Interaction with Nedd8, a Ubiquitin-like Protein, Enhances the Transcriptional Activity of the Aryl Hydrocarbon Receptor*

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The aryl hydrocarbon receptor (AhR) is a ligand-activated member of the basic helix-loop-helix period aryl hydrocarbon nuclear translocator single-minded (PAS) transcription factor family. This receptor has been shown to be important in various aspects of growth and development as demonstrated by AhR-null mice. A yeast two-hybrid screen of a mouse embryonic day 11 library for AhR-interacting proteins revealed Nedd8 as a candidate. The interaction was confirmed in a cell-free system and in mammalian cells by co-immunoprecipitation; however, in vitro neddylation assays showed that Nedd8 does not covalently modify AhR. Transfection of Nedd8 into a cell line stably transfected with a dioxin response element linked to a chloramphenicol acetyltransferase reporter gene demonstrated that Nedd8 amplified ligand-induced transcription. Deletion of the Gly-76 residue in the carboxyl terminus of Nedd8 abolished this effect and prevented AhR-Nedd8 interaction. Nedd8 overexpression also resulted in accumulation of AhR protein in the nucleus, independent of exogenous ligand. These studies demonstrate that the AhR interacts with Nedd8 and suggest that this interaction enhances the transcriptional activity of the receptor, perhaps involving increased nuclear accumulation or retention. Immunohistochemistry performed on embryonic day 11.5 mouse sections indicated Nedd8 and AhR localize to overlapping areas in the heart and spinal ganglia, raising the possibility that this interaction may play a role in organogenesis.

The aryl hydrocarbon receptor (AhR)† is a ligand-activated transcription factor belonging to the basic helix-loop-helix (bHLH)/period aryl hydrocarbon nuclear translocator single-minded (PAS) family of heterodimeric transcriptional regula-

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† The abbreviations used are: AhR, aryl hydrocarbon receptor; TCDD, 2,3,7,8-tetrachlorodibenzo-(p)-dioxin; DRE, dioxin response element; ARNT, AhR nuclear translocator; HA, hemagglutinin; bHLH, basic helix-loop-helix; CAT, chloramphenicol acetyltransferase; E, embryonic day; Cul, cullin; PBS, phosphate-buffered saline; E1, ubiquitin-activating enzyme; E2, ubiquitin conjugating enzyme; ER, estrogen receptor; HIF-1α, hypoxia-inducible factor 1α.

¶ The biological effects of 2,3,7,8-tetrachlorodibenzo-(p)-dioxin (TCDD) and related environmental contaminants are mediated by AhR and promote carcinogenesis, immune suppression, hepatotoxicity, cardiac toxicity, and impairment of reproductive function (as reviewed by Hankinson (1)). Unliganded AhR resides in the cytoplasm as part of an inactive multi-protein complex that contains 90-kDa heat shock proteins (HSP90), immunophilin-like protein XP2 (also known as AhR-associated protein 9 (ARAP9) or AhR-interacting protein (AIP)), and p23 (2, 3). Upon binding ligand, the two HSP90 molecules dissociate, and the receptor translocates to the nucleus and heterodimerizes with another bHLH member protein, the aryl hydrocarbon receptor nuclear translocator (ARNT) protein. This protein complex then binds to cognate regulatory elements, referred to as dioxin-responsive elements (DREs), located in the promoter region of a number of known target genes, which include the phase I cytochromes P4501B1, P4501A1, P4501A2, and P4501B1. Activated AhR is exported from the nucleus for degradation in the cytosol by the ubiquitin/proteasome pathway (4).

The molecular events leading to the activation of AhR in the presence of TCDD are generally well understood in a toxicological context. However, AhR signaling pathways in the absence of exogenous ligands remain relatively unknown. The phenotypes of three independent mouse strains with targeted disruption of the AhR strongly suggest a physiological role for this receptor in homeostasis, liver development, and immune system function as well as a role in cell proliferation and differentiation (5, 6). The AhR is highly conserved in a number of species including fruit flies, zebrafish, worms, chickens, mice, and humans. The function and regulation of AhR during development are currently being addressed in each of these model systems.

In an effort to identify proteins that are a part of the AhR complex and that might modulate its activity, we used a yeast two-hybrid system to screen for proteins that interact with the AhR. Studies of the developmental expression of AhR in C57BL/6 mouse embryos using in situ hybridization and/or immunohistochemical analysis have shown that AhR expression is not detectable until embryonic day (E) 10 onward, suggesting that this is the first time during development that AhR protein is expressed (7). We therefore used a mouse E11 cDNA library in our yeast two-hybrid system. We report here the isolation of a cDNA clone encoding Nedd8 (neurally expressed developmentally down-regulated protein 8) that interacts with AhR in a ligand-independent manner.

Nedd8 is a ubiquitin-like protein, initially identified by Kumar et al. (8) in a subtractive cloning approach to identify genes involved in the development of the mammalian central nervous system, that covalently modifies target proteins that reside in the nucleus. Known targets of Nedd8 modulation include
p27\textsuperscript{kip1} and all members of the human cullin family of proteins (9). In this study we provide novel evidence showing that Nedd8 enhances the action of the AhR.

**EXPERIMENTAL PROCEDURES**

**Yeast Two-hybrid Screen**—An expression vector containing a full-length mouse AhR cDNA (pcDNA1-AhR) was kindly provided by Dr. Oliver Hankinson (University of California, Los Angeles, CA). To screen for AhR-interacting proteins, a fragment of the AhR cDNA corresponding to amino acids 4–494 was excised using HindIII/NotI and EheI endonuclease digestion and inserted into the Smal/NcoI sites of the pGBK7T7 bait plasmid of the MATCHMAKER Two-Hybrid System 3 (Clontech). The exact insertion of the yeast two-hybrid system was confirmed by a sequencing-verified. The resulting pGBK7T7-AhR construct encoded a fusion protein containing the DNA binding domain of the yeast transcription factor GAL4 linked to the AhR lacking the Q-rich transactivational domain. pGBK7T7-AhR bait vector was transformed into yeast strain AH109, and transformants were screened to ensure the bait vector alone did not induce expression of the His3, Ade2, or LacZ reporter genes. Addition of 3-amino-1,2,4-triazole (2.5 mM) into the selection medium was necessary to suppress nonspecific His3 induction. AH109 pGBK7T7-AhR transformants were then transformed with plasmids amplified and isolated from a mouse 11-day embryo MATCHMAKER cDNA library (pGADT7) (Clontech). All other procedures involving the yeast two-hybrid system were performed as directed by the manufacturer (Clontech). The transformation mixtures were plated on selection medium + 2.5 mM 3-amino-1,2,4-triazole for sequential selection of both Ade2 and His3 reporter gene expression. After further selection using a colony-lift filter assay for β-galactosidase activity, positive clones were collected. Prey vectors (pACT2) containing the GAL4 cDNA were isolated as described previously (10). Briefly, yeast from each clone were collected from a 2-ml liquid culture and resuspended in 50 μl of lysis buffer (50 mM Tris–Cl, pH 7.5, 1.2 mM sorbitol, 10 mM EDTA, and 10 mM β-mercaptoethanol). The cell wall of the yeast was digested with 30 units of lyticase (Sigma) overnight at 37 °C. The lysates were centrifuged for 5 min at 10,000 × g, and the supernatant was discarded. Plasmid DNA was isolated from the pellet using the Wizard Plus SV Miniprep DNA purification System (Promega, Madison, WI) and transformed into E. coli, amplified, and sequenced.

**Expression Vectors**—A full-length Nedd8 cDNA was excised from the pcACT2 library vector using BumH/Del and inserted in-frame into pGADT7 (Clontech) to generate pGADT7-Nedd8 for in vitro expression of HA-tagged Nedd8 protein. An expression plasmid containing mouse Nedd8 in pcDNA1 (pcDNA3-HA-Nedd8) was kindly provided by Dr. E. Yeh (University of Texas). Dr. Bert O’Malley (Baylor College of Medicine) kindly provided pcDNA3-HA-Nedd8G, which has a carboxy-terminal deletion from Gly-76 to Gln-81 and is therefore unable to form a GAG, or empty pcDNA3 vector. A Myc epitope-tagged Nedd8 expression vector in pcDNA3 and a HA epitope-tagged human cullin 1 (Cul-1) expression vector in pcDNA3 were kindly provided by Dr. M. Furukawa (University of North Carolina).

**In Vitro Transcription Translation and Co-immunoprecipitation**—\textsuperscript{[\textsuperscript{35}S]Methionine (Amersham Biosciences)-labeled AhR and AhR proteins were generated from pGADT7-Nedd8 and pcDNA3-AhR, respectively, using a TNT Coupled Rabbit Reticulocyte Lysate System as directed by the manufacturer (Promega). A MATCHMAKER Co-immunoprecipitation Kit (Clontech) was used to independently confirm AhR and Nedd8 protein interaction via in vitro co-immunoprecipitation. In vitro-translated amounts of \textsuperscript{35}S-labeled AhR and Nedd8 protein were mixed and incubated for 1 h at 30 °C. To each sample, 470 μl of co-immunoprecipitation buffer (20 mM Tris–Cl, pH 7.5, 150 mM NaCl, and 1 mM dithiothreitol) containing protease inhibitor (Complete protease inhibitor tablets; Roche Molecular Biochemicals) and 1 μg/ml AhR antibody (11) (kindly provided by Dr. Pollenz, Medical University of South Carolina) or HA-tagged polyclonal antibody (Clontech) were added and incubated for 2 h at 4 °C. Protein A-agarose (10 μl) was then added to each sample. After incubation for an additional 45 min at 4 °C, the samples were centrifuged and washed four times with TBST (20 mM Tris–Cl, pH 7.5, 150 mM NaCl, and 0.1% Tween 20 (v/v)). After the final wash, the pellets were resuspended in SDS gel loading buffer, boiled for 5 min, and subjected to electrophoresis on a 4–20% gradient Tris glycine gel. The gel was fixed, exposed to Amplify (Amersham Pharmacia Biotech), and transferred to polyvinylidene difluoride membrane (Pall Gelman Laboratory) and probed with either an antibody recognizing the biotinylated secondary antibody to Nedd8. Controls in- cluded omission of the primary antibodies. After incubation, cells were washed with PBS and then washed with PBS containing 1 μg/ml 4′,6-diamidino-2-phenylindole (Sigma). Coverslips were mounted onto the glass slides with Vectashield (Vector Laboratories, Burlingame, CA) for viewing under a Olympus IX70 deconvolution microscope using DeltaVision Software for analysis/image capture (Applied Precision Inc.) with an excitation wavelength of 562 nm for Cy3 and 496 nm for Cy2.

**Co-localization Immunocytochemistry**—T47D cells were cultured on glass coverslips for 2 days and treated with 10 μM TCDD (Sapelco, Ontario, Canada) or 0.1% ethanol for 90 min and then fixed with a 1:1 mixture of acetone and methanol at −20 °C for 10 min. Cells were washed with 70% ethanol and PBS (137 mM NaCl, 2.68 mM KCl, 10 mM Na2HPO4, and 1.76 mM KH2PO4, pH 7.4) containing 0.5% Triton X-100 (PBST) for 15 min. For double-labeling experiments, cells were blocked with 10% (v/v) horse serum in PBS and incubated with rabbit anti-AhR and a Cy3-conjugated donkey anti-rabbit IgG. Cy2-conjugated streptavidin was used as a secondary antibody to the biotinylated secondary antibody to Nedd8. Controls included omission of the primary antibodies. After incubation, cells were washed with PBST and then washed with PBST containing 1 μg/ml 4′,6-diamidino-2-phenylindole (Sigma). Coverslips were mounted onto the glass slides with Vectashield (Vector Laboratories, Burlingame, CA) for viewing under a Olympus IX70 deconvolution microscope using DeltaVision Software for analysis/image capture (Applied Precision Inc.) with an excitation wavelength of 562 nm for Cy3 and 496 nm for Cy2.

**CAT Expression Assay**—DRE82 cells were seeded into 6-well plates at a density of 2.5 × 10\textsuperscript{5} cells/well 1 day before transfection with pcDNA3-HA-Nedd8, pcDNA3-HA-Nedd8G, or empty pcDNA3 expression vector. The total amount of plasmid DNA used was normalized to 1200 ng/well by the addition of empty expression plasmid. 18 h after transfection, cells were treated with fresh medium containing 1 μM TCDD or vehicle (0.1% ethanol). After 24 h, the cells were harvested, and CAT activity was determined using a CAT enzyme-linked immuno- precipitation assay kit (Roche Molecular Biochemicals) according to the manufacturer’s instructions. Values were normalized to β-galactosidase activity, measured as described in Ref. 13, and are reported as the means ± S.E. relative to 1.0 for empty expression vector alone. Transfections were performed in triplicate, with each experiment repeated three to four times. Significant differences between values were determined by Student-Newman-Keuls test (Sigma Stat 1.0; Jandel Corp.).

**In Vitro Neddylation Assay**—In vitro-translated \textsuperscript{35}S-labeled AhR or human cul-1 were incubated with 0.4 mg/ml recombinant Nedd8 or an equimolar amount of in vitro-translated Myc-tagged Nedd8, an energy-
regenerating system (20 mM Tris, pH 7.4, 2 mM ATP, 5 mM MgCl₂, 40 mM creatine phosphate, and 0.5 µg/µl creatine kinase) and 20 µg of Hela S100 fraction (S-100) (14). Reactions were supplemented with 0.01 M MgATP solution. All components for the neddylation assay were purchased from Boston Biochem (Boston, MA). Reactions were adjusted to 50 mM Tris-HCl, pH 7.5, in a total volume of 20 µl and incubated at 30 °C for 2 h. Reactions were stopped by the addition of co-immunoprecipitation buffer, and immunoprecipitation was performed with anti-HA antibody or anti-AhR antibody as described above. Immunoprecipitates were subjected to 6% SDS-PAGE, dried under vacuum, and exposed to Kodak x-ray film.

Preparation of Cell Lysates, Cytosol, and Nuclear Lysates—After treatment, cell monolayers were washed with PBS and lysed in ice-cold EYD lysis buffer, as described above. Cytosol and total nuclear lysates were prepared as described previously (4). Protein concentrations were determined by the bovine serum albumin standard as described after the Western blot analysis, the ECL exposures were scanned and quantified using Image Quant 5.0 software for Windows NT (Molecular Dynamics). The raw level of AhR protein was then divided by a Pierce S-stained protein band to generate normalized values for the concentration of AhR in each sample as described previously (15).

Immunohistochemistry—Mouse ICR (Charles River Canada, St-Constant, Quebec, Canada) embryos at day 11.5 were harvested and fixed overnight in 4% paraformaldehyde in PBS. The embryos were dehydrated through a series of ethanol solutions, cleared in toluene, and embedded in paraffin wax. Thin (5-µm) sections were cut and adhered to Superfrost-Plus microscope slides (Fisher Scientific Canada, Nepean, Ontario, Canada). AhR and Ned8 immunoperoxidase staining was performed on adjacent sections as described by Savouret al. (16). AhR protein was visualized using a 1:100 dilution of rabbit anti-AhR, and Ned8 was visualized using a 1:50 antibody dilution (Santa Cruz Biotechnology, Inc.). Vectastain ABC kit (Vector Laboratories) was used as directed by the manufacturer. The peroxidase reaction was developed using 3,3-diaminobenzidine (3.5 mg/5 ml in PBS buffer) in the presence of 0.03% H₂O₂. The sections were lightly counterstained with Harris hematoxylin solution and examined by light microscopy.

RESULTS

Two-hybrid Screen—To gain an understanding of the physiological role of AhR in development, we sought proteins that interact with AhR. The AhR cDNA sequence lacking the transactivation domain fused with the Gal4 DNA binding domain was used as bait. A total of 1.1 × 10⁹ clones were screened, of which 32 clones expressed potential interacting proteins. One clone, which we report here, encoded a 0.6-kb open reading frame DNA that showed 97% identity with mouse Ned8, a ubiquitin-like protein. The ability of these two-hybrid proteins to interact was confirmed by co-transformation of yeast AH109 cells with each of these plasmids and assessment for activation of reporter constructs by growth on His-/Ade-/3-amino-1,2,4-triazole medium and by measuring β-galactosidase activity using the colony-lift assay (data not shown).

AhR Interaction with Ned8 in Vitro—The interaction between full-length AhR and Ned8 was further demonstrated by the transcription and translation of epitope-tagged fusion proteins in vitro followed by co-immunoprecipitation using anti-HA or anti-AhR antibody (Fig. 1). Full-length AhR (with the transactivation domain intact) was synthesized and mixed with equimolar amounts of HA-tagged Ned8 protein or rabbit reticulocyte lysate. In the absence of AhR, an antibody to HA precipitated only Ned8 (~9 kDa) (Fig. 1, lane 1). In the absence of Ned8 protein, an antibody to AhR precipitated only AhR protein, which migrated at ~95 kDa (Fig. 1, lane 2). As expected, antibody to the HA epitope did not precipitate AhR protein in the absence of Ned8 (Fig. 1, lane 6). However, either AhR or HA antibody immunoprecipitated both Ned8 and AhR when an equimolar mixture of the two proteins was used (Fig. 1, lanes 3 and 4), thus confirming the interaction of these proteins. The AhR–Nedd8 interaction does not appear to be a covalent modification because this complex disassociated under reducing conditions and ran as two distinct protein bands. The addition of 10 nM TCDD to the mixture of lysates did not affect the association of AhR and Ned8 in vitro (Fig. 1, lane 5), suggesting that the conformational changes that may occur by binding of ligand to the receptor do not affect its interaction with Ned8. Additional bands represent nonspecific proteins present in the reticulocyte lysate.

Interaction of Ned8 with Full-length AhR in Mammalian Cells—The carboxyl terminus of Ned8 is efficiently processed to expose Gly-76, which is required for conjugation to target proteins via a range of specific activating and conjugating enzymes (17). Use of a Ned8 mutant lacking Gly-76 (Nedd8ΔG) should thus eliminate or attenuate the interaction between Ned8 and AhR. Expression vectors encoding AhR, HA-tagged Ned8, and HA-tagged Nedd8ΔG were transiently transfected into COS cells, and cell lysates were immunoprecipitated with anti-HA antibody. As shown in lane 5 of Fig. 2A, AhR was detected by Western blot analysis of the immunoprecipitates in cells transfected with both AhR and Ned8 expression vectors. AhR was not detected in immunoprecipitates from cells transfected with AhR or Ned8 expression vector alone (Fig. 2A, lanes 1–4). As predicted, AhR was not detected in immunoprecipitates from cells expressing both AhR and mutant Ned8 (Fig. 2A, lane 6), which is consistent with the role of this residue in formation of Ned8 conjugates.

Unlike COS cells, T47D cells express abundant levels of endogenous AhR protein and low levels of Ned8 as determined by immunocytochemistry and quantitative reverse transcription-PCR (data not shown). To determine whether endogenously expressed Ned8 and AhR interact, co-immunoprecipitation experiments were performed with protein lysates of nontransfected T47D cells (Fig. 2B). 7.5, 10, and 35 mg of T47D protein lysates (Fig. 2B, lanes 2–4, respectively) were immunoprecipitated with anti-Nedd8 antibody or anti-HA antibody (10 mg of protein, Fig. 2B, lane 5). The resultant precipitate was subjected to SDS-PAGE and transferred to polyvinylidene difluoride membrane. The immunoblot was analyzed using a rabbit polyclonal antibody to AhR. AhR protein expression in T47D lysates served as a positive control (Fig. 2B, lane 1). The
Treated with or without 10 nM TCDD for 90 min. Immunocytochemistry of AhR and Nedd8 in T47D cells. Cells were plated in chambers and low to moderate levels of Nedd8 protein (data not shown).

Immunoglobulin heavy chain. T47D cells expressed high levels of AhR and -lanes 2 and 4, AhR and Nedd8; lane 7, control, not transfected. Asterisk depicts the immunoglobulin heavy chain. B, whole cell extracts from nontransfected T47D cells were immunoprecipitated with anti-Nedd8 antibody overnight, followed by incubation with protein A-agarose. The immunoprecipitate (lanes 2–4) was analyzed by immunoblotting with a rabbit anti-AhR antibody. Lane 1, pcDNA3 vector; lane 2, Nedd8; lane 3, Nedd8ΔG; lane 4, AhR; lane 5, AhR and Nedd8; lane 6, AhR and Nedd8ΔG; lane 7, control, not transfected. Asterisk depicts the immunoglobulin heavy chain. B, whole cell extracts from nontransfected T47D cells were immunoprecipitated with anti-Nedd8 antibody overnight, followed by incubation with protein A-agarose. The immunoprecipitate (lanes 2–4) was analyzed by immunoblotting with a rabbit anti-AhR antibody. Lane 1, pcDNA3 vector; lane 2, Nedd8; lane 3, Nedd8ΔG; lane 4, AhR; lane 5, AhR and Nedd8; lane 6, AhR and Nedd8ΔG; lane 7, control, not transfected. Asterisk depicts the immunoglobulin heavy chain. B, whole cell extracts from nontransfected T47D cells were immunoprecipitated with anti-Nedd8 antibody overnight, followed by incubation with protein A-agarose. The immunoprecipitate (lanes 2–4) was analyzed by immunoblotting with a rabbit anti-AhR antibody. Lane 1, pcDNA3 vector; lane 2, Nedd8; lane 3, Nedd8ΔG; lane 4, AhR; lane 5, AhR and Nedd8; lane 6, AhR and Nedd8ΔG; lane 7, control, not transfected. Asterisk depicts the immunoglobulin heavy chain.

Fig. 2. Interaction between full-length AhR and Nedd8 in mammalian cells. A, COS cells were transiently transfected with AhR and various HA-tagged Nedd8 expression vectors. The amount of DNA transfected was kept constant by the addition of vector plasmids. Whole cell extracts were subjected to immunoprecipitation with anti-HA antibody, and immunoprecipitates were subsequently analyzed by immune-blotting with a rabbit anti-AhR antibody. Lane 1, pcDNA3 vector; lane 2, Nedd8; lane 3, Nedd8ΔG; lane 4, AhR; lane 5, AhR and Nedd8; lane 6, AhR and Nedd8ΔG; lane 7, control, not transfected. Asterisk depicts the immunoglobulin heavy chain. B, whole cell extracts from nontransfected T47D cells were immunoprecipitated with anti-Nedd8 antibody overnight, followed by incubation with protein A-agarose. The lysates were subjected to SDS-PAGE, transferred to nitrocellulose membrane, and subsequently analyzed with an anti-AhR antibody. Lane 1, positive control input lysate for AhR expression in T47D cells; lanes 2–4, varying quantities of T47D lysates (7.5, 10, and 35 mg, respectively) were immunoprecipitated with a Nedd8 antibody; lane 5, a nonspecific antibody (anti-HA) was used as a negative control for immunoprecipitation. Western blot shows expression of AhR in the Nedd8 immunoprecipitate (lanes 2–4, arrow). Asterisk depicts the immunoglobulin heavy chain. T47D cells expressed high levels of AhR and low to moderate levels of Nedd8 protein (data not shown). C, localization of AhR and Nedd8 in T47D cells. Cells were plated in chambers and treated with or without 10 nM TCDD for 90 min. Immunocytochemistry using AhR or Nedd8 antibody was performed. The top panels demonstrate protein localization in the absence of TCDD. The middle panels demonstrate the translocation of AhR into the nucleus after TCDD treatment. Primary antibodies were eliminated to serve as control for nonspecific staining (bottom panels).

Detection of AhR protein in the anti-Nedd8 immunoprecipitate (Fig. 2B, lanes 2–4) demonstrated the interaction of endogenous Nedd8 with AhR. Use of a nonspecific antibody (anti-HA), as a negative control did not precipitate AhR (Fig. 2B, lane 5).

Subcellular Localization of Endogenous AhR and Nedd8 in Mammalian T47D Cells—For Nedd8 to interact with AhR in vivo, both proteins should be at least transiently found in the same subcellular compartment of the cell. To determine if this occurs, the subcellular localization of Nedd8 and AhR protein was determined using double-labeling immunocytochemistry. AhR immunostaining was predominately cytoplasmic in the absence of TCDD (Fig. 2C); whereas primarily nuclear localization was observed after the addition of 10 nM TCDD. Some cytoplasmic staining was observed even in the presence of TCDD. Nedd8 immunostaining was also observed in the cytoplasm; however, staining was predominately nuclear, as has been shown previously (17). Treatment of cells with TCDD did not appear to affect the distribution of Nedd8 protein. Overlapping of the Cy2 and Cy3 staining, which results in prominent yellow areas, suggests that AhR and Nedd8 are physically in similar compartments of the cell, predominately in the nucleus. 4′,6-Diamidino-2-phenylindole staining, shown in the left panels of Fig. 2C, depicts the nuclear compartment of the cell and was used as a reference to distinguish between nuclear and cytoplasmic staining. The control panel demonstrates that staining was not evident in the absence of the primary antibody.

Overexpression of Nedd8 Potentiates AhR Action—To examine the influence of Nedd8 overexpression on AhR-dependent transactivation, DRE82 cells were transiently transfected with HA-tagged Nedd8 or HA-tagged Nedd8ΔG expression vectors. Transfected cells were tested for CAT expression after treatment with 1 nM TCDD or ethanol vehicle. Fig. 3A depicts the overexpression of Nedd8 protein (detected with anti-HA tag antibody) in DRE82 cells after transient transfection with Nedd8 expression vector (lane 2). Very low levels of CAT expression were measured in DRE82 cells transfected with vector and treated with ethanol vehicle (control). Treatment of these cells with TCDD caused a 6-fold induction of CAT expression above ethanol-treated cells (Fig. 3B). Cells transfected with the Nedd8 expression vector and treated with ethanol exhibited increased CAT expression (2.5-fold increase as compared with controls); however, this increase did not attain statistical significance. Overexpression of Nedd8 combined with TCDD treatment resulted in a 17-fold induction over controls. The significantly greater effect of TCDD in the presence of Nedd8 expression indicates that Nedd8 potentiated the action of AhR in these cells. Significant amplification of reported gene induction was not observed in cells overexpressing Nedd8ΔG, which is consistent with our finding that Nedd8ΔG did not interact with AhR. Additional experiments demonstrated a dose-dependent effect of Nedd8 overexpression on TCDD-induced CAT expression levels (Fig. 3C). A strong trend toward increased expression in the absence of exogenous ligand was observed; however, this failed to attain statistical significance (p = 0.057).

AhR Is Not Covalently Modified by Nedd8 in Vitro—To determine whether the association between Nedd8 and AhR represented classical neddylation, we performed an in vitro neddylation assay as described by Ohh et al. (14). Human Cul-1 was included in the assay as a positive control for neddylation because it has been previously shown that Nedd8 modifies several members of the cullin family (18). Consistent with previous reports (19), in vitro-synthesized wild-type Cul-1 migrated as two forms: a faster-migrating major band that corresponds to unmodified Cul-1, and a slower-migrating minor form corresponding to Nedd8-conjugated Cul-1 (Fig. 4, lanes 2 and 3). Incorporation of a myc epitope-tagged Nedd8 translated protein in the assay resulted in the appearance of a third, slower-migrating Cul-1 band (Fig. 4, lane 4). In contrast, in vitro-synthesized AhR migrated as only one major band, regardless of the presence of Nedd8 (recombinant or in vitro-transcribed), indicating that AhR is not a direct substrate of neddylation (Fig. 4, lanes 7–9).

Nedd8 Causes Nuclear Accumulation of AhR—The effect of overexpressing Nedd8 and treatment of T47D cells with TCDD on AhR protein levels was analyzed by Western blot of total cell lysates. After a 5-h treatment with 1 nM TCDD, analysis of T47D total cell lysates (~40 µg of protein) by immunoblotting revealed a 50% decrease in AhR protein content (Fig. 5A). Cells overexpressing Nedd8 and exposed to TCDD for the same
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**Fig. 3.** AhR-dependent transactivation is stimulated by Nedd8 overexpression. DRE82 cells were used to examine the effect of Nedd8 overexpression on AhR-mediated transactivation. A, lysates from transfected cells were resolved on a 12% SDS-PAGE, transferred to nitrocellulose, and analyzed with an anti-HA antibody to verify the overexpression of Nedd8 protein. Lane 1, vector; lane 2, Nedd8. B, cells were transiently transfected with Nedd8 or Nedd8G and treated with ethanol (vehicle) or 1 nM TCDD 16 h after transfection. In cells transfected with vector, TCDD activated the reporter gene ~6-fold. In the presence of Nedd8, there was a modest increase in CAT reporter gene compared with ethanol-treated controls. With TCDD treatment, there was a 17-fold increase in induction. Overexpression of Nedd8G eliminated the significant induction of the reporter gene in the presence of TCDD. C, expression of Nedd8 enhanced AhR-dependent transactivation in a dose-dependent fashion in the absence or presence of 1 nM TCDD. Amounts of Nedd8 transfected were 0, 100, 250, and 500 ng, respectively. Bars represent the mean ± S.E. of three independent cell cultures performed in triplicate for both B and C. Bars with different letters are statistically different from one another as determined by the Student-Newman-Keuls method. CAT levels in control cells ranged from 0.824 to 1.406.

length of time had ~70% less AhR protein in lysates than controls (Fig. 5B). To confirm the Western blotting results, the subcellular distribution of AhR was evaluated in the presence of TCDD and Nedd8. 100 μg of nuclear and cytoplasmic extracts were examined by immunoblotting (Fig. 5C). Results are consistent with AhR translocation to the nucleus after TCDD administration (compare Fig. 5C, lanes 2 and 4). Overexpression of Nedd8 in the absence of ligand led to an accumulation of AhR protein in the nuclear lysate fractions by 5h (compare Fig. 5C, lanes 2 and 6). The presence of TCDD with Nedd8 overexpression led to an accumulation or retention of AhR predominately in the nuclear fraction compared with the control.

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blots of Nedd8 expression in mouse embryos by Kamitani et al. (17) have shown that Nedd8 is expressed at highest levels at E11 and appears to be down-regulated thereafter. However, the localization of Nedd8 within tissues of a mouse embryo has not been reported. We examined the localization of AhR and Nedd8 in mouse E10–E12 embryos by immunohistochemistry to determine whether these proteins exhibit overlapping tissue distributions during development. AhR was abundantly expressed at E11.5 in the neuroepithelium, branchial arches, cranial nerves, liver, heart (Fig. 6, A and D), and spinal ganglia (Fig. 6, F and G). A similar distribution was observed at E10 and E12. The most prominent areas that expressed Nedd8 immunoreactivity were the heart (Fig. 6, B and E) and spinal ganglia (Fig. 6H). Thus, remarkable complementary expression patterns of Nedd8 and AhR are present in the heart and spinal ganglia at E10–E12 of mouse development. Immunostaining of both proteins appeared to be predominately cytoplasmic; however, some nuclear staining in these tissues was also observed. AhR protein expression was more widespread throughout this developmental stage. Nedd8 immunostaining was restricted to only the tissues shown and therefore was not always co-expressed with AhR.

**DISCUSSION**

This study identifies Nedd8 as a novel interacting partner of the AhR. This interaction, initially identified by our yeast two-hybrid screen, was confirmed both in a cell-free system and in mammalian cells by immunoprecipitation and occurred independent of exogenous ligand. Moreover, the demonstration that endogenously expressed Nedd8 and AhR interact in T47D human breast cancer cells clearly establishes this ubiquitin-like protein as a physiologically relevant partner and potential modulator of AhR.

Nedd8 is the mammalian functional homologue of Rub1 in the budding yeast *Saccharomyces cerevisiae*. The ligation of Nedd8/Rub1 to proteins is likely to have important growth-regulatory roles in plants, mammals, and presumably many other organisms (18). Nedd8 conjugation involves a pathway that parallels that of ubiquitination, including activation by a distinct E1-like enzyme and conjugation mediated by a dedicated E2 enzyme (20). The Nedd8 modification (neddylation) pathway utilizes a unique activating enzyme complex (UBA3/APP-BP1) and conjugation enzyme (UBC12). In mammalian cells, Nedd8 has been shown to modify a limited subset of cellular proteins in vivo, all of which belong to the cullin family. Human cul-1 is a major component of the SCF complex that is responsible for ubiquitination of a multitude of proteins that
regulate various biologically important processes such as cell cycle progression and signal transduction (21, 22). These include IκBα, β-catenin, and p27 (23, 24).

One of the speculated functions of AhR is to regulate gene expression in a ligand-dependent fashion; therefore, we investigated the effect of overexpression of Nedd8 on the transcriptional activity of AhR. Our data demonstrate Nedd8 overexpression enhanced AhR-mediated transactivation of a DRE-tk-CAT reporter gene in a dose-dependent manner in cells exposed to TCDD. Interestingly, a similar pattern was observed in the absence of TCDD treatment, suggesting that NEDD8 may promote AhR action in the absence of ligand or enhance its action in the presence of ligand endogenous to the culture system. The mechanism by which Nedd8 amplifies the transcriptional activity of AhR remains to be determined. One possibility is that it functions as a nuclear receptor coactivator, bridging the AhR complex with general transcriptional factors and/or histone acetyltransferases. Alternatively, association with Nedd8 could influence the nuclear localization and/or stability of the receptor complex and prolong the action of the AhR complex at the DRE.

Interaction of Nedd8 has not been reported previously for any of the nuclear receptors or hHLH/PAS family members. However, UBA3 was recently shown to interact directly with both estrogen receptor (ER)-α and β in a ligand-dependent manner and to suppress ER-mediated transactivation in mammalian cells (25). Neddylation has been shown to enhance targeting of proteins for ubiquitination and subsequent degradation at the proteasome (26). Fan et al. (25) therefore suggest that neddylation may play a role in attenuating ER action by promoting receptor turnover; however, association of ER with Nedd8 was not demonstrated. Two studies have independently shown that AhR degradation is dependent on the ubiquitin-proteasome pathway (4, 27). Ligand binding enhances down-regulation of AhR and is a typical occurrence for a number of nuclear receptors (28). We found that overexpression of Nedd8 decreased overall levels of AhR protein. Nonetheless, Nedd8 overexpression also increased the accumulation of nuclear AhR. Thus, it is possible that the interaction between AhR and Nedd8 prolongs nuclear localization of the receptor, perhaps at the DRE, and subsequently facilitates receptor ubiquitination and degradation. Nuclear accumulation was also observed in the absence of exogenous ligand and is therefore consistent with the effects of Nedd8 overexpression on CAT reporter gene activity in the absence of TCDD treatment.

The results of our in vitro neddylation assay demonstrate that AhR is not covalently modified by Nedd8. Whereas the
interaction between these two proteins may be noncovalent, the possibility that the interaction is mediated through an association with a common protein partner that is constitutively present in coupled reticulocyte lysate, yeast, and T47D cells cannot be excluded. Interestingly, it has been suggested that neddylation of Cul-2 may affect the degradation of hypoxia-inducible factor 1α (HIF-1α) (29), which is also a bHLH protein family member that heterodimerizes with ARNT. HIF-1α is rapidly degraded in the presence of oxygen, and Maxwell et al. (30) demonstrated that this degradation can be directed by the von Hippel-Lindau tumor suppressor protein (pVHL). The pVHL complex associates with Cul-2 that is directly modified by Ned8. The degradation of HIF-1α may serve as an example of how neddylation of an accessory protein, in this case a cululin family member, may affect the transcriptional activity of the HIF-1α-ARNT heterodimeric complex. It remains to be determined whether such accessory proteins exist in the AhR-ARNT heterodimeric complex and whether they include any members of the cululin family. Research into the identification of these proteins is currently under way.

Co-immunoprecipitation experiments with a mutant clone of Ned8 lacking the carboxyl-terminal amino acids including Gly-76 indicate that one or more of these residues is critical for interaction with AhR. Overexpression of this mutant also failed to enhance AhR transactivation. Previous studies have shown that Gly-76 plays a key role in the neddylation pathway, forming a thiol-ester linkage with a cysteine residue of UBA3/APPBP1 (31). Thus, it is probable that the absence of Gly-76 in the Ned8 mutant construct is responsible for the loss of AhR interaction.

Involvement of bHLH-containing proteins, such as AhR, ARNT, and HIF-1α, in embryo development is well established (32, 33). AhR-null mice are resistant to TCD2 toxicity but display severe abnormal phenotypes, including liver defects, immune system impairment, and reproductive defects (6, 33). These observations suggest that AhR influences normal cell proliferation and differentiation. There is recent evidence suggesting that Ned8 also plays a regulatory role in cell proliferation and development (34). UBA3-deficient mice, generated to investigate the role of Ned8 in mammals, die in utero at the perimplantation stage (E6–E7). The inner mass cells of these periimplantation stage (E6–E7) embryos undergo apoptosis, and the trophoblast cells fail to enter the S phase of the endoreduplication cycle, suggesting that Ned8 is essential for mitotic spindle function and endoreduplicative cell cycle progression (34). The function of Ned8 during Caenorhabditis elegans embryogenesis was similarly determined to be important for cytoskeletal regulation during pronuclear migration and cytokinesis (35).

In the mouse embryo, the pattern of AhR expression has been shown to be time- and tissue-specific (7, 36). Ned8 mRNA is expressed at its highest level at embryonic day 11, and transcript levels decrease thereafter as development continues. Characterization of the tissue distribution of Ned8 suggests that nuclear proteins expressed in heart and skeletal muscle or in early development would be candidate proteins for Ned8 modification (17). Interestingly, the interaction of Ned8 and AhR was identified in screening a mouse day E11 library. At this point of development, AhR is ubiquitously expressed, whereas Ned8 protein appears to have a more restricted pattern of expression. The expression of AhR in tissues without Ned8 expression indicates that interaction with Ned8 is not essential for the actions of this transcription factor. However, the overlap of expression in the heart and spinal ganglia at E10–E12 suggests that the interaction of these two proteins may play an important role in cardiogenesis and neural development.

At present, the function of the AhR and Ned8 interaction during development is not clear. Based on our data, we propose that Ned8 regulates the activation of AhR and AhR-mediated signal transduction by enhancing accumulation of the receptor in the cell nucleus. Additionally, by acting to facilitate ubiquitination, this association could assist in receptor turnover. Thus the interaction with Ned8 could represent a developmentally important, tissue-specific posttranslational modification of the AhR. Moreover, this interaction may be involved in modulating the toxicological effects of polycyclic aromatic hydrocarbons. Additional studies of the functional interaction of Ned8 with AhR should enhance our understanding of AhR function in vivo.

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REFERENCES

1. Hankinson, O. (1985) Annu. Rev. Pharmacol. Toxicol. 25, 307–340
2. Petralia, J. R., Hrdy, N. K., and Perdue, G. H. (2000) J. Biol. Chem. 275, 37448–37453
3. Ma, Q., and Whitlock, J. P., Jr. (1997) J. Biol. Chem. 272, 8787–8884
4. Roberts, B. J., and Whitelaw, M. L. (1999) J. Biol. Chem. 274, 36351–36356
5. Fernandez-Salgueiro, P., Pineau, T., Hibert, D. M., McPhail, T., Lee, S. S., Kimura, S., Nebert, D. W., Rudikoff, S., Ward, J. M., and Gonzalez, F. J. (1995) Science 268, 722–725
6. Schmidt, J. V., Su, G. H., Reddy, K. J., Simon, M. C., and Bradfield, C. A. (1996) Proc. Natl. Acad. Sci. U.S.A. 93, 6731–6736
7. Abbott, B. D., Birnbaum, L. S., and Perdue, G. H. (1995) Dev. Dyn. 204, 133–143
8. Kumar, S., Tomoka, Y., and Noda, M. (1992) Biochem. Biophys. Res. Commun. 185, 1155–1161
9. Hort, T., Osaka, F., Chiba, T., Miyamoto, C., Okabayashi, K., Shimbara, N., Kato, S., and Tanaka, R. (1999) Oncogene 18, 6829–6834
10. Nicklas, G. P. D., Fournier, C., Galian, C., and Lecomte, M.-C. (2001) in Promega eNotes: Applications, www.promega.com/enotes/applications/ag0112e.htm
11. Holmes, J. L., and Pollenz, R. S. (1997) Mol. Pharmacol. 52, 202–211
12. Casper, R. F., Antenos, M., Quesne, M., Shirota, T., Jolivet, A., Milgrom, E., and Savouret, J. F. (1999) Mol. Pharmacol. 56, 784–790
13. Sambrook, J., and Russell, D. W. (2001) Molecular Cloning: A Laboratory Manual, 3rd Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
14. Ohh, M., Kim, W. Y., Moslehi, J. J., Chen, Y., Chua, V., Read, M. A., Simon, M. C., and Bradfield, C. A. (2001) Nature 410, 683–687
15. Ma, Q., and Baldwin, K. T. (2000) J. Biol. Chem. 275, 34983–34989
16. Laney, J. D., and Hochstrasser, M. (1999) Cell 97, 427–430
17. Fan, M., Long, X., Bailey, J. A., Reed, C. A., Osborne, E., Gize, A. E., Kirk, E. A., Bigsby, R. M., and Nephew, K. P. (2002) Mol. Endocrinol. 16, 315–330
18. Kamitani, T., Kito, K., Fukuda-Kamitani, T., and Yeh, E. T. (2001) J. Biol. Chem. 276, 28557–28562
19. Hochstrasser, M. (1998) Genes Dev. 12, 901–907
20. Okino, S. T., and Whitlock, J. P., Jr. (2000) Vitam. Horm. 59, 241–286
21. Ivan, M., and Kazin, W. G., Jr. (2001) Curr. Opin. Genet. Dev. 11, 27–34
22. Quinlan, B. J., Fall, W. K., Ricker, D. W., and Young, C. Y. (1997) Cancer Res. 59, 5882–5895
23. Kamitani, T., Kito, K., Yang, H.-P., and Yeh, E. T. (1997) J. Biol. Chem. 272, 28557–28562
24. Laney, J. D., and Hochstrasser, M. (1999) Cell 97, 427–430
25. Fan, M., Long, X., Bailey, J. A., Reed, C. A., Osborne, E., Gize, A. E., Kirk, E. A., Bigsby, R. M., and Nephew, K. P. (2002) Mol. Endocrinol. 16, 315–330
26. Kamitani, T., Kito, K., Fukuda-Kamitani, T., and Yeh, E. T. (2001) J. Biol. Chem. 276, 46655–46660
27. Ma, Q., and Baldwin, R. T. (2000) J. Biol. Chem. 275, 8432–8438
28. Okino, S. T., and Whitlock, J. P., Jr. (2000) Vitam. Horm. 59, 241–286
29. Ivan, M., and Kazin, W. G., Jr. (2001) Curr. Opin. Genet. Dev. 11, 27–34
30. Maxwell, P. H., Wiesener, M. S., Chang, G. W., Buchanan, D. L., Cooke, P. S., and Peterson, R. E. (2001) J. Toxicol. Environ. Health 64, 327–342
31. Gonzalez, F. J., and Fernandez-Salgueiro, P. (1998) Drug Metab. Dispos. 26, 1194–1198
32. Takahashi, K., Omata, M., Tanaka, K., and Chiba, T. (2001) J. Cell Biol. 151, 571–580
33. Kurz, T., Pintard, L., Willis, J. H., Hamill, D. R., Gonzalez, P., Peter, M., and Bowserman, B. (2002) Science 295, 1294–1298
34. Jain, S., Matteo, E. S., Lu, M. M., Simon, C., and Bradfield, C. A. (1998) Mech. Dev. 73, 117–123
