Aryl Hydrocarbon Receptor (AhR)/AhR Nuclear Translocator (ARNT) Activity Is Unaltered by Phosphorylation of a Periodicity/ARNT/Single-Minded (PAS)-Region Serine Residue

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This paper is available online at http://molpharm.aspetjournals.org

ABSTRACT

The aryl hydrocarbon nuclear translocator (ARNT) protein belongs to the family of basic helix-loop-helix (HLH)-periodicity/ARNT/single-minded [Per/ARNT/Sim (PAS)] transcription factors and regulates a range of cellular processes by either homodimerizing or heterodimerizing with other basic HLH-PAS proteins. To date, it has been shown that both the HLH and PAS domains are required for aryl hydrocarbon receptor (AhR) ARNT heterodimerization and that phosphorylation of ARNT is also required for this heterodimerization. Presently, regulation of ARNT with respect to phosphorylation is poorly understood. In an earlier study, murine ARNT was shown to be a phosphoprotein, to display charge heterogeneity, and to have a shift in its predominant isoforms after heterodimerization with the AhR. It was hypothesized that this shift may represent a change in ARNT phosphorylation status. Metabolic [^{32}P]orthophosphate labeling of human ARNT-transfected COS-1 cells, in conjunction with phosphoamino acid analysis, Edman degradation, and phosphopeptide mapping, demonstrated that ARNT is predominantly phosphorylated on serine residues and that serine 348 (S348) in the PAS domain is phosphorylated. Alanine and glutamic acid substitutions were used to demonstrate that loss of phosphorylation at this site did not influence AhR-mediated xenobiotic response elements-driven or ARNT-mediated class B E-box–driven signaling. Additionally, the phosphorylation pattern of ARNT was unaltered after AhR heterodimerization. Although phosphorylation of S348 did not modulate AhR-ARNT or ARNT-ARNT signaling, phosphorylation of this PAS-region serine residue may be important in other ARNT-mediated gene expression systems.

ARNT is a bHLH factor that contains a highly conserved PAS domain (Hoffman et al., 1991) which has been identified in dozens of signal transduction molecules in animals, plants, and prokaryotes (Gu et al., 2000). The formation of ARNT heterodimers involves the HLH and PAS domains (Reisz-Porszasz et al., 1994). In response to exposure to selected polycyclic aromatic hydrocarbons and halogenated aromatic hydrocarbons, ARNT forms a heterodimer with the AhR after the ligand-dependent translocation of AhR to the nucleus (Reyes et al., 1992; Hankinson, 1995). Subcellular fractionation and immunohistochemical studies have shown that the AhR resides in the cytoplasm bound to two molecules of the 90-kDa heat-shock protein (Perdew, 1988) and a single molecule of AhR interacting protein/hepatitis B virus X-associated protein/AhR-activated 9 (AIP/XAP2/ARA9; Carver and Bradfield, 1997; Ma and Whitlock, 1997; Meyer et al., 1998). In contrast to the AhR, ARNT is a constitutively nuclear protein (Hord and Perdew, 1994). After translocation to the nucleus, AhR heterodimerizes with ARNT, and the AhR-ARNT heterodimer binds to XREs, causing transactivation of genes encoding xenobiotic metabolizing enzymes (Hankinson, 1995).

The HIF1α protein is a second bHLH-PAS protein; it is responsible for sensing the chemical environment and heterodimerizing with ARNT (Gu et al., 2000). Through an anal-

ABBREVIATIONS: ARNT, aryl hydrocarbon receptor nuclear translocator protein; bHLH, basic helix-loop-helix; HIF-1α, hypoxia inducible factor 1α; PAS, Per/ARNT/Sim (periodicity/aryl hydrocarbon receptor nuclear translocator/single-minded); HLH, helix-loop-helix; AhR, aryl hydrocarbon receptor; XRE, xenobiotic response element; Sim, single-minded; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; hAhR, human aryl hydrocarbon receptor; hARNT, human aryl hydrocarbon receptor nuclear translocator; FBS, fetal bovine serum; MEM, minimum essential medium; PCR, polymerase chain reaction; NP-40, nonidet P-40; MENG, MOPS/EDTA/NaN$_3$/glycerol; MOPS, 3-(*N*-morpholino)propanesulfonic acid; MENGPI, MENG containing sodium pyrophosphate, sodium molybdate, sodium fluoride, and sodium vanadate; DMSO, dimethyl sulfoxide; mAb, monoclonal antibody; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; PAGE, polyacrylamide gel electrophoresis; TLC, thin-layer chromatography; CDTA, *trans*-1,2-diaminocyclohexane-*N*,*N*,*N*′,*N*′-tetraacetic acid; RLUs, relative light units; ANOVA, analysis of variance; mARNT, murine aryl hydrocarbon receptor nuclear translocator.

ogous signal transduction pathway to the AhR, reduced cellular oxygen tension leads to increased stability of HIF1α and heterodimerizes with ARNT. Like the AhR, HIF1α is constitutively cytoplasmic; however, upon stabilization, it translocates to the nucleus (Guillemin and Krasnow, 1997). The HIF1α-ARNT heterodimer binds to hypoxia response elements and up-regulates genes involved in adaptation to hypoxia (Wang et al, 1995). The characterization of the HIF1α-ARNT heterodimer established the role of ARNT as a central dimerization partner with other bHLH-PAS proteins that are responsible for sensing and adapting to environmental change.

In addition to heterodimerizing with the AhR and HIF1α, ARNT heterodimerizes with endothelial PAS domain protein-1 and Sim (Schmidt et al., 1996). Endothelial PAS-1–ARNT heterodimers are involved in response to hypoxia and the Sim-ARNT heterodimer is involved in mammalian central midline development (Yamaki et al., 1996). Recently, an ARNT2 factor has been described that shares 63% identity with ARNT (Drutel et al., 1996) and can also heterodimerize with Sim (Michaud et al., 2000). ARNT2 has been implicated in nervous system development (Drutel et al., 1999) as well as the development of neuroendocrine lineages (Michaud et al., 2000). In addition to heterodimerizing with numerous factors, ARNT is also able to homodimerize; this homodimer can bind to and activate class B E-box–driven reporter constructs (Gupta et al., 1993; Antonsson et al., 1995). Presently, it is known that E-box binding factors are important in the regulation of cell differentiation and proliferation (Jan and Jan, 1993; Kadesch, 1993; Weintraub, 1993; Dorshkind, 1994). Although the role of the ARNT homodimer in developmental processes is unknown, ARNT expression is important for normal development. Mouse embryos lacking ARNT died after 10.5 days of development (Maltepe et al., 1997). Lack of ARNT expression resulted in defective angiogenesis of the yolk sack and branchial arches (Maltepe et al., 1997). The abnormalities were characteristic of the angiogenic abnormalities for mice lacking vascular endothelial growth factor (Carmeliet et al., 1996a; Ferrara et al., 1996) or tissue factor (Carmeliet et al., 1996b).

Phosphorylation of transcription factors is a regulatory mechanism that can control signal transduction in several potential ways. Protein phosphorylation can modulate DNA binding activity, translocation to the nucleus, and protein-protein interactions. A previous investigation revealed the importance of AhR-ARNT phosphorylation status on activation of the heterodimer into to its active DNA-binding form (Pongratz et al., 1991). It was reported that incubating transformed cytosolic AhR with acid phosphatase abolished the ability of the receptor to bind XREs. Currently, it is known that DNA binding by human and mouse AhR-ARNT heterodimers requires phosphorylation of both proteins, whereas formation of AhR-ARNT heterodimers requires phosphorylation of only ARNT (Berghard et al., 1993). Putative AhR phosphorylation sites have been localized to two regions in the C-terminal half of the mouse protein. One putative phosphorylated region is within or adjacent to a DNA-binding repressor domain, which prevents constitutive XRE binding by AhR-ARNT complexes (Dolwick et al., 1993); a second putative phosphorylated region is located within the glutamine-rich C terminus (Mahon and Gasiewicz, 1995).

The phosphorylation status of HIF1α has also been shown to modulate HIF1α-dependent activity.

Activation of signal transduction by a protein can be controlled at the structural level as a result of the addition or removal of a phosphate group(s) (Roach, 1991). Consequently, an array of phosphorylation sites could represent a distribution of differentially activated proteins that vary in their functional abilities. Previously, Tsai and Perdew (1997) demonstrated that a shift in pI of ARNT toward the basic occurs during dimerization with AhR and/or after AhR-ARNT DNA binding. This shift in pI occurs during dimerization and/or after binding to DNA and was hypothesized to represent a change in the phosphorylation status of ARNT. To date, there has been no report characterizing the functional significance of site-specific phosphorylation sites on ARNT or the influence of TCDD-dependent heterodimerization with the AhR on the phosphorylation pattern of ARNT. The current study has identified an ARNT phosphorylation site in the PAS domain and has examined its influence on AhR-ARNT signaling and ARNT homodimer signaling. Additionally, the phosphorylation pattern of ARNT after heterodimerization with the AhR was examined.

Experimental Procedures

Materials. The ARNT expression vector pSV-Sport1-hARNT was obtained from Christopher Bradfield at the University of Wisconsin (Madison, WI) (Dolwick et al., 1993). The hARNT/474-Flag construct was generated by Jo Tsai (Tsai, 1997) and the pCI/hAhR/Flag was generated with the same method by M. Pray-Grant in our laboratory. The Hepa 1 c4 cell line was obtained from Oliver Hankinson (University of California, Los Angeles, Los Angeles, CA). COS-1 cells were purchased from the American Type Culture Collection (Manassas, VA). FBS was purchased from Hyclone Lab (Logan, UT). Acrylamide, ammonium persulfate, acetonitrile, hydrochloric acid, and 100-μM thin-layer chromatography plates were purchased from Fisher (Pittsburgh, PA). Vent DNA polymerase, restriction endonucleases, and other DNA modifying enzymes were purchased from New England Biolabs (Beverly, MA). [^{32}P]Orthophosphate (370 MBq/ml; 10 mCi/ml) and goat anti-mouse [^{125}I]IgG were purchased from Amersham Pharmacia Biotech (Piscataway, NJ). pcDNA3 vector was purchased from Invitrogen (Carlsbad, CA). The Vector VIP substrate kit for immunoblots was purchased from Vector Laboratories Inc. (Burlingame, CA). Oligonucleotides for polymerase chain amplifications were purchased from Operon (Alameda, CA). LipofectAMINE reagent, opti-MEM, and *Escherichia coli* DH5α were purchased from Life Technologies (Gaithersburg, MD). Sequelon-AA arylamine disks were purchased from Perspective Biosystems (Bedford, MA). Nitrocellulose and SDS were purchased from Bio-Rad (Hercules, CA). Tricine, glycine, Tris, and CHAPS were purchased from Research Organics (Cleveland, OH). The luciferase assay system, sequencing grade trypsin, and restriction endonucleases were purchased from Promega (Madison, WI). The bicinchoninic acid protein assay reagents were purchased from Pierce (Rockford, IL). TCDD was obtained from Steven Safe (Texas A & M University, College Station, TX). All other chemicals were purchased from Sigma (St. Louis, MO).

Plasmid Construction. hARNT/Flag cDNA was amplified by polymerase chain reaction (PCR) using pSV-Sport1/hARNT as a template. The forward primer, including the start site (5′- CCCAAGCTTGGGGATGGCGGCGACTACTGCCAACCCC-3′) and an ARNT/Flag reverse primer (5′- CCGCTCGAGCGGCTACTTGTCATCGTCGTCCTTGTAGTCTTCTGAAAAGGGGGGGAAACAT-3′), were used to amplify and add a Flag sequence to the 3′ terminus of the hARNT gene using standard PCR techniques. The resulting PCR product was gel-purified, digested with *Hin*dIII and *Xho*I, and subcloned into the *Hin*dIII/*Xho*I sites of

pcDNA3. All plasmids were propagated in *Escherichia coli* strain DH5α. The nucleotide sequence of hARNT/Flag, was confirmed with an automated DNA sequencer at the DNA Core Facility at Pennsylvania State University.

Site-Directed Mutagenesis. Site-directed mutagenesis of hARNT constructs was performed using the Stratagene Quick-change site-directed mutagenesis procedure, according to the manufacturers instructions. The nucleotide sequence of hARNT mutants was confirmed with an automated DNA sequencer at the DNA core facility at the Pennsylvania State University.

Transient Transfection with hARNT and [^{32}P]Orthophosphate Labeling of Transfected Cells. COS-1 cells were cultured in α-MEM supplemented with 10% FBS, 100 IU/ml penicillin, and 0.1 mg/ml streptomycin at 37°C in 95% air/5% CO_2. COS-1 cells (80% confluent) were transiently transfected with pcDNA3/hARNT/474-Flag or pcDNA3/hARNT/Flag using the LipofectAMINE procedure. For each transfection, 9 μg of DNA/30 μl of LipofectAMINE complex was added to cells in a 10-cm^2 plate with serum-free opti-MEM. After 8 h of incubation with the DNA-LipofectAMINE complexes, the cells were rinsed twice with PBS and then incubated with α-MEM supplemented with 10% FBS, 100 IU/ml penicillin, and 0.1 mg/ml streptomycin at 37°C for 24 h. Transfected COS-1 cells were rinsed three times with phosphate-free Eagle's MEM, and then preincubated in phosphate-free Eagle's MEM supplemented with 10% dialyzed FBS, 100 IU/ml penicillin, and 0.1 mg/ml streptomycin at 37°C. After the 1-h preincubation, the media was replaced and cells were incubated with [^{32}P]orthophosphate (1 mCi/ml) for 4 h. After aspirating the [^{32}P]orthophosphate media, cells were rinsed with PBS, scraped from the dishes, and lysed with 1% NP-40 in 25 mM MOPS, 2 mM EDTA, 0.02% NaN$_3$, and 10% glycerol, pH 7.4 (MENG) containing 10 mM sodium pyrophosphate, 20 mM sodium molybdate, 10 mM sodium fluoride, 0.4 mM sodium vanadate, (MENGPI) and a protease inhibitor cocktail (2 mM 4-(2-aminoethyl)benzenesulfonyl fluoride, 2 μM *trans*-epoxysuccinyl-L-leucylamido(4-guanidino)butane, 2 μM bestatin, 2 μM leupeptin, 2 μM aprotinin, and 2 mM sodium EDTA) over 15 min on ice. The cell homogenate was centrifuged at 105,000g for 1 h and the supernatant was collected for immunoprecipitation.

In Vitro Transformation of AhR with TCDD. AhR can be induced to form a heterodimer with ARNT when treated with TCDD in vitro as well as in vivo. To examine the influence of hAhR heterodimerization on the phosphorylation pattern of hARNT, COS-1 cells (80% confluent) in 10-cm^2 plates were cotransfected with 4.5 μg of pSV-Sport1/hARNT and 4.5 μg of pCI/hAhR/Flag or, as a reference control, with 9 μg of pcDNA3/hARNT/Flag using the LipofectAMINE procedure. Twenty-four hours after transfection, cells were washed three times with phosphate-free Eagle's MEM, and then preincubated in phosphate-free Eagle's MEM supplemented with 10% dialyzed FBS, 100 IU/ml penicillin, and 0.1 mg/ml streptomycin at 37°C. After the 1-h preincubation, the media were replaced and cells were incubated with [^{32}P]orthophosphate (1 mCi/ml) for 4 h. During the final 1 h of the [^{32}P]orthophosphate labeling, COS-1 cells cotransfected with hAhR/Flag and hARNT were treated with 10 nM TCDD (dissolved in DMSO, 1 μg/ml) and cells transfected with ARNT/Flag alone were treated only with carrier (DMSO, 1 mg/ml). After aspirating the [^{32}P]orthophosphate media, cells were rinsed with PBS, and lysed in MENGPI/1% NP-40 containing 1× protease inhibitor on ice for 15 min with frequent vortexing. To isolate nuclei, the MENGPI/1% NP-40 lysate was centrifuged at 1,000g at 4°C for 15 min. Nuclei were washed three times with MENGPI before extraction with 500 mM NaCl, in MENGPI for 1 h on ice. Lysed nuclei were centrifuged at 105,000g at 4°C for 1 h and the supernatant was taken as the high-salt nuclear extract for coimmunoprecipitation. In the control experiment, the MENGPI/1% NP-40 lysate was centrifuged at 105,000g at 4°C for 1 h and the supernatant was collected for immunoprecipitation.

Immunoprecipitation and Gel Purification of Radiolabeled hARNT. Nuclear extracts form COS-1 cells cotransfected with hAhR/Flag and hARNT were coimmunoprecipitated with 50 μl of anti-Flag M2 mAb affinity gel. Similarly, cytosolic lysates from hARNT/474-Flag and hARNT/Flag-transfected COS-1 cells were immunoprecipitated with 50 μl of anti-Flag M2 mAb affinity gel. The final buffer composition during immunoprecipitation of hARNT/Flag was MENGPI, 1% CHAPS, and 250 mM NaCl, pH 7.4, and 1× protease inhibitor cocktail. The final buffer composition during immunoprecipitation of the hAhR/Flag-hARNT complexes extracted from nuclei was MENGPI, 250 mM NaCl, pH 7.4, and 1× protease inhibitor cocktail. In separate experiments, designed to demonstrate the specificity of the Flag-tagged hARNT proteins for the anti-Flag M2 mAb affinity gel, the gel was preincubated for several hours with 90 nmol of the Flag peptide (DYKDDDK) before the cell lysate was added. Immunoprecipitations were run overnight on ice. The immunoprecipitations were washed twice with 1 ml of MENGPI + 500 mM NaCl. For cytosolic extracts, the gel was then incubated with MENGPI + 500 mM NaCl + 2% NP-40 + 0.5% SDS for 1 h, with rocking on ice, and washed twice with MENGPI before gel purification by SDS-PAGE on an 8% Tricine gel. For nuclear extracts, the gel was incubated for 1 h with MENGPI + 500 mM NaCl, and then washed twice with MENGPI before SDS-PAGE on an 8% Tricine gel. Gels were transferred to nitrocellulose at 15 V for 3 h at 4°C in a Genie Blotting unit (Idea Scientific, Minneapolis, MN).

Phosphopeptide Mapping and Phosphoamino Acid Analysis of hARNT. For phosphopeptide mapping, polyacrylamide gels containing radiolabeled hARNT/Flag were transferred to nitrocellulose. After autoradiography, the hARNT/Flag band was excised from the membrane, transferred to a microcentrifuge tube, washed five times with 1 ml of water, and immediately blocked with 0.5% polyvinylpyrrolidone-360 in 100 mM acetic acid for 30 min at 37°C. The membrane was then washed five times with 1 ml of water and then once with freshly made 0.05 M ammonium bicarbonate. The membrane was resuspended in 200 μl of 0.05 M ammonium bicarbonate and 10 μg of tosylphenylalanine chloromethyl ketone-treated trypsin was added and incubated at 37°C for 16 h. At the end of the digestion, 300 μl of water was added to the digestion, the membrane was vortexed for 30 s, the digestion was centrifuged at 10,000g for 10 min, transferred to a new microcentrifuge tube, and the digestion was dried in a speed-vac. The dried sample was resuspended in 300 μl of electrophoresis buffer I, pH 1.9 (15% acetic acid, 5% formic acid), dried, resuspended in 10 μl of pH 1.9 buffer, and spotted onto a 20- × 20-cm cellulose TLC plate and electrophoresed at 1.2 kV for 35 min with a Hunter Thin Layer Electrophoresis System model HTLE-7000 (CBS Scientific, INC., Del Mar, CA). Plates were then air-dried, subjected to ascending chromatography in the second dimension for 16 h in phosphochromatography buffer (37.5% *n*-butanol, 25% pyridine, 7.5% acetic acid, and 30% deionized water), air dried, and exposed to film (Boyle et al., 1991). Phosphopeptides were visualized by autoradiography at −80°C using an intensifying screen.

For phosphoamino acid analysis, a portion of the original trypsin digest was dried down and hydrolyzed in 5.7 N HCl by heating at 110°C for 1 h. The sample was then diluted with 300 μl of water, centrifuged at 10,000g for 10 min, the hydroxylate was transferred to a new microcentrifuge tube, 1 μl each of phosphoserine, phosphothreonine, and phosphotyrosine standards were added from a 10 mM stock and then dried in a speed-vac. The hydroxylate was spotted onto thin-layer cellulose and electrophoresis was carried out in the first dimension at 1.2 kV for 30 min in pH 1.9 buffer and in the second dimension at 1.2 kV for 25 min in pH 3.5 buffer (5% acetic acid, 0.5% pyridine, 94.5% deionized water). Phosphoamino acid standards were visualized by staining with 0.25% (w/v) ninhydrin in methanol and ^{32}P-labeled phosphoamino acid residues were visualized by autoradiography at −80°C using an intensifying screen. For the analysis of individual phosphopeptides, the phosphopeptide was scraped from the TLC plate and eluted from the cellulose as described by Boyle et al. (1991). The hydroxylate was prepared, electrophoresed, standards were visualized, and autoradiography was performed at −80° using an intensifying screen.

Phosphate Release by Manual Edman Degradation. To determine the number and position of phosphorylated residues of a selected phosphopeptide, manual Edman degradation was performed by the method of Sullivan and Wong (1991). Phosphopeptides were scraped from that TLC plate and eluted from the cellulose as described by Boyle et al. (1991), dried down, dissolved in 25 μl of 50% acetonitrile, and covalently linked to an arylamine-Sequelon disk. The disk was then subjected to Edman degradation for 10 min at 50°C with 0.5 ml of coupling reagent [methanol/water/triethylamine/phenylisothiocyanate; 7:1:1:1 (v/v/v/v)], followed by six washes with 1 ml of methanol, and reheated at 50°C for 6 min with 0.5 ml of trifluoroacetic acid to cleave the N-terminal residue. The disk was then washed with 1 ml of trifluoroacetic acid and 42.5% phosphoric acid (9:1) and combined with the 0.5 ml of trifluoroacetic acid. The combined disk washes were dried under a stream of nitrogen, 10 ml of scintillation cocktail was added, and then they were quantitated for ^{32}P. The disk was subsequently washed five times with 1 ml of methanol before the next cycle was started.

Functionality of hARNT Mutants and Protein Expression. Comparison of the ability of hARNT and hARNT mutants to *trans*-activate XRE-driven luciferase activity was made in ARNT-deficient Hepa-1 c4 cells using the LipofectAMINE procedure. Hepa-1 c4 cells (80% confluent) in six-well plates were cotransfected with 2.5 ng of pSV-Sport1/hARNT, 100 ng of pGudLuc 6.1, 100 ng of pSV-β-galactosidase, and the total amount of DNA was equalized to 1.5 μg with pSV-Sport1. Control transfections contained 100 ng of pGudLuc 6.1, 100 ng of pSV-β-galactosidase, and the total amount of DNA was equalized to 1.5 μg with empty pSV-Sport1. Twenty-four hours after transfection, cells were incubated with either DMSO (1 μl/ml) or 10 nM TCDD (1 μl/ml) for 8 h. At the end of the exposure period, cells were lysed with a lysis buffer (containing 25 mM Tris-phosphate, 2 mM dithiothreitol, 2 mM CDTA, 10% glycerol, and 1% Triton X-100) and assayed for luciferase activity using the Promega luciferase assay system. Luciferase activity was measured with a Turner Instruments TD-20e luminometer (Sunnyvale, CA) and is expressed as RLUs. Luciferase activity was normalized against β-galactosidase activity and hARNT expression levels.

For quantification of hARNT expression levels in Hepa-1 c4 cells, cells in 10-cm^2 plates (80% confluent) were transfected with 9 μg of pSV-Sport1/hARNT, or pSV-Sport1/hARNT mutants with LipofectAMINE. Thirty-two hours after transfection, cells were washed with PBS, trypsinized, washed once with PBS, and lysed with 0.5 ml MENG lysis buffer (MENG, 1% NP-40, 500 mM NaCl, and 1× protease inhibitor cocktail). Cell debris was pelleted by ultracentrifugation at 105,000*g* for 1 h and the protein concentration was determined with the bicinchoninic acid protein assay. One hundred micrograms of lysate was resolved by SDS-PAGE on an 8% Tricine gel and transferred to a polyvinylidene difluoride membrane. Blots were blocked for 1 h at room temperature in a buffer containing 3% (w/v) bovine serum albumin in PBS containing 10 mM Na$_2$HPO$_4$, 0.05% (v/v) Tween 20, pH 7.4 at 25°C. The membrane was rinsed once in blot wash buffer consisting of 0.1% (w/v) bovine serum albumin in PBS containing 0.5% (v/v) Tween 20. The ARNT-specific mAb 2B10 was used to detect the wild-type ARNT protein and mutant ARNT proteins (Hord and Perdew, 1994). The anti-p50 (mcdc37) mAb C1p50 (Perdew et al., 1997) was used as a loading control for normalization of ARNT protein levels. Blots were incubated with mAb 2B10 at a dilution of 1:1000 and mAb C1p50 and given five 10-min rinses in wash buffer. For quantitative blots, mAb 2B10/hARNT complexes were detected with 1 μCi/ml ^{125}I-labeled sheep anti-mouse IgG. Blots were given five 10-min rinses in wash buffer and ^{125}I-IgG was visualized using a Bio-Rad GS-363 Molecular Imager System PhosphorImager. For qualitative hARNT blots, anti-Flag (M2) specific mAb was used at a dilution of 1:1000 for 1 h at 25°C followed by five 5-min rinses in wash buffer at 25°C. The mAb M2-probed blots were incubated with goat anti-mouse horseradish peroxidase-IgG conjugate at a dilution of 1:1000 for 1 h at 25°C followed by five 5-min rinses in wash buffer. horseradish peroxidase antibody conjugate probed blots were then visualized with using the Vector VIP substrate kit for peroxidase staining as specified by the manufacturer.

Comparison of the ability of hARNT and hARNT mutants to *trans*-activate E box-driven luciferase activity was assessed in transiently transfected COS-1 cells and Hepa-1 c4 cells. For each transfection, 1 μg of pSV-Sport1/hARNT, 0.1 μg of pSV-β-galactosidase, and either 0.4 μg of pMyc0E1bLuc or 0.4 μg of pMyc3E1bLuc were cotransfected into six-well plates using the LipofectAMINE procedure. The pMyc0E1bLuc and the pMyc3E1bLuc reporter constructs consist of zero and three E-boxes, respectively, located 5′ to the *luc+* gene; construction of these plasmids (gifts from R. Davis, University of Massachusetts Medical School, Worcester, MA) was described previously (Gupta et al., 1993). Control transfections contained 0.4 μg of pMyc0E1bLuc or 0.1 μg of pMyc3E1bLuc and 0.1 μg of pSV-β-galactosidase and were equalized to 1.5 μg of DNA with pSV-Sport1. Thirty-two hours after transfection, cell lysates were used to measure luciferase activity using the Promega luciferase assay system. Luciferase activity is expressed as RLUs and was normalized against β-galactosidase activity.

Statistical Analyses. Values are reported as means ± S.E.M. ANOVA with Duncan's multiple range test were performed to test for differences between treatment groups ($\alpha = 0.05$). All statistical tests were performed with SAS (ver. 8; SAS Institute, Cary, NC) on an IBM microcomputer.

Results

hARNT Is a Phosphoprotein. Flag-tagged truncated hARNT containing the N-terminal 474 amino acid residues (hARNT/474-Flag), as well as a Flag-tagged full-length hARNT (hARNT/Flag) (Fig. 1A) in transfected COS-1 cells incubated with [^{32}P]orthophosphate, demonstrated that hARNT is a phosphoprotein and that anti-Flag M2 affinity gel specifically immunoprecipitates each of these Flag-tagged proteins (Fig. 1B and 1C). Phosphoamino acid analysis was performed to determine whether hARNT is phosphorylated on serine, threonine, and tyrosine residues. This analysis demonstrated that both hARNT/474-Flag and hARNT/Flag are predominantly phosphorylated on serine residues (Fig. 1D).

The Phosphorylation Pattern of hARNT Is Unaltered after hAhR Heterodimerization. To assess the influence of heterodimerization with the AhR on the phosphorylation pattern of ARNT, COS-1 cells were either cotransfected with hAhR/Flag and hARNT and treated with 10 nM TCDD in DMSO or transfected with hARNT/Flag alone and treated only with DMSO. Twenty-four hours after transfection, COS-1 cells were [^{32}P]orthophosphate-labeled for 4 h. COS-1 cells cotransfected with hAhR/Flag and hARNT were treated with 10 nM TCDD during the final 1 h of [^{32}P]orthophosphate labeling and the hAhR/Flag-hARNT complex was co-immunoprecipitated with anti-Flag M2 mAb affinity gel from nuclear extracts. In the reference control, COS-1 cells transfected with hARNT/Flag alone were treated with DMSO during the final 1 h of [^{32}P]orthophosphate labeling and hARNT/Flag was immunoprecipitated with anti-Flag M2 affinity gel from cytosolic lysate. Two-dimensional phosphopeptide maps were then generated to compare the phosphorylation pattern of hARNT heterodimerized with the hAhR or in its unheterodimerized form. Based on visual analysis of two-dimensional phosphopeptide maps, it seems that the phosphorylation pattern of hARNT was unaltered whether it was heterodimerized with the hAhR or not (Fig. 2). However,

because a stoichiometric comparison was not made between phosphopeptides on these two maps, differences between the stoichiometry of particular hARNT sites in unknown.

Serine 348 Is Phosphorylated. Solid-phase sequencing of tryptic phosphopeptides isolated from a two-dimensional phosphopeptide map of hARNT/474-Flag (Fig. 3A) revealed that the predominant tryptic phosphopeptides were labeled either three or six amino acid residues away from the N terminus (Figs. 3B-I). The hARNT/474-Flag construct was chosen because it expresses the N-terminal amino acid sequence through the end of the PAS domain. Table 1 summarizes the list of candidate hARNT tryptic peptides that have a serine residue at either three or six amino acid residues from the N terminus. Site-directed mutagenesis was used to change each of these candidate serines to an alanine in the hARNT/474-Flag and hARNT/Flag cDNAs. This screening procedure revealed that serine 348 (S348) is phosphorylated in the hARNT/474-Flag construct (Fig. 4A) and in the full-length hARNT/Flag construct (Fig. 4B). In addition, this screening procedure indicated that not any of the other can-

didate serine residues in Table 1 are phosphorylated. Figure 4A illustrates the disappearance of a cluster of phosphopeptides on the hARNT/474-Flag S348A map. This cluster of missing phosphopeptides on the hARNT/474-Flag map may represent incomplete digestion products that each contain S348. Alternatively, the cluster may represent different oxidation states of either the cysteine residue and/or methionine residue in the tryptic peptide, which contains S348. This cluster was not apparent in the hARNT/Flag map; rather, the S348 containing peptide migrated as a single peptide evidenced by the disappearance of a single spot (Fig. 4B). Changing S348 to a threonine (S348T) did not restore phosphorylation of the missing peptide (Fig. 4B). Phosphoamino acid analysis of the tryptic phosphopeptide that contains S348 demonstrated that this phosphopeptide is exclusively phosphorylated on serine (Fig. 4C).

Functionality of Mutant hARNT cDNAs Expressed in Cell Lines. Phosphorylation of a protein causes an increase in negative charge. Consequently, it is possible that the effects of phosphorylation on protein function could be repro-
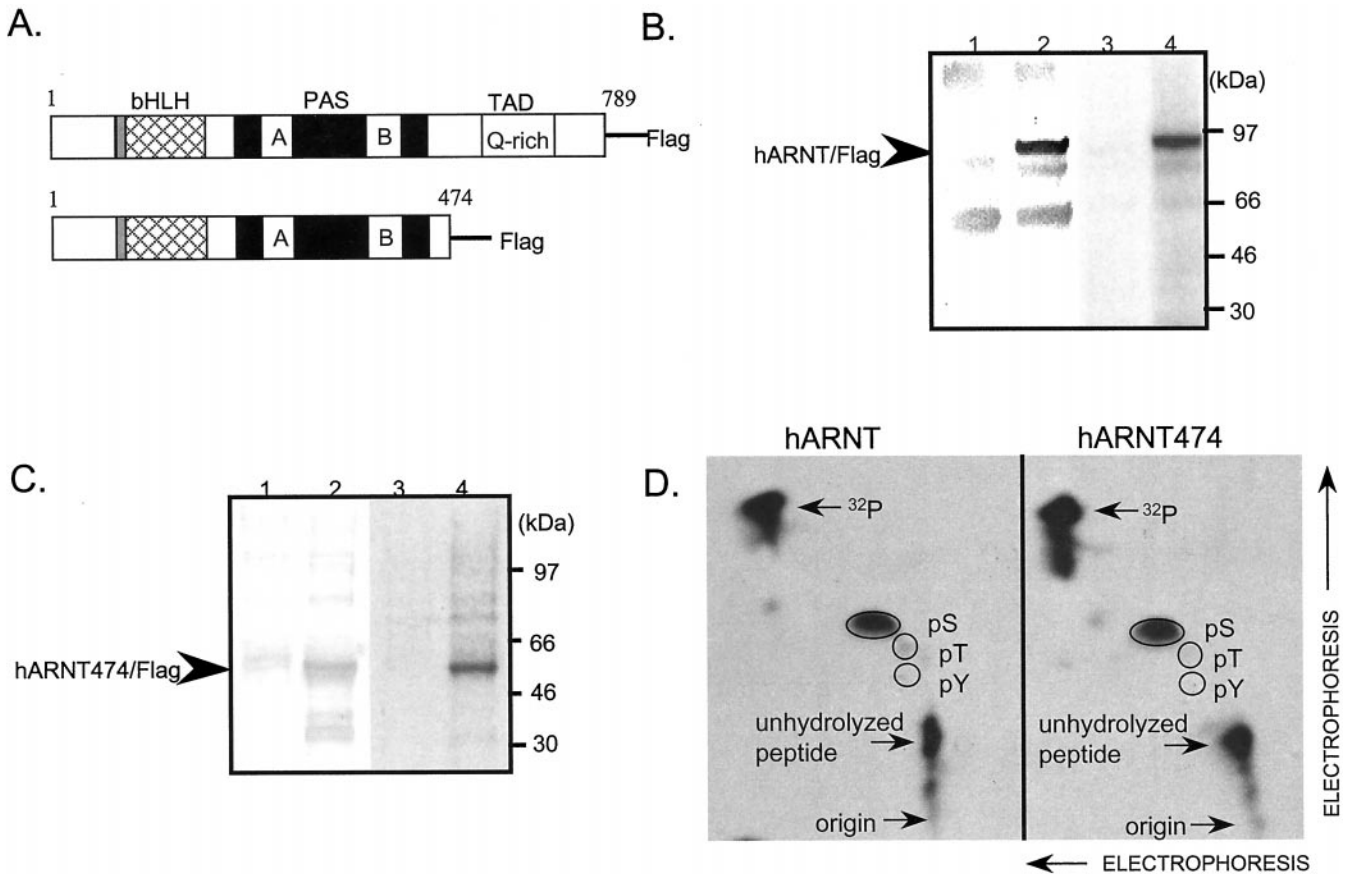

Fig. 1. hARNT is a phosphoprotein that is predominantly phosphorylated on serine residues. A, Flag-tagged hARNT cDNAs used for [^{32}P]orthophosphate labeling studies. hARNT/474-Flag is comprised of the N-terminal 474 amino acids of hARNT and contains the bHLH and PAS domains. B and C, expression of and phosphorylation of wild-type pcDNA3/hARNT/Flag and pcDNA3/hARNT474/Flag in COS-1 cells. COS-1 cells were transiently transfected with hARNT constructs and labeled for 4 h, with 1 mCi/ml [^{32}P]orthophosphate. COS-1 cytosol was immunoprecipitated with anti-Flag M2 affinity gel, resolved by SDS-PAGE on an 8% Tricine gel, transferred to nitrocellulose, probed with an anti-Flag specific mAb and autoradiographed as described under *Experimental Procedures*. B and C, lanes 2 and 4, demonstrate immunodetection of the hARNT proteins with the anti-Flag specific mAb antibody and the autoradiograph of the orthophosphate labeled hARNT proteins, respectively. The hARNT/Flag proteins are labeled with an arrowhead. Lanes 1 and 3 demonstrate the specificity of the anti-Flag M2 gel for the Flag-tagged hARNT proteins; the anti-Flag M2 gel was incubated with Flag peptide before the cell lysates were added. D, phosphoamino acid analysis of pcDNA3/hARNT/Flag and pcDNA3/hARNT474/Flag from [^{32}P]orthophosphate labeled (1 mCi/ml) COS-1 cells. SDS-PAGE–purified hARNT was hydrolyzed in 5.7 N HCl at 110°C for 1 h and mixed with phosphoamino standards [phospho-serine (pS); phospho-threonine (pT); and phospho-tyrosine (pY)] and vacuum dried. Hydroxylates were separated electrophoretically in two dimensions on cellulose plates, stained with ninhydrin to visualize standards (shown by outlined circles), and autoradiography was performed.

duced experimentally by the addition of a negative charge at the phosphorylation site. To test this hypothesis, we constructed hARNT expression vectors in which S348 was changed to either the neutrally charged alanine residue
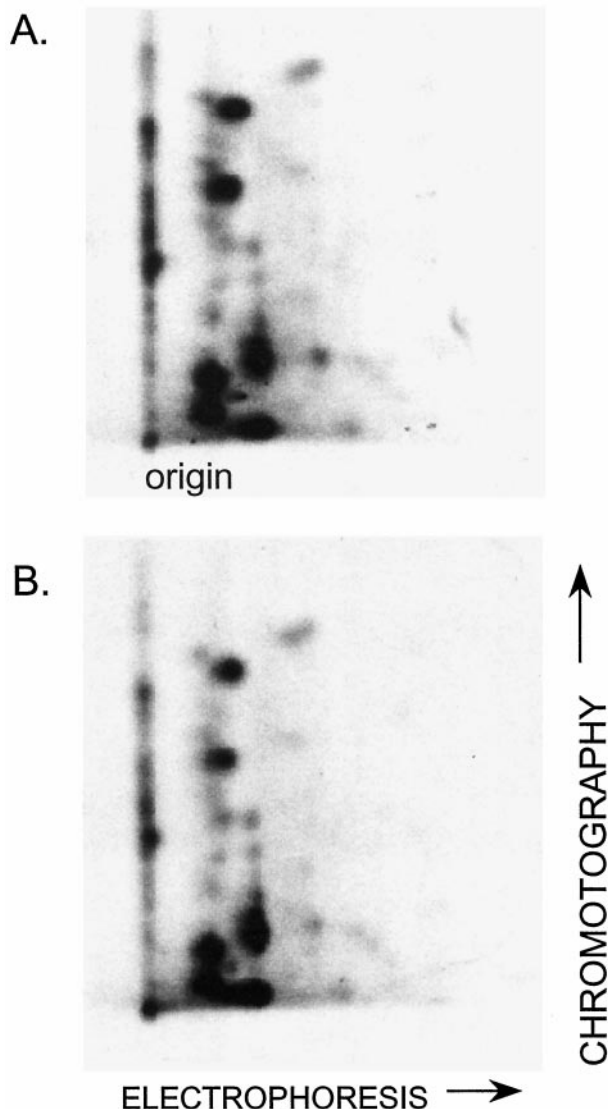

Fig. 2. Heterodimerization with the hAhR does not alter the hARNT phosphorylation pattern. A, two-dimensional phosphopeptide map of hARNT/Flag. COS-1 cells were transiently transfected with 9 μg of pcDNA3/hARNT/Flag in 10-cm^2 dishes. Cells were labeled for 4 h with 1 mCi/ml [^{32}P]orthophosphate. Cytosolic lysates were prepared as described under *Experimental Procedures* and hARNT/Flag was immunoprecipitated with anti-Flag M2 affinity gel. hARNT/Flag was resolved by SDS-PAGE on an 8% Tricine gel, transferred to nitrocellulose, and digested with trypsin. Resulting peptides were resolved in two dimensions by electrophoresis and chromatography on TLC plates, and phosphorylated peptides were visualized by autoradiography. B, two-dimensional phosphopeptide map of hARNT that was coimmunoprecipitated with AhR/Flag from nuclear extract. COS-1 cells were transiently transfected with 4.5 μg of pSV-Sport1/hARNT and 4.5 μg of pCI/hAhR/Flag in 10-cm^2 dishes and labeled for 4 h with 4 mCi/ml [^{32}P]orthophosphate and during the final hour of [^{32}P]orthophosphate labeling, 10 nM TCDD was introduced to the culture media. Nuclear extracts were prepared as described under *Experimental Procedures* and AhR/Flag-ARNT complexes were immunoprecipitated with anti-Flag M2 gel. SDS-PAGE purified hARNT was transferred to nitrocellulose and digested with trypsin. Resulting tryptic peptides were resolved in two dimensions by electrophoresis and chromatography on TLC plates, and the phosphorylated peptides were visualized by autoradiography.

(S348A) or the negatively charged glutamic acid residue (S348E) to assess the functional role of phosphoserine 348. Therefore, a comparison between the activity of S348A and S348E can provide a basis for determining whether a phosphorylation site has a positive or negative functional role. Hepa-1 c4 cells were used for this comparison because they do not express ARNT at the protein level. Figure 5A shows that both S348A and S348E mutated hARNT proteins *trans*-activated XRE driven reporter activity in Hepa-1 c4 cells, in the presence and absence of TCDD, to an equal level as S348. Figure 5B shows the protein expression level of each of the transfected hARNT constructs in Hepa-1 c4 cells. XRE driven reporter activity was normalized against hARNT expression levels and β-galactosidase activity. Western blot analysis confirmed that mock-transfected Hepa-1 c4 cells did not express detectable levels of ARNT and that each of the transfected hARNT constructs express at similar levels when normalized to p50 expression levels.

Previously, it was demonstrated that ARNT is capable of forming a homodimer that binds class B E-boxes and can *trans*-activate class B E-box–driven reporter constructs (Antonsson et al., 1995; Swanson et al., 1995; Long et al., 1999). To further assess the functional importance of phosphorylation of serine 348, the ability of the S348A and S348E mutants to activate the class B E-box reporter was tested in COS-1 cells and Hepa-1 c4 cells. These cell lines were transiently transfected with either the pMyc0E1bLuc or pMyc3E1bLuc reporter constructs, containing zero and three class B E-box 5′ to the *luc*$^+$ gene, respectively, alone or in the presence of hARNT cDNAs. The wild-type and mutant hARNT cDNAs (S348, S348A, and S348E) activated pMyc3E1bLuc reporter activity in COS-1 cells and Hepa-1 c4 cells to an equal extent (Fig. 6). In COS-1 cells, there was a small but significant increase in pMyc0E1bLuc reporter activity for cells cotransfected with pMyc0E1bLuc and wild-type hARNT. Induction of the pMyc0E1bLuc reporter construct in COS-1 cells, cotransfected with ARNT, is consistent with previous reports and is believed to result from E-box–like sequences 5′ to the *luc*+ gene (Long et al., 1999 and references within). Because the Hepa-1 c4 cells do not express ARNT, an increase in pMyc0E1bLuc reporter activity for cells cotransfected with pMyc0E1bLuc and wild-type hARNT did not occur in this cell line.

Discussion

An earlier investigation in our laboratory characterized the level of charge heterogeneity displayed by mARNT (Tsai and Perdew, 1997). A comparison between mARNT as a monomer and mARNT heterodimerized with the murine AhR, demonstrated that a significant shift in the pI of ARNT occurs just before or after heterodimerization in the nucleus. A movement of the predominant ARNT isoforms toward the basic end characterized this shift in charge heterogeneity. Furthermore, this shift in charge heterogeneity was believed to be related in part to a change in ARNT phosphorylation or an alteration in another type of secondary modification. For example, one type of modification that could influence ARNT charge heterogeneity is glycosylation, which can block phosphorylation of specific sites. Previously, it was shown that the bHLH protein c-Myc can be either phosphorylated or glycosylated at threonine 58, which is located in the *trans*-

activation domain (Gupta et al., 1993; Chou et al., 1995). Additionally, it was determined that phosphorylation of threonine 58 can regulate c-MYC signaling (Gupta et al., 1993). Consequently, it was proposed that reciprocal glycosylation and phosphorylation at threonine 58 could play an important role in the regulation of c-MYC activity. Whether hARNT residues can be glycosylated is unknown. However, the possibility exists that glycosylation could potentially influence ARNT charge heterogeneity, phosphorylation status, and activity. Presently, the significance of the aforementioned shift in ARNT charge heterogeneity after heterodimerization with the AhR is unknown. Despite the shift in ARNT charge heterogeneity after heterodimerization with the AhR, het-

erodimerization does not seem to influence the general phosphorylation pattern of hARNT (Fig. 2). However, because the stoichiometry of each hARNT phosphorylation site was not assessed, it is unknown whether heterodimerization with the AhR significantly influences the stoichiometry of individual phosphorylation sites.

An earlier study that investigated the functional role of phosphorylation in the interaction between AhR and ARNT demonstrated that phosphorylation of ARNT is required for formation of the AhR-ARNT heterodimer (Berghard et al., 1993). In a later study, it was shown that the HLH and PAS domains of ARNT mediate dimerization (Reisz-Porszasz et al., 1994). Taken together, the results from these two studies
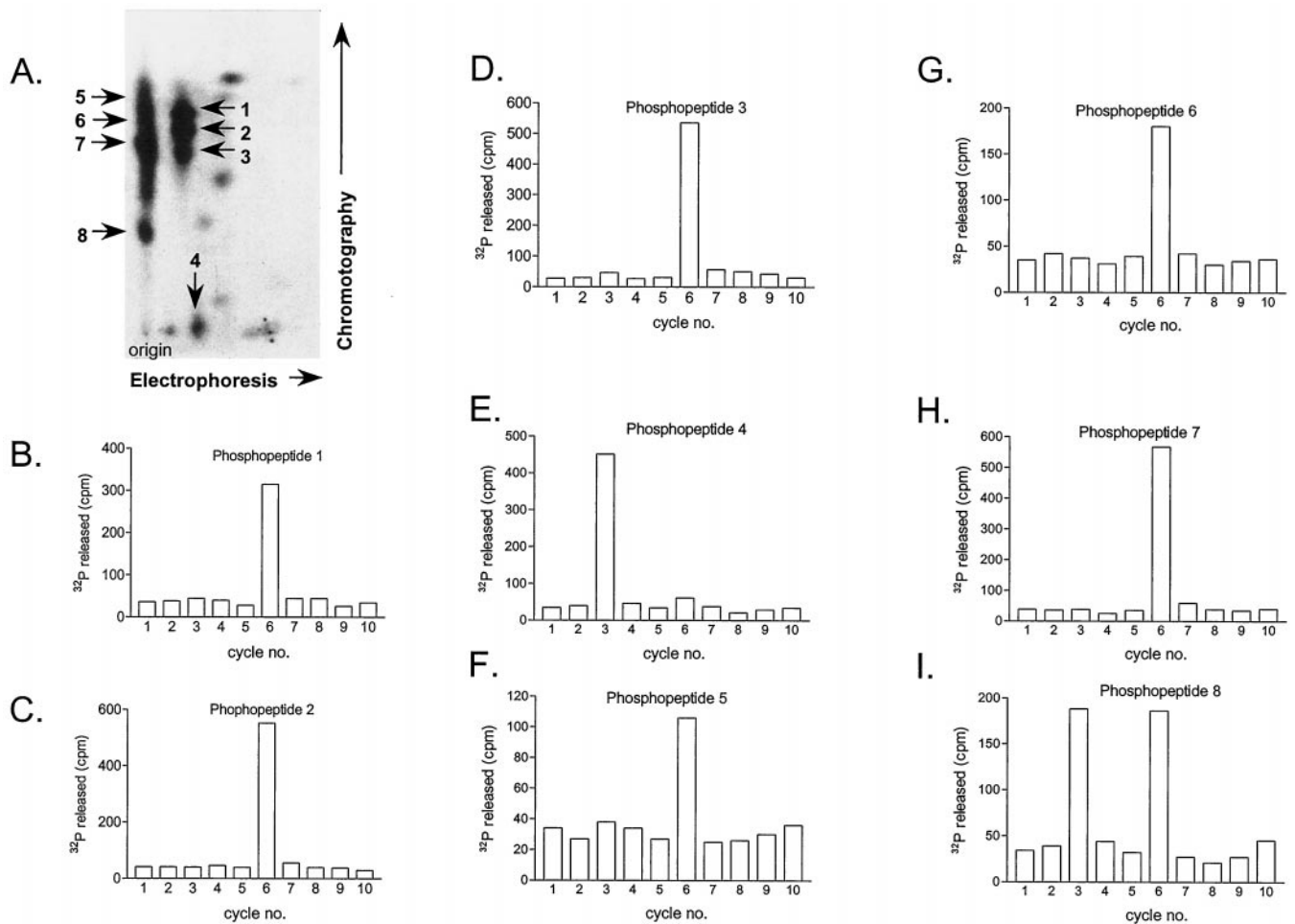

Fig. 3. Manual Edman degradation of tryptic phosphopeptides to determine cycles of [^{32}P]orthophosphate release. A, tryptic phosphopeptides were generated from pcDNA3/hARNT/474-Flag- transfected COS-1 cells and were separated by electrophoresis and chromatography on a TLC plate. B to I, individual phosphopeptides were eluted from the cellulose and resuspended in 50% acetonitrile. Phosphopeptides were covalently linked to arylamine disks and subjected to a modification of Edman degradation. The counts of [^{32}P]orthophosphate in the released amino acid were determined after each cycle by Cerenkov counting.

TABLE 1

Candidate tryptic phosphopeptides as determined by manual Edman degradation

The underlined serine represents predicted phosphorylation sites.

Number of residues from N terminus	Tryptic Peptide (amino acids)	Amino Acid Number	Tryptic Peptide Sequence[a]	Region
6	144–165	149	GTGNTSTDGSYKPSFLTDQELK	HLH
6	223–235	228	EQLSTSENALTGR	PAS
6	247–254	252	EGQQSSMR	PAS
3	269–281	272	CGSSSVDPVSVNR	PAS
6	343–366	348	LQVTSSPNCTDMSNVCQPTEFISR	PAS
3	441–465	443	TSSFQNPYSDEIEYIICTNTNVKNSSQEPR	PAS

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merited our use of the truncated hARNT/474-Flag construct to search for a phosphorylation site(s) that may be involved in ARNT heterodimerization and homodimerization. Pho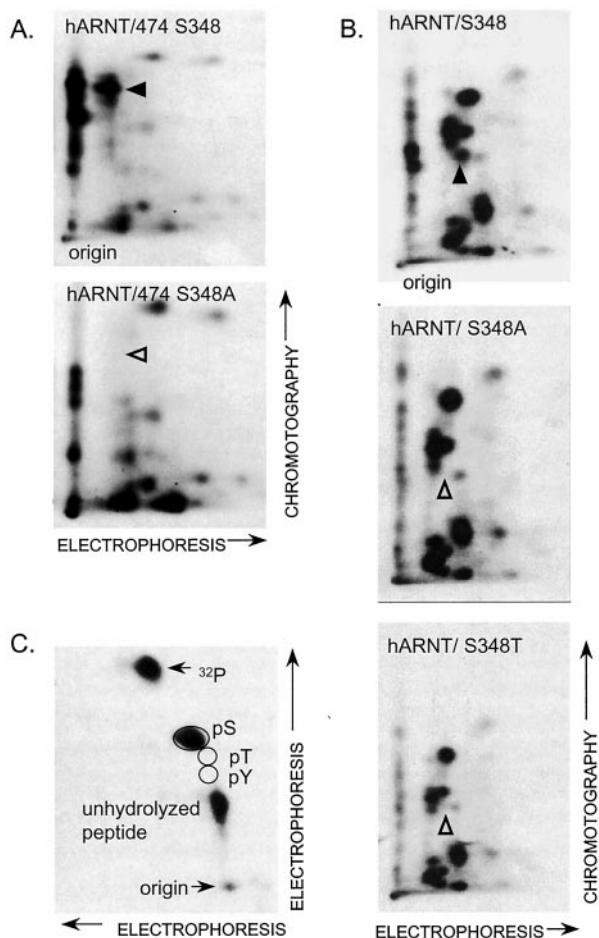sphoamino acid analysis indicated that hARNT/474-Flag is phosphorylated exclusively on serine residues and that hARNT/Flag is phosphorylated almost exclusively on serine residues. Solid-phase sequencing of tryptic phosphopeptides isolated from two-dimensional maps of hARNT/474-Flag identified five candidate serine containing tryptic peptides located in the PAS domain and identified one candidate serine containing peptide located in the distal end of the HLH region (Table 1). By changing each of these candidate serine residues to alanine residues, we were able to identify S348 as a phosphorylation site and verify that the other five candidate sites were not phosphorylated. The functional role of S348 was assessed with two ARNT signaling pathways. The first signaling pathway examined the role of S348 in AhR-ARNT signaling by comparing the ability of S348, S348A, and S348E to *trans*-activate XRE-driven luciferase activity in an ARNT-deficient cell line. The second signaling pathway examined the role of S348 on ARNT-ARNT homodimer signaling by comparing the ability of S348, S348A, and S348E to *trans*-activate class B E-box-driven luciferase activity in the ARNT deficient cell line and COS-1 cells. These analyses have demonstrated that phosphorylation of S348 apparently does not influence ARNT heterodimerization or homodimerization and therefore does not influence AhR-hARNT or hARNT homodimer mediated gene expression.
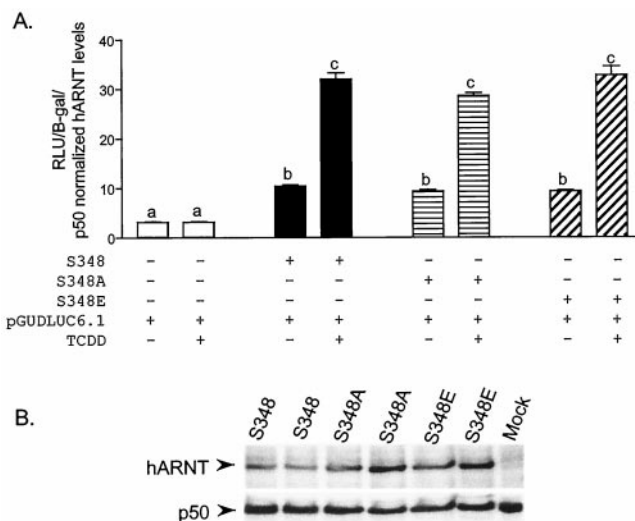

Fig. 4. Serine 348 is a phosphorylation site on the hARNT protein. A, two-dimensional tryptic phosphopeptide map of pcDNA3/hARNT/474-Flag and pcDNA3/hARNT/474-Flag S348A, which contains a serine-to-alanine substitution at position 348. COS-1 cells transiently expressing pcDNA3/hARNT/474-Flag cDNAs were labeled for 4 h with 1 mCi/ml [^{32}P]orthophosphate, and cytosol was immunoprecipitated with anti-Flag M2 affinity gel. SDS-PAGE–purified proteins were transferred to nitrocellulose and digested with trypsin. Resulting peptides were resolved by electrophoresis and chromatography, and the phosphorylated peptides were visualized by autoradiography. The filled arrowhead identifies the phosphopeptides that contain S348 and the open arrowheads identify the location of the missing phosphopeptides. B, two-dimensional tryptic phosphopeptide maps of pcDNA3/hARNT/Flag (S348) and mutant pcDNA3/hARNT/Flag proteins (S348A and S348T). COS-1 cells transiently expressing hARNT/Flag cDNAs were labeled for 4 h with 1 mCi/ml [^{32}P]orthophosphate, and cytosol was immunoprecipitated with anti-Flag M2 affinity gel. SDS-PAGE–purified proteins were transferred to nitrocellulose and digested with trypsin. Resulting peptides were resolved by electrophoresis and chromatography, and the phosphorylated peptides were visualized by autoradiography. The filled arrowhead identifies the phosphopeptide that contains S348 and the open arrowhead identifies the location of the missing phosphopeptide. C, phosphoamino acid analysis of the phosphopeptide representing phosphoserine 348. The phosphopeptide containing phosphoserine 348 in B (marked with a filled arrow) was scraped from the TLC plate and eluted from the cellulose as described under *Experimental Procedures*. The eluent was vacuum-dried and hydrolyzed in 5.7 N HCl at 110°C for 1 h. The hydrolyate was mixed with phosphoamino standards [phospho-serine (pS); phospho-threonine (pT); and phospho-tyrosine (pY)], separated electrophoretically in two dimensions on cellulose plates, stained with ninhydrin to visualize standards (shown by outlined circles), and autoradiography was performed.

Fig. 5. Changing serine at amino acid position 348 (S348) to an alanine (S348A) or a glutamic acid (S348E) does not influence XRE driven luciferase activity in Hepa-1 c4 cells. A, Hepa-1 c4 cells were transiently transfected with either pSV-Sport1/hARNT/S348, pSV-Sport1/hARNT/S348A, pSV-Sport1/hARNT/S348E (2.5 ng μg), pGudLuc 6.1 (100 ng), and a β-galactosidase expression vector (100 ng) in a 6-well plate. The total amount of DNA transfected per well was 1.5 μg and equalized with pSV-Sport1 expression vector. Twenty-four hours after transfection, cells were treated with 10 nM TCDD for 8 h and cells were lysed to measure luciferase activity; luciferase activity is reported as RLUs normalized against β-galactosidase expression. Transfection groups are presented as mean ± S.E.M. (*n* = 6) and groups with the same letter were not significantly different as determined with ANOVA and Duncan's multiple range test (α = 0.05). B, expression of hARNT in Hepa-1 c4 cells. Hepa-1 c4 cells were transiently transfected with the pSV-Sport1/hARNT cDNA (9 μg per 10-cm² dish) and whole-cell extracts were prepared 36 h after transfection. The control transfection (mock transfected) received 9 μg of pSV-Sport1 expression vector. SDS-PAGE and immunoblotting were conducted as described under *Experimental Procedures*. hARNT expression levels were normalized against p50 protein levels and then normalized hARNT levels were used along with β-galactosidase activity to adjust XRE-driven luciferase activity reported in A.

In contrast to the study by Berghard et al. (1993), which compared a phosphorylated form of ARNT with a dephosphorylated form of ARNT through the use of alkaline phosphatase, the results of the present study were generated by examining the role of a single phosphorylation site in a functional domain. Therefore, it is difficult to draw a comparison between the results from the present study with those of Berghard et al. (1993). However, an unpublished result from our laboratory demonstrated that the phosphatase inhibitor okadaic acid increased hARNT-hARNT–mediated E-box activity by 2-fold in hARNT transfected COS-1 cells. This 2-fold increase in E-box activity was accompanied by a greater than 10-fold increase in [^{32}P]orthophosphate incorporation into hARNT without influencing hARNT expression levels in COS-1 cells. The results from our laboratory, along with the results of Berghard et al. (1993), suggest that overall phosphorylation status, or a cluster of sites, rather than site-specific phosphorylation, may be crucial in the regulation of ARNT-mediated signaling.

This study has shown that although hARNT is a phosphoprotein, the phosphorylation of a PAS-region serine residue does not modulate AhR-ARNT and ARNT-ARNT mediated signaling. In addition, the phosphorylation pattern of

hARNT seems to be unaltered whether it is a monomer or dimerized with the hAhR. Although phosphorylation of S348 was shown not to modulate AhR and ARNT-ARNT signaling, the phosphorylation status of S348 may play a functional role with other ARNT dimerization partners in their respective systems. Future investigations will identify phosphorylation sites distal to the PAS domain and determine whether S348 plays a functional role in combination with these other hARNT phosphorylation sites in ARNT-mediated signaling systems.

Fig. 6. Changing serine at amino acid position 348 (S348) to an alanine (S348A) or a glutamic acid (S348E) does not influence class B E-box–driven luciferase activity Hepa-1 c4 cells and COS-1 cells. Hepa-1 c4 cells and COS-1 cells were transiently transfected with either pSV-Sport1/hARNT/S348, pSV-Sport1/hARNT/S348A, pSV-Sport1/hARNT/S348E cDNAs (1.0 μg), either pMyc3E1bluc or pMyc3E1bluc E-box reporter construct (0.4 μg), and a β-galactosidase expression vector (0.1 μg) in a 6-well plate. The total amount of DNA transfected per well was 1.5 μg, and for control groups the amount of DNA was equalized with empty pSV-Sport1 expression vector. The pMyc0E1bLuc and the pMyc3E1bLuc reporter constructs consist of zero and three E-boxes, respectively, located 5′ to $luc+$. Thirty-six hours after transfection, cells were lysed and luciferase activity was measured. Class B E-box–driven luciferase activity is reported as RLUs normalized against β-galactosidase expression. Transfection groups are presented as mean $\pm$ S.E.M. ($n = 6$) and groups with the same letter were not significantly different as determined with ANOVA and Duncan's multiple range test ($\alpha = 0.05$).

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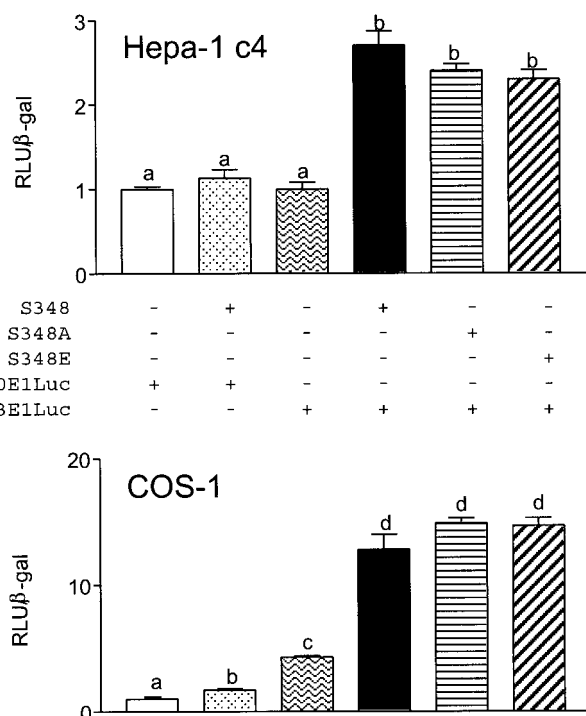
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