Subunit Composition of the Heteromorphic Cytosolic Aryl Hydrocarbon Receptor Complex*

(Received for publication, July 18, 1994, and in revised form, August 31, 1994)

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In a previous cross-linking study we have shown that the cytosolic aryl hydrocarbon receptor (AhR) complex has a heterotetrameric structure (Perdew, G. H. (1992) Biochem. Biophys. Res. Commun. 182, 55-62). In this report, both cross-linked and [35S]methionine-labeled Hepa 1c1c7 cytosol were used to characterize the subunit composition of the AhR complex by immunoprecipitation with an AhR polyclonal antibody followed by immunonochemical analysis using antibodies against the AhR and 90-kDa heat shock proteins (hsp90). Results indicated that the four subunits found in cross-linking experiments were composed of three species: the AhR ligand binding subunit, hsp90, and an unknown 43-kDa protein. The stoichiometry of hsp90 present in each AhR complex was determined in two separate experiments: 1) from cross-linking experiments, stoichiometry was determined by quantitative immunoblotting with anti-AhR and anti-hsp90 antibodies followed by quantitation with [35S]-counterantibody on protein blots; 2) using [35S]-labeled Hepa 1 cytosol, the hsp90/AhR stoichiometry was determined by immunoprecipitating receptor complexes, and the amount of [35S]-labeled AhR and hsp90 was assessed. The stoichiometry values obtained were 2.4 and 1.72 mol of hsp90/mol of AhR using each experimental approach, respectively.

2,3,7,8-Tetrachlorodibenzo-p-dioxin and similar compounds (e.g. polychlorinated biphenyl) are environmental contaminants. Their toxic and biological effects occur via the aryl hydrocarbon receptor (AhR) mediated signal transduction pathway. After binding to the agonist the ligand-activated AhR is believed to transform from a 9 S form to a DNA binding form (6 S form). The 6 S AhR complex is apparently composed of the AhR and Arnt; this heterodimer is capable of binding to dioxin response elements, resulting in increased transcription of several genes involved in xenobiotic metabolism, such as cyp1a1 and cyp1a2. The cDNA for both the AhR and Arnt have been cloned; each protein belongs to a family of basic region/helix-loop-helix (bHLH) transcription factors (1). The transformation process involves dissociation of hsp90 from the AhR followed by formation of a heterodimer with Arnt (2, 3, 4). However, the sequence of events and all possible proteins involved in receptor transformation remain to be delineated. Determination of the subunit structure of the AhR is important to help unravel the molecular mechanism of AhR action and regulation.

After cellular disruption the untransformed 9 S form is found predominantly in cytosolic extracts. Our previous cross-linking experiments utilized [125I]N3Br,DpD-labeled Hepa 1 cytosol, and the results revealed a tetrameric receptor complex consisting of a ligand-binding subunit and three other proteins of approximately 96, 88, and 46 kDa relative molecular masses (5). The 90-kDa heat shock protein (hsp90) has been identified as one of the proteins associated with the cytosolic form of the AhR complex (6, 7). Since hsp90 is an abundant cytosolic protein (1-2% of total cytosolic proteins) only occurring in a dimeric 180-kDa form under nondenaturing conditions (8, 9), it is reasonable to propose that the tetrameric AhR receptor complex may contain two hsp90 subunits. Recent studies of the glucocorticoid and progesterone receptor support this possibility. Bresnick et al. (10) have shown that the 9 S form of the glucocorticoid receptor contains two hsp90 subunits. In addition, cross-linking experiments of progesterone receptor indicated a heterotetrameric structure composed of a hsp90 dimer and a 59-kDa protein (11). However, there is no direct evidence that the AhR complex contains a dimer of hsp90. In this report, the immunopurified AhR complex from both cross-linked cytosol and [35S]methionine-labeled cytosol were utilized to establish the subunit composition and determine if a dimer of hsp90 is associated with the ligand-binding subunit. Cross-linking studies can be used to determine the number of subunits present in each AhR oligomeric complex. Direct immunoprecipitation of the [35S]methionine-labeled cytosol will reveal the molecular weight of each subunit in the protein complex. The subunits can be further identified by immunonochemical staining of the immunoprecipitates after resolution by SDS-PAGE and electrophoretic transfer to membrane. These two distinct approaches to compositional analysis are complimentary in the analysis of oligomeric protein complexes. This is the first published report on the subunit composition of the cytosolic 9 S form of the AhR.

EXPERIMENTAL PROCEDURES

Materials

[35S]Methionine was purchased from Amersham. Goat anti-mouse IgG/Fc, goat anti-mouse IgG peroxidase conjugate, peroxidase-conjugated streptavidin, biotin-SP-conjugated donkey anti-rabbit IgG, and biotin-SP-conjugated goat anti-mouse IgG were purchased from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA). Piperazine diethylamide and Aff-Gel 15 were obtained from Bio-Rad. Acrylamide was obtained from Research Organics (Cleveland, OH). Marine polyclonal antibodies against an N-terminal peptide sequence of AhR were

* This work was supported in part by National Institute of Environmental Health Science Grant ES-04869. This is technical paper 14,311, which the AhR is altered from a non-DNA binding form (9 S) to a DNA binding form (6 S).

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1 The abbreviations used are: AhR, Ah receptor (aryl hydrocarbon receptor); cyp1a1 and cyp1a2, structural gene for cytochrome P-4501A1 and cytochrome P-4501A2, respectively; hsp90, 90-kDa heat shock protein; Arnt, Ah receptor nuclear translocator protein; [125I]N3Br,DpD, [125I]-iodo-3-azido-7,8-dibromodibenzo-p-dioxin; Hepa 1 or Hepa 1c1c7, mouse hepatoma cell line 1c1c7; GAM, goat anti-mouse; PAGE, polyacrylamide gel electrophoresis; PVDF, polyvinylidene fluoride; c2, Hepa 1 mutant cell line containing a low concentration of AhR; mAb, monoclonal antibody; Mops, 4-morpholinepropanesulfonic acid.

In this report, transformation or transform refers to the process by which the AhR is altered from a non-DNA binding form (9 S) to a DNA binding form (6 S).
produced and characterized elsewhere (12). The anti-AhR monoclonal antibody Rpt-1 was produced and utilized as previously described (13). Hepa 1 clone 7 and c1 mutant cell lines were obtained from Dr. James P. Whitlock, Jr. and Dr. Oliver Hankinson, respectively. Fetal bovine serum was obtained from Hyclone Lab (Logan, UT). DMP and all other chemicals were from Sigma unless otherwise noted.

Cell Culture and Extracts
Hepa 1 clone 7 and c1 mutant cell lines were grown in a minimum essential medium supplemented with 8% fetal bovine serum, 100 international units/ml penicillin, and 0.1 µg/ml streptomycin at 37 °C in 94% air, 6% CO2. Hepa 1 cells were grown in a 25 cm2 culture flask, harvested using trypsin/EDTA, and washed once in Dulbecco's phosphate-buffered saline. The cells were resuspended in MENG (25 mM Mops, 2 mM EDTA, 0.02% NaN3, 10% glycerol, pH 7.5) with 20 µg/ml leupeptin and homogenized in a Dounce homogenizer. The cell homogenate was centrifuged at 1000 x g for 20 min, and the supernatant was removed and centrifuged at 100,000 x g for 60 min to yield the cytosolic fraction. For [35S]methionine-labeling experiments, cells were grown for 18 h in the following medium: 80% minimum essential medium with no methionine, 1 x nonessential amino acids, and 20% minimum essential medium. To this medium was added dialysed fetal bovine serum (final concentration, 11%) and [35S]methionine at a final concentration of 30 µCi/ml. The final concentration of methionine was 20 µM. Long term incubation of cells was tested in this study to attain a steady-state level of the proteins by increasing the total methionine concentration to 100 µM and incubation time to 48 h. Cells apparently underwent radiation damage and then adjusted by dividing the weighted ratio of trimer to dimer. The weighted ratio was determined by dividing cpm of AhR in the trimer by that in the dimer.

Chemical Cross-linking
To 100 µl of cytosol at a protein concentration of 1 mg/ml was added 10 µl of 2.2 µM triethanolamine, pH 8, to adjust the pH. Then 10 µl of 0.1 M dimethyl pimelimidate freshly prepared in dimethyl sulfoxide was added. This mixture was incubated at 10 °C for various time periods. The reaction was stopped by the addition of 10 µl of 1 M l-lysine, and the cross-linked cytosol was used immediately in immunoprecipitation experiments.

Immunoprecipitation and Gel Electrophoresis
Affinity-purified goat anti-mouse (GAM) IgG(Fc) was coupled to Affigel 15 (1 mg of antibody/ml of gel). To 50 µl of GAM IgG-Sepharose was added 5 µg of polyclonal anti-AhR antibody or control murine polyclonal IgG fraction, and the mixtures were incubated for 1 h on ice followed by two washes with MENG, 500 mM NaCl (the gel is collected each time by centrifugation). Then 1.2 ml of cross-linked Hepa 1 cytosol (667 µg/ml in MENG, 500 mM NaCl, 1% Nonidet P-40) was added to the gel, and the mixture was gently agitated for 3 h on ice. This sample was washed two times with MENG, 500 mM NaCl. The affinity gel was overlaid on 1 ml of sucrose solution (MENG, 1 x sucrose, 500 mM NaCl, 1% Nonidet P-40) and subjected to centrifugation. The gel was washed twice with MENG, 500 mM NaCl, 4% Nonidet P-40 and once with MENG. The gel was then solubilized in 2X LDS sample buffer (4% LDS, 24% glycerol, 100 mM Tris-HCl, 2 mM EDTA, 20 mM dithiothreitol, pH 6.8) and heated at 95 °C for 4 min. Samples were subjected to polyacrylamide gel electrophoresis on 3.8% acrylamide, 0.18% bisacrylamide Tricine gel essentially as described (14). Electrophoresis was performed at room temperature at 30 V for 2 h followed by 60 V for 14 h.

Immunoblotting
Immunoblotting was carried out by transferring proteins from polyacrylamide gels to PVDF or nitrocellulose membranes at 8 V for 6 h. After blocking the nonspecific binding sites at room temperature for 1 h with 3% bovine serum albumin, 0.05% Tween 20, in phosphate-buffered saline, the blot was incubated with mAb Rpt-1 (1:10,000 diluted ascites) or rabbit polyclonal anti-hsp84/86 antibody (1 µg/ml). The immunoblotted proteins were detected by incubation with biotin-SP-conjugated GAM IgG or donkey anti-rabbit IgG followed by incubation with streptavidin-conjugated GAM IgG or by incubation with mouse anti-rabbit IgG followed by incubation with 125I-conjugated GAM IgG. The resulting blots were subjected to autoradiography, and the appropriate protein bands on the membrane were then excised and counted for radioactivity in a γ-counter. During the experiment, we found that although the PVDF membrane exhibited superior protein retention, nitrocellulose membrane (Bio-Rad) gave better relative intensity of the blotted proteins in cross-linking experiments.

Measurement of Receptor and hsp90 Stoichiometry

Method 1—Stoichiometry was measured by quantitative immunoblotting with [35S]methionine-specific antibodies. The amount of each receptor-specific species was determined by subtracting the cpm of control band from the cpm of its corresponding receptor species. The molar ratio of the hsp90 in the trimer and dimer bands of the AhR complex was first calculated by dividing counts of hsp90 in the trimer by that in the dimer and then adjusted by dividing the weighted ratio of trimer to dimer. The weighted ratio was determined by dividing cpm of AhR in the trimer by that in the dimer.

Method 2—The amounts of receptor and hsp90 in receptor complex were determined by labeling Hepa 1 cells for 18 h with [35S]methionine. The immunoadsorbed proteins were resolved by SDS-PAGE and transferred onto PVDF membrane. The blot was used to perform phosphoimage analysis. Relative intensity of each species was analyzed by a Fuji image analyzer (BAS2000). The relative intensity of the AhR or hsp90 was obtained by subtracting the relative intensity of the immunospecific bands from that of their corresponding bands in the control lane and then correcting for the methionine content of AhR and hsp90. Assuming 17.7 methionine residues for each hsp90 (adjusted from the relative concentrations of hsp86/84 in Hepa 1 cytosol (15, 16) and 15 methionine residues for each AhR (17, 18), the stoichiometry of AhR and hsp90 was obtained by multiplying the AhR:hsp90 relative intensity ratio by 1.18:1, thus correcting for differing methionine content.

RESULTS AND DISCUSSION
In a previous report from our laboratory the number and approximate size of each subunit of the 9 S AhR were established using cross-linking techniques (5). The studies described here will use a combination of protein-protein cross-linking, immunoprecipitation, and immunochemical analysis to identify the proteins found in the 9 S AhR complex. The concentration of dimethyl pimelimidate that was employed in cross-linking incubations of cytosolic extracts was established in preliminary studies. Treatment of Hepa 1 cytosolic extracts with decreasing DMP concentrations resulted in a progressive increase in the immunoprecipitation efficiency of the cross-linked AhR complex. The minimum concentration of DMP that was capable of forming a cross-linked tetrameric AhR complex was 0.01 M and thus was used in all immunoprecipitation experiments. After cross-linking for 2 h, the immunopurified AhR complexes were resolved by continuous SDS-PAGE and transferred to the PVDF membrane. The blot was stained with mAb Rpt-1 to visualize the AhR. The results shown in Fig. 1 confirmed our previous study, which revealed that cross-linking of the cytosolic AhR complex yielded monomeric, dimeric, trimeric, and tetrameric complexes with molecular masses of 97, 185, 281, and 327 kDa, respectively. In order to further ascertain whether the hsp90 is present in these complexes, we used rabbit anti-hsp84 and anti-hsp86 polyclonal antibodies to probe the immunopurified AhR complex. These antibodies have been

3 H.-S. Chen and G. H. Perdew, unpublished data.
FIG. 1. Western blot analysis of the immunoadsorbed, cross-linked aryl hydrocarbon receptor complex. The crude Hepa 1 cytosol extract was cross-linked with DMP for 2 h and immunoadsorbed with anti-AhR polyclonal antibody or nonimmune IgG. After washing, the immunoadsorbed proteins were resolved by SDS-PAGE and protein blotting. The blot was incubated with primary antibody against AhR or hsp90 and stained as described under “Experimental Procedures.” Lanes: 1, cross-linked cytosol; 2 and 4, cross-linked cytosols adsorbed with nonimmune IgG; 3 and 5, cross-linked cytosol adsorbed with anti-AhR polyclonal antibody. Monomer indicates the position of non-cross-linked AhR.

FIG. 2. Stoichiometric analysis of AhR and hsp90 by quantitative immunoblotting with 125I-labeled counterantibody. Samples were prepared as in Fig. 1. Cross-linking reactions were incubated for 45 min. The blot was stained with the 125I-labeled counterantibody. Lanes 1 and 3, cross-linked cytosols adsorbed with nonimmune IgG; lanes 2 and 4, cross-linked cytosol adsorbed with anti-AhR polyclonal antibody.

shown to be highly specific to each form of hsp90 in Hepa 1 cytosol (16). Stringent washing procedures were used in this experiment to minimize the nonspecific binding of hsp90 from cross-linked cytosol immunoadsorbed with nonimmune IgG. By increasing the Nonidet P-40 in washing buffer from 1 to 4%, the majority of nonspecific bound hsp90 was washed off, whereas the receptor complex still remained bound to the immune complex. As shown in Fig. 1, hsp90 is present in the dimeric, trimeric, and tetrameric complexes and exhibited higher relative staining intensity in the trimeric and tetramer complexes when compared with the AhR species (Fig. 1, lanes 3 and 5). This would suggest that a considerable stoichiometric excess of hsp90 exists in the trimeric and tetrameric AhR complexes, which is in accordance with the assumption that hsp90 exists dimerically in the AhR complex. The hsp90 monomer band shown in Fig. 1, lane 5, can be explained by the noncross-linked hsp90 dissociated from the dimeric AhR complex during the solubilization in SDS sample buffer.

There are two separate gene products of hsp90 present in Hepa 1 cells, referred to as hsp84 and hsp86; they are present at different cellular concentrations and differ in how their expression is regulated (Refs. 15, 16, and references therein). Whether hsp86 and hsp84 differ in their ability to associate with other proteins or the ligand-binding subunit of the AhR complex is not known. One advantage of the cross-linking reaction is that it can provide information about the spatial relationship that exists between two subunits. To address the above question, immunoochemical staining of the immunopurified cross-linking products was performed by using antibody against each form of hsp90. Results revealed that the hsp84 and hsp86 can be detected in both dimer and trimer, and a higher intensity was shown on the blot probed with anti-hsp84 antibody, which corresponded to the higher concentration of hsp84 in Hepa 1 cells compared with the hsp86 (16). These results implied that either there is no preferential interaction of hsp84/hsp86 with AhR or both hsp90 subunits directly interact with AhR. However, determining whether only one or both hsp90 subunits directly interact with the AhR will require additional studies.

To further demonstrate that the hsp90 exists as dimer in the AhR complex, we used a quantitative radioimmunochemical visualization method to determine the stoichiometry of hsp90 and AhR in each cross-linked complex. Hepa 1 cytosol extract was cross-linked for 30 min to obtain a high ratio of dimeric and trimeric AhR complexes. The immunoadsorbed AhR complexes were subjected to SDS-PAGE followed by transfer to PVDF membrane and incubated with antibody against AhR or hsp84/hsp86. The primary antibodies were visualized by incubation with 125I-counterantibody. Autoradiography of the resulting blot is shown in Fig. 2. The immunoreactive bands were cut out and counted for radioactivity. Immunospecific AhR and hsp90 cpm were calculated by subtracting the value obtained with nonimmune mouse IgG from the cpm obtained with anti-AhR or a mixture of hsp84/hsp86 antibody. The stoichiometry was measured by dividing the ratio of trimer to dimer from hsp90 visualization into the ratio of trimer to dimer from AhR visualization. In this experiment, the stoichiometry was determined to be 2.4 mol of hsp90/mol of AhR. In the method used for estimating stoichiometry in Fig. 2, we assumed that all epitopes of the subunits in the immunoblot are accessible to the antibody, which may not be true when such a large cross-linked complex is blotted onto the membrane.

Immunoprecipitation of the [35S]methionine labeled, non-cross-linked AhR complex not only gives the information of subunit composition but also provides a direct way to measure the relative amount of hsp90 and AhR in the complex (19). Hepa 1 cells were cultured in medium containing [35S]methi-
AhR complex has been shown to be highly specific toward AhR, as determined from standard molecular mass markers their molecular weight band was identified as the AhR, and the doublet corresponding to hsp86 and hsp84. The more intensely stained hsp90 bands in the control lane (Fig. 3, panel B, lane 1) illustrates the ability of hsp90 to nonspecifically bind to the gel matrix. The low intensity shown in the fourth subunit may indicate that this protein has a low methionine content or is partially lost during the immunoadsorption and washing procedures, although it should be pointed out that the p43 band was consistently seen under a variety of immunoprecipitation conditions.

To determine the relative amount of [35S]methionine present in the AhR and hsp90, a phosphor image of the blot was analyzed using an image analyzer. Stoichiometry was calculated as described in method 2 (see "Experimental Procedures"). The mean stoichiometry from duplicate samples of three separate experiments was 1.72 mol of hsp90/mol of AhR. Thus, using the two methods employed in this study, 2.4 and 1.7 mol of hsp90/mol of AhR were obtained, supporting the hypothesis that a dimer of hsp90 is bound to the AhR.

Three possible proteins were possible candidates for the unidentified subunit (43-kDa protein) in the AhR complex: a p56-59 (FKBP), p50, and a 40-kDa cyclophilin. The immunophilin p56-59 has been shown to be transiently associated with the untransformed steroid receptor (21, 22). p50 was found to be associated with hsp90-p60cow and Hsp90-Raf complexes (23, 24). Moreover, both p56-59 and p50 were detected in receptor-free hsp90 complexes (25). However, antibody against p56-59 (EC-1 antigen) failed to shift the cytosolic untransformed AhR complex in sucrose gradients (26). The 40-kDa protein appeared to be a reasonable candidate since it has a mobility on SDS-PAGE similar to that of p43 and was found to be a component of the unactivated estrogen receptor, designated estrogen receptor-binding cyclophilin. This cyclophilin is a member of a distinct group of immunophilins with a specificity for cyclosporin A (27). In a series of experiments antibodies against these three proteins (JP-1, mAb 1B5p50 (21), and anti-cyclophilin 40) were used to detect p43 in AhR-specific immunoprecipitates of the cross-linked or [35S]methionine-labeled cytosolic preparations. None of the antibodies was able to detect the 43-kDa protein. In addition, immunoochemical staining with these antibodies localized a protein of higher Mr, in total Hepa 1 cytosolic protein than p43 found in the AhR complex. These results would suggest that p56-59, p50, or p40 are not associated with the AhR complex and that the p43 subunit may be a unique protein.

In our previous cross-linking study, two other heteromeric intermediates of the cross-linked AhR species were observed with molecular masses of 146 and 230 kDa (5). The composition of these two cross-linked intermediates may be 1) a 97-kDa AhR and a 49-kDa protein; or 2) a 97-kDa AhR plus an 88-kDa protein and a 45 kDa protein, respectively. These two species were also observed in the immunoprecipitates of the cross-linked Hepa 1 cytosol as very faint bands shown in Fig. 1, lanes 1 and 3. Apparently the 88-kDa protein is a monomer of hsp90, and the 45 kDa protein is the fourth unidentified subunit. This finding suggests that the fourth subunit may be loosely bound to the AhR. This differs from the association of p56-59 with steroid receptors since this protein appears to interact with hsp90 and is not directly associated with the receptors, as determined from cross-linking experiments (27, 28). In addition, the p43 bound to the AhR complex, unlike p56-59, is not associated with the cytosolic hsp90-heteromeric complexes previously observed in Hepa 1 cytosol (25). The 185-kDa dimer resulting from the cross-linking reaction provides direct evidence that at least one hsp90 directly interacts with the AhR. However, determining the precise orientation of the second hsp90 and p43 in the AhR complex will require more experimentation. The AhR shares many similar biochemical properties with the

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4 Hankinson and co-workers (20) developed mutant clones of the wild type Hepa 1c1c77 parent cell line, which exhibited varying degrees of deficiency in aryl hydrocarbon hydroxylase inducibility. We have utilized the c7 mutant cell line that contains extremely low concentrations of the AhR relative to Hepa 1 cells.
glucocorticoid receptor: molecular size, surface charge, sedimentation coefficient, and the presence of hsp90 in the untransformed complex. The untransformed murine AhR complex is a highly stable complex, with or without ligand binding, when compared to the glucocorticoid receptor complex (29). The mechanism for this high degree of stability is not clear, but it is possible that p43 may play a role in the stabilization of the 9 S AhR complex.

This report has established the presence of four subunits in each AhR complex. In the AhR immunoprecipitation studies from [S35 methionine-labeled Hepa 1 cytosol, results indicated that the AhR complex was composed of three individual protein species. We propose that the cytosolic AhR complex contains one ligand binding protein, two hsp90 proteins, and a p43 subunit. Future studies will be directed at the purification and characterization of the p43 protein.

Acknowledgments—We thank Marcia H. Perdew and Dr. Shoo S. Singh for critically reading this manuscript.

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