ERK Kinase Inhibition Stabilizes the Aryl Hydrocarbon Receptor

IMPLICATIONS FOR TRANSCRIPTIONAL ACTIVATION AND PROTEIN DEGRADATION*

Shujuan Chen, Theresa Operaña, Jessica Bonzo, Nghia Nguyen, and Robert H. Tukey‡

From the Laboratory of Environmental Toxicology, Department of Pharmacology, Chemistry & Biochemistry, University of California, San Diego, La Jolla, California 92039

The ultimate carcinogen and metabolite of benzo-[a]pyrene-7,8-dihydriodiol, benzo[a]pyrene-7,8-dihydrodiol-7,9,10-epoxide (±), stimulates apoptosis, and this process can be blocked by extracellular signal-regulated kinase (Erk) kinase inhibitors. However, we show here that Erk kinase inhibitors were unable to prevent B[a]P-7,8-dihydriodiol-induced apoptosis, leading us to speculate that Erk kinases are linked to regulation of the aryl hydrocarbon (Ah) receptor. Cotreatment of hepa1c1c7 cells with 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and Erk kinase inhibitor PD98059, U0126, or SL327 led to enhanced nuclear accumulation of Ah receptor but with a reduced capacity to complement TCDD induction of Cyp1a1. This is explained in part by the ability of Erk kinase inhibitors to alter the steady-state levels of cellular Ah receptor, a result that leads to a dramatic induction in detectable receptor levels. These changes in cellular Ah receptor levels are associated with delayed degradation of the Ah receptor because TCDD-initiated degradation is reversed when cells are co-treated with TCDD and Erk kinase inhibitors. Erk kinase is linked to Ah receptor expression, as demonstrated by reductions in total Ah receptor levels after overexpression of constitutively active MEK1. In addition, Erk kinase activity modulates the transcriptional response because MEK1 overexpression enhances TCDD-initiated transactivation potential of the receptor. Thus, Erk kinase activity facilitates ligand-initiated transcriptional activation while targeting the Ah receptor for degradation. Immunoprecipitation experiments of the Ah receptor indicate that Erk kinase activity is associated with the receptor. It is interesting that the carboxyl region of the Ah receptor is associated with the transactivation region as well as the site for ubiquitination, indicating that Erk kinase-dependent phosphorylation targets the carboxyl region of the receptor.

The dioxin or Ah receptor is a basic helix-loop-helix transcription factor that resides in the cytosol associated with the 90-kDa heat shock protein (hsp90),1 the hsp90-interacting protein p23, and the immunophilin-like protein XAP2 (1–4). Upon association with ligand, the Ah receptor rapidly migrates to the nucleus, where it partners with another basic helix-loop-helix protein, Arnt, to form a transcriptionally active complex that is capable of binding to enhancer sequences. Initiated by ligand binding, conformational changes in hsp90 and the hsp90-interacting protein p23 lead to unmasking of a nuclear localization sequence that triggers uptake of the Ah receptor to the nucleus (5, 6). Once in the nucleus, partnering of the Ah receptor with Arnt leads to displacement of hsp90 and the formation of a transcriptional complex that associates with xenobiotic-responsive elements (XREs) followed by transcriptional activation of target genes (7). The carboxyl region of the Ah receptor contains the transactivation domain responsible for initiating ligand-dependent transcription (8, 9). It has been speculated that the carboxyl region of the Ah receptor may serve as a target region for phosphorylation because inhibition of protein kinase C activity leads to loss of TCDD-initiated induction of CYP1A1 as well as the elimination of transactivation potential of the receptor in reporter gene assays (10, 11). The end result of ligand-dependent Ah receptor activation and nuclear transport is controlled degradation of the receptor through proteolysis by the 26 S proteasome complex (12); the carboxyl region of the Ah receptor serves as the region for ubiquitination (13, 14). Little is known regarding the cellular or signaling events that lead to ligand-dependent proteolysis. Because the carboxyl end of the Ah receptor plays a critical role in both transcriptional potential and cellular half-life, it is quite possible that this region of the receptor contains important target sequences that are subject to cellular signaling events required for function.

Along with the mechanics of ligand binding and nuclear translocation, evidence exists that intracellular signaling pathways play an important role in Ah receptor activation. Ah receptor ligands such as TCDD are known to activate mitogen-activated protein (MAP) kinases, such as the Erk1/2 MAP kinases, the p38 MAP kinases, and stress-activated protein kinase/c-Jun NH2-terminal kinase (15–17). Cellular cross-talk between these kinases and the cellular mechanisms underlying gene expression through Ah receptor activation seem evident because chemical inhibitors of Erk, c-Jun NH2-terminal kinase, and p38 kinases interfere with TCDD-mediated transcriptional activation of Cyp1 genes (18, 19). It has been demonstrated that the treatment of mouse hepa cells with Erk kinase inhibitors PD98059 (20) and U0126 (21) or the c-Jun NH2-terminal kinase inhibitor SB202190 inhibit Ah receptor-activated induction of Cyp1a1 (15). Recent evidence has also demonstrated that pyridinyl imidazole compounds, inhibitors of p38 MAP

PARP, poly(ADP-ribose) polymerase; DTT, dithiothreitol; EMSA, electrophoretic mobility shift assay; GRE, glucocorticoid-responsive element; GRDBD, glucocorticoid receptor containing the DNA-binding domain; AhR, aryl hydrocarbon receptor.

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‡ To whom correspondence should be addressed: Leichtag Biomedical Research Bldg., La Jolla, CA 92039-0722. Tel.: 858-822-0288; Fax: 858-822-0363; E-mail: rtukey@ucsd.edu.
1 The abbreviations used are: hsp90, 90-kDa heat shock protein; Arnt, aryl hydrocarbon receptor nuclear translocator; XRE, xenobiotic-responsive element; MAP, mitogen-activated protein; TCDD, 2,3,7,8-tetrachlorodibenzo-p-dioxin; B[a]P, benzo[a]pyrene; BPDE-2, B[a]P-7,8-dihydrodiol-9,10-epoxide; TCDF, 2,3,7,8-tetrachlorodibenzo-furan;
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kinase activity, weakly inhibit histone acetyltransferase activity, potentially inhibiting TCDD induction of Cyp1a1 transcription (22). However, the underlying mechanisms linking kinase control and Ah receptor directed gene transcription are blurred by findings that most kinase inhibitors also inhibit ligand binding to the Ah receptor (23, 24). For example, PD98059, an Erk kinase inhibitor with structural similarity to Ah receptor inhibitory flavonoids (25), directly interferes with TCDD binding to the Ah receptor (24). Similar findings have been observed with the pyridyl imidazole compounds that are used as tools to inhibit p38 MAP kinase. Thus, definitive evidence that MAP kinase activity participates in Ah receptor-mediated control is lacking.

We have recently demonstrated that the cellular and molecular actions associated with the carcinogenic actions of polycyclic aromatic hydrocarbons such as benzo[a]pyrene (B[a]P) can be attributed in part to involvement of the Ah receptor as well as MAP kinases (26). Benzo[a]pyrene activates the Ah receptor, leading to induction of CYP1 proteins and the resulting metabolism of B[a]P to B[a]P-7,8-epoxide. The formation of B[a]P-7,8-epoxide becomes a substrate for epoxide hydrolase and the formation of B[a]P-7,8-dihydrodiol has limited mutagenic potential, metabolism by the CYP1 proteins leads to the generation of the ultimate carcino- gen, B[a]P-7,8-dihydrodiol-9,10-epoxide (BPDE-2). It is interesting that B[a]P-7,8-dihydrodiol is an efficient Ah receptor ligand that can activate the receptor to a DNA binding form, resulting in the induction of microsomal CYP1A1. It is assumed that the induction of CYP1A1 facilitates the metabolism of B[a]P-7,8-dihydrodiol to BPDE-2. The intracellular formation of BPDE-2 culminates in cellular apoptosis.

Because apoptosis and Ah receptor control have been linked to the actions of the MAP kinase family of proteins, we became interested in examining the contribution of cellular signaling events as modulators of B[a]P-7,8-dihydrodiol-initiated apoptosis. Exposure of human HepG2 cells and mouse hep1a1c7 cells to either B[a]P-7,8-dihydrodiol or BPDE-2 leads to the activation of Erk kinase as well as p38 (26). The inhibition of Erk1/2 phosphorylation by MAP kinase inhibitors U0126 and PD98059 and the blockage of p38 activation by SB203580 each prevented BPDE-2-induced apoptosis in mouse hep1a1c7 cells. It is noteworthy that p38 activation has been implicated in apoptosis, particularly with agents that lead to nuclear stress and DNA damage. This was verified when p38-deficient mouse embryonic fibroblasts (p38−/−) were shown to be resistant to the apoptotic actions of BPDE-2 (26). Hence, there are important ties linking the actions of cellular MAP kinases to Ah receptor-mediated metabolism of B[a]P and the onset of an apoptotic episode.

These findings led us to initiate a series of experiments examining in greater detail the potential link between Erk kinase and apoptosis. We will present evidence that activation of the Ah receptor by B[a]P-7,8-dihydrodiol targets apoptosis independent of the actions of Erk kinase and BPDE-2-initiated apoptosis. However, the actions of Erk kinase have a dramatic effect on the functional parameters of the Ah receptor, linking phosphorylation as an important signaling pathway in the transactivation potential of the receptor as well as its susceptibility and targeting for ubiquitination.

MATERIALS AND METHODS

Chemicals and Reagents—U0126 (1,2-diamino-2,3-dicyano-1,4-bis[2-aminophenylthio]butadiene) was purchased from Cell Signaling (Beverly, MA). PD98059 (2′-amino-3′-methoxyflavone) and SL327 (E/Z)-a-(aminofluoromethyl)thio)methylene-2′-(trifluoromethyl)benzene- acetonitrile) were from Calbiochem. PD184352 (2′-(2-chloro-4-iodo-phenylamino)-5-cyclopropylmethoxy-3,4-difluoro-benzamide) was a generous gift from Dr. John J. Reiners, Jr. (Wayne State University, Detroit, MI). TCDD (2,3,7,8-tetrachlorodibenzo-p-dioxin) was from Wellington Laboratory Incorporation (Ontario, Canada). [3H]TCDD (specific activity, 27.5 Ci/mmol) was purchased from EaglePicher Pharmaceutical (Lenexa, KS) and 2,3,7,8-tetrachlorodibenzo-furan (TCDF) was from Cambridge Isotope Laboratories (Andover, MA). All of the chemicals were dissolved in Me2SO as 1000× stocks. The final concentrations of Me2SO in cell culture experiments were 0.1%. All other chemicals were obtained through standard suppliers. The transfection reagent Lipofectamine 2000 was the product of Invitrogen. The anti-human/mouse phosphorylated Erk1/2 was purchased from Cell Signal (Beverly, MA). Mouse anti-PARP antibody was from BD Pharmingen. Rabbit anti-human CYP1A1 was a generous gift from Dr. Fred Guengerich (Vanderbilt University, Nashville, TN), and the rabbit anti-mouse Ah receptor (27) was a generous gift from Dr. Christopher Bradford (University of Wisconsin, Madison, WI). The horseradish peroxidase-conju- gated secondary antibodies were from Cell Signaling.

Cell Culture—The human hepatoma HepG2 and human embryonic kidney 293 cell lines were obtained from the American Type Culture Collection. Wild-type mouse hep1a1c7 and Arnt-defective BPRc1 cells were a generous gift from Dr. James Whitlock (Stanford University, Stanford, CA). All cell lines in this study were cultured in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum and supplemented with 100 units/ml penicillin and 100 µg/ml streptomycin.

Whole Cell Extracts and Nuclear Extracts—For total cellular protein preparations, cells were collected from a single 100 mm tissue culture plate and lysed in a buffer containing 0.5 M Tris-HCl, pH 7.4, 0.15 M NaCl, 0.25% deoxycholic acid, and 1% Nonidet P-40 with a complement of protease and phosphatase mixture inhibitors (Sigma). After incubation, this mixture was clarified by centrifu- gation at 30,000 g for 10 min. The supernatant was centrifuged for 20 min in a refrigerated Eppendorf centrifuge at 16,000 × g. The supernatant was collected and used for Western blot studies.

To obtain nuclear protein preparations (10), cells were grown and treated in P150 plates, the media removed and the cells washed twice with 10 ml HEPES buffer, pH 7.5, before being collected by scraping into 1 ml of MDH buffer (3 mM MgCl2, 1 mM DTT, and 25 mM HEPES, pH 7.5). These cells were then homogenized with 30 strokes in a mortar-Elvehjem power-driven tissue homogenizer. The homogenate was centrifuged at 2,500 × g for 5 min, and the pellet was washed twice in 1 ml of MDHK buffer (3 mM MgCl2, 1 mM DTT, 25 mM HEPES, pH 7.5, and 0.1% Triton X-100). This pellet was then suspended in 100 µl of HDK buffer (25 mM HEPES, pH 7.5, 1 mM DTT, and 0.4% Triton X-100) and lysed on ice for 30 min. This solution was centrifuged for 20 min in a refrigerated Eppendorf microcentrifuge at 105,000 × g. The resulting supernatant was collected and diluted with an equal volume of 20 mM HEPES, pH 7.5, and 150 mM NaCl, 0.5% Nonidet P-40, and centrifuged at 100,000 × g for 90 min. The supernatant was collected and used in Western blot studies.

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Detection of apoptotic oligonucleosomal DNA Fragmentation—DNA fragmentation was performed as described previously (26). HepG2 cells were exposed to different concentrations of B[a]P-7,8-dihydrodiol for 24 h, and the cells were collected after trypsinization and centrifugation at 1,000 × g for 5 min. Cell pellets were resuspended in 55 µl of lysis buffer (20 mM EDTA, 10 mM Tris-HCl, pH 8.0, and 0.8% SDS) and treated with 20 µl of RNase A (10 mg/ml) at 37 °C for 1 h, followed by the addition of 20 µl of proteinase K (20 mg/ml) for incubation at 55 °C overnight. Lysates were extracted by phenol/chloroform/isoamyl alcohol (25:24:1), and the DNA was precipitated after the addition of 2 volumes of ice-cold ethyl alcohol. The DNA was collected in an Eppendorf microcentrifuge, the alcohol solution was removed, and the pellet was resuspended in 20 µl of 10 mM Tris-HCl, pH 8.0, containing 10 mM EDTA. The DNA samples were subjected to electrophoresis in 1.5% agarose gels containing ethidium bromide.

Reverse Transcription of RNA Followed by PCR Analysis for Cyp1a1 RNA Content—Total RNA was extracted from cells using acidic phenol/ guanidinium isothiocyanate solution (TRizol; Invitrogen). Using Omniscript Reverse Transcriptase reagents (Qiagen), ~2 µg of total RNA was used for the generation of cDNA, as outlined by the manufacturer. After synthesis of cDNA, amplification of mouse Cyp1a1 was performed with sense 5′-CTGGCTGTCACCCTAGTCTTC-3′ and antisense 5′-GGGTATCCAGGGCTGAACTG-3′ primers at a final concentration of 1 µM (28). Using 2 µl of the cDNA reaction, PCR analysis was conducted using HotStarTaq (Qiagen) reagents, as outlined by the manufacturer. Cycling was carried out at 95 °C for 15 min, followed by 35 cycles of 15 s at 95 °C, 30 s at 58 °C (30 s), and 30 s at 72 °C (60 s) for 35 cycles. After 10 cycles, mouse actin oligonucleotides (sense, 5′-ATGGCACCTGCGCCATCTC-3′; anti-
sense, 5’-GGTACATGTTGTTACCC-3’ were added to a final concentration of 0.5 μM. The protocol was preceded by an extended elution time of 10 min at 72 °C.

**Western Blot Analysis**—All Western blots were performed using NuPAGE gel electrophoresis as outlined by the supplier (Invitrogen). Protein was heated at 70 °C for 10 min in loading buffer and resolved on appropriate gels under denaturing conditions, and the proteins were transferred to polyvinylidene difluoride membrane using a semidyry transfer system (Norvex). The membrane was blocked with 5% nonfat dry milk in Tris-buffered saline overnight at 4 °C. Membranes were washed and exposed to horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature. Each membrane was again washed, and the conjugated horseradish peroxidase was detected using the ECL Plus Western blotting detection system and scanned with a Storm 840 scanner (both from Amersham Biosciences).

**Electrophoretic Mobility Shift Assay (EMSA)**—A complementary pair of synthetic DNA oligonucleotides containing the sequence 5’-GATCCG-GGTCTTGTGGACCACTCCGCTA-3’ and 5’-GATCCGTTGGCAGAGGCGCCT-3’ (10) were synthesized, annealed, and labeled at their 5’ ends by using Klenow and [α-32P]dCTP (Amersham Biosciences). DNA binding was measured by EMSA. The binding reaction was performed with 14 μg of protein in 10 μl of loading buffer (100 mM Tris, pH 7.5, 20 mM KCl, 75 mM NaCl, 0.5 mM EDTA, and 5% glycerol) as a running buffer. The protein-DNA complexes were visualized using a Storm 840 scanner.

**Western Blot Analysis**—Western blots were performed using 4.0% Bis-Tris gels, as outlined for Western blot analysis.

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**Ethoxyresorufin-O-deethylase Assay**—Ethoxyresorufin-O-deethylase analysis was carried out as described previously (29). In brief, cells were seeded at 2.5 × 105 cells in 6-well plates and treated overnight. Media were replaced containing 1.5% salicilamide and 2.5 μM ethoxyresorufin, and Ethoxyresorufin-O-deethylase (EROD) activity was determined by liquid scintillation counting. Specific binding of [3H]TCDD to the Ah-receptor was calculated as a percentage of the total [3H]TCDD bound in control samples containing no competitor. For transient expression studies, hepG2c7 cells were seeded into 6-well tissue culture plates in medium supplemented with 10% FBS. After 24 h of culture, 4 μl of plasmid was transfected into each well using Lipofectamine 2000. After incubation for 24 h, whole cell extracts were prepared and used in Western blot analysis for the detection of Ah receptor and phosphorylated Erk1/2.

Plasmids pCMV/GRDBD/AhR and pGRE1, T015Luc were kindly provided by Dr. Lawrence Pawliger. (Karolinska Institute, Stockholm, Sweden). The plasmids pCMV/GRDBD/AhR contains N-terminal zinc finger DNA-binding domain of the glucocorticoid receptor linked to the C-terminal amino acids 83–805 of the Ah receptor. This construct can be activated by ligand as measured by GRE-driven luciferase activity when cotransfected with the pGRE1, T015Luc plasmid (8). Human embryonic kidney 293 cells were transfected with 2 μg of pCMV/GRDBD/AhR and 4 μg of pGRE1, T015Luc plasmid; after 24 h, the cells were treated with TCDD or Erk kinase inhibitors. To examine the effect of MEK1 expression, 2 μg of the MEK1 wild-type or constitutively active expression plasmids were also included.

**Luciferase Activity Assay**—Luciferase assays were carried out as described previously (10). Cells were treated and lysed on plates in a buffer containing 1% Triton X-100, 25 mM Tricine, pH 7.8, 15 mM MgSO4, 4 mM EDTA, and 1 mM DTT. Cell lysates were centrifuged at 14,000 × g for 10 min at 4 °C, and supernatants were used for luciferase and protein assays. A cell extract aliquot of 10 μl was mixed with 100 μl of reaction mixture, which contained 15 mM potassium phosphate buffer, pH 7.8, 15 mM MgSO4, 2 mM ATP, 4 mM EDTA, 25 mM Tricine, and 1 mM DTT. Reactions were started by adding 30 μl of a solution of luciferin (0.3 mg/ml) and light output measured for 10 s at 24 °C using a LMax II1144 luminescence (Molecular Devices). The results were normalized by protein concentrations.

**Common precipitation with Anti-Ah Receptor Antibody**—Cells were treated with B[a]P-7,8-dihydrodiol or B[a]P-7,8-dihydrodiol-induced Apoptosis. Expression of MAP Kinases and the Ah Receptor

**Actions of Erk Kinase Inhibitors on BPDE-2 and B[a]P-7,8-dihydrodiol-induced Apoptosis**—Treatment of HepG2 cells with B[a]P-7,8-dihydrodiol leads to Ah receptor activation, as previously demonstrated by induction of CYP1A1 (26). Because B[a]P-7,8-dihydrodiol is metabolized to BPDE-2 after induction of CYP1A1, BPDE-2 is believed to be the active metabolite that stimulates the apoptotic episode. The ultimate carcinogen, BPDE-2, induces apoptosis through Ah receptor-independent processes (26) and can be blocked when cells are treated with the Erk kinase inhibitors PD98059 and U0126 (Fig. 1A), as demonstrated by inhibition of BPDE-2-induced cleavage of PARP-1. Because treatment of HepG2 cells with B[a]P-7,8-dihydrodiol leads to the formation of BPDE-2, the results in Fig. 1A indicate that MAP kinase inhibitors would also block B[a]P-7,8-dihydrodiol-induced apoptosis. When HepG2 cells were treated with B[a]P-7,8-dihydrodiol and PARP-1 cleavage inhibitors, apoptosis was completely inhibited with PD98059 (Fig. 1B). This can be explained in part because PD98059 is a competitive inhibitor of the Ah receptor (24). However, treatment with U0126 did not block B[a]P-7,8-dihydrodiol-induced apoptosis, as determined by DNA fragmentation and PARP-1 cleavage (Fig. 1B).

We were surprised to observe that U0126 was unable to prevent B[a]P-7,8-dihydrodiol-induced apoptosis because it was clear that inhibition of Erk kinase activity by U0126 prevented BPDE-2-induced apoptosis. It has been suggested that activation of the Ah receptor by polycyclic aromatic hydrocarbons leads to induction of Bax in oocytes, a process that triggers

**Results**

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cellular apoptosis (33, 34). The process of Ah receptor activation by polycyclic aromatic hydrocarbons leads to a unique form of the receptor capable of inducing the \( \text{Bax} \) gene. However, it was originally demonstrated by Tan et al. (15) that U0126 induces Cyp1a1 and this result stems from activation of the Ah receptor. If Ah receptor activation by U0126 were occurring mechanistically in a fashion similar to that of \( \text{B[\text{a}]P-7,8-dihydriodiol} \) initiated activation, we would have expected U0126 treatment to also induce apoptosis. However, U0126 treatment did not trigger cellular apoptosis (Fig. 1). Thus, we hypothesized that U0126 may be influencing the regulation of the Ah receptor independent of those mechanisms that are controlled by \( \text{B[\text{a}]P-7,8-dihydriodiol} \). Because U0126 is a conventional Erk kinase inhibitor and has been shown to induce Cyp1a1, experiments were undertaken to investigate the actions of U0126 and other Erk kinase inhibitors on Ah receptor function.

**U0126 Is Capable of Activating the Ah Receptor**—To examine the potential impact of U0126 on Ah receptor function, initial experiments focused on the ability of U0126 to activate the Ah receptor. Treatment of hepatic1c7 cells with U0126 and TCDD led to induction of nuclear protein that bound to the Ah receptor XR sequences, as shown by EMSA (Fig. 2A). This binding was Ah receptor-dependent, because binding to XR sequences was not observed in the \( \text{Arnt} \)-deficient mouse BPRc1 cells. The treatment of hepatic1c7 cells with U0126 promoted Ah receptor-

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**FIG. 1.** The effects of Erk kinase inhibitors on BPDE-2 and \( \text{B[\text{a}]P-7,8-dihydriodiol} \) induced apoptosis in HepG2 cells. A, HepG2 cells were treated with BPDE-2 (1 \( \mu \text{M} \)) in the absence and the presence of an Erk kinase inhibitor (PD98059 (20 \( \mu \text{M} \)), U0126 (10 \( \mu \text{M} \)), or SL327 (10 \( \mu \text{M} \)) for 24 h. Total cell extracts were collected, and PARP-1, an apoptotic biomarker, was determined by Western blot. Antibodies to PARP-1 detected both intact protein at 116 kDa and its caspase-activated cleavage product of 85 kDa. B, HepG2 cells were treated with \( \text{B[\text{a}]P-7,8-dihydriodiol} \) and cotreated with the same Erk kinase inhibitors as listed in A. Cells were collected either for DNA fragmentation analysis or for the detection of PARP-1 by Western blot.
dependent induction of Cyp1a1, as demonstrated by analysis of Cyp1a1 mRNA by RT-PCR (Fig. 2B), Cyp1a1 protein by Western blot analysis (Fig. 2C), and ethoxyresorufin-O-deethylase activity (Fig. 2D). When comparing Cyp1a1 induction and Ah receptor activation, these data suggested that U0126 may be an agonist of the Ah receptor leading to induction of Cyp1a1 through a mechanism similar to TCDD induction of Cyp1a1.

U0126 Does Not Displace TCDD Binding to the Ah Receptor—To examine whether U0126 is an agonist for the Ah receptor, receptor binding experiments with [3H]TCDD were initiated. Using cytosol from hepa1c1c7 cells, [3H]TCDD was shown to bind to the Ah receptor in a concentration-dependent manner from 1 to 10 nM TCDD, as demonstrated by sedimentation of specific protein binding through linear sucrose gradients (Fig. 3A). Specificity for [3H]TCDD binding to the receptor was confirmed by complete inhibition of binding when cytosolic preparations were incubated with 1 μM TCDF, a competitive antagonist toward TCDD binding. Although PD98059 is a potent inhibitor of Erk kinase, it has been demonstrated to be a competitive antagonist of the Ah receptor (24). A dose-dependent inhibition of [3H]TCDD binding by PD98059 confirms this observation (Fig. 3B). Other known inhibitors of TCDD binding to the Ah receptor, such as β-naphthoflavone, have also been confirmed (data not shown). Because TCDF, PD98059 and β-naphthoflavone could block [3H]TCDD binding in a concentration-dependent manner, these results indicate that these compounds were competing for [3H]TCDD at the active site of the receptor.

Under conditions that demonstrate linear binding of [3H]TCDD to cytosolic Ah receptor, there was virtually no interference of [3H]TCDD binding to the Ah receptor when conditions included concentrations of U0126 ranging from 1 to 100 μM (Fig. 3C). Only a slight inhibition of [3H]TCDD binding, less than 5%, was observed when 100 μM U0126 was included in the binding reaction. Because high concentrations of U0126 were needed to interfere with TCDD binding, it was not possible to determine whether the minimal interference was specific. Thus, the finding that U0126 was unable to interfere with [3H]TCDD binding while activating the Ah receptor and inducing Cyp1a1 suggests that U0126 activation of the receptor does not occur through association with the TCDD binding site.

Actions of Erk Kinase Inhibitors on Nuclear Ah Receptor Accumulation and Induction of Cyp1a1—The inability of U0126 to compete for [3H]TCDD binding while displaying the potential to activate the Ah receptor led us to examine the impact of U0126 on TCDD-elicited activation of the Ah receptor. The rationale for this experiment was to examine whether synergy in the activation response existed when cells were cotreated with both inducers. To examine Ah receptor activation, nuclear accumulation of the receptor was quantitated. When hepa1c1c7 cells were treated with either U0126 or TCDD alone, induction of nuclear Ah receptor was observed (Fig. 4A). However, when co-treated with U0126 and TCDD, accumulation of the Ah receptor in the nucleus was increased more than 5-fold.

To examine whether synergy in nuclear accumulation of the Ah receptor by U0126 was linked to Erk kinase activity, analysis of nuclear Ah receptor accumulation and DNA binding was examined when cells were cotreated with TCDD and either U0126, PD98059, or SL327. Treatment with U0126 leads to induction of nuclear Ah receptor and DNA binding activity (Fig. 4B). However, the treatment of hepa1c1c7 cells with PD98059 and SL327 did not significantly activate the Ah receptor as displayed by a lack of nuclear Ah receptor accumulation and DNA binding potential. Yet cotreatment of hepa1c1c7 cells with TCDD and each of the Erk kinase inhibitors led to dramatic nuclear accumulation of the Ah receptor, a property that was also reflected in enhanced DNA binding to XRE sequences.

Accumulation of Ah receptor resulting from cotreatment with the Erk kinase inhibitors and TCDD led us to speculate that an increase in receptor concentration would lead to comparable increases in induction of Cyp1a1 activity. However,
when we evaluated the levels of induced Cyp1a1 by Western blot analysis and ethoxyresorufin O-deethylase activity (Fig. 4, C and D), cotreatment of hepa1c1c7 cells resulted in a decrease in Cyp1a1 induction compared with TCDD elicited induction of Cyp1a1. The large synergistic increase in nuclear Ah receptor after cotreatment with the reduction in Cyp1a1 induction indicates that Erk kinase inhibition facilitates nuclear uptake of a DNA binding form of the receptor but alters the ability of the receptor to efficiently function in eliciting enhanced transcription of the Cyp1a1 gene.

U0126 Rescues TCDD-induced Degradation of the Ah Receptor—Because nuclear Ah receptor levels increased when hepa1c1c7 cells were cotreated with U0126 and TCDD, we reasoned that U0126 may be activating a form of the Ah receptor with different functional/physical properties than the TCDD-activated receptor. As an agonist and activator of the Ah receptor, TCDD has been shown to initiate ubiquitin-mediated degradation of the Ah receptor (13, 14). When hepa1c1c7 cells were treated with several different concentrations of TCDD over an 18-h period, a concentration- and time-dependent reduction in total cellular Ah receptor was observed (Fig. 5A). The agonist-dependent 26 S proteasome initiated degradation of the Ah receptor does not require dimerization with Arnt, because degradation is also observed in BPRc1 cells (Fig. 5A). Because the Ah receptor agonist 3H9252-naphthoflavone stimulates proteolysis, whereas the structurally related Ah receptor antagonist 3H9251-naphthoflavone (β-NF) were used to treat Hepa1c1c7 cells at different concentrations (1, 5, 10, 20, and 50 μM). Me2SO-treated cells were used as control samples. After 24 h, whole cell extracts were prepared and used in Western blot analysis to detect levels of Ah receptor. C, Hepa1c1c7 cells were treated with U0126 (1 and 10 μM) in the absence and the presence of TCDD (1 nM) for either 4 or 24 h. Whole cell extracts were prepared and used for Western blot analysis to detect Ah receptor levels.

MAP Kinases and the Ah Receptor
properties as an Erk kinase inhibitor (Fig. 6). Treatment of hepa1c1c7 cells with U0124 and U0125, structural analogs of U0126 that elicit reduced Erk kinase inhibition, are not capable of inducing the Ah receptor. However, when hepa1c1c7 cells are treated with PD98059, a potent inhibitor of Erk kinase, induction of Ah receptor levels are shown to be concordant with the increasing concentrations of PD98059. It is noteworthy that although PD98059 inhibits TCDD binding to the Ah receptor and is speculated to be a competitive inhibitor (Fig. 3), treatment of hepa1c1c7 cells dramatically induces Ah receptor levels. Other Erk kinase inhibitors, such as PD184352 and SL327, show similar capabilities in inducing Ah receptor protein.

Role of Erk Kinase in Ah Receptor Stabilization and Transactivation Potential—The ability of Erk kinase inhibitors to induce the Ah receptor and to prevent degradation strongly suggests that the actions of the Erk kinase inhibitors are modifying the steady-state levels of the Ah receptor. To demonstrate this possibility, we overexpressed in hepa1c1c7 cells a variant of MEK1 that is constitutively active and over phosphorylates Erk 1/2 (Fig. 7A). Through transient expression of the constitutively active MEK1 for 48 h, both wildtype and constitutively active MEK1 were detected by Western blot analysis using an antibody to the HA tag sequence. Wild-type expression leads to limited Erk1/2 phosphorylation, but overexpression of the constitutively active variant generates significant phosphorylation of Erk1/2. The activation of MEK1, as represented in the overexpression of the constitutively active MEK1, is followed by a dramatic reduction in cellular Ah receptor levels, indicating that activated Erk kinase plays an important role in stimulating proteolysis.
of the receptor. We can predict that because the Ah receptor is targeted for ubiquitination in the carboxyl region, phosphorylation by MEK1 in this region may provide the signal for the initiation of ubiquitination.

It is also evident that the treatment of hepa1c1c7 cells with Erk kinase inhibitors diminishes the ability of the activated receptor to induce Cyp1a1 (Fig. 4C). Because binding of the Ah receptor to DNA in cells cotreated with TCDD and Erk kinase inhibitors is not compromised, the reduced transcripational activation of the Cyp1a1 gene may result from diminished transactivation potential of the receptor. To examine this possibility, an Ah receptor cDNA lacking the coding region of the N-terminal basic helix-loop-helix domain fused to a functional glucocorticoid receptor containing the DNA-binding domain (GRBD) was transfected with a luciferase reporter construct under control of the glucocorticoid-binding GRE sequences. When the GRBD portion of the fusion protein binds to the GRE, the transactivation domain on the Ah receptor stimulates transactivation of the GRE-luciferase reporter construct (8). When human embryonic kidney 293 cells are treated with TCDD, the GRBD/AhR-directed transactivation is observed (Fig. 7B). However, when treated with TCDD and Erk kinase inhibitors, transactivation is blocked, indicating that Erk kinase-dependent phosphorylation is essential in main-
taining the functional properties of the receptor. This observation is reinforced by studies demonstrating that overexpression of the constitutively active form of MEK1 synergizes the transactivation response after TCDD treatment. This would indicate that the location on the Ah receptor for Erk kinase dependent phosphorylation is the carboxyl region, which contains the transactivation domain (8, 9).

The impact of Erk kinase on cellular Ah receptor levels and TCDD-initiated transcriptional activation indicates that Erk kinase is physically associated with the Ah receptor. To examine this possibility, Ah receptor antibody was used to immunoprecipitate the Ah receptor complex followed by analysis of phosphorylated Erk1/2 (Fig. 8). With whole-cell extracts, phosphorylated Erk1/2 was present in cells treated with Me2SO and TCDD, but not detectable in SL327-treated cells. When the Ah receptor antibody was employed to precipitate total cellular Ah Receptor complex, Western blot analysis of the precipitate with anti-Ah receptor antibody. A Western blot was prepared using WCE as well as samples from immunoprecipitation. Phosphorylated Erk1/2 was detected from the Western blot using an anti-phospho-Erk1/2 antibody.

**Fig. 8.** Detection of phosphorylated Erk1/2 by immunoprecipitation of Ah receptor. Hepa1c1c7 cells were treated with MeSO (D), TCDD (T), or SL327 (SL) for 5 h. Whole cell extracts (WCE) were prepared for immunoprecipitation (IP) as outlined under “Materials and Methods,” and protein complexes were immunoprecipitated with anti-Ah receptor antibody. A Western blot was prepared using WCE as well as samples from immunoprecipitation. Phosphorylated Erk1/2 was detected from the Western blot using an anti-phospho-Erk1/2 antibody.

**DISCUSSION**

The product of B[a]P-7,8-dihydropdiol metabolism after Cyp1a1 induction leads to the ultimate carcinogen, BPDE-2. We have shown that BPDE-2 induces apoptosis as demonstrated by caspases activation and DNA fragmentation and that this activity can be blocked by Erk kinase inhibitors such as PD98059 and U0126 (26). Thus, we anticipated that when the Ah receptor was activated and Cyp1a1 was induced with B[a]P-7,8-dihydropdiol, inhibition of Erk kinase activity with U0126 and PD98059 would result in the inhibition of the apoptotic response. PD98059 blocks apoptosis, but this can be explained by the findings that PD98059 is a competitive antagonist of the Ah receptor and through this mechanism inhibits Cyp1a1 induction. Although U0126 completely inhibits BPDE-2 induced apoptosis, it has no ability to prevent cell death when cells are treated with B[a]P-7,8-dihydropdiol. Thus, induction of the Ah receptor by B[a]P-7,8-dihydropdiol may in part underlie the cellular mechanism leading to apoptosis. Indeed, because B[a]P-7,8-dihydropdiol is not capable of initiating apoptosis in Arnt-deficient hepa cells (26), functional Ah receptor is a requirement. However, the treatment of cells with U0126, a mild activator of the Ah receptor, or TCDD, a potent activator of the receptor, has no effect on cell death compared with the actions of B[a]P-7,8-dihydropdiol. Thus, it can be speculated that activation of the Ah receptor by B[a]P-7,8-dihydropdiol is initiating an alternative cellular mechanism that results in cell death. Because Erk kinase inhibitors do not block B[a]P-7,8-dihydropdiol-induced apoptosis, the Ah receptor-mediated induction of xenobiotic metabolizing proteins may lead to the formation of additional metabolites that can promote cell death independently of those actions carried out by BPDE-2.

In evaluating the actions of Erk kinase inhibitors on Ah receptor function, U0126 was shown to activate and induce nuclear accumulation of a DNA binding form of the receptor that was capable of initiating induction of Cyp1a1. Previous work has concluded that U0126 is an Ah receptor ligand (35), yet evidence that U0126 is capable of interacting at the ligand-binding site has not been documented. Identification of TCDD-like ligand specificity of Ah receptor ligands is typically demonstrated by competitive binding experiments. Under experimental conditions developed to examine [3H]TCDD binding, agents such as β-naphthoflavone, PD98059, and TCDF were shown to selectively antagonize [3H]TCDD binding, confirming observations that have been documented previously (24). Because PD98059 is unable to activate the cytosolic Ah receptor, it is considered an antagonist, whereas β-naphthoflavone and TCDF are known agonists. Careful analysis of the inhibitory potential of U0126 to interfere with binding of [3H]TCDD convincingly demonstrated that U0126 was not able to block binding of TCDD to the receptor. Because U0126 was unable to compete for [3H]TCDD binding, it can be concluded that U0126 does not associate at the TCDD binding site of the Ah receptor. Therefore, the actions of U0126 that lead to activation of the Ah receptor into a DNA binding complex must be controlled through an alternate binding site or by modifying the confirmation of partner proteins associated with the Ah receptor (36, 37). These findings indicate that U0126 treatment leads to exposure of the nuclear localization sequence, which is necessary for trafficking of the receptor to the nucleus. It is interesting to note that along with U0126, PD98059 and SL327 stimulate a mild degree of receptor accumulation in the nucleus. This result supports findings that phosphorylation of the nuclear localization sequence is important in nuclear uptake of the Ah receptor (38).

One of the more surprising observations was the excellent correlation between inhibition of Erk kinase activity and induction of Ah receptor protein. It is well known that the Ah receptor is rapidly degraded after activation with ligands such as TCDD, benzo[a]pyrene, 3-methylcholanthrene, and β-naphthoflavone (13, 39–41) and confirmed in our studies by monitoring Ah receptor levels by Western blot analysis. This downregulation of Ah receptor is associated with ubiquitination via the 26 S proteasome pathway. Proteasome inhibitors MG-132 or lactacystin, or the overexpression of a dominant-negative ubiquitin mutant, UbK48R, blocks Ah receptor degradation by TCDD leading to an increase in gene expression as demonstrated by enhanced binding to DNA and CYP1A1 induction (13, 42–44). This result implies that changes in the steady-state levels of the Ah receptor initiated by blocking 26 S proteasome-dependent proteolysis results in enhancement of functional activity. Inhibition of Erk kinase activity leads to induction of the Ah receptor, mimicking the effect of blocking 26 S proteasome activity. However, in the presence of ligand, the Erk kinase-dependent induction of the receptor is followed by a reduction in functional activity. This finding indicates that
alterations in the steady-state concentrations of the receptor are from impaired ubiquitination, not inhibition of the proteolytic process by 26 S proteasome.

Ligand binding to the Ah receptor stimulates both transactivation and proteolysis, functional properties of the receptor that can be controlled by Erk kinase. It is interesting that the carboxyl region of the Ah receptor plays a crucial role in both transcriptional activation (9, 45) and protein stability (14), a finding that may indicate that this region has the dual function of controlling transcriptional activation of target genes while serving as a substrate for ubiquitination. These results implicate a role for Erk kinase in control and expression of the Ah receptor.

Immunoprecipitation of total cellular Ah receptor and analysis by Western blot of Erk1/2 in the precipitate demonstrated that Erk kinase is coimmunoprecipitated with the receptor and that this activity can be inhibited when cells are treated with Ah receptor inhibitors. Thus, the possibility exists that Erk kinase interacts directly with the carboxyl region of the Ah receptor and, after activation by exposure to Ah receptor ligands, initiates a phosphorylation pattern that is critical for initiating transcriptional activation of target genes as well as providing a signal to control removal of the receptor through proteasome-dependent pathways.

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ERK Kinase Inhibition Stabilizes the Aryl Hydrocarbon Receptor: IMPLICATIONS FOR TRANSCRIPTIONAL ACTIVATION AND PROTEIN DEGRADATION

Shujuan Chen, Theresa Operaña, Jessica Bonzo, Nghia Nguyen and Robert H. Tukey

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