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Chemical Cross-Linking of the Cytosolic and Nuclear Forms of the Ah Receptor in Hepatoma Cell Line 1c1c7

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SUMMARY: Both cytosolic and high salt nuclear extracts were isolated from Hepa 1c1c7 cells incubated with 2-azido-3[^{125}I]iodo-7,8-dibromo-dibenzo-*p*-dioxin ([^{125}I]N_3Br_2DpD). The [^{125}I]N_3Br_2DpD-labeled cytosolic fraction was subjected to chemical cross-linking with dimethyl pimelimidate and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Chemical cross-linking of the cytosolic form of the AhR revealed monomeric (97 kDa), dimeric (185 kDa), trimeric (281 kDa), and tetrameric (327 kDa) complexes. In a time course of exposure to the cross-linking reagent, the largest form given above became the predominant AhR form observed in the cytosolic extracts. The 327 kDa cytosolic species apparently consists of a 97 kDa AhR, an ~88 kDa protein, an ~96 kDa protein, and an ~46 kDa protein. Nuclear extracts from [^{125}I]N_3Br_2DpD-labeled Hepa 1c1c7 cells were applied to sucrose density gradients. The 6 S nuclear receptor peak fractions were pooled and subjected to chemical cross-linking. Analysis by SDS-PAGE revealed a monomeric (97 kDa) ligand binding protein and a dimeric (182 kDa) complex. This would suggest that the nuclear 6 S AhR consists of a 97 kDa AhR and an ~85 kDa protein. These findings would indicate that the AhR exists in cytosol as a tetrameric species, while in the nucleus the AhR exists as a heterodimer.

The AhR plays a central role in the biological response to planar polycyclic aromatic hydrocarbons (e.g. TCDD, 1,2-benzanthracene), an important class of environmental contaminants. A wide range of pleiotropic responses are believed to be mediated by the AhR, these include; thymic atrophy, hepatotoxicity, wasting syndrome, chloracne and hyperkeratosis (1,2). Perhaps the most studied action of the AhR is the induction of cytochrome P_{450}1A1 transcription. After binding TCDD the AhR apparently translocates into the nucleus. The TCDD/AhR complex is capable of binding directly to a dioxin-responsive enhancer that is located upstream from the CYP1A1 gene (3).

Abbreviations: AhR, Aryl hydrocarbon receptor; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; M_r, relative molecular weight; HSP90, 90 kDa heat shock protein; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; [^{125}I]N_3Br_2DpD, [^{125}I]2-iodo-3-azido-7,8-dibromo-*p*-dioxin; PDA, piperazine diacrylamide; MOPS, 3-(N-morpholino)propanesulfonic acid; DMP, dimethyl pimelimidate.

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The sequence of events that occur after a ligand binds to the AhR and until the receptor binds to the dioxin-responsive enhancer element are not well understood. In addition, the subcellular location of the AhR has not been firmly established. A number of studies have suggested that the unliganded AhR is predominantly associated with the cytosolic fraction (4,5). In contrast, Whitlock and Galeazzi reported that the presence of the AhR in the cytosolic fraction was due to homogenizing cells in dilute buffer (6). Recently, the 9 S liganded AhR has also been shown to be present in the nucleus (7,8). Nevertheless, the current model of receptor action is based on dioxin binding the cytosolic AhR followed by translocation into the nucleus and binding to the nuclear material. In the cytosol prepared from Hepa 1 cells the AhR exists as an 8-9 S highly asymmetric oligomeric species with an estimated M_r of 260-300 kDa (9,10). Analysis of high salt extracts has revealed an ~6 S AhR species, with a M_r of 176 kDa, which is larger than the cytosolic AhR transformed by high salt in vitro (9). This would indicate that the ~6 S species extracted from the nucleus of Hepa 1 cells is composed of more than a monomer of the ligand binding AhR.

Both the AhR and the glucocorticoid receptor have the following biochemical properties in common: 1) they contain HSP90 in a cytosolic complex (11,12,13); 2) have similar molecular weights and isoelectric points under denaturing conditions (14,15); 3) they are DNA binding proteins (6,16); they are asymmetric and have similar M_r values for the cytosolic form of each receptor (10). Chemical cross-linking and SDS-PAGE have been used to examine the protein composition of the glucocorticoid receptor (17). In this report, similar methodology was used to examine the composition of both the nuclear and cytosolic forms of the AhR. This report establishes for the first time the relative molecular weights and the number of the proteins present in the ~9 S cytosolic and ~6 S nuclear forms of the AhR.

MATERIALS AND METHODS

Chemicals. [^{125}I]N_3Br_2D*p*D was synthesized as described (18). Fetal bovine serum was obtained from Hyclone Lab. (Logan, Utah). Acrylamide and PDA were obtained from Research Organics (Cleveland, Ohio) and Bio-Rad (Richmond, CA), respectively. DMP and all other chemicals were from Sigma unless otherwise noted.

Cell Culture. Hepa 1c1c7 cells, obtained from Dr. J.P. Whitlock, were grown in α-minimum essential medium containing 8% fetal calf serum, 100 IU/ml penicillin, and 0.1 mg/ml streptomycin at 37°C. Hepa 1 cells were grown to 90% confluency in a 75-cm^2 culture flask, 26 pmol (2176 Ci/mol, 1 x 10^8 cpm) of [^{125}I]N_3Br_2D*p*D was added in 10 ml of serum-free media and incubated for 1 h.

Cell Extracts. Hepa 1 cells were harvested and homogenized as previously described (8). The cell homogenate was centrifuged at 1,000 x g for 20 min, the supernatant removed and centrifuged at 100,000 x g for 60 min to yield the cytosolic fraction. The 1,000 x g pellet (nuclear pellet) was resuspended and washed two times with MENG. The nuclear pellet was washed once with MENG + 100 mM KCl, followed by resuspension in 300 μl of MENG + 400 mM KCl and gently shaken at 4°C for 1 h. This mixture was centrifuged at 100,000 x g for

60 min, the resulting nuclear extract was either used immediately or stored in liquid nitrogen.
Cross-Linking of Receptor. The cross-linking reagent DMP (520 μg) was added to 100 μl of [^{125}I]N_3Br_2DpD-labeled cytosol at a protein concentration of 1 mg/ml. This mixture was incubated at 10°C for various time periods. The reaction was stopped by the addition of 200 μl of 50 mM hydroxylamine. To this mixture was added 100 μg of soybean trypsin inhibitor (to aid acetone precipitation) and 2 ml of acetone. The samples were stored at -20°C overnight, followed by centrifugation at 3,000 x g for 10 min. The resulting precipitate pellets were solublized in SDS sample buffer and were applied to 3.3% acrylamide/0.11% PDA SDS continuous gels as described in Technical Bulletin No. MWS-877 (Sigma). The following molecular weight standards were used; cross-linked hemocyanin (70 and 140 kDa), and cross-linked phosphorylase B (97, 194, 292, 389 kDa). Gels were stained with Coomassie Blue, destained, and dried as previously described (14). Each gel was subjected to autoradiography (19). Dried gels were also cut into 2 mm slices and counted in a gamma counter.
Sucrose density gradient analysis and cross-linking of the nuclear AhR. Sucose density gradients were used as a technique to separate the 6 S AhR from the 9 S AhR for use in cross-linking studies. Nuclear extracts of Hepa 1 cells previously incubated with [^{125}I]N_3Br_2DpD were layered on 5.1 ml 10-30% sucrose gradients prepared in MENG plus 400 mM KCl. The sealed tubes were centrifuged in a Beckman VTi65.2 at 416,000 x g_{max} for 135 min. Each tube was fractionated with an Isco Model 640 density gradient fractionator and counted in a TmAnalytic 1191 gamma counter (Elk Grove Village, IL). Fractions containing the ~6 S receptor were pooled and used in cross-linking studies.

RESULTS AND DISCUSSION

Recently we have shown that [^{125}I]N_3Br_2DpD, a photoaffinity ligand, added to Hepa 1 cell culture, is capable of binding to the AhR with a high level of specificity (14). Hepa 1 cytosol subjected to SDS-PAGE revealed a single radiolabeled band. Application of cytosol from [^{125}I]N_3Br_2DpD-labeled Hepa 1 cells to sucrose density gradients revealed a single peak of ~9 S. The [^{125}I]N_3Br_2DpD/AhR complex is capable of translocating into the nucleus and binding to the nuclear matrix. High salt extracts of [^{125}I]N_3Br_2DpD-labeled Hepa 1 nuclear fraction were applied to sucrose density gradients. Analysis of the fractionated sucrose density gradient revealed both an ~6 S and an ~9 S peak (8). In this study chemical cross-linking was used to further define the protein composition of the cytosolic 9 S and nuclear 6 S forms of the AhR. Homobifunctional imidoesters like DMP selectively react with amino groups and thus would be selective for proteins. The cytosol from [^{125}I]N_3Br_2DpD-labeled Hepa 1 cells was subjected to cross-linking with DMP over time. Over a three hour period a steady increase in larger cross-linked AhR species was observed (Fig.1). The monomeric AhR was labeled a, and the three larger abundant species were labeled b, c, and d. Cross-linking for 3 h, followed by the addition of more DMP and subsequent incubation for an additional 3 h resulted in 63% of the AhR being found in peak d (Fig 2). Thus the cytosolic AhR exists as a 327 kDa complex. Cross-linking for 1 h was optimal for observing every cross-linked species (Fig. 1). But, each radiolabeled species detected was consistently observed over a wide range of protein and cross-linker concentrations. Hepa 1 cytosol was applied to a sucrose gradient and the 9 S peak was pooled and subjected to

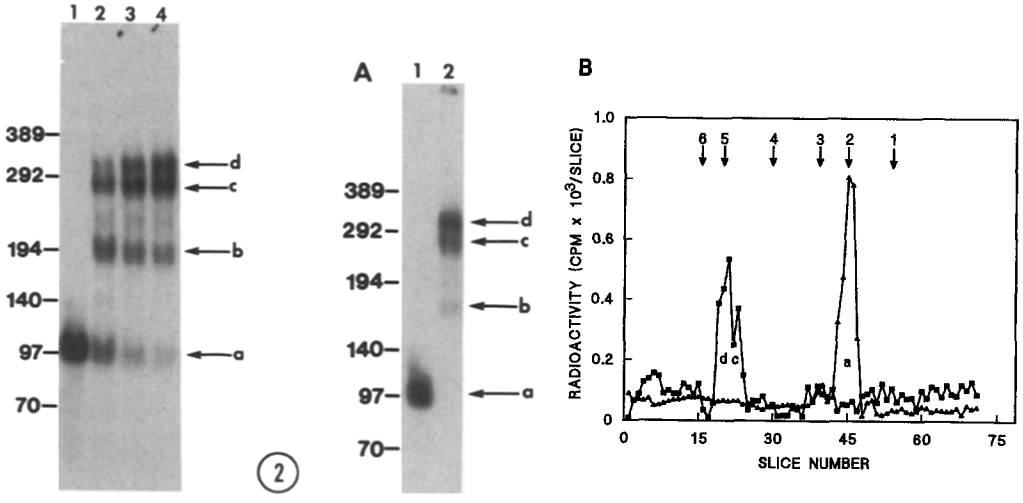

Figure 1. SDS-PAGE of cross-linked cytosolic extracts from [^{125}I]N_3Br_2D*p*D-labeled Hepa 1 cells. Cross-linking was performed for 0 h (lane 1), 1 h (lane 2), 2 h (lane 3), and 3 h (lane 4).

Figure 2. SDS-PAGE of cytosolic extract from [^{125}I]N_3Br_2D*p*D-labeled Hepa 1 cells cross-linked for 6 h. Autoradiogram of gel (panel A), and the gel cut into 2 mm slices and counted (panel B). [^{125}I]N_3Br_2D*p*D-labeled cytosol with no cross-linker added (panel A, lane 1, or panel B, peak a). [^{125}I]N_3Br_2D*p*D-labeled cytosol with the addition of cross-linker for 6 h (panel A, lane 2, or panel B, peak c and e).

chemical cross-linking. The cross-linked forms obtained were the same as unfractionated cytosol[1].

Analysis of high salt nuclear extracts from Hepa 1 cells incubated with [^{3}H]TCDD revealed only a 6 S species which is apparently the DNA binding form of the AhR in vivo (7,9). High salt nuclear extracts were isolated from rat, hamster, and guinea pig liver after the animals were injected with [^{3}H]TCDD. These extracts were analyzed using sucrose density gradients, the results revealed a single peak sedimenting between 6-6.6 S (20). In order to perform cross-linking studies on only the 6 S [^{125}I]N_3Br_2D*p*D-labeled AhR nuclear extracts were applied to sucrose gradients. After fractionation, the 6 S AhR peak was pooled and used in cross-linking experiments. Over a 1 h cross-linking time course an increase in an additional radiolabeled receptor species was detected at 182 kDa, while no other cross-linked species were detected (Fig. 3). This result would suggest that the 6 S nuclear AhR is a dimeric protein complex. The monomeric AhR has an M_r of 97 kDa, if the 6 S nuclear form of the AhR was a homodimer the expected M_r would be 194 kDa. In this report the dimeric nuclear AhR has an M_r of 182 kDa, 12 kDa less than a theoretical homodimer. In Fig. 3 cross-linked cytosolic receptor was applied to the same polyacrylamide gel with nuclear receptor to provide an additional sizing reference

[1]Unpublished data.

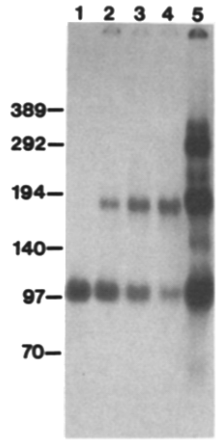

Figure 3. SDS-PAGE of cross-linked nuclear and cytosolic extracts from [^{125}I]N_3Br_2D*p*D-labeled Hepa 1 cells. Nuclear extract was applied to sucrose gradient and the ~6 S peak fractions were pooled and used for cross-linking. Nuclear fractions were cross-linked for 0 min (lane 1), 15 min (lane 2), 30 min (lane 3), 60 min (lane 4). Cytosolic extract was cross-linked for 1 h (lane 5).

point. The dimeric cross-linked receptor in lane 4 is slightly lower in molecular weight compared to the cytosolic band b in lane 5. These results provide evidence for a heterodimeric nuclear AhR containing the 97 kDa ligand binding receptor and an ~85 kDa protein. Hankinson and coworkers have recently cloned a gene corresponding to a human 87 kDa protein (Arnt) that may be associated with the nuclear form of the AhR (21). This would suggest that the ~85 kDa protein found cross-linked to the Hepa 1 nuclear AhR in this report may be the same protein as Arnt. The experiments shown in Figs. 1 and 3 were repeated four times. The relative molecular weight of each radiolabeled receptor species was calculated, the results are summarized in Table 1.

Table 2 summarizes an interpretation of the data outlined in Table 1. Other investigators have determined the M_r of the Hepa 1 cytosolic AhR to be between 260-300 kDa using gel filtration

Table 1

Summary of cross-linking experiments: M_r data[1]

Cytosolic (9S) receptor		Nuclear (6S) receptor	
a	97,000 ± 1,000	a	97,000 ± 1,000
b	185,000 ± 5,000	e	182,000 ± 2,000
c	281,000 ± 6,000		
d	327,000 ± 6,000		

M_r values were determined on SDS gels. Mean and range values reported here were determined from 4 separate experiments. The letters a to e refer to radiolabeled bands in fig. 1-4.
[1]All M_r values are expressed in daltons.

Table 2

Interpretation of M_r data[1]

	Cross-linked receptor species
a	M_r = 97,000: AhR
b	M_r = 185,000: AhR + p88
c	M_r = 281,000: AhR + p88 + p96
d	M_r = 327,000: AhR + p88 + p96 + p46
e	M_r = 182,000: AhR + p85

The letters a to e refer to radiolabeled bands in fig. 1-4.
[1]All M_r values are expressed in daltons.

and sucrose density gradients (9,10). This M_r value is lower than the M_r of 327 kDa observed for the tetrameric species d observed here. The reason for this difference may be due to the different sizing techniques used. In contrast, the M_r of 176 kDa for the Hepa 1 salt extracted nuclear form of the AhR was determined by Prokipcak and Okey (9) and is similar to the 182 kDa value obtained in this study. The detection of a number of intermediate cross-linked receptor species at different molecular weights would indicate that the cytosolic AhR is a tetrameric complex containing the following polypeptides; one 97 kDa dioxin binding subunit, one ~88 kDa subunit, one ~96 kDa subunit, and one ~46 kDa subunit. The cross-linking data obtained here for the AhR is strikingly similar to results obtained in glucocorticoid receptor cross-linking experiments (17). Chemical cross-linking of the glucocorticoid receptor in intact mouse lymphoma cells was achieved, yielding essentially the same results as those obtained in vitro. However, attempts to chemically cross-link the AhR in Hepa 1 cells have been unsuccessful.

Two ~90 kDa proteins were detected in the cytosolic AhR complex. Anti-HSP90 antibodies have been used previously to show that the cytosolic AhR is associated with HSP90 (11,12). Whether both p88 and p96, detected as part of the 9 S AhR complex, reflects the presence of a dimer of HSP90 will require additional experimentation. Studies on the glucocorticoid receptor have revealed that a dimer of HSP90 is bound to the cytosolic ~9 S form of this receptor (20,21). There are two previously characterized proteins that may possibly be the ~46 kDa protein observed bound to the cytosolic form of the Ah receptor. The first possibility is a 56-59 kDa protein (EC-1 antigen[2]) that has been observed bound to the untransformed steroid receptors (24,25). The cytosolic form of the Ah receptor in rabbit liver apparently is not associated with

[2]EC-1 antigen refers to a 59 kDa protein that has been found to be associated with several of the steroid hormone receptors. KN 382/EC-1 is a monoclonal antibody that recognizes this protein.

the EC-1 antigen associated with the glucocorticoid receptor (26). However, further studies will be needed to conclusively rule out the possible association of EC-1 antigen with the Ah receptor. The EC-1 antigen has been shown to directly bind to HSP90 (27,28). A 50 kDa phosphoprotein has been shown to be bound to HSP90 in Hepa 1 cells (29,30).

Recently, rat cytosolic AhR was transformed in vitro with 2,3,7,8-tetrachlorodibenzo-*p*-dioxin and incubated with bromodeoxyuridine-substituted DNA recognition motif (31). The receptor/DNA complex was cross-linked using ultraviolet irradiation and analyzed by SDS-PAGE. Two proteins with M_r of 110 and 100 kDa bound to the DNA motif were detected. In this report the M_r of each protein in the DNA binding form of the AhR was determined by salt extraction of a nuclear fraction from Hepa 1 cells previously incubated with [^{125}I]N_3Br_2DpD, followed by chemical cross-linking. The M_r of each of the two proteins in the 6 S nuclear receptor reported here were 97 and 85 kDa. The reason for the differences between the results of these two studies is not known. In conclusion, these studies have established that the AhR exists in cytosol as a tetrameric protein complex, and in the nucleus as a dimeric protein complex bound to DNA.

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