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Analysis of Photoaffinity-Labeled Aryl Hydrocarbon Receptor Heterogeneity by Two-Dimensional Gel Electrophoresis†

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ABSTRACT: The level of charge heterogeneity in the aryl hydrocarbon receptor (AhR) was examined by high-resolution denaturing two-dimensional (2D) gel electrophoresis. Hepa 1c1c7 cell cytosolic fraction was photoaffinity-labeled with 2-azido-3-[^{125}I]iodo-7,8-dibromodibenzo-*p*-dioxin and applied to isoelectric focusing (IEF) tube gels. After optimization of focusing conditions a broad peak of radioactivity was detected in the apparent p*I* range of 5.2–5.7. IEF tube gels were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis followed by visualization of the radiolabeled AhR by autoradiography; three distinct isoforms were detected. The same 2D electrophoretic isoform pattern was obtained when the AhR from Hepa 1c1c7 was photoaffinity-labeled in cell culture. BP^{r}C1 cells, a mutant line derived from Hepa 1c1c7 cells, contain an AhR that is unable to bind to DNA. Photoaffinity-labeled BP^{r}Cl cytosolic fractions were subjected to 2D gel electrophoretic analysis resulting in essentially the same molecular weight and isoform pattern as seen in Hepa 1c1c7 cytosol. This result would suggest that if a mutation is present in the BP^{r}Cl AhR it has not caused a significant change in its IEF pattern, although a small shift in the p*I* values was observed. Two-dimensional gel electrophoresis of photoaffinity-labeled cytosolic fractions from HeLa cells, the rat liver tumor cell line McA-RH7777, and buffalo rat thymus revealed three isoforms, essentially the same isoform pattern as in Hepa 1c1c7 cells. This would indicate that despite the considerable molecular weight polymorphism between species the level of charge heterogeneity is highly conserved.

The effects of both halogenated and nonhalogenated polycyclic aromatic hydrocarbons are of concern because of their widespread distribution as contaminants in the environment (Young et al., 1983). For example, the use of brominated fire retardants in the manufacturing of carpets, plastics, and textiles is a major source of waste products taken to municipal incinerators. During combustion of these products brominated dioxins and dibenzofurans are produced and can be found in the fly ash, usually disposed of in landfills (Sovocool et al., 1988). The biological response to halogenated polycyclic aromatic hydrocarbons (e.g., TCDD, TCDF)[1] is thought to be mediated by the *Ah* receptor (AhR) (Safe, 1988). This response is both species- and tissue-specific (Poland et al.,

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[1] Abbreviations: TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; TCDF, 2,3,7,8-tetrachlorodibenzofuran; AhR, Aryl hydrocarbon receptor; HSP90, 90-kDa heat-shock protein; M_r, relative mass; MOPS, 3-(*N*-morpholino)propanesulfonic acid; Hepa 1, mouse hepatoma cell line 1c1c7; NP-40, nonidet P-40; IEF, isoelectric focusing; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; PDA, piperazine diacrylamide; CHAPS, (3-[(3-cholamidopropyl)dimethylammonio] 1-propanesulfonate; IEF–SDS–PAGE, isoelectric focusing in the first dimension, followed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis in the second dimension.

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1982). A wide range of effects have been noted upon exposure of test animals to TCDD, these include the following: a slow wasting syndrome, thymic atrophy, teratogenesis, immunotoxicity, and hepatotoxicity.

Recently the AhR has been shown to be associated with the 90-kDa heat-shock protein (HSP90) (Denis et al., 1988; Perdew, 1988). It has been determined that HSP90 exists as a dimer both free in the cytosol and complexed with the progesterone receptor (Radanyi et al., 1989). This would suggest that the AhR is composed, at least in part, of a dimer of HSP90 and one ligand/DNA binding protein. This hypothesis would be consistent with the published M_r value of 270000 for the murine AhR (Cuthill et al., 1987). Determination of the complete composition of the 9S form of the AhR will require additional studies. After binding ligand in vivo, the AhR translocates into the nucleus and binds to specific DNA binding sites, during this process the AhR is converted from an 9S to a 5–6S form. The ligand-occupied nuclear form of the AhR exists as a 6S species (Prokipcak & Okey, 1988); in contrast, salt-induced transformation of the AhR in vitro results in a change in sedimentation from 9 S to 5 S. This result would suggest that in vivo the nuclear-binding AhR complex contains the ligand-binding protein plus an additional factor(s). The AhR is much more resistant to salt-induced disaggregation in mouse liver or Hepa 1 cell cytosol compared to rat cytosol (Denison et al., 1986). The apparent molecular weight of the murine liver AhR ligand-binding polypeptide under denaturing conditions is approximately 95 kDa (Poland & Glover, 1986), a considerable degree of molecular weight polymorphism has been observed between species (Poland & Glover, 1987). Despite this physicochemical difference between species, little interspecies differences in the AhR function have been described (Gasiewicz & Rucci, 1984). However, the human AhR apparently has a 10-fold lower affinity for TCDD than the murine AhR (Harper et al., 1988; Manchester et al., 1987). Because of the lack of the proper biochemical tools (e.g., antibodies) and extremely low AhR concentrations, no direct information is available on the structural properties of the ligand-binding receptor protein (e.g., posttranslational modifications, functional domains, etc.).

An ^{125}I photoaffinity ligand has been developed that binds with a high degree of specificity to the AhR (Poland et al., 1986). With this tool and high-resolution denaturing two-dimensional gel electrophoresis one can examine the charge heterogeneity in the AhR and its possible significance. In this report, the conditions for determining the isoforms of the AhR are presented and their use to compare the charge heterogeneity in several different cell lines and in rat thymic cytosol is described.

Experimental Procedures

Animals and Preparation of Cytosolic Extracts. Three-week-old Buffalo rats were obtained from Harlan Sprague-Dawley Inc., Indianapolis, IN. The thymus was removed from three animals and immediately homogenized in 9 volumes of MEN buffer (25 mM MOPS, 1 mM EDTA, 0.02% NaN_3, pH 7.5) + 10% glycerol. The homogenate was centrifuged at 10000*g* for 20 min at 4 °C. The supernatant fraction was then centrifuged at 100000*g* for 1 h. The resulting cytosolic fraction was stored at −80 °C.

Cells and Media. Hepa 1c1c7 (Hepa 1) and BP^{r}C1 cells were obtained from Dr. James P. Whitlock (Department of Pharmacology, Stanford, CA) and maintained in α-minimal essential medium (Sigma) containing 8% fetal calf serum, 100 IU/mL penicillin, and 0.1 mg/mL streptomycin at 37 °C in 94% air and 6% CO_2. McA-RH7777 rat hepatoma cells were obtained from the American Type Culture Collection (Rockville, MD) and maintained in Swims S-77 media (Sigma) modified to contain phenol red, 11 mg/L, 4 mM L-glutamine, 1.8 mM $CaCl_2$, and 0.1 mM L-cystine, and supplemented with 20% horse serum and 5% fetal bovine serum. HeLa cells were obtained from Dr. Jeffrey Ross (McArdle Laboratory for Cancer Research, University of Wisconsin—Madison) and were grown in α-minimal essential medium supplemented with 10% fetal calf serum.

Sample Preparation. Hepa 1 or BP^{r}C1 cells were harvested by scraping and washed twice in phosphate-buffered saline. Cells were resuspended in MEN + 10% glycerol (5×10^7 cells/mL) and homogenized with 15 strokes in a Dura-Grind dounce tissue grinder (Wheaton Instruments, Millville, NJ). The cytosolic fraction was isolated from homogenate as described for rat thymus. Cytosolic protein samples (400 μg/mL) were photoaffinity-labeled with 2-azido-3-[^{125}I]iodo-7,8-dibromodibenzo-*p*-dioxin as previously described (Perdew, 1988), except the photoaffinity ligand concentration for each cytosol was as follows: Hepa 1, 0.468 pmol/mL, rat thymus and McA-RH7777, 0.936 pmol/mL, and HeLa, 1.4 pmol/mL. The photoaffinity ligand was synthesized as described (Poland et al., 1986) starting with 2-amino-7,8-dibromodibenzo-*p*-dioxin, kindly provided by Dr. Alan Poland (McArdle Laboratory for Cancer Research, University of Wisconsin). One volume of cytosol (0.4 mg/mL) was added to 2 volumes of lysis buffer [3% NP-40, 2.0% pH 3.0–10 ampholytes (Pharmacia LKB Biotechnology, Piscataway, NJ), 9.5 M urea, 70 mM dithiothreitol]. Urea was added to the sample until the saturation point was reached. Samples were centrifuged in a microfuge for 5 min just prior to application to the tube gel.

Photoaffinity Labeling of Cells in Culture. Hepa 1 cells were grown to 90% confluency in a 75-cm^3 flask as described above. To this flask was added 5.2 pmol (2176 Ci/mol, 2×10^7 cpm) of 2-azido-3-[^{125}I]iodo-7,8-dibromodibenzo-*p*-dioxin for 2 h at 37 °C. A second flask of cells was incubated with photoaffinity ligand and 100-fold molar excess of TCDF. Each flask of cells was rinsed twice with phosphate-buffered saline followed by irradiation with two 15-W lamps (UV Products, >302 nm) at a distance of 8 cm for 4 min. The cytosolic fraction was isolated as described above.

Isoelectric Focusing and Polyacrylamide Gel Electrophoresis. IEF gels were formed with the following final composition: 2.0% pH 3.0–10 ampholytes, 2% NP-40 (w/v), 9 M urea, 3.0% acrylamide, 0.085% piperazine diacrylamide (Bio-Rad, Rockville Centre, New York). The 3.0 mm i.d. × 11 cm gels were placed in a DE 102 tube gel unit (Hoefer Scientific Instruments). Samples were applied to the acidic end, overlayed with sample overlay buffer (1.0% ampholytes, 8 M urea, 70 mM dithiothreitol), and finally overlayed with 25 mM H_3PO_4. The lower tank was completely filled with deaerated 50 mM NaOH. The gels were run at 200 V for 2 h, followed by 300 V for 14 h, and then 800 V for 2 h, using a programmable Model 1000/500 power supply (Bio-Rad, Rockville Centre, New York). Focused gels were pushed out of the glass tubes with a pipet bulb. Each gel was then transferred into 10 mL of the equilibration buffer (2.8% SDS, 0.5 g/L bromophenol blue, 75 mM Tris-HCl, pH 6.8) and gently agitated for 10 min. The IEF gels were sealed with 1% hot agarose in equilibration buffer on top of a 1.5 mM thick, 7.5% sodium dodecylsulfate–polyacrylamide gel with a 2-cm, 3% stacking gel and ran at 25 mA/gel, essentially as described by O'Farrell (1975). One-dimensional gel electrophoresis was performed as previously described (Perdew, 1988). The gel electrophoresis units were maintained at 10

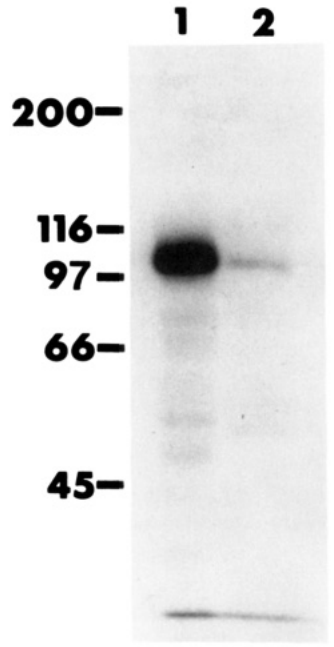

FIGURE 1: One-dimensional SDS–PAGE of cytosol from Hepta 1 cells ^{125}I-photoaffinity-labeled in culture. Lane 1, 200 μg of cytosolic protein from Hepa 1 cells incubated with photoaffinity ligand. Lane 2, 200 μg of cytosolic protein from cells incubated with photoaffinity ligand plus 100-fold molar excess of TCDF. The positions of the following standard proteins are given to the left of the autoradiogram: myosin (200 kDa), β-galactosidase (116 kDa), phosphorylase b (97 kDa), bovine serum albumin (66 kDa), and ovalbumin (45 kDa).

°C with a recirculating water bath.

The electrophoretic gels were then fixed and stained with 25% isopropyl alcohol, 10% acetic acid, 0.025% Coomassie Brilliant Blue R, followed by destaining in 10% isopropyl alcohol, 10% acetic acid, 10% glycerol. After being dried under vacuum, the gels were exposed to preflashed Kodak XAR-5 film backed with an intensifying screen and stored at −80 °C until film development (Laskey & Mills, 1977). The pH gradients in the isoelectric focusing tube gels were measured as described elsewhere (Perdew et al., 1983).

Safety Precautions. Both TCDD and TCDF are extremely toxic compounds that require special handling procedures, which have been previously described (Knutson & Poland, 1982).

Results and Discussion

Both Hepa 1 cell cytosol and cells in culture can be covalently labeled with a high degree of specificity with the photoaffinity ligand 2-azido-3-[^{125}I]iodo-7,8-dibromodibenzo-*p*-dioxin. For example, cytosol from photoaffinity-labeled Hepa 1 cells exhibited only one radiolabeled band on a 7.5% polyacrylamide gel (Figure 1, lane 1) and was greater than 95% blocked by a 100-fold molar excess of TCDF (Figure 1, lane 2). The cytosolic form of the AhR labeled with the photoaffinity ligand in Hepa 1 cell culture is in the 9S form on sucrose gradients,[2] these data are similar to the results of Miller et al. (1983), who labeled the AhR in Hepa 1 cells with [^{3}H]TCDD.

Initial studies were undertaken to optimize the resolution of the photoaffinity-labeled AhR from Hepa 1 cells by denaturing isoelectric focusing. A number of different isoelectric-focusing conditions were tested, including the following: application to either the acidic or basic end, concentration and type of cross-linker, type of detergent (e.g., CHAPS, NP-40),

[2] Unpublished observation.

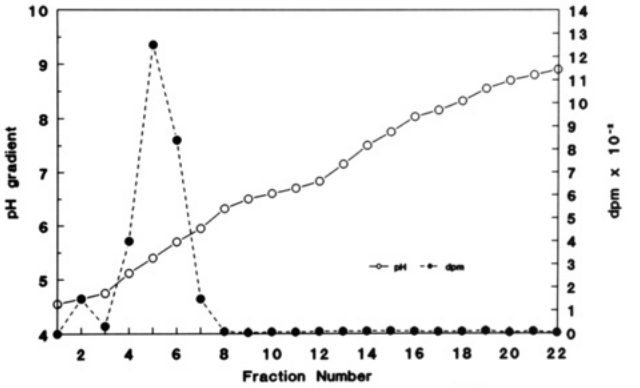

FIGURE 2: Isoelectric focusing of the denatured ^{125}I-photoaffinity-labeled *Ah* receptor. Isoelectric focusing was carried out as described under Experimental Procedures. The gel was sliced into 0.5-cm segments and placed in 1.5-mL microfuge tubes with 0.4 mL of deionized water. The closed circles indicate the radioactivity in each segment. The set of tubes was allowed to set for 24 h followed by measurement of the pH values (open circles).

amount of protein applied, slope of pH gradient, and number of volt-hours applied. The slope of the pH gradient was particularly important; attempts to use a pH gradient with a decreased slope to further resolve the photoaffinity-labeled AhR isoforms resulted in an unacceptable level of streaking. Earlier attempts at IEF using bis(acrylamide) as a cross-linking reagent in the polyacrylamide gels required the gels to contain a minimum of 3.5% acrylamide to have sufficient structural integrity to be used. The use of 3.5% acrylamide/0.1% bis(acrylamide) gels resulted in detection of only two isoforms instead of three isoforms reported here with 3% acrylamide/0.085% PDA. The use of 3.5% acrylamide/0.1% PDA gels also reduced resolution; thus, the advantage of PDA is that it allows the use of 3% acrylamide gels. Previously we have used the detergent CHAPS to enhance resolution of membrane proteins during IEF (Perdew et al. 1983); we have noted no difference between CHAPS and NP-40 in the ability to resolve the AhR. Application of small amounts of protein increased resolution of the AhR isoforms; only 5 μg of protein was applied to each gel in this report. Finally, we have observed that shorter focusing volt-hours than described under Experimental Procedures resulted in incomplete resolution of the three isoforms. The isoelectric point of the Hepa 1 AhR was determined to be between 5.2 and 5.7 (Figure 2), approximately 80–90% of the photoaffinity-labeled Hepa 1 receptor entered and focused in this region of the IEF gel. The small number of counts present in fraction 2 (Figure 2) probably represents free photoaffinity ligand or nonspecifically bound ligand. The three AhR isoforms, described below on IEF–SDS–PAGE, are apparently between fractions 4 and 7. Routinely six separate IEF tube gels were applied to polyacrylamide gels from a single photoaffinity-labeled cytosolic preparation. In addition, several cytosolic preparations isolated at different times were also subjected to IEF–SDS–PAGE. From these experiments we have determined that there are three different photoaffinity-labeled AhR isoforms, differing in their overall charge in Hepa 1 cytosol (Figure 3A). When Hepa 1 cells were photoaffinity-labeled in culture, the level of AhR charge heterogeneity was identical (Figure 3B). The isoelectric point of each isoform was estimated to be approximately 5.3, 5.45, and 5.6. The ratio of radioactivity in the three isoforms varied somewhat from one gel to another when the same sample was subjected to IEF–SDS–PAGE (data not shown). In cells incubated with the photoaffinity ligand, the AhR is capable of translocating to the nucleus and binds tightly to DNA. Only 8% of the total photoaffinity-labeled AhR is

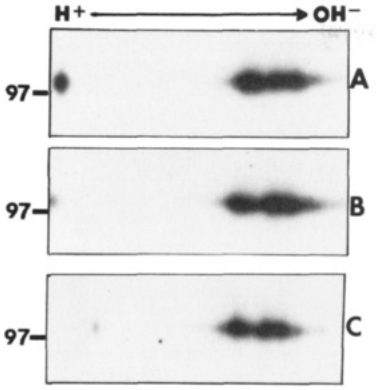

FIGURE 3: IEF–SDS–PAGE of ^{125}I-photoaffinity-labeled cytosolic fraction from Hepa 1 and BP^{r}Cl cells. The iodinated *Ah* receptor was visualized by autoradiography. The position of phosphorylase b (97 kDa) is marked to the left of each autoradiogram. IEF–SDS–PAGE of Hepa 1 cell cytosol photoaffinity-labeled in vitro (panel A), cytosol isolated from Hepa 1 cells photoaffinity-labeled in cell culture (panel B), BP^{r}Cl cytosol photoaffinity-labeled in vitro (panel C).

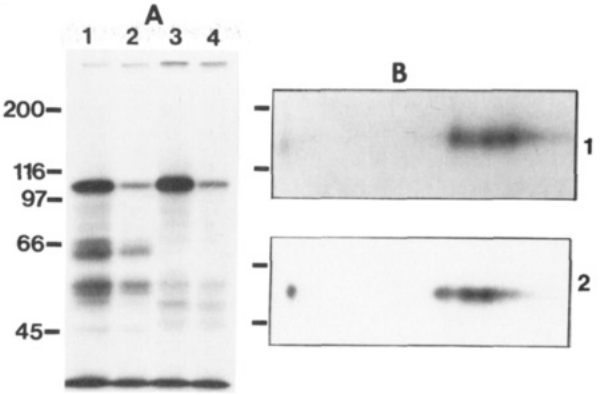

FIGURE 4: Comparison of ^{125}I-photoaffinity-labeled buffalo rat thymus McA-RH7777 tumor cell *Ah* receptor by one- and two-dimensional gel electrophoresis. In panel A buffalo rat thymus (lanes 1 and 2) and McA-RH7777 cytosolic fractions (lanes 3 and 4) were labeled with the ^{125}I-photoaffinity ligand in the absence (lanes 1 and 3) or presence (lanes 2 and 4) of a 20-fold molar excess of TCDF and subjected to SDS–PAGE. Both of the cytosolic fractions labeled in the absence of TCDF were subjected to IEF–SDS–PAGE: buffalo rat thymus (panel B1), and McA-RH7777 tumor cell cytosol (panel B2). The molecular weight markers in panel B correspond to β-galactosidase (116 kDa) and phosphorylase b (97 kDa).

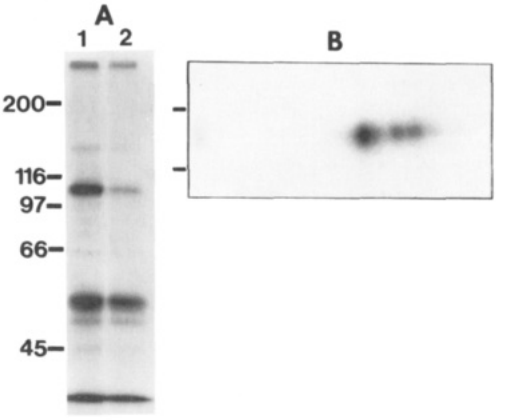

FIGURE 5: One- and two-dimensional gel electrophoresis of ^{125}I-photoaffinity-labeled HeLa cell cytosolic fraction. HeLa cell cytosolic fraction was labeled with the ^{125}I-photoaffinity ligand in the absence (panel A1) or presence (panel A2) of a 20-fold molar excess of TCDF. HeLa cell cytosol labeled in the absence of TCDF was also subjected to IEF–SDS–PAGE (panel B). The molecular weight markers in panel B correspond to β-galactosidase (116 kDa) and phosphorylase b (97 kDa).

extracted with 400 mM KCl from the nuclear pellet; the remaining 92% is in the cytosolic fraction. Attempts to focus the AhR in nuclear salt extracts have resulted in excessive streaking, making interpretation difficult.[2]

The mutant cell line BP^{r}Cl derived from Hepa 1 cells was photoaffinity-labeled and resolved by IEF–SDS–PAGE. This mutant contains an AhR that is capable of binding ligand but is unable to bind with high affinity to DNA (Cuthill & Poellinger, 1988; Whitlock & Galeazzi, 1984). Results presented here reveal that the AhR in BP^{r}Cl cells have the same apparent molecular weight and isoform pattern as wild-type cells (Figure 3C), although it is important to note that a shift of all three isoforms in p*I* of approximately 0.1–0.2 pH unit (0.5 mm on the 11-cm tube gels) toward the acidic end was observed. A more accurate comparison of the p*I* of various Hepa 1 mutants will require IEF slab gels for lane to lane comparisons.

We were interested in comparing the observed level of AhR heterogeneity seen in Hepa 1 cell cytosol to other cell lines derived from other species and in tissue cytosolic extracts. The tumor cell line McA-RH7777 was selected as a source of rat AhR because it has a relatively high receptor concentration, as judged by photoaffinity-labeling and SDS–PAGE (Figure 4A), compared to H4-II-E rat liver tumor cells (data not shown). McA-RH7777 cells, derived from a buffalo rat liver tumor, apparently have a functional AhR because aryl hydroxylase activity is highly inducible by TCDD (McManus et al., 1986). Analysis of photoaffinity-labeled rat liver cytosol (Figure 4A, lanes 1 and 2) revealed several radiolabeled protein bands between the 66- and 45-kDa protein standards that were at least partially blocked with TCDF. These protein bands may be proteolytic products of the AhR; this would be consistent with results of Poland and Glover (1988), who have shown that the photoaffinity-labeled AhR in mouse liver extracts contain proteolysis fragments of the intact receptor. We decided to compare the isoform pattern in McA-RH7777 cells to buffalo rat thymus cytosol to determine if the AhR has a different level of charge heterogeneity in vivo. As can be seen in Figure 4B, there was no apparent difference in the isoform pattern for the photoaffinity-labeled AhR from rat thymus. Photoaffinity-labeled HeLa cell cytosol was also subjected to SDS–PAGE and IEF–SDS–PAGE, and the results (Figure 5) reveal that the human AhR in HeLa cells has the same level of charge heterogeneity as was seen in the other species tested. It is interesting to note that a major nonspecifically radiolabeled protein at 58 kDa was observed in HeLa cell cytosol (Figure 5A), the identity of this protein is unknown. These results taken together would indicate that the three isoforms detected are conserved, and the apparent isoelectric points were similar despite the observed M_r differences between each species (Poland & Glover, 1987). There are two possible explanations for the observed charge heterogeneity: (1) The isoforms could be the products of two different allelelic forms of the AhR, or (2) the charge difference may be due to a posttranslational modification (e.g., phosphorylation, glycosylation). The first explanation could possibly be supported by the presence of three complementation groups involved in AhR function (Karenlampi et al., 1988); this may indicate that there is more than one structural gene for the AhR. However, further work will be necessary to determine the reason for the three isoforms observed. A method to isoelectric focus the nondenatured AhR labeled with [^{3}H]TCDD has been developed (Carlstedt-Duke et al., 1978). This method requires the AhR to undergo partial proteolysis before application to IEF polyacrylamide gels; proteolysis is necessary to obtain

proper focusing. The p*I* values for the AhR from rat liver and human leukocytes were determined to be 5.2 and 6.0, respectively (Carlstedt-Duke, 1979; Gillner et al., 1989). Nondenaturing isoelectric focusing usually does not result in sharp resolution on isoforms of a given protein in a complex mixture; one reason is because of protein aggregation. The AhR in cytosol is bound to a 90-kDa heat-shock protein and perhaps is aggregated with other proteins, whereas under the highly denaturing IEF conditions employed here, the ligand-binding 95-kDa AhR protein should be in a monomeric form.

The AhR and the glucocorticoid receptor (GR) have a number of biochemical properties in common (Cuthill et al., 1987). For example, they have a similar molecular weight under both denaturing and nondenaturing conditions, and both are present in cytosol in a "nontransformed" state bound to HSP90 (Lefebvre et al., 1989; Perdew, 1988). Both receptors are transformed from an 8–9S form to a 4–6S form upon binding an agonist and subsequent binding to specific DNA sequences. With this background it is useful to compare the apparent charge heterogeneity of the AhR and GR. The human GR has been reported to have two isoforms in crude cytosol preparations, with the approximate p*I* values of $\simeq$5.7 and $\simeq$6.0–6.5 (Harmon et al., 1989), and perhaps a third more acidic form (p*I* $\simeq$ 5.2) also exists (Smith & Harmon, 1985). This result would indicate a similar level of charge heterogeneity compared to the AhR. Attempts to use the isoelectric-focusing method described by Smith and Harmon (1985) resulted in an unacceptable level of streaking (data not shown) of the photoaffinity-labeled AhR. It is interesting to note that two of the isoforms described for the GR vary in their ability to bind to DNA (Smith et al., 1986), and it will be important to determine if this is also true for the AhR.

We have described a method for examining the level of charge heterogeneity for the AhR that should be useful in determining the possible role of charge heterogeneity in AhR function. In addition, the methods described here for isoelectric focusing may also be useful for other soluble receptors (e.g., progesterone, estrogen). Future studies will be directed at examining the functional significance of the three isoforms of the AhR.

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