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PARTIAL PURIFICATION AND CHARACTERIZATION OF A RAT LIVER POLYCHLORINATED BIPHENYL (PCB) BINDING PROTEIN

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Abstract—A protein capable of specifically binding polychlorinated biphenyls (PCB) was partially purified from rat liver cytosol. After labeling with [^{3}H]2,2′,4,4′,5,5′-hexachlorobiphenyl (6-CB), protein enrichment was guided by monitoring the protein-bound radioactivity through a sequence of purification steps including ion exchange chromatography and preparative gel electrophoresis. In addition, specific binding tests of individual fractions were carried out. An average 100-fold enrichment of the 40 kDa protein was achieved. A variety of ligands were tested in competitive binding studies with 6-CB. Whereas penta- and hexachloro-PCB congeners are high affinity competitors, the 3,3′,4,4′-tetrachlorobiphenyl congener does not compete for 6-CB binding. Studies on the species and tissue distribution suggest a prevalence of the binding protein in tissues of the rat. Since the natural physiological ligand of the protein has not yet been identified, the function of the protein can only be speculated on.

Research on the mammalian toxicology of polychlorinated biphenyls (PCB†) has focused on the role these environmental pollutants play in carcinogenesis and tumor promotion [1–3]. A thoroughly studied system in tumor promotion is the 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD)-triggered translocation of the dioxin receptor protein (the *Ah*-receptor) from the cell cytosol into the nucleus and subsequent interaction with DNA [4–6]. The structural and stereochemical similarity with TCDD made PCB congeners early candidate ligands for the *Ah*-receptor. Indeed, structure-dependent and high affinity binding of PCB to the *Ah*-receptor has been demonstrated [7, 8]. Whereas the biochemistry of the "TCDD receptor" is fairly well established, the linkage of cause and effect, i.e. of TCDD receptor binding and animal toxicity, remains obscure [6, 9]. This is all the more true for the effect of PCB *Ah*-receptor interaction and PCB toxicity. There is much room left for alternative mechanisms, primarily with respect to PCB congeners with steric ambitions such as 2,2′,4,4′,5,5′-hexachlorobiphenyl.

Recently, two papers addressed the biochemical mode of PCB action in liver cells from a different point of view. First, PCBs were shown to lead to an altered pattern of oncogene expression in rat liver [10]. More precisely, feeding a PCB-supplemented diet increased *raf* protooncogene expression at the transcription level [10]. Second, a binding protein for chlorinated biphenyls has been described in rat liver cytosol [11]. By labeling liver cytosol with the PCB congener [^{3}H]2,2′,4,4′,5,5′-hexachlorobiphenyl (6-CB) some binding properties of this protein were reported. It has been shown that 6-CB binding was saturable, of high affinity, specific and different from any other known PCB binding protein [11].

The present paper provides further data on this protein. A route of purification of the 40 kDa protein is described. Experiments with competing ligands determine the specificity of binding. The physiological function of this protein must further account for the observed species and tissue distribution of this PCB binding protein.

MATERIALS AND METHODS

Chemicals and materials. The synthesis of the [^{3}H]-labeled PCB congener, 2,2′4,4′5,5′-hexachlorobiphenyl (6-CB) has been reported [11]; the specific radioactivity of aliquots from stock used in the experiments averaged at 0.4 TBq/mmol = 11 Ci/mmol. Samples of 2,2′4,4′5,5′-hexachlorobiphenyl, 3,3′4,4′5-pentachlorobiphenyl, 3,3′4,4′-tetrachlorobiphenyl and TCCD were obtained from Promochem (Wesel). Biphenyl was obtained from Riedel-de-Haën (Seelze), and the reagents listed in Table 3 were from the Sigma Chemical Co. (Taufkirchen, F.R.G.).

Hydroxyapatite was obtained from Bio-Rad (München, F.R.G.). Phenyl-Sepharose CL-4B, DEAE-Sepharose and the reagents for polyacrylamide gel electrophoresis were from Pharmacia LKB (Freiburg, F.R.G.). All reagents were of analytical grade.

Biological tissues. Samples of human liver were obtained through the courtesy of Dr C. Bauer, Institut für Rechtsmedizin der Universität München; autopsy was within 10 hr post-mortem. Female adipose tissue was collected on the occasion of cosmetic surgery through Dr V. Mallinckrodt, Rot-Kreuz-Krankenhaus München. Skin keratinocytes were kindly provided by Dr G. Michel, Der-

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† Abbreviations: PCB, polychlorinated biphenyls; 6-CB, [^{3}H]2,2′,4,4′,5,5′-hexachlorobiphenyl; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; HIC, hydrophobic interaction chromatography; HAP, hydroxyapatite; PAGE, polyacrylamide gel electrophoresis.

matologisches Institut der Universität München. Other biological material was obtained from intramural GSF sources.

Preparation of rat liver cytosol. Liver cytosol from 6–8 week old Sprague–Dawley rats was prepared as described [11]. Briefly, the livers of the anesthesized animals were minced, rinsed and homogenized in KP-buffer (10 mM potassium phosphate, 5 mM $MgCl_2$, 1 mM EDTA, 1 mM dithiothreitol, 0.24 M sucrose; pH 7.5). The 105,000 *g* cytosol supernatant was prepared and stored at −80°. Protein was determined according to Bradford's method [12] with the "Bio-Rad" protein assay. All steps were carried out at 4°.

Ammonium sulfate fractionation. A buffered (50 mM KP-buffer) saturated solution of $(NH_4)_2SO_4$ was slowly added at 4° to stirred samples of liver cytosol (typically 900 mg of protein) to a final salt concentration of 48%. The solution was stirred for 45 min, centrifuged (10,000 *g*, 20 min), and the supernatant used directly for hydrophobic interaction chromatography (HIC).

HIC. A phenyl-Sepharose column (70-mL bed volume) was equilibrated with 4 M $(NH_4)_2SO_4$. The high salt supernatant was loaded onto the column and adsorption was completed with additional 10 mL of 0.6 M $(NH_4)_2SO_4$. Then, protein elution was started with 10 mM potassium phosphate buffer (pH 7.5; room temperature). Five fractions/hr were collected; the fraction volume was 9–10 mL. The protein-containing fractions of the eluate were concentrated to about 6 mL for further purification. The sample was then incubated (4°, overnight) with 400,000 cpm of 6-CB (≈ 10 nM) to radiolabel the 6-CB binding protein.

Hydroxyapatite (HAP) chromatography. The labeled protein solution was applied onto a HAP column (57-mL bed volume, equilibrated with 10 mM of KP-buffer) for ion exchange chromatography. Proteins were eluted by means of a linear potassium phosphate gradient (10–600 mM potassium phosphate). Six fractions/hr were collected; the fraction volume was 6–7 mL. Fractions were analysed for protein and ^3H-radioactivity; development of the gradient was monitored by conductivity measurement. Protein fractions containing bound radioactivity were eluted by 40–50 mM KP-buffer. These fractions were pooled, concentrated by ultrafiltration (Amicon PM10 filter), and dialysed against 10 mM KP-buffer.

DEAE chromatography. Further purification was by DEAE anion exchange chromatography. The samples were loaded onto a DEAE-Sepharose column (5-mL bed volume, equilibrated with 10 mM KP-buffer). After a column wash with 10 mL of 15 mM KP-buffer (containing 10 mM of NaCl), proteins were eluted with a linear NaCl gradient (10–500 mM NaCl in 15 mM KP-buffer). Three fractions/hr were collected; the fraction volume was 3 mL. The PCB-binding protein was eluted by 90–110 mM NaCl.

Non-denaturing gel electrophoresis. The procedure of protein purification by polyacrylamide gel electrophoresis (PAGE) under non-denaturing conditions followed essentially the standard conditions described [11, 13] with some minor modifications: electrophoresis was in 3-mm thick slab gels of 9% polyacrylamide gels lacking SDS. For analysis of migration, protein was visualized by fixing and Coomassie Blue staining of a reference lane. In the preparative mode, adjacent lanes were cut into 5-mm slices. The gel matrix was broken by squeezing the gel pieces through a standard hypodermic needle into a test tube. Protein was eluted by shaking these broken gel slices at 4° overnight in a small volume of buffer (approx. 2 mL). Recovery of individual proteins was approx. 35% (as judged from recovery of bovine serum albumin); radioactivity was determined in aliquots.

HPLC. The binding protein was further enriched and electrophoretic trace contaminants were removed by HPLC on a Beckman HPLC unit, equipped with a Zorbax® GF-250 gel filtration column. The eluent was 0.2 M potassium phosphate buffer (pH 7.5) at a flow rate of 0.7 mL/min; chromatography was at 4°. Protein elution was monitored photometrically at 280 nm.

SDS–PAGE. Electrophoresis followed the method of Laemmli [13] with some modifications: 1.5-mm thick slab gels with 4% acrylamide in the stacking gel, 10% acrylamide in the resolving gel and 0.1% of SDS; up to 100 μg of protein was applied to each slot. Following electrophoresis at 8° to a length of 8–10 cm, the gels were fixed and stained with Coomassie Brilliant Blue. For quick analysis, mini gels (Biometra, Göttingen, F.R.G.; 10 μg of protein/well of 1-mm thick gels) were used under similar conditions.

Binding analysis. Routine binding analysis was as described [11], using a miniature technique of the above HAP protocol. Briefly, 40,000 cpm of 6-CB (6–10 nM) and the non-radioactive candidate competitor (10^{-9}–5×10^{-5} M) were incubated at 4° overnight in a solution of 0.6 mg of protein in 0.25 mL of KP-buffer. After incubation, 50 μL samples were withdrawn to estimate the actual concentration of 6-CB. The remaining 0.2 mL were applied onto small HAP columns (0.25-mL bed volume), previously equilibrated with 10 mM of KP-buffer. The columns were washed with 2 × 0.4 mL of 15 mM KP-buffer, binding activity was recovered by a one-step elution with 0.8 mL of 70 mM KP-buffer.

RESULTS

Purification sequence

The 6-CB binding protein was purified from rat liver cytosol. Initially, an excess of non-binding protein was removed by salt precipitation at 48% ammonium sulfate saturation. The high-salt supernatant was then passed over a 75-mL Phenyl-Sepharose CL-4B column under conditions of hydrophobic interaction. Elution with low salt buffer (10 mM potassium phosphate) mobilized a single large protein fraction (Fig. 1A) that contained all specific 6-CB binding activity. This fraction was collected and concentrated by ultrafiltration.

At this stage, the binding protein was labeled with the radioactive ligand 6-CB (cf. Materials and Methods). Previous experiments had demonstrated that a protein fraction containing specific 6-CB

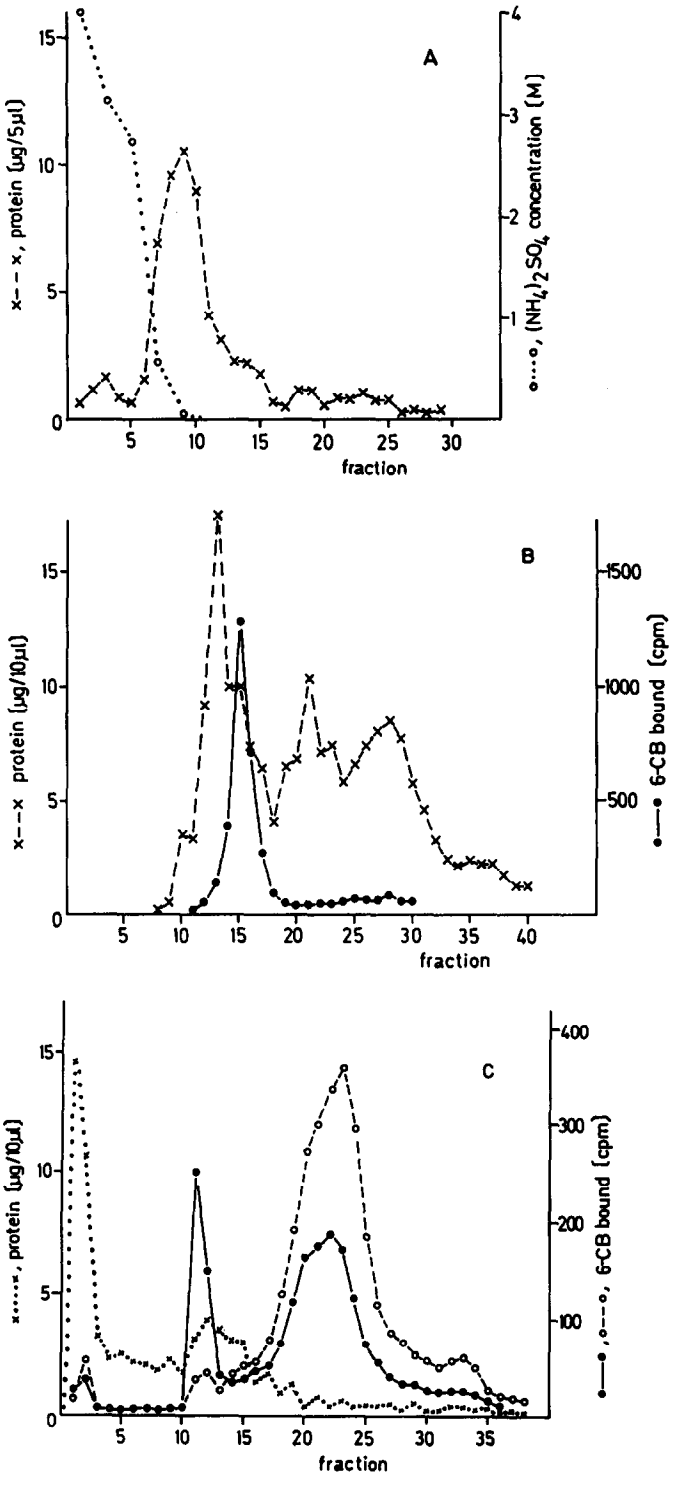

Fig. 1. Enrichment of the 6-CB binding protein by column chromatography. Liver cytosol (900 mg of protein) was pretreated with ammonium sulfate to 48% salt saturation. The supernatant was applied onto a Phenyl-Sepharose CL-4B column for HIC (panel A). The protein eluate (×——×, 225 mg of protein in fractions 8–13) was concentrated by ultrafiltration. After labeling with 400,000 cpm of 6-CB, the solution was applied onto a HAP column for ion exchange chromatography (panel B). The labeled eluate (●——●, 42 mg of protein in fractions 14–17) was collected and concentrated, desalted by ultrafiltration and applied onto a DEAE column for anion exchange chromatography (panel C). The labeled 6-CB binding protein (●——●, 2.5 mg of protein) was eluted in fractions 11 + 12. Further experimental details are given in Materials and Methods; for clarity, the salt gradient is not drawn above all in the figures. This protocol was carried out about 10 times.

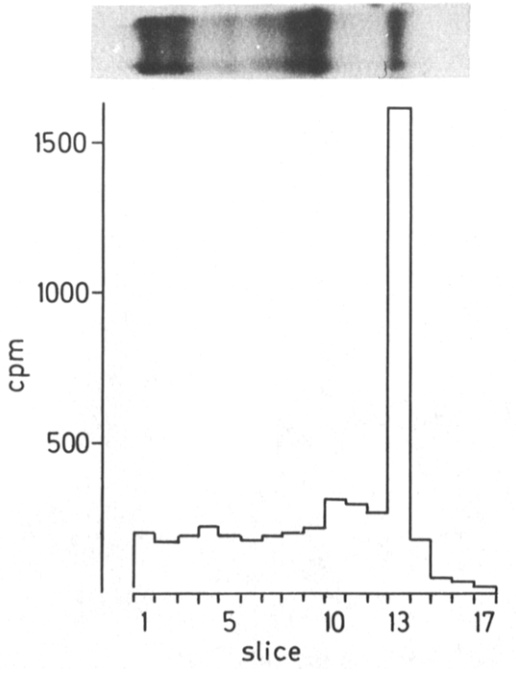

Fig. 2. Preparative PAGE of the 6-CB binding protein following DEAE chromatography. Protein fractions 11 + 12 of the DEAE eluate were combined and subjected to non-denaturing "native" PAGE on two slab gels run in parallel. After separation, one gel was stained for visualizing protein (upper panel); one lane of the other gel was sliced for detecting radioactivity (lower panel). Slice 13 of the remaining lanes were combined for protein extraction. For experimental details see Materials and Methods.

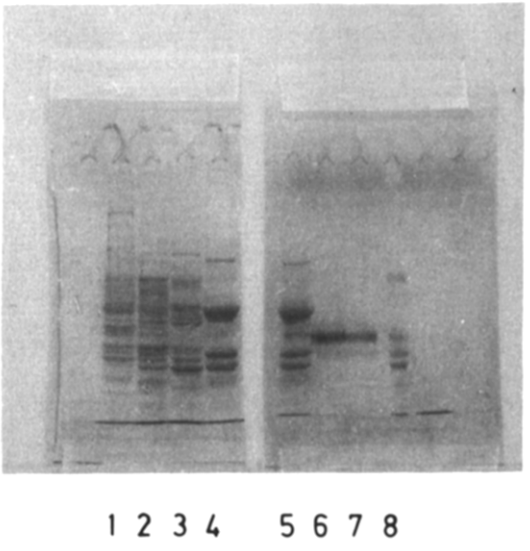

Fig. 3. SDS–PAGE of the 6-CB binding protein. Enrichment of the 6-CB binding protein was checked by SDS–PAGE. The figure shows the Coomassie Blue staining pattern of two slab gels. Lane 1: cytosol; lane 2: HIC; lane 3: HAP; lane 4: DEAE. Lane 5: DEAE; lane 6: "native" PAGE; lane 7: HPLC; lane 8: marker proteins with molecular mass 66,000, 42,000, 36,000, 29,000 Da (from top).

binding could be separated by ion exchange chromatography on HAP [11]. The method was scaled up on a 75-mL HAP column for preparative isolation. Using a linear potassium phosphate gradient, the 6-CB labeled protein was eluted at 40–50 mM potassium phosphate within the tailing edge of the first protein peak (Fig. 1B).

The labeled fractions were collected, concentrated, desalted by ultrafiltration, and applied onto a 5-mL DEAE-Sepharose column. Using a linear sodium chloride gradient the active binding protein was eluted at 80–100 mM NaCl (Fig. 1C, fraction Nos 10 + 11). The burden of non-specifically bound ligand and free ligand 6-CB were eluted in distant fractions.

The binding protein was further purified by preparative non-denaturing gel electrophoresis. Following electrophoresis the gels were immediately sliced in order to minimize protein spreading due to diffusion in the "native" gel. The position of the labeled protein was determined by counting ^{3}H-radioactivity of a sliced reference gel lane (Fig. 2). The radioactive slices (No. 13 of the adjacent lanes run in parallel) were combined and the protein eluted with buffer. The eluate was concentrated to a small volume and subjected to HPLC to remove contaminating trace compounds of electrophoresis.

The purification of the binding protein was controlled by analytical SDS–PAGE (Fig. 3). Protein composition of cytosol (lane 1), of the HIC eluate (lane 2), of HAP (lane 3), and of DEAE (lane 4) are shown in the left panel. Preparative gel electrophoresis under non-denaturing conditions yielded a rather pure protein fraction (Fig. 3, right panel, lane 6) which could subsequently be recovered after HPLC (lane 7). For comparison, the DEAE eluate (lanes 4 + 5) and standard marker proteins (lane 8) were used.

To follow up enrichment by additional means, samples of chromatographic fractions were assayed in a quick binding test (referred to as 'miniature binding technique" [11]). Using this technique, enrichment of the binding protein was approximately 50-fold (Table 1). In some preparations an apparent initial increase of binding recovery was observed, supposedly as a consequence of removal of endogenous inhibiting substances (lipid?) during purification.

The result of purification was also calculated from isolated chromatographic fractions. By neglecting the small amount of non-specific binding (cf. Fig. 1A), a more than 100-fold enrichment of the binding protein was derived (Table 2).

The data accumulated from the experiments shown are fairly consistent with respect to enrichment. The factor of 100 could rather underestimate the actual factor of enrichment if measurable loss of the bound radioactive ligand was to occur in the course of purification. The high affinity of binding of the lipophilic ligand ($K_d \approx 3$ nM; [11]) obviously prevents

Table 1. Enrichment of 6-CB binding activity

Purification step	Protein (mg)	Protein recovery (%)	Specific binding (pg 6-CB/mg)	Binding recovery (%)
Cytosol	1550		200	
HIC	410	26.5	1600	212
HAP	81	5.2	3900	102
DEAE	4	0.25	9500	12

The binding protein of rat liver cytosol was enriched by consecutive column chromatography. Samples of individual preparations from cytosol through DEAE chromatography were assayed for specific 6-CB binding activity by means of the miniature binding technique. One test (out of eight) is shown.

Table 2. Monitoring of protein enrichment by radioactive labeling

	Protein (mg)		Protein recovery (%)		Binding (pg 6-CB/mg protein)	
Fraction	I	II	I	II	I	II
Cytosol	785	1225			25	
HIC	350	335	45	27	50	
HAP	56	94	7	7.7	250	300
DEAE	2.5	4	0.3	0.35	1500	8000
HPLC		0.03		0.002		40,000

After labeling with 6-CB, the binding protein of rat liver cytosol was purified by sequential column chromatography. Total 6-CB binding activity of fractions was calculated from radioactivity and protein content. The figures for HPLC include the preceding "native" PAGE purification step. The results of two (out of four) complete purification protocols (I and II) are shown.

a large rate of dissociation of the complex in aqueous systems.

Binding specificity

We had previously shown [11] that 6-CB binding is specific, saturable ($B_{max} \approx 600$ pg/mg protein) and of high affinity ($K_D \approx 3$ nM). Concentration-dependent inhibition of 6-CB binding was demonstrated for 3,3',4,4',5-pentachlorobiphenyl and the thyroid hormones thyroxine and 3,5,3'-triiodothyronine [11]. Additional experiments showed that lower chlorinated biphenyls such as 3,3'4,4'-tetrachlorobiphenyl did not compete (Table 3). Moreover, a number of candidate competitors such as steroid hormones and hormone antagonists, retinol, cytochrome P450 inducers, and aromatic hydrocarbons did also not compete for 6-CB binding. *In vivo* and *in vitro* binding inhibition by high concentrations of triglyceride [11] is of a different nature: the very high IC_{50} indicated inhibition of 6-CB binding by non-specific protein–ligand interaction.

Tissue distribution

The species and tissue distribution of the binding protein (Table 4) is suggestive of a rather unique prevalence of the binding protein in rat tissues. A protein with identical biochemical properties was absent in all the other tissue samples searched.

DISCUSSION

These and other previous results [11] describe the biochemical properties of a soluble protein in rat liver that is capable of binding chlorinated biphenyls with high specificity and affinity. Any reflections about the physiological function of this protein must account for these results which are compiled as:

1. A soluble protein with an apparent molecular mass of 40 kDa and an isoelectric point around pH 5.5.
2. High affinity to PCB congeners with more than four chlorine atoms.
3. High affinity towards thyroid hormones.
4. Lack of affinity towards steroid hormones and various compounds including polycyclic aromatic hydrocarbons.
5. Near ubiquitous distribution in tissues of the rat (rodents?).
6. Absence in human tissue (liver, fat, serum) and cultured cells.

From the introductory remarks, it is obvious to address the potential role of the 6-CB binding protein in the PCB tumor promoting activity. There are broadly two alternative mechanisms. Binding of the promoting chemical to a cytosol receptor protein may trigger a signal transduction cascade to the nucleus in a hormone-like fashion. Alternatively, the chemical may interfere with post-translational modification of proteins or may even directly modify enzyme activity. This mechanism is illustrated by the interaction of 12-*O*-tetradecanoyl-phorbol-13-acetate and protein kinase C and may be ranked as an epigenetic or non-genotoxic effect.

Some additional experimental evidence obtained may support speculations on the potential role of the 6-CB binding protein in tumor promotion. For example: the 6-CB binding protein is localized exclusively in the cell cytosol, extracts of cell nuclei are devoid of the 6-CB binding protein, there is no evidence of 6-CB binding-induced protein translocation from the cytosol into the nucleus (E. Glitz and K. Buff, in preparation). There is no common binding site for 6-CB and inducing agents such as 3-methylcholanthrene and phenobarbital, as

Table 3. Ligand competition experiments

Competing ligand	Binding competition	IC_{50} (μM)
2,2′,4,4′,5,5′-Hexachlorobiphenyl*	+	1
3,3′,4,4′,5-Pentachlorobiphenyl*	+	1
3,3′,4,4′-Tetrachlorobiphenyl	−	
Biphenyl*	−	
TCDD	−	
Benzo[*a*]pyrene	−	
Thyroxine*	+	0.05
3,5,3′-Triiodothyronine*	+	1
Dexamethasone*	−	
Estrogen	−	
Tamoxifen	−	
Retinol	−	
3-Methylcholanthrene	−	
Phenobarbital	−	
Triglyceride*	(+)	≈500

Protein of the HIC eluate was incubated with 6-CB (10–12 nM) in the presence of various concentrations of unlabeled ligands (cf. Materials and Methods and Ref. 11). Binding competition was revealed by a concentration-dependent decrease of 6-CB binding. Ligand competition or its absence are indicated by the + or − sign, respectively. If +, the ligand concentration required to cause 50% inhibition (IC_{50}) is given. For a comprehensive view, data reported in Ref. 11 are included and are marked with an asterisk.

Table 4. Specific 6-CB binding to liver cytosol protein from various mammalian cells

Species	Sex	Source of cytosol	Specific 6-CB binding (pg/mg protein)	Result of competition
Rat	F	Liver	300	+
	M	Liver	240	+
	F	Kidney	2500	+
	F	Lung	14,000	+
	F	Spleen	2700	+
	F	Adrenals	100	−
	F	Blood serum	≤0	−
Weanling Rat	F	Liver	250	+
Pig	F	Liver	200	−*
Human	M	Liver	10	−
	F	Liver	5	−
	F	Adipose tissue	≤0	−
	M	Blood serum	≤0	−
Rat		Zydela liver cells	≤0	−
Human		Chang liver cells	100	−†
		HepG2 liver cells	15	−†
		Skin keratinocytes	0	−

Cytosol was prepared from a number of mammalian tissues and cell cultures as described for the preparation of rat liver cytosol (Materials and Methods). Specific 6-CB binding is the difference of 6-CB binding in the absence and presence of a 500-fold excess of the unlabeled congener.

F, female; M, male.

* Rapid loss of "specific" binding in subsequent DEAE chromatography.

† Specific binding not detected in subsequent analysis.

well as for polycyclic aromatic hydrocarbons. Ligand binding, nuclear exclusion and/or a different molecular mass discriminate the 6-CB binding protein from any other binding protein of aromatic carcinogens [14–16]. The 6-CB binding protein also has ligand binding affinities different from the *Ah*-receptor: TCDD and 6-CB do not cross-bind to their mutual receptor protein; pentachlorobiphenyls do compete for either receptor protein.

The only physiological ligands competing for 6-

CB binding found at present are thyroid hormones. At first glance, the high-affinity competition data suggested an apparent similarity to the thyroid hormone binding protein [17] or the thyroxine transport protein [18]. However, some properties including molecular mass, tissue and intracellular distribution, and the reversed order of binding affinity for thyroxine and triiodothyronine discriminate the 6-CB binding protein from any thyroid hormone binding protein.

The experimental evidence obtained, such as tissue distribution, comparative binding profile and apparent molecular mass, also discriminate the 6-CB binding protein of rat liver from the well-known PCB binding protein in rat lung cells [19]. The latter is a cytosolic binding protein with high affinity and specificity for methyl sulfone derivatives of PCB. Remarkably, 2,2′,4,4′,5,5′-hexachlorobiphenyl is also bound with an affinity in the range of 3 μM [20].

Lastly, the 6-CB binding protein may of course have a physiological function of its own that remains to be identified. There is no current evidence indicating the possible role in cell metabolism. It would be best to initiate experiments with the purified protein. Determinations of the partial amino acid sequence would enable molecular cloning and over-expression of the protein in suitable cell lines. The combined techniques of molecular biology and membrane enzymology may then help in clarifying the natural function and significance of the PCB binding protein.

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