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Purification and Characterization of an Ah Receptor Binding Factor in Chromatin

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ABSTRACT. Dioxin induces biological responses through interaction with a specific intracellular receptor, the Ah receptor, and the subsequent interaction of the Ah receptor with chromatin. We previously reported the binding of the Ah receptor, partially purified from rabbit liver, to receptor binding factors (termed AhRBFs) in chromatin. Rabbit liver chromatin proteins (CP) were isolated by adsorption of chromatin to hydroxylapatite followed by sequential extraction with 3 M NaCl and 1–8 M guanidine hydrochloride (GdnHCl). In the present study, we continued the purification of the CP5 fraction, which exhibited AhRBF activity. The proteins in CP5 were separated by CL-Sepharose 6B column chromatography resolving lower molecular weight fractions. To assay for receptor binding, a portion of each CL-Sepharose 6B fraction was reconstituted to rabbit double-stranded DNA (dsDNA) using a reverse gradient dialysis of 7.5 to 0.0 M GdnHCl. These reconstituted chromatins were then examined for binding to [^{3}H]-2,3,7,8-tetrachlorodibenzo-*p*-dioxin ([^{3}H]TCDD)-receptor complexes by the streptomycin filter binding assay. Two protein fractions with a molecular weight in the range of 10,000–14,000 demonstrated high affinity binding to the Ah receptor. The binding of AhRBFs reconstituted to dsDNA was shown, by competition experiments with Ah receptor bound by unlabeled TCDD (TCDD-R), to be >90% specific for [^{3}H]TCDD-R. Further purification was achieved by preparative SDS–PAGE, and AhRBF activity was attributed to two fractions with molecular weights between 12,000 and 10,000. A 12 kDa protein with AhRBF activity was found to have an isoelectric point (pI) of ≥10. The 12 kDa AhRBF was sequenced by Edman degradation after cyanogen bromide cleavage and identified as histone H4. Although histone H4 has been postulated to interact with transcription factors in a variety of systems, this is the first report of a specific interaction of AhR with histone H4. BIOCHEM PHARMACOL 51;4:437–445, 1996.

KEY WORDS. Ah receptor; dioxin; chromatin; histones; chromosomal proteins; nucleosomal proteins

There has been a great deal of interest in elucidating the mechanism of action of the well studied environmental contaminant TCDD† and related compounds. It is indeed surprising that these compounds, TCDD in particular, are capable of inducing such a myriad of biological responses. Responses to TCDD range from a generalized "wasting syndrome," indicative of overt toxicity, to quite specific effects such as the induction of drug-metabolizing enzymes [1]. Enzyme induction, particularly the P4501A1 monooxygenase system, is by far the most well studied of the actions of TCDD. The effects of TCDD are believed to be mediated by a soluble intracellular protein known as the AhR. The AhR is a basic region helix-loop-helix transcription factor [2–4] unique among this class of transcriptional activators in that it is the only one known to be ligand activated.

The events downstream of ligand binding to the Ah receptor are still incompletely resolved but most likely include dissociation of hsp 90, nuclear translocation, heterodimerization with ARNT, and sequence specific binding to DNA [5–9]. Certain key events in the mechanism of action of the Ah receptor following ligand binding occur in the nuclear compartment where the interaction of the AhR:ARNT heterodimeric complex with chromatin occurs. Pollenz *et al.* [10] have proposed that Ah and ARNT reside in the cytoplasm and nucleus, respectively, and that only after ligand binding does the AhR translocate to the nucleus to dimerize with ARNT. Once inside the nucleus, the receptor complex must access DNA and bind specific sequences known as DREs near inducible genes. Evidence exists which suggests that the AhR:ARNT receptor complex is capable of disrupting the nucleosomal structure [11]. As a result of these studies, it was proposed that binding of the AhR:ARNT complex to DREs within the enhancer mediates an alteration of local chromatin structure, thereby allowing increased promoter accessibility and transcriptional activation [12]. Our laboratory has suggested that certain chromosomal proteins are components of the nuclear binding sites for the Ah receptor [13]. These pro-

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† *Abbreviations:* TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; TCDD-R, TCDD-receptor complex(es); TCDF, 2,3,7,8-tetrachlorodibenzofuran; AhR, aryl hydrocarbon receptor; ARNT, Ah receptor nuclear translocator; DRE, dioxin responsive element; AhRBF, Ah receptor binding factor; GdnHCl, guanidine hydrochloride; PMSF, phenylmethylsulfonyl fluoride; dsDNA, double-stranded DNA; and CP, chromatin protein.

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teins have been termed AhRBFs. We report here the isolation and characterization of one AhRBF from rabbit liver chromatin with a molecular weight of ~12,000, which exhibited specific binding to the AhR partially purified from rabbit liver.

MATERIALS AND METHODS

Chemicals and Biochemicals

TCDD, [^{3}H]TCDD (25 Ci/mmol), and TCDF were obtained from Dr. S. Safe (Texas A&M University). Ultrapure GdnHCl was obtained from Amresco (Solon, OH). Hydroxylapatite was Bio-Gel HTP from Bio-Rad (Richmond, CA). DEAE cellulose was obtained from Whatman (Maidstone, England). Buffer A = 10 mM Tris, 1 mM EDTA, 10 mM monothioglycerol, 5% glycerol (v/v), and 5 mM PMSF, pH 7.5. Buffer B = 7 M GdnHCl, 5 mM potassium phosphate, pH 6.0. Buffer C = 7.5 M GdnHCl, 10 mM EDTA, 50 mM sodium acetate, 2 mM PMSF, and 1 mM β-mercaptoethanol, pH 6.0. Buffer D = 1 mM EDTA, 5 mM sodium acetate, 1 mM PMSF, and 1 mM β-mercaptoethanol, pH 6.0. Buffer E = 0.02% streptomycin sulfate in 2 mM Tris/0.1 mM EDTA, pH 7.5.

Preparation and Partial Purification of Ah Receptor

Ah receptor was obtained from male New Zealand white rabbits. Briefly, rabbits were killed, and the liver was perfused *in situ* with 500 mL buffer A at 4° via cannulation of the hepatic portal vein. The tissue was then homogenized in buffer A (1 mL/g tissue) using a Kontes No. 24 glass/glass homogenizer. The homogenate was then centrifuged at 200,000 *g* for 30 min (Type 70.1 Ti rotor, Beckman) to obtain a cytosolic fraction. All steps were performed at 4°. Ligands were dissolved in DMSO and added to cytosol preparations (≤10 μL/mL cytosol) and allowed to incubate for 2 hr at 20°. Partial purification of Ah receptor complexes was carried out by KCl step-elution from DEAE-cellulose, and specific binding was monitored by sucrose density gradient analysis as previously described [13].

Preparation of DNA and Chromatin Proteins

The isolation of chromatin from rabbit liver was as previously described [13] except that the CP fractions were obtained from 950 mg chromatin prepared from 6.1 kg rabbit liver. Rabbit spleen DNA was prepared as previously described [13].

Further purification of the chromosomal protein fraction extracted with 5 M GdnHCl (CP5) was achieved by molecular sieve chromatography. Approximately 180 mg of the CP5 fraction was dissolved in buffer B and allowed to hydrate overnight. The dissolved protein was then separated by a 200 mL (2.5 × 33 cm) CL-Sepharose 6B column previously equilibrated in buffer B. Fractions (5 mL) were collected, and the protein concentration was assayed in each fraction by a modification of the Bradford assay [14]. AhRBF activity was determined with the streptomycin filter binding assay (see below). Protein profiles were monitored by SDS–PAGE [15] using 16% acrylamide mini-gels (1.0 mm × 7 cm × 10 wells) (Novex, San Diego, CA). The gels were silver stained (ICN Radiochemicals, Irvine, CA) for visualization of banding patterns.

To further purify Ah receptor binding factors, aliquots of the fractions from the CL-Sepharose 6B column that gave specific AhRBF activity for the [^{3}H]TCDD-R were subjected to preparative SDS–PAGE (15% acrylamide for the separating gel, 1.5 mm × 20 cm, 15 well capacity, according to the method of Laemmli [15]). The proteins were electroblotted onto 2 sheets of Immobilon-P as described by Matsudaira [16]. The second Immobilon sheet was stained with colloidal gold (Bio-Rad) to visualize the bands and used as a template for cutting specific bands from the first sheet. The first sheet was cut into sections corresponding to the protein bands on the second sheet, and each section was eluted with 1–2 mL of 70% isopropanol, 5% trifluoroacetic acid, 25% water for 2 hr. The samples were then dialyzed in bags made from Spectra/Por No. 7 dialysis tubing (Spectrum, Houston, TX, molecular weight cutoff 6000–8000) in 100 vol. distilled water for 24 hr with three changes. The dialysis was then continued in fresh distilled water containing 5% acetonitrile for 2 hr. The proteins were freeze-dried and then solubilized in 1.0 mL buffer B. AhRBF activity was determined by the streptomycin filter binding assay.

Cell Free Receptor Binding Assay

Briefly, isolated chromosomal protein fractions in buffer C were reconstituted to rabbit dsDNA at various ratios of protein:DNA using reverse gradient dialysis in reconstitution buffer D, reducing the GdnHCl concentration from 7.5 to 0 M over a 24-hr time period to produce a reconstituted chromatin preparation. This preparation was centrifuged for 20 hr at 150,000 *g*, and the pellets were resuspended in 1.2 mL of 10 mM KCl in a 2 mM Tris/0.1 mM EDTA (pH 7.5) buffer. To test for AhRBF activity, DEAE partially purified [^{3}H]TCDD-R (0.5 pmol) were added to approximately 70 μg chromatin-DNA in a final assay volume of 500 μL (150 mM KCl). After incubation on ice for 1 hr with frequent vortexing, 100 μL of 1.2% streptomycin sulfate was added to precipitate the receptor–chromatin complexes, and incubation was continued for 30 min. The assay tubes were centrifuged at 800 *g* for 10 min, and the supernatant was aspirated. The pellets were resuspended and washed two times with 1 mL buffer E, then suspended in 1 mL buffer E and poured over Millipore (HAWP) filters under vacuum. The filters were washed three times with 1 mL buffer E, air dried for 15 min, and then dried under a Fisher IR lamp for 15 min. The radioactivity on the filters was counted in 10 mL scintillation fluid (0.4% Omnifluor/xylene), the filters were removed and dried, and the DNA content was determined [17]. Binding to DNA alone was subtracted from all values.

Specificity Experiments

Cytosol was incubated with 40 nM [^{3}H]TCDD or 40 nM TCDD and subjected to DEAE-cellulose chromatography to partially purify the Ah receptor as above. Also prepared was

cytosol that had been heated to 34° for 75 min and partially purified to obtain a denatured receptor (dR) preparation. The denatured receptor preparation was examined for binding by [^{3}H]TCDD, and no binding was measurable. Binding of [^{3}H]TCDD-R to CP5/CLS/14-15 reconstituted to dsDNA was performed in the presence of [^{3}H]TCDD-R plus 2-fold excess denatured receptor (to keep protein concentration constant) for total binding, or in the presence of [^{3}H]TCDD-R plus 2-fold excess TCDD-R for nonspecific binding. The TCDD-R preparation was exactly as described for the [^{3}H]TCDD-R, except that a portion of the same cytosol preparation was incubated with 40 nM unlabeled TCDD. The same fraction of DEAE-purified receptor was used for both preparations. Such a protocol should result in the same concentration of ligand-bound Ah receptors in the binding assay. Experiments were also performed to measure binding to DNA alone.

Determination of the Isoelectric Point (pI) of Ah Receptor Binding Factors

An aliquot (5.5 mg) of the CL-Sepharose 6B fractions with AhRBF activity was dissolved in deionized 6 M urea and 2.5% Bio-Lyte (Bio-Rad) carrier ampholytes (pH range 3–10) for a total volume of 55 mL and loaded onto the isoelectric focusing cell (Bio-Rad) and focused for 4 hr at 12 W power and 4°. After focusing was complete, the pH of each fraction was determined. The fractions were then dialyzed against 100 vol. of 1 M NaCl at 4° for 1 hr and then against fresh 1 M NaCl at 4° overnight for removal of ampholytes. Dialysis was continued for 48 hr with changes to fresh 1 M NaCl every 12 hr to ensure complete removal of ampholytes. Dialysis was then continued against deionized water with three changes over 48 hr followed by dialysis against 5% acetonitrile in deionized water for 2 hr. The fractions were then lyophilized, solubilized in SDS solubilization buffer, and separated on SDS–PAGE (0.75 mm × 20 cm, 15-well capacity). Proteins were stained with Coomassie brilliant blue or transferred to Immobilon-P and stained with colloidal gold.

Protein Sequencing Analysis

CP5/CLS/14-15 was solubilized in SDS-solubilization buffer and separated on tricine SDS–PAGE (30 μg/lane × 6 lanes) (1.5 mm × 20 cm × 15 wells, 0.1 M tricine in cathode buffer). The proteins were transferred to a Problott membrane (Applied Biosystems, Foster City, CA) and stained with Coomassie brilliant blue. AhRBFs were subjected to protein sequencing analysis directly from the membrane by the Edman degradation reaction using an Applied Biosystems model 477 protein sequencer [18, 19]. Proteins that were blocked at the N-terminus were subjected to cleavage with cyanogen bromide prior to Edman degradation.

Immunoblot Analysis of AhRBF(s)

CL-Sepharose 6B fractions that contained AhRBFs were separated on preparative SDS–PAGE and electroblotted onto Immobilon-P. The membrane was rinsed three times in Tris-buffered saline (TBS = 20 mM Tris, 100 mM NaCl, pH 7.5) for 10 min. After the third rinse, the membrane was incubated overnight in 50 mL of 4% milk in TBS at 4° to block nonspecific proteins. After blocking, the Immobilon sheets were rinsed in TBS and then placed in cellophane bags. Monoclonal mouse anti-phosphotyrosine antibody (Zymed Laboratories, Inc., South San Francisco, CA) (1 μg/μL) at a 1:2000 dilution in 10 mL TBS was added to each bag, which was then sealed and incubated at 37° for 2 hr. After incubation, the sheets were washed extensively in TTBS (TBS containing 0.3% Tween). The incubation with secondary antibody (anti-mouse IgG, HRP linked, 1:40 dilution) was for 1 hr at room temperature followed by sequential rinses in TTBS and TBS and incubation with ECL (Enhanced Chemiluminescence, Amersham) detection reagents. The sheets were then blotted and exposed to film (Hyperfilm-MP, Amersham). Specificity was determined by competition experiments with phosphotyrosine or mouse IgG as a nonspecific primary antibody.

RESULTS

Reconstitution of chromosomal proteins to DNA aids in the renaturation of these chromosomal proteins in low salt buffers and allows for radiolabeled receptor binding assays using partially purified dilute receptor preparations. In a previous study [13] using chromatin prepared from sufficient quantities of rabbit liver to allow for initial detection and characterization of AhRBF activity, we reported that chromosomal protein fractions CP2, CP5, and CP7 demonstrated the greatest AhRBF activity for Ah receptor bound by [^{3}H]TCDD when these protein fractions were reconstituted to rabbit dsDNA. Since the CP5 fraction contained AhRBF activity and sufficient quantities of protein necessary to carry out further purification, we focused our present studies on this particular fraction. We demonstrated in a previous study [13] that the partially purified AhR was in the 6S form in 0.15 M KCl, the salt concentration used in the binding assay. The DNA binding form of the receptor *in vivo* has been reported to be 6S [20–22]. It has also been shown that nuclear salt-extractable Ah receptors from a variety of species sediment at 6S [23, 24]. Additionally, we confirmed that our cytosolic AhR was in the DNA binding form by gel mobility shift analysis with a ^{32}P-labeled synthetic DRE [25] (not shown).

CL-Sepharose 6B Separation of AhRBFs

We continued the purification of AhRBFs in the CP5 fraction utilizing molecular sieve chromatography. The protein profile of the separation of the proteins in CP5 through a 200-mL CL-Sepharose 6B column is shown in Fig. 1A. AhRBF activity in aliquots of each fraction was determined with the streptomycin filter binding assay, and the results from fractions 12–17 are shown in Fig. 1B. Fractions 14 and 15 demonstrated AhRBF activity for the Ah receptor bound by [^{3}H]TCDD. No significant AhRBF activity was detected in the other fractions from the CL-Sepharose 6B separation. Aliquots of each frac-

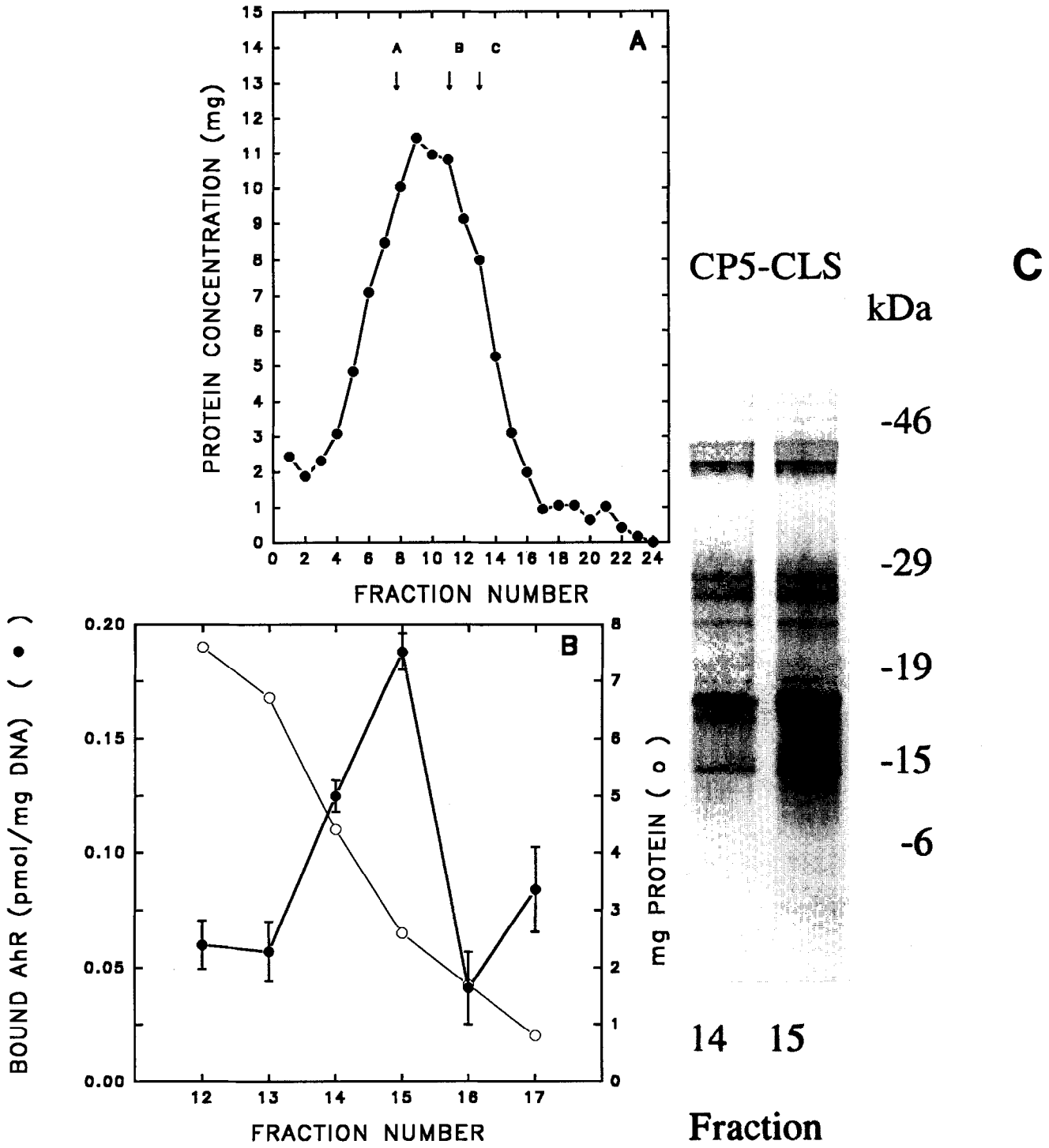

FIG. 1. Molecular sieve chromatography of CP5. (A) Protein profile of the separation of rabbit liver CP5 on a 200-mL CL-Sepharose 6B column. CP5 protein (180 mg) was dissolved in 10 mL buffer B and then loaded on the column; 5-mL fractions were collected. Protein was assayed in each fraction by a modification of the method of Bradford [14]. The column was calibrated with the following molecular weight standards: A = BSA (68 kDa), B = chymotrypsinogen (25 kDa), C = cytochrome c (12.4 kDa). (B) Measurement of the binding of AhR complexes to CL-Sepharose 6B fractions 12–17 reconstituted to dsDNA (●). Data are expressed as means ± SEM for 4 determinations. Samples of the CL-Sepharose 6B fractions 12–17 were reconstituted to dsDNA by reverse gradient dialysis to generate reconstituted chromatins. Each preparation was probed with [^{3}H]TCDD-R to assay for Ah receptor binding factor activity using the streptomycin filter assay. Binding to DNA alone was subtracted from these values. Also shown (○) is the protein profile of the separation of CP5 on the CL-Sepharose 6B column fractions 12–17. Protein was assayed using a modification of the Bradford assay [14]. (C) SDS–PAGE profile of CP5 fractions 14 and 15 eluted from 200-mL CL-Sepharose 6B column. A portion of the proteins in CL-Sepharose 6B fractions 14 and 15 was dialyzed, lyophilized, solubilized in sample buffer equivalent to 1 μg protein/μL sample buffer, and run on a modified Laemmli 16% acrylamide mini-gel system. The proteins were transferred to Immobilon-P [15] and stained with colloidal gold for analysis of banding patterns.

tion were dialyzed to remove the GdnHCl, solubilized, and separated on SDS–PAGE. SDS–PAGE minigel analysis of aliquots from fractions 14 and 15 used to monitor the purification steps is shown in Fig. 1C.

Specificity of Ah Receptor Binding to Receptor Binding Factors in Chromatin

For the continued purification and characterization of the AhRBFs, fractions 14 and 15 from the CL-Sepharose 6B separation were combined and designated as CP5/CLS/14-15. To investigate the specificity of Ah receptor binding to AhRBFs, competition experiments were performed. [^{3}H]TCDD-R in the presence of 2-fold excess heat-denatured Ah receptor preparation, to keep the protein concentration constant, were incubated with CP5/CLS/14-15 reconstituted to dsDNA. Simultaneous experiments were also performed using [^{3}H]TCDD-R plus a 2-fold excess of unlabeled TCDD-R. Under these conditions, the binding of [^{3}H]TCDD-R to AhRBFs was decreased by nearly 67%, which is the theoretical maximal reduction expected given a 2-fold excess addition of unlabeled receptor complexes if the binding were 100% specific (Fig. 2). Therefore, specific binding was >90%. Substantially lower binding was observed using DNA alone, and no competition was observed in the binding of [^{3}H]TCDD-R to DNA alone (data not shown).

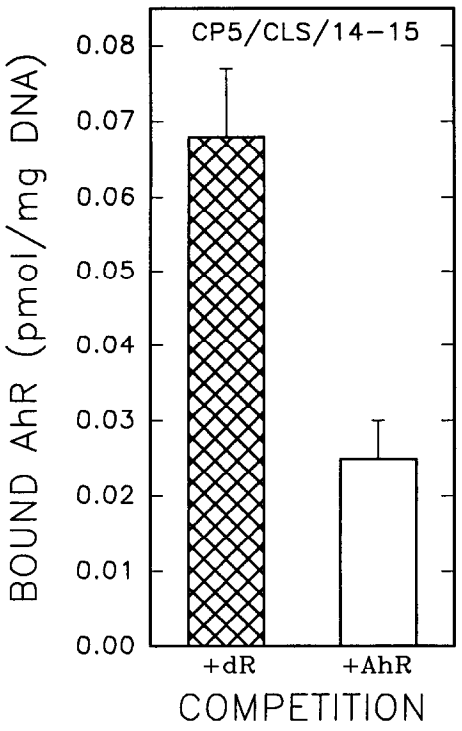

FIG. 2. Competitive binding of AhR complexes to reconstituted chromatin from CP5/CLS/14-15. Competition experiments were performed in which partially purified [^{3}H]TCDD-R were used to determine binding to this reconstituted chromatin preparation in the absence and in the presence of 2-fold excess unlabeled TCDD-R. Protein content was kept constant with heat-denatured receptor complexes (dR). Binding to DNA alone was subtracted from each of the values. Data are expressed as means ± SEM of 4–5 determinations.

SDS–PAGE Purification of AhRBFs

A portion (1.6 mg) of the combined CP5/CLS/14-15 was solubilized and separated on preparative SDS–PAGE and transferred to Immobilon-P. The Immobilon sheet was cut into sections (some sections contained individual protein bands), and the proteins were extracted and reconstituted to rabbit dsDNA. The reconstituted chromatin was then screened for AhRBF activity using the streptomycin filter binding assay. An Immobilon strip corresponding to the strips used to elute the proteins was subjected to staining with colloidal gold and calibrated with molecular weight standards run in parallel (Fig. 3A). Binding by [^{3}H]TCDD-R was observed in fractions 8, 9, and 10 (Fig. 3B). The binding observed in fractions 9 and 10 corresponded to two bands of molecular weight 12,000 and 10,000, respectively. Parallel experiments were also performed with cytosol prepared with [^{3}H]TCDD + 100-fold excess TCDF. No specific binding was detected, indicating that [^{3}H]TCDD-R was specifically bound in fractions 8–10, i.e. there was no non-specific binding by free [^{3}H]TCDD or [^{3}H]TCDD bound to non-receptor proteins.

Isoelectric Focusing of AhRBFs

An aliquot of pooled CL-Sepharose fractions containing AhRBFs (5.5 mg) was solubilized in 6 M urea and Biolyte carrier ampholytes (pH range 3–10) and focused. At equilibrium, twenty fractions (~2.0 mL each) were collected, and the pH of each fraction was determined. Following extensive dialysis against 1 M NaCl to remove ampholytes, and dialysis against deionized water to remove salts, each fraction was lyophilized, solubilized, and subjected to SDS–PAGE analysis. Since each lane of the gel corresponds to a specific fraction from the isoelectric focusing, the isoelectric point (pI) of specific bands could be determined. The 12 kDa protein was only present in the pH range $\geq$10 (Fig. 4). The 10 kDa protein had a pI of approximately 6.0 (not shown).

Protein Sequencing Analysis

To determine the amino acid sequence of the AhRBF(s), protein sequence analysis was performed. An aliquot of the pooled CL-Sepharose 6B fractions containing AhRBF(s) was separated by SDS–PAGE and transferred to a Problott membrane. Protein bands were detected with Coomassie brilliant blue and excised. Sequencing was performed directly from the Problott membrane by Edman degradation. Several attempts to sequence the 12 kDa AhRBF (fraction 9, Fig. 3B) revealed that the N-terminus was blocked. The 12 kDa AhRBF was then subjected to cyanogen bromide treatment, which cleaves peptide bonds on the C-terminal side of methionine, followed by direct sequencing from the membrane. A peptide of 12 amino acids was identified. This sequence was analyzed by computer

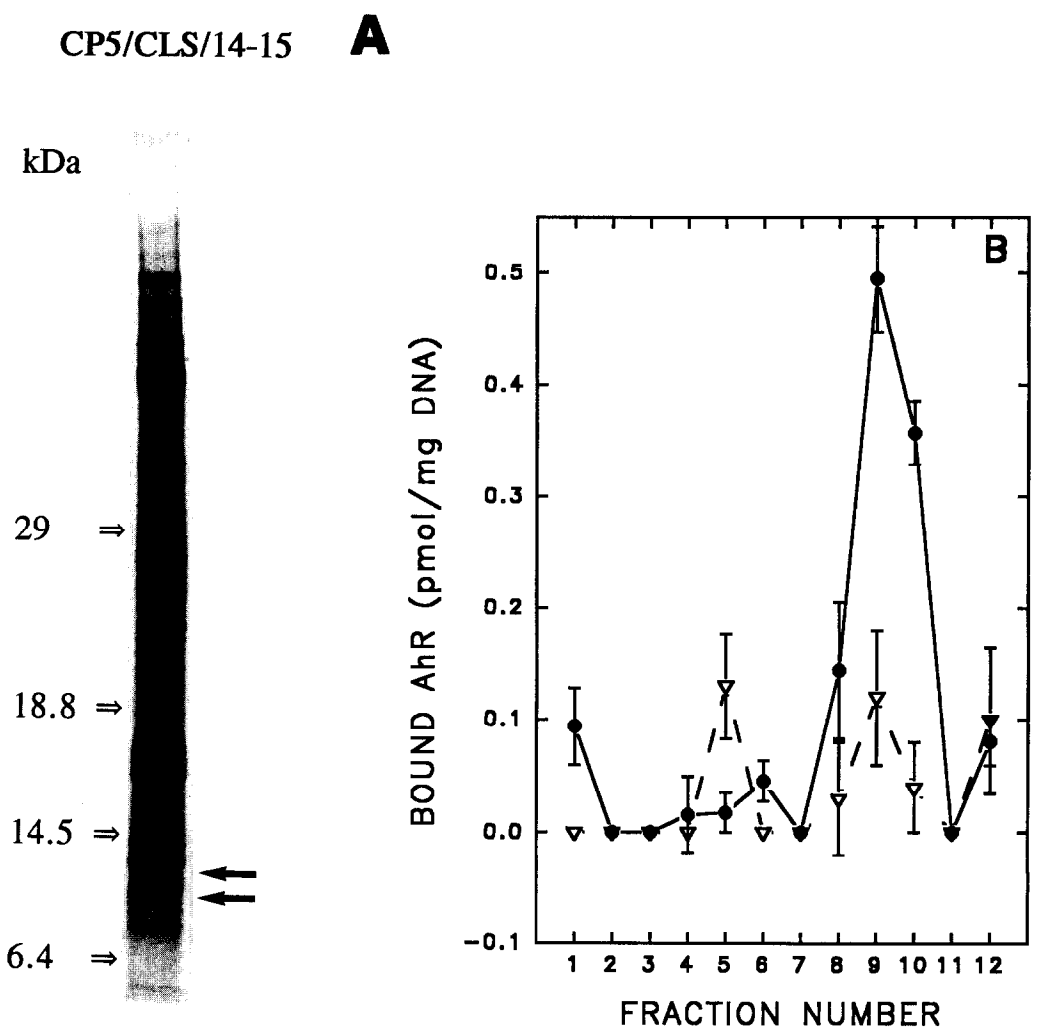

FIG. 3. Purification of AhRBFs with preparative SDS–PAGE. Fractions 14 and 15 from the CP5 fractionation on CL Sepharose were combined (CP5/CLS/14-15), dialyzed, lyophilized, solubilized in sample buffer, and run on a 15% acrylamide gel. The proteins were electro-transferred onto 2 sheets of Immobilon-P. (A) Colloidal gold staining of an Immobilon strip corresponding to the strips used to elute the proteins is shown. Solid arrows at right denote protein fractions 9 and 10, which contained AhRBF activity. (B) Multiple lanes were cut into strips, some containing individual protein bands as determined from a colloidal gold-stained second Immobilon sheet used as a template. The proteins were extracted with 70% isopropanol—5% trifluoroacetic acid and then dialyzed, lyophilized, and hydrated in buffer B. AhRBF activity of the protein fractions was determined after reconstitution to dsDNA (protein:DNA = 1:200). AhRBF activity was assayed with partially purified Ah receptor complexes bound by [^{3}H]TCDD in the absence (●) or presence (△) of 100-fold excess TCDF.

using the FASTA program [26]. A region of 100% homology was identified between the 12 kDa AhRBF and histone H4. The sequence analysis was repeated, and similar results were obtained. Histone H4 (mol. wt 11,300) contains a single methionine at residue number 84 [27–29]. The region of identity between the 12 kDa AhRBF and histone H4 lies in the region just C-terminal to the methionine at position 84. Sequence analysis of the 10 kDa protein band (fraction 10, Fig. 3B) revealed that this protein band contained two proteins, histone H4 and a cytoplasmic contaminant identified by sequence analysis as β-2 microglobulin. Thus, the AhRBF activity in fraction 10 (Fig. 3B) is also attributable to H4.

Immunoblot Analysis of AhRBF(s)

Since histone H4 contains multiple tyrosines [28], one of which is located at an apparent consensus site for tyrosine phosphorylation [30], experiments were performed to determine if the H4 AhRBF is a tyrosine phosphoprotein. An aliquot of CP5/CLS/14-15 was separated on preparative SDS–PAGE and transferred to Immobilon-P (see Fig. 3A for protein banding pattern). The membrane was then probed with a monoclonal anti-phosphotyrosine antibody. The 12 kDa AhRBF exhibited a positive reaction with the anti-phosphotyrosine antibody, suggesting that H4 is a phosphoprotein (Fig.

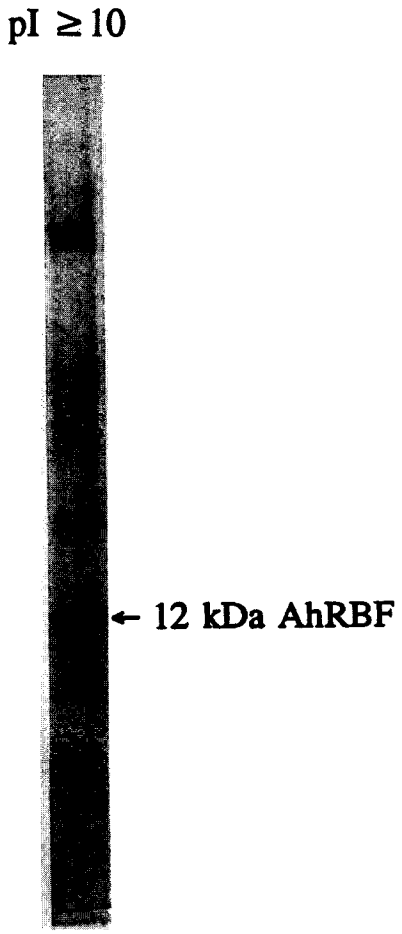

FIG. 4. Isoelectric focusing analysis of AhRBFs from rabbit liver chromatin. An aliquot (5.5 mg) of combined CL-Sepharose 6B fractions containing AhRBF activity was subjected to isoelectric focusing followed by SDS–PAGE of each focused fraction. Shown is a Coomassie stained lane containing the 12 kDa AhRBF.

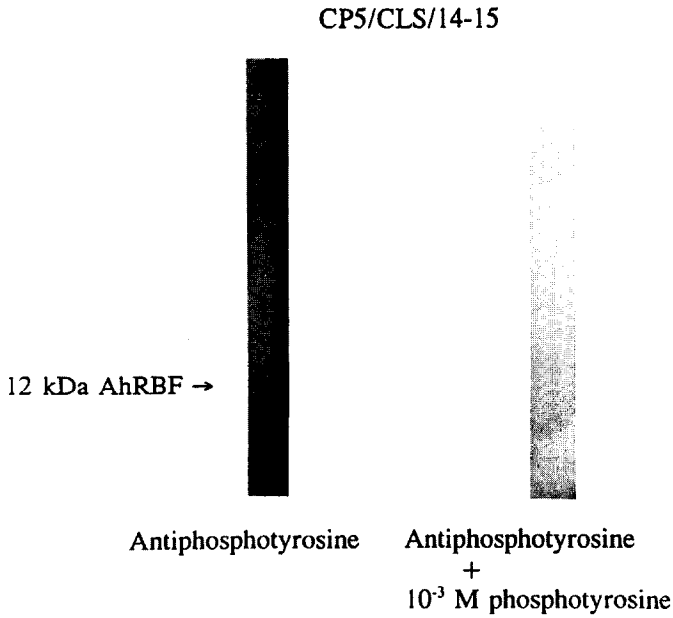

FIG. 5. Phosphotyrosine analysis of AhRBFs. An aliquot of CP5/CLS/14-15 was separated on SDS–PAGE and transferred to Immobilon-P and probed with anti-phosphotyrosine monoclonal antibody. Development was with ECL. The interaction of the antibody with the 12 kDa AhRBF is depicted (left). Competition with excess phosphotyrosine is also shown (right).

5, left). This protein was the only protein in this fraction identified as a tyrosine phosphoprotein. Competition experiments with excess phosphotyrosine were performed which determined that the interaction of the antibody with the 12 kDa AhRBF was indeed specific (Fig. 5, right). Additionally, immunoblot analysis was performed with mouse IgG, and no interaction with AhRBF was observed (not shown).

DISCUSSION

We have previously reported the binding of the AhR to chromosomal proteins, termed AhRBFs, which demonstrated saturability, specificity, and a requirement for intact transformed Ah receptor. Three chromosomal protein fractions, CP2, CP5, and CP7, obtained by extraction of chromatin with 2, 5, and 7 M GdnHCl, exhibited a high degree of AhRBF activity in a binding assay using [^{3}H]TCDD-R titrated over a 10-fold range. K_d values for the reconstituted chromatins were in the nanomolar range, and the sites per cell were ~2000 [13]. These hydrophobic proteins, tightly bound to DNA *in vivo*, conferred high affinity and specific binding of the [^{3}H]TCDD-bound Ah receptor to genomic DNA *in vitro* [13]. These AhRBFs may act as docking proteins in chromatin to which the AhR complex binds, thus directing, by DNA looping, interaction with a DRE. These chromosomal protein/AhR/ARNT interactions may also be important for making accessible the promotor region of induced genes by allowing alterations in chromatin structure, possibly through nucleosome disruption. In the present study, we purified and identified one AhRBF isolated from the rabbit liver chromatin CP5 fraction after sequential DNA affinity chromatography, molecular sieve chromatography, and preparative SDS–PAGE.

The methods used in our studies for reannealing protein to DNA have been used to reconstitute chromatin, which resembles native chromatin with respect to thermal denaturation, template capacity for DNA-dependent RNA synthesis, protein composition, and nucleosome formation [31, 32]. This approach has permitted us to investigate the interaction of chromosomal proteins tightly bound to DNA in chromatin with transformed AhR. The specificity of the interaction has been established by competition experiments in both partially purified [13] and purified fractions of chromatin protein.

The sequence of events that lead to Ah receptor mediated gene induction has been studied in detail and involves, at least, ligand binding [2], receptor transformation [33], translocation [34], heterodimerization [6], and sequence specific DNA binding [8, 9]. In terms of DNA binding, evidence exists that the AhR complex interaction with DNA is accompanied by changes in chromatin structure involving nucleosome disruption and increases in the accessibility of the promoter [11]. Our present study is the first to report a high affinity interac-

tion of the AhR complex with histone H4. Others have shown that treatment of cells with TCDD results in the rapid disruption of nucleosomal structure [12], and that this disruption is dependent on the presence of functional AhR:ARNT heterodimeric complexes [12]. Our demonstration of an interaction between the AhR complex and histone H4 suggests that AhR may facilitate the disruption of nucleosomal structure by altering the conformation of H4, which could lead to destabilization of the nucleosomal core structure. The finding that the 12 kDa AhRBF was histone H4 was intriguing albeit somewhat unexpected, since we performed not only a series of 3 M NaCl extractions intended to remove histones but several extractions with increasing molarities of GdnHCl. It has been reported that histones are relatively easy to extract in NaCl concentrations of 1 M and less [35]. This suggests that the 12 kDa AhRBF/histone H4 differs from histones that are typically extracted under much less stringent conditions. Thus, we believe that we have selected for a subset of histone H4 that was tightly bound to DNA and resistant to salt extraction. This particular histone H4 may be a variant form since variant forms of H4 have been demonstrated in certain systems [29]. Alternatively, it is possible that the H4 reported here is modified in some way as there are several modifications of H4 that have been reported [36].

Precedent does exist for the interaction of various transcription factors with both the nucleosome as a whole and individual histones. Both glucocorticoid and progesterone receptor can bind reconstituted mouse mammary tumor virus (MMTV) nucleosomes, and this binding increases the accessibility of the NF-1 binding site to exonuclease III [37]. Additionally, glucocorticoid receptor binding to glucocorticoid response elements (GREs) disrupts nucleosomes, allowing the binding of both NF-1 and TFIID to their recognition sites [38]. In a study of reconstituted nucleosomal cores, utilizing a 196-bp region of the MMTV upstream sequence and H1-depleted histones, it was shown that organization of the MMTV DNA into nucleosomes resulted in a 60- to 70-fold increase in the selective binding of the glucocorticoid receptor to a specific binding site [39]. Interaction between estrogen receptor and histones has also been shown [40].

Histone H4 has been implicated in both the activation and repression of gene expression in several systems [reviewed in Ref. 41]. In studies of the GAL1 (galactokinase) gene in yeast, which is inducible up to 1000-fold if yeast is treated with galactose, it was found that deletion of the N-terminal region of H4 results in significant reduction of GAL1 gene induction *in vivo* [42]. N-Terminal deletions of the other core histones did not lead to significant reductions in GAL1 induction. In another study, Workman and Kingston [43] demonstrated that destabilization of nucleosome cores is the direct result of binding of the regulatory factor GAL4.

It has been postulated that binding of the AhR:ARNT complex to DREs within the enhancer region of the CYP1A1 gene mediates an alteration of local chromatin structure [44]. The basis for this proposal arose from *in vivo* studies on the accessibility of the enhancer region of the CYP1A1 gene which revealed that prior to treatment of cells with TCDD the major grove was largely devoid of interacting proteins. However, upon treatment with TCDD, six DREs each bound a heterodimer of AhR:ARNT [11]. These results indicated that TCDD mediated an increased accessibility to enhancer regions of the CYP1A1 gene via AhR:ARNT independently of transcription. Additionally, it has been observed that the enhancer region of the uninduced CYP1A1 gene is in a nucleosomal configuration which necessitates that the AhR:ARNT complex interact with nucleosomes [11, 12]. Whitlock has proposed that the interaction of the AhR complex with nucleosomal proteins could lead to the functioning of or recruitment of histone acetylase [44], which could modify the properties of H4 (i.e. decreasing N-terminal charge density and consequently N-terminal DNA affinity), and subsequently mediate increases in enhancer accessibility. It is also possible that interaction of the AhR:ARNT complex with H4 might stabilize the binding of the receptor complex to dioxin-responsive elements leading to activation of TCDD-inducible gene expression. Future studies will determine the nature of the modification(s) of the 12 kDa AhRBF/histone H4, which provides for the high affinity interaction with the AhR.

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