Suspension-mediated Induction of Hepa 1c1c7 Cyp1a-1 Expression Is Dependent on the Ah Receptor Signal Transduction Pathway*

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We have recently demonstrated that release of normal human epithelial cells from cell-substratum and/or cell-cell adhesion generates cellular signals that induce the expression of CYP1A1 in the absence of xenobiotic polycyclic aromatic hydrocarbons (Sadek, C. M., and Allen-Hoffmann, B. L. (1994) J. Biol. Chem. 169, 16067-16074). To directly test the involvement of the Ah receptor signal transduction pathway in CYP1A1 induction following suspension of epithelial cells, we analyzed wild-type Hepa 1c1c7 cells, a subclone of the Hepa-1c1c7 mouse hepatoma line, and two mutant Hepa 1c1c7 lines, Class I and Class II. Suspension of wild-type Hepa 1c1c7 cells for 4 h led to an induction of steady state levels of CYP1A1 mRNA, similar to that obtained following treatment of adherent cells with 10-9 M 2,3,7,8-tetrachlorodibenzo-p-dioxin. Mutants of the Hepa 1c1c7 cells defective in different aspects of the Ah receptor signal transduction pathway exhibited negligible (Class I) or no (Class II) suspension-mediated induction of CYP1A1 mRNA. Gel mobility shift analysis of nuclear extracts from suspended or 2,3,7,8-tetrachlorodibenzo-p-dioxin-treated wild-type cells showed that both treatments produced identical shifts in the mobility of an XRE-containing probe. Antibody supershift experiments confirmed that the Ah receptor was a component of the DNA-protein complex from suspended wild-type Hepa 1c1c7 cells. These data directly demonstrate that suspension of wild-type Hepa 1c1c7 cells leads to nuclear localization and activation of the Ah receptor to a DNA-binding form.

Ligand-activated transcription factors regulate the expression of a number of different gene products, including members of the cytochrome P450 monooxygenase superfamily. One member of this enzyme family, CYP1A1, is substrate-inducible and has been shown to be activated by exposure to xenobiotic polycyclic aromatic hydrocarbons, such as 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD). TCDD-activated induction of CYP1A1 occurs via a signal transduction pathway involving the aromatic hydrocarbon (Ah) receptor (Burbach et al., 1992), a 90-kDa heat shock protein (hsp90) (Perdew, 1988), and the Ah receptor nuclear translocator protein (Arnt) (Hoffman et al., 1991; Reyes et al., 1992). Studies to date indicate that TCDD enters the cytosol where it binds an Ah receptors-hsp90 complex. This complex then interacts with Arnt, concomitantly displacing hsp90 (McGuire et al., 1994). The liganded Ah receptor-Arnt heterodimer forms an active transcription factor which binds to xenobiotic responsive elements (XREs) in the 5′ promoter region of the CYP1A1 gene, as well as a battery of other Ah receptor-responsive genes (reviewed in Nebert et al. (1993) and Okey et al. (1994)). Aside from metabolism of xenobiotic polycyclic aromatic hydrocarbons, the role of the CYP1A1 enzyme is currently unclear, in large part because no endogenous substrate or Ah receptor ligand has been identified (reviewed in Poellinger et al. (1992)).

We recently demonstrated that both CYP1A1 mRNA and enzyme activity are induced in cultured human keratinocytes, but not dermal fibroblasts, in the absence of polycyclic aromatic hydrocarbon treatment by suspending adherent cells (Sadek and Allen-Hoffmann, 1994). In this previous study, normal skin keratinocytes were suspended in serum-free and growth factor-free medium made semi-solid with an inert polymer, methylcellulose, to prevent cell-cell adhesion and alter cell shape. Suspension of adherent normal human epithelial cell types, such as keratinocytes, induces CYP1A1 mRNA and enzyme activity in the absence of serum, calcium, as well as a number of growth promoting agents such as epidermal growth factor, hydrocortisone, insulin, and cholera toxin. A similar induction has also been noted following suspension of cultured normal human thymic epithelial cells. Addition of methylcellulose does not alter the osmolarity of the medium and replacement of methylcellulose with colloidal silica (Percoll) in medium also supports CYP1A1 induction. Furthermore, suspension in medium alone is sufficient to elicit a response in cultured normal keratinocytes, thus ruling out any potential contaminant unique to methylcellulose as the inducing agent (Sadek and Allen-Hoffmann, 1994).

As reported in our earlier work, other members of the Ah receptor gene battery responded to either suspension or TCDD treatment in a similar manner. Therefore, we hypothesized that suspension of epithelial cells may initiate cellular events which activate the Ah receptor pathway. To test this hypothesis, we have used an epithelial cell line, Hepa 1c1c7, derived from a mouse liver tumor. In this report we have analyzed mutants of the Hepa 1c1c7 cells defective in different aspects of the Ah receptor signal transduction pathway. Our findings confirm that suspension of wild-type Hepa 1c1c7 cells leads to an induction of Cyp1a-1 via an Ah receptor-dependent pathway.

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1 The abbreviations used are: CYP, cytochrome P450 as recommended by the Committee on Cytochrome P450 Nomenclature (Nelson et al., 1993); the italicized CYP indicates the human gene and cDNA, Cyp1a-1 indicates the murine gene and cDNA, the human and murine mRNA and protein are written as the non-italicized symbol CYP, an Arabic number designates the P450 family, the subfamily is identified by a letter when two or more subfamilies exist within a family, and an Arabic number represents the specific gene or protein; α-MEM, α-minimal essential medium; TCDD, 2,3,7,8-tetrachlorodibenzo-p-dioxin; XRE, xenobiotic responsive element; MOPS, 4-morpholinepropanesulfonic acid.

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Methylcellulose and a magnetic stir bar were autoclaved in a 250-ml flask. Sterile methylcellulose-containing medium was prepared as described previously (Sadek and Allen-Hoffmann, 1994). Briefly, 3.37 g of a-MEM containing 1.68% methylcellulose (4000 centipoises; Fisher Scientific) and 0.5 mM EDTA, 0.1% trypsin, washed with serum-containing medium, were added to the mixture in a volume of 5 μl containing 10 mM MgCl₂, 1 mM DTT. Nuclei were lysed by the addition of 2 μl of Buffer C (25 mM Hepes (pH 7.5), 0.3 mM MgCl₂, 1 μM dithiobitol) and were incubated for 20 min at 4 °C. The noncoding strand was then added and the mixture was incubated for 4 °C for 1 h. To remove undissolved methylcellulose fibers, 100 μl aliquots of methylcellulose were centrifuged at 16,000 × g for 90 min at 4 °C.

For suspension studies, cells were removed from tissue culture plates with 0.5 μg/ml EDTA, 0.1% trypsin, washed with serum-containing medium to inactivate residual trypsin, then with serum-free medium, and finally suspended in serum-free methylcellulose-containing medium at a density of approximately 2 × 10⁶ cells/ml (100-mm dish/4 ml) in sterile 50-ml polypropylene tubes. Suspended cells were incubated at 37 °C in a humidified 5% CO₂ atmosphere. Cells were recovered from suspension by repeated dilution with serum-free medium followed by centrifugation at 1000 × g. Adherent cells used for controls were rinsed and treated with 4 ml of αMEM or αMEM containing 10⁻⁹ M TCDD per 100-mm dish for the same time as suspended cells.

Northern Analysis—Poly(A)⁺ RNA was isolated from logistically growing cells according to the method of Badley et al. (1988). Poly(A)⁺ RNA was electrophoresed in a 1.2% agarose gel containing formaldehyde and electrophoretically transferred to a Zeta probe membrane (Bio-Rad). The membrane was prehybridized and hybridized under the presence of a random primer. ³²P-labeled cDNA probe as recommended by the supplier. The probes used were a 1269-base pair cDNA for rat glyceraldehyde-3-phosphate dehydrogenase, GAPDH, untreated; SUSP, suspended.

Cell Cultures—Hepa 1c1c7 wild-type cells, Class I and Class II mutant cell lines were the kind gift of Dr. James Whitlock Jr. (Stanford University, Stanford, CA). Cells were maintained in α-minimal essential medium (α-MEM) without nucleosides supplemented with 10% fetal calf serum, 100 units penicillin, and 100 μg/ml streptomycin. All cultures were maintained at 37 °C in a humidified, 5% CO₂ atmosphere.

Experimental Procedures

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Adherent wild-type Hepa 1c1c7 cells were rinsed and treated with serum-free medium alone (Untreated, lanes 1-3), with 10^{-6} M TCDD (TCDD, lanes 4-6), or suspended in serum-free, semi-solid medium (Suspended, lanes 7-9) for 90 min. Following treatment, nuclei were isolated as described and nuclear extracts were analyzed by gel mobility shift assay using a 32P-labeled 29-mer containing the core XRE sequence. XRE-binding reactions were conducted in the absence or presence of a 1000-fold (lanes 1, 3, 6, and 7) or 5000-fold (lanes 3, 6, and 9) molar excess of unlabeled XRE probe. The positions of the XRE-binding complex (R) and the free (Free) probes are indicated.

RESULTS

Suspension-induced Increases in CYP1A1 mRNA Are Dependent on Functional Ah Receptor and Arnt—To study the involvement of the Ah receptor in suspension-induced Cyp1a-1, we used wild-type mouse hepatoma Hepa 1c1c7 cells (Hankinson, 1979) and two mutant Hepa 1c1c7 cell lines, termed Class I and II (Miller et al., 1983; Whitlock and Galeazzi, 1984). These mouse hepatoma-derived epithelial cells have been used extensively to study the mechanism of TCDD-dependent induction of CYP1A1. For this reason, they are important as a model to analyze the signal transduction mechanisms triggered by suspension of certain epithelial cell types.

The wild-type Hepa 1c1c7 hepatoma cell line exhibits the morphological features of epithelial cells (Fig. 1a). They are well spread and form cell-cell contacts that produce a characteristic cobblestone morphology. Class I mutants possess a fusiform, fibroblastic morphology and are poorly spread, with little cell-cell interaction (Fig. 1b). Class II mutants exhibit cell-cell interaction typical of foci formation in fibroblastic cell types (Fig. 1c). Neither Class I nor Class II cells possess morphology typical of cultured epithelial cells. All three cell lines contain a positive cis-acting Ah receptor-dependent promoter/enhancer region of Cyp1a-1. Wild-type cells express functional Ah receptors and Arnt protein and are highly responsive to treatment with TCDD. Both Class I and Class II mutants contain defects in the Ah receptor signal transduction pathway which result in either an attenuation or inability to activate transcription of Cyp1a-1. Class I mutants contain functional Arnt protein but contain less than 10% of normal Ah receptor levels as measured by ligand binding (Miller et al., 1983) and photoaffinity labeling (Burbach et al., 1992). Class II mutants contain normal levels of Ah receptor, however, the Ah receptor will not translocate to the nucleus following ligand binding (Whitlock and Galeazzi, 1984).

To directly test the involvement of the Ah receptor signal transduction pathway in suspension-mediated induction of Cyp1a-1, we analyzed wild-type Hepa 1c1c7 and the two mutant Hepa 1c1c7 cell lines, Class I and Class II. We first tested the responsiveness of adherent wild-type, Class I and Class II Hepa 1c1c7 cells to either TCDD treatment or suspension by Northern analysis (Fig. 2). All cells were treated for 4 h in serum-free medium with or without 10^{-6} M TCDD or by suspension in serum-free, semi-solid medium. We found wild-type cells to be responsive to both TCDD and suspension treatments to a similar degree. Suspension-mediated induction of wild-type Hepa 1c1c7 CYP1A1 mRNA also occurred in the absence of the pH indicator phenol red (data not shown). Class I cells have lower levels of Ah receptors and thus were less responsive to TCDD treatment when compared to wild-type Hepa 1c1c7 cells. Consistent with this finding, adherent untreated Class I cells did not exhibit detectable levels of CYP1A1 mRNA, whereas adherent wild-type cells have constitutive, albeit low, levels of CYP1A1 mRNA. Suspension of the Class I mutants produced barely detectable levels of steady state CYP1A1 mRNA. The Class II translocation-defective mutants were completely non-responsive to both TCDD and suspension. Because induction
FIG. 4. The Ah receptor is a component of the XRE-binding complex found in the nuclei of suspended Hepa 1c1c7 cells. Ah receptor-specific antibody (α-AhR) and preimmune serum (PIS) were used to verify the presence of the Ah receptor in the XRE-binding complexes generated by TCDD and suspension treatments. Supershift binding reactions were conducted for 20 min with no additions (lanes 1, 4, and 7), with PIS (lanes 2, 5, and 8), or α-AhR (lanes 3, 6, and 9). Ah receptor antibody-dependent retardation of the XRE complex (R*), the XRE-binding complex (R), and free probe (Free) are indicated.

The DNA Binding Activity of the Ah Receptor Is Induced by Suspension—During TCDD treatment, the Ah receptor translocates to the nucleus and forms a heterodimeric transcription factor with Arnt. This complex then binds XRE sequences in the DNA to initiate transcription. Because we observed that the suspension-induction of CYP1A1 mRNA is dependent on Ah receptor levels and nuclear translocation, we hypothesized that suspension of wild-type Hepa 1c1c7 cells would lead to Ah receptor activation similar to that observed following exposure of adherent cells to TCDD. We next performed gel mobility shift assays to determine if suspension of the wild-type Hepa 1c1c7 cells leads to formation of an active XRE-binding transcription factor. Adherent wild-type cells were treated with serum-free medium alone, with TCDD, or were suspended for 90 min. Nuclear extracts were incubated with a radiolabeled 29-mer probe containing the core XRE sequence. Both TCDD treatment and suspension of the wild-type cells led to identical shifts in probe mobility (Fig. 3, lanes 4 and 7). As expected, the 29-mer probe was not bound by nuclear extracts of untreated adherent control cells (Fig. 3, lane 1). To ensure that the XRE containing 29-mer was properly labeled and that the shifted DNA was specifically recognized by the Ah receptor complex, we also performed competition experiments using unlabeled oligomer containing the XRE. Nuclear extracts from adherent, TCDD-treated, and suspended cells were incubated with a 1000- or 5000-fold molar excess of unlabeled oligomer in addition to the radiolabeled probe. As expected, the intensity of the shifted band was reduced to background levels upon addition of cold competitor (Fig. 3, lanes 5, 6, 8, and 9). These results demonstrate that suspension of wild-type Hepa 1c1c7 cells produces a nuclear DNA-binding factor of the same size and in a similar quantity as TCDD treatment.

To confirm that the nuclear complex binding the 29-mer probe contains the Ah receptor, we conducted an antibody supershift experiment (Fig. 4). Nuclear extracts were generated from untreated (lanes 1–3), TCDD-treated (lanes 4–6), or suspended (lanes 7–9) wild-type cells and incubated with the radiolabeled XRE-containing 29-mer in the presence or absence of an antibody against the Hepa 1c1c7 Ah receptor. The A-1 antibody used in our experiments was generated against a recombinant protein (amino acids 61–419 of the AhR) encoded by a cDNA to the mouse Ah<sup><sup>0</sup></sup>-<sup><sup>1</sup></sup> allele (Pollenz et al., 1994). This...
antibody recognizes authentic Ah receptor protein in both wild-type Hepa 1clc7 cells and in both Class I and II mutants. Both TCDD treatment (lane 4) and suspension (lane 7) induced identical shifts in gel mobility, similar to the results shown in Fig. 3. Addition of the A-1 antibody to XRE-binding reactions containing the nuclear extracts from adherent TCDD-treated cells (lane 6) or suspended cells (lane 9) resulted in further retardation in gel mobility (supershift) of the radiolabeled complex relative to preimmune serum (lanes 5 and 8). As anticipated, nuclear extracts from untreated adherent wild-type cells bound little radiolabeled XRE-containing probe under any condition (lanes 1–3). These results demonstrate that TCDD treatment and suspension not only lead to a similar shift in band mobility but that activated Ah receptor is a component of the nuclear extract from both treatments. Based on our results in Fig. 2 and the identical gel shifts and supershifts produced by these two treatments, we suspect that Arnt is a component of the XRE-binding complex as well. These data directly demonstrate that suspension of wild-type Hepa 1clc7 cells leads to activation of the Ah receptor to a DNA-binding form in the absence of xenobiotics.

**DISCUSSION**

In this report, we demonstrate that the suspension-mediated induction of Cyp1a-1 requires an intact Ah receptor signaling pathway. As with human keratinocytes, suspension of adherent wild-type Hepa 1clc7 cells causes induction of Cyp1a-1 gene expression in the absence of polycyclic aromatic hydrocarbon treatment (Sadek and Allen-Hoffmann, 1994). The cellular signals responsible for suspension-mediated signal transduction through the Ah receptor pathway are currently unknown. It is possible that suspension promotes production or release of an endogenous ligand(s) thereby leading to activation of an Ah receptor to a DNA-binding form via a mechanism similar to polycyclic aromatic hydrocarbon induction. It is also reasonable to postulate that the cell shape and adhesion changes that result from suspension may disrupt the Ah receptor-hsp90 complex sufficiently to activate non-ligated Ah receptor to a DNA-binding form. The molecular chaperone hsp90 is necessary to maintain the Ah receptor in a polycyclic aromatic hydrocarbon ligand-binding conformation (Whitelaw et al., 1994). However, release of hsp90 is required for activation of the Ah receptor to a DNA-binding form. Following disruption of hsp90-Ah receptor complexes, the Ah receptor exhibits a loss of affinity for its cognate ligands (Pongratz et al., 1992). In *in vitro* studies have shown that disruption of the Ah receptor-hsp90 complex in the absence of ligand is sufficient to promote Ah receptor binding to the XRE (Pongratz et al., 1992). Recent studies by McGuire et al. (1994) suggest that release of hsp90 from the Ah receptor is dependent not only on ligand binding but also on interaction with Arnt. That is, *in vitro* activation of the Ah receptor by ligand-induced release of hsp90 appears to require interaction with functional Arnt. By analogy, suspension of epithelial cells may disrupt the association of hsp90 and the Ah receptor directly, thus promoting interaction with Arnt and consequently promoting XRE binding and *Cyp1a-1* induction. Alternatively, suspension may alter cellular pools of Ah receptor and Arnt and in this manner facilitate Arnt-mediated release of hsp90 from the Ah receptor. Therefore, the presence of an endogenous ligand may or may not be necessary for suspension-mediated induction of CYP1A1 in the epithelial models we have investigated.

Taken together, our results suggest that an endogenous ligand(s) or other cellular change resulting from suspension of adherent wild-type Hepa 1clc7 cells activates the Ah receptor to a DNA-binding form and promotes its nuclear localization. The biological role of Ah receptors in normal tissue homeostasis is a relatively unexplored area. Further studies will focus on confirming the presence of endogenous ligands as well as determining the role of Arnt in suspension-mediated induction of CYP1A1 in epithelial cell types. Isolation and characterization of putative endogenous ligands or other cellular mechanisms of Ah receptor activation will increase our understanding of this signal transduction pathway in normal developing and adult tissues as well as pathological conditions.

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