E1⁺E4 expression compared to the level for the DMSO controls and 8.1-fold inhibition compared with the level for BaP alone ($P < 0.01$) (Fig. 3C).

We confirmed the U0126 results using a second Erk1/2 pathway inhibitor. Organotypic raft cultures of CIN-612 9E cells were grown for 12 days and treated with 1.0 μ M BaP with or without 50 μ M PD98059. PD98059 was added on days 7 and 8, 9 and 10, or 11 and 12. Virus was isolated from the rafts and was used to infect HaCaT keratinocytes, and relative E1⁺E4 expression was determined as before (Fig. 1) (17). Similar to

FIG. 3. Effects of Erk1/2 pathway inhibition on viral titers. (A) Histochemical analysis. Shown are hematoxylin and eosin-stained CIN-612 9E raft culture tissue sections treated with 1.0 μ M BaP, 1.0 μ M BaP plus 20 μ M U0126, or an equivalent concentration of DMSO. U0126 treatments were performed on days 7 and 8, 9 and 10, or 11 and 12. (B) Inhibition of Erk1/2 activation. Western blot analysis was performed on lysates from rafts treated with 1.0 μ M BaP with or without 20 μ M U0126. U0126 treatments were performed on days 7 and 8, 9 and 10, or 11 and 12. Cell lysates were prepared on day 8, 10, or 12, respectively. Western blot analyses were performed using a phospho-specific Erk1/2 antibody (see Materials and Methods) or GAPDH. (C) Quantitative RT-PCR infectivity assay of U0126-treated rafts. CIN-612 9E organotypic raft cultures were treated with 1.0 μ M BaP with or without 20 μ M U0126. U0126 treatments were performed as described for panel A. Virus titers were measured by a

the U0126 results, treatment of the CIN-612 9E rafts with PD98059 reduced viral titers, with the greatest inhibition occurring when the cells were treated on days 7 and 8 (Fig. 3D). The data from both inhibitors indicated the importance of Erk1/2 pathway activity in mediating the increase in viral titers observed with BaP treatment. Furthermore, the data indicated that Erk1/2 pathway activation is most important on days 7 and 8 of raft growth.

Given the importance of Erk1/2 pathway activity to the BaP-mediated increase in viral titers, we wished to assess the potential contribution of Erk1/2 signaling to basal virus production. To do this, we measured viral titers from 12-day CIN-612 9E organotypic raft cultures treated with 20 μ M U0126 or an equivalent concentration of DMSO in the absence of BaP treatment. Relative E1^E4 transcript levels were measured by quantitative RT-PCR as before (Fig. 3C), and the data are represented as fold changes in E1^E4 expression compared with the level for the DMSO-only control, which was set to 1. No significant change in E1^E4 transcript abundance was observed following U0126 treatment (Fig. 3C). U0126 treatment on days 7 and 8 resulted in a 1.3-fold change, treatment on days 9 and 10 resulted in no change, and treatment on days 11 and 12 resulted in a 1.6-fold increase in viral titer compared with the level for the DMSO controls (Fig. 3C). Taken together, the U0126 studies indicated that Erk1/2 pathway activation is necessary for the increased viral titers observed following BaP treatment but is not required for basal virus production.

BaP-mediated Erk1/2 pathway activation has no effect on HPV31b early- or late-promoter activity. Once activated by upstream factors, Erk1/2 has been shown to possess the ability to activate a large number of transcription factors. One potential mechanism for explaining the increased HPV titers following BaP treatment would be that the increased activity of Erk1/2 signaling following BaP treatment leads to the activation of the early and/or the late HPV31b promoter elements. Therefore, luciferase assays were performed using HPV31b promoter luciferase constructs to assess the effects of BaP treatment on HPV promoter activity. The luciferase plasmids pGL2B-630 and pGL2B-1706 were generated and used by our laboratory previously to examine HPV31b early- and late-promoter activity (6). The pGL2B-630 plasmid contains only the late (p742) HPV31b promoter cloned upstream of the luciferase gene, while pGL2B-1706 contains both the early (p99) and the late (p742) HPV31b promoters cloned upstream of the luciferase gene. Either the HPV31b promoter construct or empty pGL2-Basic was transfected into CIN-612 9E cells, and the cells were treated with 1.0 μ M BaP or an equivalent concentration of DMSO and were cultured in 1.6% methylcellulose for 48 h to induce differentiation. Cells were harvested, and luciferase assays were performed three times to assess promoter activity. The relative luciferase activity for each sam-

ple was determined and is represented in Fig. 4A as fold increase in activation over the basal luciferase activity obtained from cells transfected with empty pGL2-Basic. The data obtained for DMSO control samples are generally consistent with our previous report based on the use of these constructs (6). Promoter activity from the pGL2B-1706 plasmid was 196-fold above the level for the empty-vector controls, while promoter activity from the pGL2B-630 plasmid was 70-fold above the level for the vector controls. Interestingly, the promoter activity of both plasmids was unchanged by treatment with 1.0 μ M BaP. BaP treatment of cells transfected with the pGL2B-1706 plasmid resulted in a 196-fold increase in activation over the level for the empty-vector controls, a level identical to that of the activation seen with DMSO treatment alone (Fig. 4A). Similarly, BaP treatment of cells transfected with the pGL2B-630 plasmid resulted in an 84-fold increase in activation, which is similar to the 70-fold increase in activation measured in comparison with the level for the DMSO-treated controls. These data revealed that BaP treatment of CIN-612 9E cells in the context of methylcellulose-induced differentiation failed to affect HPV promoter activity.

The activation of the Erk1/2 signaling pathway by BaP has no effect on the abundance of HPV31b late transcripts. We extended our observations of the effects of BaP treatment on transcription by measuring the abundance of HPV31b late transcripts following BaP treatment. Although the luciferase assays demonstrated that BaP treatment did not affect HPV31b promoter activity, a measurement of transcript abundance would have the additional effect of determining whether there was a change in the stability of HPV late transcripts in response to BaP treatment. Organotypic raft cultures of CIN-612 9E cells were grown for 12 days and were treated with 1.0 μ M BaP or an equivalent concentration of DMSO, as before. RNA was isolated from the raft cultures and was converted to cDNA. The abundance of HPV31b L1 or L2 cDNAs was determined by SYBR green quantitative PCR as described above. The threshold cycle (C_T) values for each sample were normalized to the level for TBP and were plotted as fold increases in activation over the level for the DMSO controls. As shown in Fig. 4B and C, treatment of the raft cultures with 1.0 μ M BaP resulted in a 1.55-fold change in L1 transcript abundance (Fig. 4B) and a 1.31-fold change in L2 abundance (Fig. 4C), indicating that BaP does not significantly affect the abundance of HPV late transcripts.

BaP treatment promotes p90RSK activation. Given that BaP treatment of CIN-612 9E cells does not affect transcription, we examined the effects of BaP treatment on other downstream Erk1/2 substrates. Previous studies indicated that activation of the Erk1/2 signaling pathway can lead to the phosphorylation and activation of p90RSK (14, 71). Activated p90RSK, in turn, has the ability to regulate transcription (12,

quantitative RT-PCR infectivity assay. Data are presented as fold increases in activation of E1^E4 transcript abundance compared with the level for DMSO treatment alone. Separately, CIN-612 9E organotypic raft cultures were grown without 1.0 μ M BaP with or without 20 μ M U0126. U0126 treatments were performed as described for panel A. Virus titers were measured as before and are presented as fold increases in activation of E1^E4 transcript abundance compared with the level for DMSO treatment alone. (D) Quantitative RT-PCR infectivity assay of PD98059-treated rafts. CIN-612 9E organotypic raft cultures were treated with 1.0 μ M BaP with or without 50 μ M PD98059. Virus titers were measured and reported as described for panel C.

FIG. 4. (A) HPV31b early/late-promoter activity. CIN-612 9E cells were transfected with pGL2-Basic, pGL2B-630, or pGL2B-1706 and were grown in 1.6% methylcellulose with 1.0 μ M BaP or an equivalent concentration of DMSO for 48 h. Cells were harvested, and promoter activity was determined by a luciferase assay. Data are represented as fold increases in activation over the level for the pGL2-Basic empty-vector control. (B and C) HPV31b late-transcript stability. CIN-612 9E organotypic raft cultures were grown for 12 days with 1.0 μ M BaP or an equivalent concentration of DMSO. Total RNA was isolated and used in quantitative RT-PCR analysis with primers specific for HPV31b L1 (B) or L2 (C) transcripts. Data are represented as fold increases in activation over the level for the DMSO controls.

14, 20, 53, 81), translation (10, 65), and the cell cycle (23, 59). To assess the effects of BaP treatment on p90RSK activation, organotypic raft cultures of CIN-612 9E cells were treated with 1.0 μ M BaP with or without 20 μ M U0126 or an equivalent concentration of DMSO. U0126 treatment was performed on days 11 and 12. Total protein extracts were prepared and used in Western blot analysis. A Western blot for total p90RSK revealed an increase over the level for the DMSO control in the abundance of p90RSK with the addition of 1.0 μ M BaP (Fig. 5). Treatment with BaP and U0126 together resulted in the appearance of a faster-migrating band compared with the level for the BaP-only sample, suggesting the presence of an unphosphorylated or hypophosphorylated form of p90RSK following U0126 inhibition (Fig. 5). To confirm this, Western blot analysis was performed using a phosphospecific antibody that recognizes phosphorylation of p90RSK at threonine residue 573, an event indicative of activation (19). DMSO control rafts displayed limited background phosphorylation of p90RSK; however, treatment with 1.0 μ M BaP led to an in-

FIG. 5. p90RSK activation. CIN-612 9E organotypic raft cultures were grown for 12 days with 1.0 μ M BaP, BaP with 20 μ M U0126, or an equivalent concentration of DMSO. U0126 treatments were performed on days 11 and 12. Cell lysates were generated and used in Western blot analysis with an antibody specific for total p90RSK and a phosphospecific p90RSK antibody that recognizes p90RSK phosphorylated at Thr573, indicative of activation.

FIG. 6. U0126 inhibition of CDK1 kinase activity. Kinase assays were performed with cell lysates from rafts treated with 1.0 μ M BaP with or without 20 μ M U0126 or an equivalent concentration of DMSO as a control. U0126 treatments were performed on days 7 and 8, 9 and 10, or 11 and 12. Histone H1 was used as a CDK1 substrate.

creased phosphorylation (Fig. 5). Treatment of the raft cultures with BaP and U0126 inhibited p90RSK phosphorylation to background, DMSO-treated-control levels (Fig. 5). These data indicated that treatment of organotypic raft cultures with BaP activated p90RSK via the Erk1/2 signaling pathway. These Western blot analyses have been repeated multiple times, with reproducible results.

BaP-mediated Erk1/2 signaling promotes CDK1 kinase activity. One downstream target of activated p90RSK is the cell cycle (23, 59). We have found that CDK1 kinase activity increases in raft cultures treated with BaP (1). Furthermore, we found that CDK1 inhibition with purvalanol A in BaP-treated raft cultures dramatically reduced infectivity (1). Since CDK1 can be activated by p90RSK (59), we explored the possibility that BaP treatment activates CDK1 through the Erk1/2-p90RSK pathway. Organotypic raft cultures of CIN-612 9E cells were grown for 12 days and were treated with 1.0 μ M BaP with or without 20 μ M U0126 or an equivalent concentration of DMSO. U0126 treatment was performed on days 7 and 8, 9 and 10, or 11 and 12. Total protein extracts were prepared, and 200 μ g of each sample was used in immunoprecipitations with a CDK1 antibody. The activity of CDK1 was determined by a kinase assay, with histone H1 as a substrate. Treatment of the rafts with BaP alone increased CDK1 kinase activity nearly 4-fold (Fig. 6), similarly to what we have found previously (1). However, treatment of the raft cultures with BaP and U0126 together inhibited the activity of CDK1, with the most obvious inhibition occurring in cells treated with U0126 at later time points (Fig. 6). Treatment of the raft cultures with BaP and U0126 on days 11 and 12 diminished CDK1 kinase activity to 90% of the level for the DMSO controls (Fig. 6).

DISCUSSION

Epidemiological data support a strong correlation between cigarette smoking and increased incidence of cervical cancer, implicating cigarette smoking as a cofactor in cervical carcinogenesis (4, 24, 43, 55, 70, 82, 85). However, a mechanism for explaining this correlation has yet to be determined. Here, we explore this phenomenon in greater detail, with an emphasis on the effects of BaP on the cellular environment that promote increased infectivity.

We previously demonstrated that a range of BaP concentrations from 0.001 μ M to 1.0 μ M affected multiple steps in the HPV life cycle (2, 11). Among the affected steps was virion synthesis. Treatment of CIN-612 9E cells with 1.0 μ M BaP promoted a roughly 10-fold increase in HPV31b viral titers as determined by endpoint RT-PCR analysis (2). To substantiate these data, we performed a quantitative RT-PCR infectivity assay using virus isolated from CIN-612 9E cells treated with 1.0 μ M BaP or an equivalent concentration of DMSO. This experiment revealed a 6-fold increase in HPV31b titer in response to BaP treatment, thus confirming the BaP-mediated increase in viral titers observed previously (2) and providing a more quantitative readout. Most HPV-induced dysplasias are cleared spontaneously by the host immune system (7, 21, 28). However, increased virion production may increase the carcinogenic potential of an HPV infection by increasing viral persistence, which has been suggested to be necessary for carcinogenic progression (83, 85). The increased viral titers associated with BaP exposure may therefore increase the risk of carcinogenic progression through increased virus persistence.

Numerous studies have reported the activation of cell signaling pathways by BaP treatment. These analyses were conducted in multiple cell contexts over a range of BaP concentrations and demonstrated the activation of both MAPK and PI3K/Akt signaling pathways (8, 9, 60). Among the MAPK pathways activated was the Erk1/2 signaling pathway (8, 42, 60). Given the number of studies demonstrating Erk1/2 activation by BaP, we focused our studies of BaP signaling in CIN-612 9E raft cultures on the Erk1/2 pathway. Treatment of raft cultures with 1.0 μ M BaP led to the phosphorylation and activation of Erk1/2, indicating pathway activation. Erk1/2 pathway activation has been shown to occur via the Ras-Raf-Mek1/2-Erk1/2 pathway as well as PKC α -Raf-Mek1/2-Erk1/2 (35, 46). To distinguish between the two pathways leading to Erk1/2 activation, we also assessed the activation of Ras, Raf, and Mek1/2. BaP treatment promoted increases in both Ras and Raf as well as the phosphorylation of Mek1/2, indicating the activation of the Ras-Raf-Mek1/2-Erk1/2 pathway in response to BaP treatment. These data do not preclude the involvement of PKC signaling in the observed effects of BaP on viral titer, however, and we have designed subsequent studies to examine the effects of the PKC pathway on the HPV life cycle. The observed increase in Erk1/2 activity may correlate with the carcinogenic potential of the tissue. Increased levels of phospho-Erk1/2 have been linked to tumor progression in cervical neoplasms, suggesting the possibility that BaP exposure may establish a cellular environment conducive to tumor growth (15).

Once activated, the Erk1/2 signaling pathway affects multiple downstream substrates, including transcription factors, p90RSK, cell cycle regulators, and apoptosis regulators (13, 14, 26, 40, 41, 52, 54, 64, 71, 87). Previous data indicated that there are multiple potential transcription factor binding sites throughout the HPV early and late promoter elements (29, 39, 56, 84). Some of these transcription factor binding sites, such as C/EBP- β and AP-1, are activated by Erk1/2 signaling, suggesting that Erk1/2 activation by BaP may directly affect transcription of HPV early and/or late genes (13, 52, 54, 87). Increased expression of HPV late genes may facilitate the

production and assembly of elevated levels of virions. We have demonstrated by Western blot analysis that treatment of CIN-612 9E cells with 1.0 μ M BaP leads to greatly increased levels of L1 major capsid protein (Fig. 1B) (2). To examine the effects of BaP treatment on viral transcription, we performed luciferase assays to quantitate early- and late-HPV31b-promoter activity with or without 1.0 μ M BaP. These analyses demonstrated that BaP treatment had no effects on the activity of the early or late promoters, indicating that the effects of BaP on the virus life cycle occur at a posttranscriptional step.

Since HPV promoter activity was unchanged, we examined the possibility that BaP treatment led to changes in HPV late-transcript stability. Changes to transcript stability can lead to rapid, transient changes in gene expression. For example, PMA stimulation led to an increased stability of nucleolin mRNAs via activation of the Erk signaling pathway within the context of peripheral blood mononuclear cells (PBMCs) (78). Furthermore, Sokolowski et al. demonstrated the existence of an mRNA instability element in the HPV16 L2 coding region that limits gene expression (69). Given this information, we examined HPV31b late-transcript abundance to quantitate any changes that may occur in response to BaP treatment. Neither L1 nor L2 transcript abundance changed following BaP treatment, indicating that BaP affects a later step in the virus life cycle.

Our initial hypothesis was that BaP-mediated activation of the Erk1/2 signaling pathway promoted increased expression of L1. However, promoter and transcript abundance studies indicated that the Erk1/2 signaling pathway did not affect L1 expression on a transcriptional level. It is possible that the increased L1 protein abundance that we have observed (Fig. 1B) (2) is the result of increased translation of L1 transcripts or increased half-life of L1 proteins. This effect may be mediated by regulation of the translation machinery or through control of proteasomal-mediated degradation, respectively. Additional studies will be necessary to identify these signals.

Once we determined that BaP treatment does not affect gene expression on a transcriptional level, we focused our attention on other downstream targets of the Erk1/2 pathway. Among these downstream targets is p90RSK. p90RSK activation by Kaposi's sarcoma-associated herpesvirus (KSHV) open reading frame 45 (Orf45) was shown to promote lytic gene expression and was necessary for the production of progeny virions (37, 38). p90RSK activation by BaP treatment may also affect the HPV life cycle, leading to increased infectivity of progeny virions. In addition, the role of p90RSK in cellular transformation is being elucidated, and p90RSK has been shown to function as a tumor promoter (32, 68). Activation of p90RSK by BaP treatment may therefore contribute to cervical cancer progression through both increased virus infectivity and activation of cellular signals that promote cell proliferation. We performed Western blot analysis on lysates from organotypic raft cultures treated with 1.0 μ M BaP or an equivalent concentration of DMSO. Western blotting was performed using an antibody that recognizes total p90RSK and an antibody to the phosphorylated/activated form of p90RSK. This analysis demonstrated the activation of p90RSK by BaP treatment compared with the level for the DMSO controls and implicated the Erk1/2 signaling pathway in that activation (Fig. 5).

We have found that treatment of CIN-612 9E raft cultures

with BaP leads to the activation of CDK1 (1). This activation is an important step in the virus life cycle of BaP-treated rafts, since inhibition of CDK1 with purvalanol A dramatically reduced infectivity (1). Here, we have confirmed the activation of CDK1 by BaP treatment (Fig. 6) but took that a step further by demonstrating the involvement of Erk1/2 signaling in CDK1 activation by BaP. Based upon our earlier study (1), the most likely step to be affected by CDK1 is viral maturation. It is possible that CDK1 kinase activity regulates the phosphorylation of L1. This phosphorylation may regulate the conformation and/or stability of the protein and could explain the increased viral infectivity that we have observed following BaP treatment. However, CDK1 activation may also provide a cellular environment that promotes tumor growth. A panel of invasive breast cancer tissue sections was analyzed for CDK1 expression and compared with patient cancer outcome (33). It was found that patients with high CDK1 levels were more likely to relapse, indicating a correlation between CDK1 activation and tumorigenesis (33).

In summary, treatment of HPV-positive CIN-612 9E raft cultures with BaP increases infectivity through activation of the Erk1/2 signaling pathway. Erk1/2 signaling, in turn, leads to the activation of CDK1. This activation of CDK1 subsequently increases the infectivity of virions grown in BaP-treated rafts.

ACKNOWLEDGMENTS

We thank Neil Christensen for the HPV16 L1 D9 primary antibody. We thank Lynn Budgeon for excellent technical assistance. We thank Eric Ryndock for critical reading of the manuscript.

This study was supported by a PHS grant from the National Institute of Allergy and Infectious Disease (R01AI057988), by Philip Morris USA, Inc., and by Philip Morris International. B.S.B. was supported in part by the Penn State College of Medicine, Department of Microbiology and Immunology, by an institutional training grant (5 T32 CA60395-12).

REFERENCES

1. Alam, S., et al. 2010. Downregulation of Cdc2/CDK1 kinase activity induces the synthesis of noninfectious human papillomavirus type 31b virions in organotypic tissues exposed to benzo[a]pyrene. *J. Virol.* **84**:4630–4645.
2. Alam, S., M. J. Conway, H. S. Chen, and C. Meyers. 2008. The cigarette smoke carcinogen benzo[a]pyrene enhances human papillomavirus synthesis. *J. Virol.* **82**:1053–1058.
3. Alam, S., E. Sen, H. Brashear, and C. Meyers. 2006. Adeno-associated virus type 2 increases proteasome-dependent degradation of p21WAF1 in a human papillomavirus type 31b-positive cervical carcinoma line. *J. Virol.* **80**:4927–4939.
4. Appleby, P., et al. 2006. Carcinoma of the cervix and tobacco smoking: collaborative reanalysis of individual data on 13,541 women with carcinoma of the cervix and 23,017 women without carcinoma of the cervix from 23 epidemiological studies. *Int. J. Cancer* **118**:1481–1495.
5. Bedell, M. A., et al. 1991. Amplification of human papillomavirus genomes in vitro is dependent on epithelial differentiation. *J. Virol.* **65**:2254–2260.
6. Bodily, J. M., and C. Meyers. 2005. Genetic analysis of the human papillomavirus type 31 differentiation-dependent late promoter. *J. Virol.* **79**:3309–3321.
7. Brinton, L. A., and J. F. Fraumeni, Jr. 1986. Epidemiology of uterine cervical cancer. *J. Chronic Dis.* **39**:1051–1065.
8. Burdick, A. D., et al. 2003. Benzo(a)pyrene quinones increase cell proliferation, generate reactive oxygen species, and transactivate the epidermal growth factor receptor in breast epithelial cells. *Cancer Res.* **63**:7825–7833.
9. Burdick, A. D., I. D. Ivitski-Steele, F. T. Lauer, and S. W. Burchiel. 2006. PYK2 mediates anti-apoptotic AKT signaling in response to benzo[a]pyrene diol epoxide in mammary epithelial cells. *Carcinogenesis* **27**:2331–2340.
10. Carriere, A., et al. 2008. Oncogenic MAPK signaling stimulates mTORC1 activity by promoting RSK-mediated raptor phosphorylation. *Curr. Biol.* **18**:1269–1277.
11. Castle, P. E., C. Meyers, S. Alam, and M. J. Conway. 2008. How does tobacco smoke contribute to cervical carcinogenesis? *J. Virol.* **82**:6084–6086.
12. Chen, R. H., C. Abate, and J. Blenis. 1993. Phosphorylation of the c-Fos

- transrepression domain by mitogen-activated protein kinase and 90-kDa ribosomal S6 kinase. *Proc. Natl. Acad. Sci. U. S. A.* **90**:10952–10956.
13. **Chen, R. H., P. C. Juo, T. Curran, and J. Blenis.** 1996. Phosphorylation of c-Fos at the C-terminus enhances its transforming activity. *Oncogene* **12**: 1493–1502.
 14. **Chen, R. H., C. Sarnecki, and J. Blenis.** 1992. Nuclear localization and regulation of erk- and rsk-encoded protein kinases. *Mol. Cell. Biol.* **12**:915–927.
 15. **Chen, T. P., et al.** 2007. Increased expression of SKP2 and phospho-MAPK/ERK1/2 and decreased expression of p27 during tumor progression of cervical neoplasms. *Gynecol. Oncol.* **104**:516–523.
 16. **Conney, A. H.** 1982. Induction of microsomal enzymes by foreign chemicals and carcinogenesis by polycyclic aromatic hydrocarbons: G. H. A. Clowes Memorial Lecture. *Cancer Res.* **42**:4875–4917.
 17. **Conway, M. J., et al.** 2009. Tissue-spanning redox gradient-dependent assembly of native human papillomavirus type 16 virions. *J. Virol.* **83**:10515–10526.
 18. **Corden, S. A., L. J. Sant-Cassia, A. J. Easton, and A. G. Morris.** 1999. The integration of HPV-18 DNA in cervical carcinoma. *Mol. Pathol.* **52**:275–282.
 19. **Dalby, K. N., N. Morrice, F. B. Caudwell, J. Avruch, and P. Cohen.** 1998. Identification of regulatory phosphorylation sites in mitogen-activated protein kinase (MAPK)-activated protein kinase-1a/p90rsk that are inducible by MAPK. *J. Biol. Chem.* **273**:1496–1505.
 20. **De Cesare, D., S. Jacquot, A. Hanauer, and P. Sassone-Corsi.** 1998. Rsk-2 activity is necessary for epidermal growth factor-induced phosphorylation of CREB protein and transcription of c-fos gene. *Proc. Natl. Acad. Sci. U. S. A.* **95**:12202–12207.
 21. **Durst, M., L. Gissmann, H. Ikenberg, and H. zur Hausen.** 1983. A papillomavirus DNA from a cervical carcinoma and its prevalence in cancer biopsy samples from different geographic regions. *Proc. Natl. Acad. Sci. U. S. A.* **80**:3812–3815.
 22. **Frattini, M. G., H. B. Lim, and L. A. Laimins.** 1996. In vitro synthesis of oncogenic human papillomaviruses requires episomal genomes for differentiation-dependent late expression. *Proc. Natl. Acad. Sci. U. S. A.* **93**:3062–3067.
 23. **Fujita, N., S. Sato, and T. Tsuruo.** 2003. Phosphorylation of p27Kip1 at threonine 198 by p90 ribosomal protein S6 kinases promotes its binding to 14-3-3 and cytoplasmic localization. *J. Biol. Chem.* **278**:49254–49260.
 24. **Garrett, L. R., N. Perez-Reyes, P. P. Smith, and J. K. McDougall.** 1993. Interaction of HPV-18 and nitrosomethylurea in the induction of squamous cell carcinoma. *Carcinogenesis* **14**:329–332.
 25. **Gomez, N., and P. Cohen.** 1991. Dissection of the protein kinase cascade by which nerve growth factor activates MAP kinases. *Nature* **353**:170–173.
 26. **Harada, H., B. Quearry, A. Ruiz-Vela, and S. J. Korsmeyer.** 2004. Survival factor-induced extracellular signal-regulated kinase phosphorylates BIM, inhibiting its association with BAX and proapoptotic activity. *Proc. Natl. Acad. Sci. U. S. A.* **101**:15313–15317.
 27. **Hecht, S. S.** 1999. Tobacco smoke carcinogens and lung cancer. *J. Natl. Cancer Inst.* **91**:1194–1210.
 28. **Herrington, C. S.** 1995. Human papillomaviruses and cervical neoplasia. II. Interaction of HPV with other factors. *J. Clin. Pathol.* **48**:1–6.
 29. **Hoppe-Seyler, F., K. Butz, and H. zur Hausen.** 1991. Repression of the human papillomavirus type 18 enhancer by the cellular transcription factor Oct-1. *J. Virol.* **65**:5613–5618.
 30. **Jeon, S., B. L. Allen-Hoffmann, and P. F. Lambert.** 1995. Integration of human papillomavirus type 16 into the human genome correlates with a selective growth advantage of cells. *J. Virol.* **69**:2989–2997.
 31. **Jyonouchi, H., S. Sun, K. Iijima, M. Wang, and S. S. Hecht.** 1999. Effects of anti-7,8-dihydroxy-9,10-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene on human small airway epithelial cells and the protective effects of myo-inositol. *Carcinogenesis* **20**:139–145.
 32. **Kang, S., et al.** 2007. FGFR3 activates RSK2 to mediate hematopoietic transformation through tyrosine phosphorylation of RSK2 and activation of the MEK/ERK pathway. *Cancer Cell* **12**:201–214.
 33. **Kim, S. J., et al.** 2008. Determination of the specific activity of CDK1 and CDK2 as a novel prognostic indicator for early breast cancer. *Ann. Oncol.* **19**:68–72.
 34. **Kim, S. O., P. Irwin, S. Katz, and S. L. Pelech.** 1998. Expression of mitogen-activated protein kinase pathways during postnatal development of rat heart. *J. Cell. Biochem.* **71**:286–301.
 35. **Kolch, W., et al.** 1993. Protein kinase C α activates RAF-1 by direct phosphorylation. *Nature* **364**:249–252.
 36. **Kortenjann, M., O. Thomae, and P. E. Shaw.** 1994. Inhibition of v-raf-dependent c-fos expression and transformation by a kinase-defective mutant of the mitogen-activated protein kinase Erk2. *Mol. Cell. Biol.* **14**:4815–4824.
 37. **Kuang, E., Q. Tang, G. G. Maul, and F. Zhu.** 2008. Activation of p90 ribosomal S6 kinase by ORF45 of Kaposi's sarcoma-associated herpesvirus and its role in viral lytic replication. *J. Virol.* **82**:1838–1850.
 38. **Kuang, E., F. Wu, and F. Zhu.** 2009. Mechanism of sustained activation of ribosomal S6 kinase (RSK) and ERK by Kaposi sarcoma-associated herpesvirus ORF45: multiprotein complexes retain active phosphorylated ERK AND RSK and protect them from dephosphorylation. *J. Biol. Chem.* **284**: 13958–13968.
 39. **Kukimoto, I., T. Takeuchi, and T. Kanda.** 2006. CCAAT/enhancer binding protein beta binds to and activates the P670 promoter of human papillomavirus type 16. *Virology* **346**:98–107.
 40. **Lavoie, J. N., G. L'Allemain, A. Brunet, R. Muller, and J. Pouyssegur.** 1996. Cyclin D1 expression is regulated positively by the p42/p44MAPK and negatively by the p38/HOGMAPK pathway. *J. Biol. Chem.* **271**:20608–20616.
 41. **Ley, R., K. Balmanno, K. Hadfield, C. Weston, and S. J. Cook.** 2003. Activation of the ERK1/2 signaling pathway promotes phosphorylation and proteasome-dependent degradation of the BH3-only protein, Bim. *J. Biol. Chem.* **278**:18811–18816.
 42. **Li, J., M. S. Tang, B. Liu, X. Shi, and C. Huang.** 2004. A critical role of PI-3K/Akt/JNKs pathway in benzo[a]pyrene diol-epoxide (B[a]PDE)-induced AP-1 transactivation in mouse epidermal Cl41 cells. *Oncogene* **23**: 3932–3944.
 43. **Li, S. L., M. S. Kim, H. M. Cherrick, J. Doniger, and N. H. Park.** 1992. Sequential combined tumorigenic effect of HPV-16 and chemical carcinogens. *Carcinogenesis* **13**:1981–1987.
 44. **Lin, D. A., and J. A. Boyce.** 2005. IL-4 regulates MEK expression required for lysophosphatidic acid-mediated chemokine generation by human mast cells. *J. Immunol.* **175**:5430–5438.
 45. **Maher, D. M., et al.** 2011. Curcumin suppresses human papillomavirus oncoproteins, restores p53, Rb, and PTPN13 proteins and inhibits benzo[a]pyrene-induced upregulation of HPV E7. *Mol. Carcinog.* **50**:47–57.
 46. **Marais, R., et al.** 1998. Requirement of Ras-GTP-Raf complexes for activation of Raf-1 by protein kinase C. *Science* **280**:109–112.
 47. **Marais, R., J. Wynne, and R. Treisman.** 1993. The SRF accessory protein Elk-1 contains a growth factor-regulated transcriptional activation domain. *Cell* **73**:381–393.
 48. **Melikian, A. A., et al.** 1999. Identification of benzo[a]pyrene metabolites in cervical mucus and DNA adducts in cervical tissues in humans by gas chromatography-mass spectrometry. *Cancer Lett.* **146**:127–134.
 49. **Meyers, C., S. Alam, M. Mane, and P. L. Hermonat.** 2001. Altered biology of adeno-associated virus type 2 and human papillomavirus during dual infection of natural host tissue. *Virology* **287**:30–39.
 50. **Meyers, C., M. G. Frattini, J. B. Hudson, and L. A. Laimins.** 1992. Biosynthesis of human papillomavirus from a continuous cell line upon epithelial differentiation. *Science* **257**:971–973.
 51. **Meyers, C., T. J. Mayer, and M. A. Ozbun.** 1997. Synthesis of infectious human papillomavirus type 18 in differentiating epithelium transfected with viral DNA. *J. Virol.* **71**:7381–7386.
 52. **Murphy, L. O., S. Smith, R. H. Chen, D. C. Fingar, and J. Blenis.** 2002. Molecular interpretation of ERK signal duration by immediate early gene products. *Nat. Cell Biol.* **4**:556–564.
 53. **Nakajima, T., et al.** 1996. The signal-dependent coactivator CBP is a nuclear target for pp90RSK. *Cell* **86**:465–474.
 54. **Nakajima, T., et al.** 1993. Phosphorylation at threonine-235 by a ras-dependent mitogen-activated protein kinase cascade is essential for transcription factor NF-IL6. *Proc. Natl. Acad. Sci. U. S. A.* **90**:2207–2211.
 55. **Nischan, P., K. Ebeling, and C. Schindler.** 1988. Smoking and invasive cervical cancer risk. Results from a case-control study. *Am. J. Epidemiol.* **128**:74–77.
 56. **Offord, E. A., and P. Beard.** 1990. A member of the activator protein 1 family found in keratinocytes but not in fibroblasts required for transcription from a human papillomavirus type 18 promoter. *J. Virol.* **64**:4792–4798.
 57. **Ozbun, M. A., and C. Meyers.** 1997. Characterization of late gene transcripts expressed during vegetative replication of human papillomavirus type 31b. *J. Virol.* **71**:5161–5172.
 58. **Ozbun, M. A., and C. Meyers.** 1998. Human papillomavirus type 31b E1 and E2 transcript expression correlates with vegetative viral genome amplification. *Virology* **248**:218–230.
 59. **Palmer, A., A. C. Gavin, and A. R. Nebreda.** 1998. A link between MAP kinase and p34(cdc2)/cyclin B during oocyte maturation: p90(rsk) phosphorylates and inactivates the p34(cdc2) inhibitory kinase Myt1. *EMBO J.* **17**: 5037–5047.
 60. **Patten Hitt, E., M. J. DeLong, and A. H. Merrill, Jr.** 2002. Benzo(a)pyrene activates extracellular signal-related and p38 mitogen-activated protein kinases in HT29 colon adenocarcinoma cells: involvement in NAD(P)H:quinone reductase activity and cell proliferation. *Toxicol. Appl. Pharmacol.* **183**:160–167.
 61. **Pfister, H.** 1987. Relationship of papillomaviruses to anogenital cancer. *Obstet. Gynecol. Clin. North Am.* **14**:349–361.
 62. **Pliskova, M., J. Vondracek, B. Vojtesek, A. Kozubik, and M. Machala.** 2005. Deregulation of cell proliferation by polycyclic aromatic hydrocarbons in human breast carcinoma MCF-7 cells reflects both genotoxic and nongenotoxic events. *Toxicol. Sci.* **83**:246–256.
 63. **Prokopczyk, B., J. E. Cox, D. Hoffmann, and S. E. Waggoner.** 1997. Identification of tobacco-specific carcinogen in the cervical mucus of smokers and nonsmokers. *J. Natl. Cancer Inst.* **89**:868–873.
 64. **Pucci, B., et al.** 2009. ERK-1 MAP kinase prevents TNF-induced apoptosis

- through bad phosphorylation and inhibition of Bax translocation in HeLa cells. *J. Cell. Biochem.* **108**:1166–1174.
65. Roux, P. P., B. A. Ballif, R. Anjum, S. P. Gygi, and J. Blenis. 2004. Tumor-promoting phorbol esters and activated Ras inactivate the tuberous sclerosis tumor suppressor complex via p90 ribosomal S6 kinase. *Proc. Natl. Acad. Sci. U. S. A.* **101**:13489–13494.
 66. Schiffman, M. H., et al. 1987. Biochemical epidemiology of cervical neoplasia: measuring cigarette smoke constituents in the cervix. *Cancer Res.* **47**:3886–3888.
 67. Schoell, W. M., M. F. Janicek, and R. Mirhashemi. 1999. Epidemiology and biology of cervical cancer. *Semin. Surg. Oncol.* **16**:203–211.
 68. Smith, J. A., et al. 2005. Identification of the first specific inhibitor of p90 ribosomal S6 kinase (RSK) reveals an unexpected role for RSK in cancer cell proliferation. *Cancer Res.* **65**:1027–1034.
 69. Sokolowski, M., W. Tan, M. Jeline, and S. Schwartz. 1998. mRNA instability elements in the human papillomavirus type 16 L2 coding region. *J. Virol.* **72**:1504–1515.
 70. Stockwell, H. G., and G. H. Lyman. 1987. Cigarette smoking and the risk of female reproductive cancer. *Am. J. Obstet. Gynecol.* **157**:35–40.
 71. Sturgill, T. W., L. B. Ray, E. Erikson, and J. L. Maller. 1988. Insulin-stimulated MAP-2 kinase phosphorylates and activates ribosomal protein S6 kinase II. *Nature* **334**:715–718.
 72. Szeliga, J., and A. Dipple. 1998. DNA adduct formation by polycyclic aromatic hydrocarbon dihydrodiol epoxides. *Chem. Res. Toxicol.* **11**:1–11.
 73. Tang, D., et al. 2001. Association between carcinogen-DNA adducts in white blood cells and lung cancer risk in the physicians health study. *Cancer Res.* **61**:6708–6712.
 74. Tannheimer, S. L., S. L. Barton, S. P. Ethier, and S. W. Burchiel. 1997. Carcinogenic polycyclic aromatic hydrocarbons increase intracellular Ca²⁺ and cell proliferation in primary human mammary epithelial cells. *Carcinogenesis* **18**:1177–1182.
 75. Tsai, K. S., R. S. Yang, and S. H. Liu. 2004. Benzo[a]pyrene regulates osteoblast proliferation through an estrogen receptor-related cyclooxygenase-2 pathway. *Chem. Res. Toxicol.* **17**:679–684.
 76. Walboomers, J. M., et al. 1999. Human papillomavirus is a necessary cause of invasive cervical cancer worldwide. *J. Pathol.* **189**:12–19.
 77. Wang, P. Y., et al. 2010. Post-transcriptional regulation of MEK-1 by polyamines through the RNA-binding protein HuR modulating intestinal epithelial apoptosis. *Biochem. J.* **426**:293–306.
 78. Westmark, C. J., and J. S. Malter. 2001. Up-regulation of nucleolin mRNA and protein in peripheral blood mononuclear cells by extracellular-regulated kinase. *J. Biol. Chem.* **276**:1119–1126.
 79. Weston, C. R., et al. 2003. Activation of ERK1/2 by deltaRaf-1:ER* represses Bim expression independently of the JNK or PI3K pathways. *Oncogene* **22**:1281–1293.
 80. White, D. O., R. J. Huebner, W. P. Rowe, and R. Traub. 1963. Studies on the virus of rabbit papilloma. 1. Methods of assay. *Aust. J. Exp. Biol. Med. Sci.* **41**:41–50.
 81. Wingate, A. D., D. G. Campbell, M. Pegg, and J. S. Arthur. 2006. Nur77 is phosphorylated in cells by RSK in response to mitogenic stimulation. *Biochem. J.* **393**:715–724.
 82. Winkelstein, W., Jr. 1977. Smoking and cancer of the uterine cervix: hypothesis. *Am. J. Epidemiol.* **106**:257–259.
 83. Woodman, C. B., S. I. Collins, and L. S. Young. 2007. The natural history of cervical HPV infection: unresolved issues. *Nat. Rev. Cancer* **7**:11–22.
 84. Wooldridge, T. R., and L. A. Laimins. 2008. Regulation of human papillomavirus type 31 gene expression during the differentiation-dependent life cycle through histone modifications and transcription factor binding. *Virology* **374**:371–380.
 85. Xi, L. F., et al. 2009. Relationship between cigarette smoking and human papilloma virus types 16 and 18 DNA load. *Cancer Epidemiol. Biomarkers Prev.* **18**:3490–3496.
 86. You, J., J. L. Croyle, A. Nishimura, K. Ozato, and P. M. Howley. 2004. Interaction of the bovine papillomavirus E2 protein with Brd4 tethers the viral DNA to host mitotic chromosomes. *Cell* **117**:349–360.
 87. Zhu, S., K. Yoon, E. Sterneck, P. F. Johnson, and R. C. Smart. 2002. CCAAT/enhancer binding protein-beta is a mediator of keratinocyte survival and skin tumorigenesis involving oncogenic Ras signaling. *Proc. Natl. Acad. Sci. U. S. A.* **99**:207–212.
 88. zur Hausen, H. 2001. Cervical carcinoma and human papillomavirus: on the road to preventing a major human cancer. *J. Natl. Cancer Inst.* **93**:252–253.
 89. zur Hausen, H. 1991. Human papillomaviruses in the pathogenesis of anogenital cancer. *Virology* **184**:9–13.
 90. zur Hausen, H. 1991. Viruses in human cancers. *Science* **254**:1167–1173.