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DEVELOPMENT OF AN AFFINITY CHROMATOGRAPHY RESIN FOR THE PURIFICATION OF CARCINOGEN BINDING PROTEINS FROM MOUSE LIVER

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Pyrene, a structural analog of benzo[a]pyrene, is an effective competing ligand for high affinity carcinogen binding proteins in mouse liver. A pyrene-derivatized Sepharose gel was prepared for affinity chromatography purification of these proteins, and adsorbs all detectable [^{3}H]B[a]P-binding activity from hepatic cytosol with the adsorption of less than 1% of total protein. Specific carcinogen binding activity is recovered from pyrene-derivatized Sepharose columns with the enrichment of a 33 kDa polypeptide. This chromatograpy resin represents a major step in the isolation of these unusual receptor-like binding proteins for aromatic hydrocarbon carcinogens.

Recent investigations have demonstrated the existence of novel proteins that bind a limited number of polycyclic aromatic hydrocarbons (PAH)[1] and chlorinated pesticides with binding features reminiscent of hormone and drug receptor interactions (1-5). Many of these ligands are potent animal carcinogens (6). Binding affinites in the low nanomolar range and a finite number of sites per cell are the primary characteristics of these receptor-like xenobiotic-binding proteins. With the exception of the TCDD-binding Ah receptor (1,7), no biological function has been discerned for these PAH-binding sites, although attempts to correlate binding affinities with carcinogenic potency or ability to induce microsomal drug metabolizing enzymes have been made

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[1]Abbreviations used are: Ah, aryl hydrocarbon; 1-AP-6B, 1-Aminopyrene Sepharose 6B; B[a]P, benzo[a]pyrene; B6, C57B1/6J mice; BSA, bovine serum albumin; CL-6B, Sepharose CL-6B; Hepes, 4-(2-hydroxyethyl)1-piperazine ethanesulfonic acid; HEDG buffer, composed of 25 mM Hepes, 5 mM EDTA, 1.0 mM dithiothreitol and 10% glycerol at pH 7.6; PAH, polycyclic aromatic hydrocarbon; SDS, sodium dodecyl sulfate, SDS-PAGE, SDS-polyacrylamide gel electrophoresis; TCDD, 2,3,7,8-tetrachlorodibenzo-p-dioxin; TLC, thin-layer chromatography.

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(2,3,8). The existence of carrier proteins that may facilitate microsomal metabolism of lipophilic substrates has been suggested (9,10), but supporting evidence remains inconclusive.

The limitations of ligand binding studies for establishing the biological significance of putative receptors and for probing their structural features in crude extracts have been discussed (11). Therefore, in order to further study the significance of these high affinity carcinogen-binding proteins distinct from the Ah receptor, their purification is required. Toward this end, we have synthesized an affinity chromatography resin for the isolation of a carcinogen binding protein (or class of proteins) from mouse liver whose properties we have previously described (5).

MATERIALS AND METHODS

ANIMALS Male B6 mice aged 6-8 weeks were obtained from Jackson Laboratories (Bar Harbor, Maine). Preparation of 100,000 x g hepatic cytosol and standard [^{3}H]B[a]P binding assays were performed as described previously (5).

CHEMICALS B[a]P, pyrene and 1-aminopyrene were obtained from Aldrich Chemical Company (Milwaukee, WI), TCDD from KOR Isotopes (Cambridge, MA), Hepes, dextran, Coomassie Blue G250, fluorescamine and epoxy-activated Sepharose 6B from Sigma Chemical Company (St. Louis, MO), Norit-A charcoal from Fisher Scientific (Pittsburgh, PA). Reagents for SDS-PAGE were electrophoretic grade and obtained from either Bio-Rad Laboratories or BRL (Rockville, MD). All other chemicals were of the highest purity available.

RADIOCHEMICALS [1,3,6-^{3}H]B[a]P (specific activity 87.5 Ci/mmole) and [methyl-^{14}C] methylated BSA were obtained from New England Nuclear Corporation (Boston, MA). [^{3}H]B[a]P was repurified before use as previously described (5). [1,6-^{3}H]TCDD (52 Ci/mmole) was obtained from ICN Radiochemicals (Irvine, CA) with purity assessed by TLC on Silica gel in benzene:methanol (4:1).

SYNTHESIS OF 1-AMINOPYRENE SEPHAROSE 6B (1-AP-6B) 1-AP-6B was prepared from epoxy-activated Sepharose 6B and 1-aminopyrene (recrystallized from cyclohexane) according to the manufacturer's instructions. The coupling reaction was carried out in p-dioxane:water (50:50), pH 9.0, at a molar ratio of 1-aminopyrene:epoxy groups of 5:1 under a nitrogen atmosphere for 20-24 hours. The derivatized resin was washed extensively with methanol (14). The solvent was periodically collected, evaporated to dryness and examined for the presence of 1-aminopyrene by TLC and UV spectra. Aside from the initial washing step with coupling buffer, none was found. Extent of derivatization was determined spectrally (15) at 292 nm by suspending the desiccated gel in 80% aqueous glycerol and found to be 6.38 $\pm$ 1.90 nmoles/mg gel, or 2.1 μmoles/ml packed gel. Comparable values have been obtained for steroid-modified Sepharose gels used in steroid hormone receptor purifications (14,16). Subsequent preparations of 1-AP-6B routinely contained 1-2 μmoles ligand/ml packed gel.

CHROMATOGRAPHY WITH 1-AMINOPYRENE SEPHAROSE 6B 1-AP-6B was equilibrated in HEDG buffer overnight. Control incubations contained an equal gram amount of identically treated Sepharose CL-6B. In batch experiments, the Sepharose was mixed with an equal volume of B6 mouse liver cytosol (final concentrations: 1.0 mg/ml

protein, 0.25 - 2.5 mg/ml Sepharose). Samples were incubated in a circular shaking bath for 2.5 hours at 20-22°C. After chilling on ice, aliquots (1.0 ml) of the suspension were added to 1.5 ml Eppendorf tubes and centrifuged 1 minute in a microcentrifuge (13,000 x g) to pellet the Sepharose. Portions of the supernatant (0.5 ml and 0.1 ml) were removed to monitor B[a]P binding activity and protein concentrations, respectively.

For column chromatography, 1-AP-6B (6.0 mg/ml) was equilibrated overnight in HEDG buffer containing 0.1 M NaCl. Typically, 150 mg of cytosolic protein (3.5 pmol binding activity/mg) were incubated with the 1-AP-6B suspension as described for batch chromatography at a gram ratio of 2.1 mg 1-AP-6B/mg protein and a final protein concentration of 2.5 mg/ml (total volume 61.0 ml). Subsequent operations were conducted at 4°C. The entire suspension was poured into a 0.5 x 9.0 cm column (bed volume = 1.8 ml) at a maximum flow rate of 0.2 ml/min. The unbound proteins were collected, then fractions (1.0 ml) were collected as the column was washed overnight with equilibration buffer (approximately 50 ml), which was subsequently pooled and concentrated on an Amicon YM5 membrane to 1.5 ml. A small amount of protein (1.15 mg) and only nonspecific binding activity was recovered. Elution of protein bound to 1-AP-6B was performed with Hepes buffer, pH 7.6, containing 2.0 M sodium thiocyanate, and fractions (1.0 ml) were collected. Bound proteins were eluted in the first 8 ml. These were pooled, dialyzed against HEDG buffer (3x1 liter), and concentrated as described above to about 1 ml. This volume was further reduced with an Amicon Centricon-10 to 400 µl (0.85 mg/ml protein).

ELECTROPHORESIS SDS-PAGE was carried out using a modification of the method of Laemmli (17) as previously described (5). Gels were stained with Coomassie Blue (18) or by silver staining (19).

RESULTS AND DISCUSSION

COMPETITION BINDING WITH PYRENE. The choice of a ligand for coupling to epoxy-Sepharose was based on its relative affinity for the carcinogen binding protein and the chemical stability of the compound. Pyrene was tested for its ability to compete for [^{3}H]B[a]P binding in competition assays and found to be effective with an IC_{50} of 35 nM (not shown), threefold weaker in affinity than B[a]P (5). While a number of hydroxy and amine derivatives of PAHs rapidly oxidize, 1-aminopyrene is a relatively stable molecule. These data suggested that 1-aminopyrene would be a good candidate for affinity chromatography development. Finally, in experiments where 1-aminopyrene was incubated directly at 1 µM with [^{3}H]B[a]P and B6 liver cytosol, specific binding was reduced to nonspecific levels (not shown).

ADSORPTION OF B[a]P-BINDING PROTEIN TO THE 1-AP-6B GEL. The structure of 1-AP-6B is shown below.

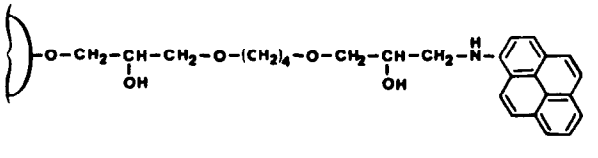

Table I. ADSORPTION OF B[a]P-BINDING ACTIVITY FROM WHOLE CYTOSOL BY 1-AMINOPYRENE-SEPHAROSE-6B RESIN

CONTROL	SPECIFIC [^{3}H]-B[a]P Bound/ml	% CONTROL
	0.535 ± 0.090	100
0.25 mg/ml CL - 6B	0.790 ± 0.030	147
2.50 mg/ml CL - 6B	0.599 ± 0.043	112
0.25 mg/ml 1-AP-6B	0.421 ± 0.040	78.7
1.25 mg/ml 1-AP-6B	0.080 ± 0.008	14.9
2.50 mg/ml 1-AP-6B	0.041 ± 0.018	7.6

The results of Table I indicate that essentially all of the B[a]P binding activity can be removed by 1-AP-6B in a concentration dependent manner, while at the highest concentration of CL-6B tested binding activity is fully recovered. No changes in supernatant protein concentrations from 1-AP-6B or CL-6B were observed. The recovery of 147% of binding activity at low CL-6B concentrations probably does not reflect significant purifcation by Sepharose CL-6B, but most likely results from our definition of 100% activity. Tierney et al. (20) have used phenyl-Sepharose 4B chromatography to partially purify a class of 3-methylcholanthrene binding proteins from rat liver cytosol. We found phenyl-Sepharose 4B to be minimally effective in adsorptive capacity for these B[a]P-binding proteins by comparison with 1-AP-6B. Despite the high degree of derivatization on phenyl-Sepharose (40 μmoles/ml packed gel vs. 1-2 μmols 1-AP/ml gel) less than 50% of [^{3}H]B[a]P binding activity was adsorbed at the highest concentration (2.3 mg/ml) of phenyl-Sepharose tested (not shown).

Samples from 1-AP-6B and CL-6B were also analyzed on 5 - 20% sucrose density gradients (Figure 1). The purpose of these experiments was to illustrate specific adsorption of the major B[a]P binding protein by 1-AP-6B, and to assess the ability of this resin to also bind the 9S Ah receptor. Figure 1a indicates that the vast majority of the 4S-sedimenting B[a]P binding protein is absent after adsorption to the affinity matrix, in agreement with the results of Table I. In Figure 1b identical samples were brought to binding equilibrium with [^{3}H]TCDD, which allows better visualization of the Ah receptor. Under these conditions more than half of the total Ah receptor content from whole cytosol was removed by 1-AP-6B. In control experiments containing CL-6B there was no

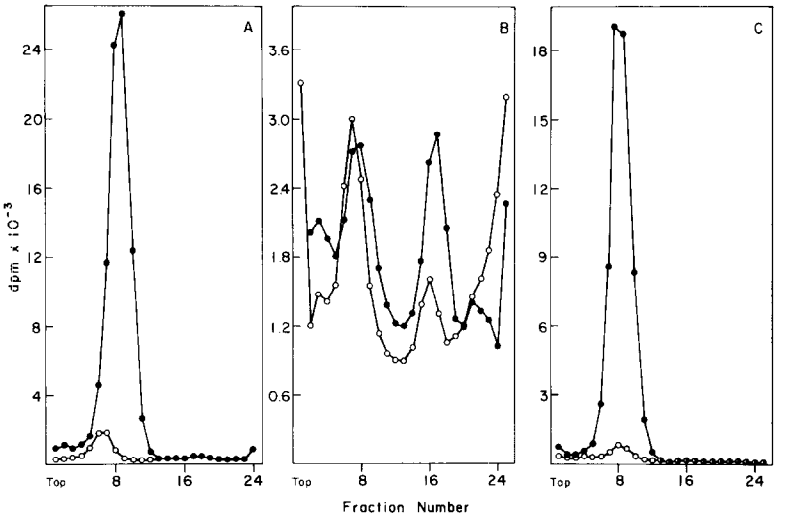

Figure 1. Sucrose density gradient fractionation of carcinogen-binding proteins. Samples were incubated with radioligands, treated with dextran-coated charcoal to remove free ligand as described (5), and centrifuged through 5-20% sucrose gradients (5.0 ml) in a SW50.1 Ti rotor at 50,000 rpm for 16 hr at 4°C. Fractions (3 drops) were collected. A: [^{3}]B[a]P (6.5 nM) binding in cytosol before (•) and after (o) 1-AP-6B incubation; 300 μg total protein. B: [^{3}H]TCDD (8.5 nM) binding as in A; 1.8 mg total protein. C: [^{3}H]B[a]P (8.5 nM) binding in salt-eluted proteins from 1-AP-6B in the absence (•) or presence (o) of 1 μM unlabeled B[a]P; 75 μg total protein.

reduction in either 4S B[a]P binding or 9S TCDD binding peaks relative to whole cytosol. Since TCDD has little affinity for this more abundant B[a]P binding species at 4S (4,5), [^{3}H]TCDD binding in this lower sedimenting region of the gradient is consistent with the notion that these sites are distinct from the 4S protein which binds polycyclics. Small differences between our assay methods and those of other investigators (7 and references therein) probably account for this 4S TCDD-binding observed in our experiments. These results indicate that 1-AP-6B has the potential to isolate both of these classes of hepatic carcinogen-binding proteins, neither of which have been purified to date.

RECOVERY OF B[a]P BINDING ACTIVITY. Proteins bound to the 1-AP-6B column were eluted and concentrated as described in Materials and Methods. Elution with sodium thiocyanate was chosen for several reasons. Thiocyanate serves as a chaotropic agent to disrupt hydrophobic interactions which we presume to play a significant part in the binding of ligands to this protein, based on structure-activity studies conducted previously (5). In addition, Bresciani (21,22) has

shown that thiocyanate-containing buffers allow recovery of the calf uterine estrogen receptor in active form from estradiol-affinity columns. Figure 1c illustrates the recovery of specific [^{3}H]B[a]P binding activity from 1-AP-6B with a peak profile identical to that observed in unfractionated cytosol (Fig. 1a). With 1 μM unlabeled B[a]P present in the incubation as competing ligand, [^{3}H]B[a]P binding is absent, suggesting that the protein is recovered in a form indistinguishable from the native cytosolic protein.

Irreversible denaturation of receptors during purification by affinity chromatography has been a significant problem. Biospecific elution strategies have diminished this difficulty (14). However, the poor solubility of B[a]P and related ligands at micromolar concentrations, and their tendency to associate with the 1-AP-6B matrix has precluded their use here. This may explain, in part, our inability to recover the Ah receptor from 1-AP-6B in active form. Modification of this procedure to include buffers containing mild detergents may overcome this problem without significant alteration of binding properties, and allow recovery of both the 9S Ah receptor as well as the more abundant 4S PAH-binding protein.

ANALYSIS OF POLYPEPTIDES BOUND TO 1-AP-6B. SDS-PAGE of proteins after chromatography on 1-AP-6B are presented in Figure 2. Most of the cytosolic proteins are not adsorbed to either 1-AP-6B or CL-6B (lanes 5 or 6) compared to samples of the unfractionated 100,000 x *g* hepatic cytosol (lanes 1 and 2). Proteins recovered from the 1-AP-6B column are displayed in lanes 3 and 4. Two major proteins of apparent molecular weights 33 kDa and 22 kDa are present. Discrete, yet less abundant, species of 35, 23, and 17 kDa are also indicated, as well as a number of minor bands. Affinity labeling studies of whole cytosol and of these affinity chromatography purified proteins suggest that the major carcinogen-binding protein in both the unfractionated cytosol and that isolated by 1-AP-6B chromatography is a 33 kDa species (Collins and Marletta, unpublished observations). Tierney et al. (20) report the partial purification of multiple binding proteins for 3-methylcholanthrene from rat liver cytosol. An abundant

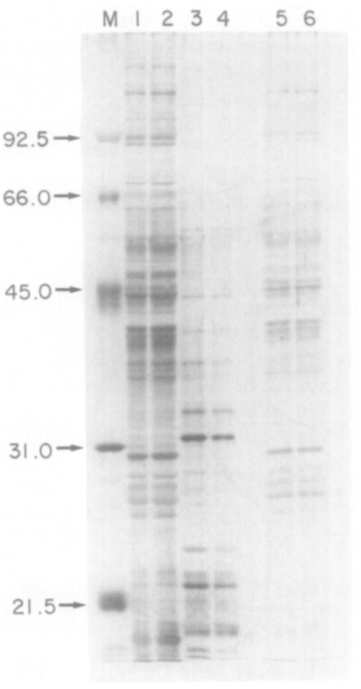

Figure 2. SDS-PAGE of proteins from 1-AP-6B chromatography. Electrophoresis was through a 12.5% polyacrylamide gel. M: molecular weight standards with M_r in kilodaltons indicated; 1: B6 mouse liver cytosol (9.4 μg); 2: same as 1 (15 μg); 3: proteins bound to 1-AP-6B (2.1 μg); 4: same as 3 (1.3 μg); 5: unbound effluent from 1-AP-6B (5 μg); 6: unbound effluent from CL-6B (5 μg).

32 kDa species in their preparation by SDS-PAGE is analogous to our findings in mouse liver.

While it is clear that additional fractionation steps are required in addition to 1-AP-6B chromatography to achieve purity, this single step procedure is effective in separating the vast majority (98-99%) of cytosolic proteins from the carcinogen-binding activity, and should greatly enhance the selectivity in purification of these novel binding proteins over more conventional methods.

REFERENCES

1. Poland, A., Glover, E., and Kende, A.S. (1976) J. Biol. Chem. 251, 4936-4946.
2. Tierney, B., Weaver, D., Heintz, N.H., Schaeffer, W.I., and Bresnick, E. (1980) Arch. Biochem. Biophys. 200, 513-523.
3. Holder, G.M., Tierney, B., and Bresnick, E. (1981) Cancer Res. 41, 4408-4414.
4. Zytkovicz, T.H. (1982) Cancer Res. 42, 4387-4393.
5. Collins, S. and Marletta, M.A. (1984) Mol. Pharmacol. 26, 353-359.

6. IARC Monographs on the Evaluation of the Carcinogenic Risk of Chemicals to Humans, Volume 32, Polynuclear Aromatic Compounds, Part I, Chemical, Environmental and Experimental Data, (1983) W.H.O., Lyon.
7. Eisen, H.J., Hannah, R.R., Legraverand, C., Okey, A.B., and Nebert, D.W. (1983) Biochem. Actions Horm. 10, 227-257.
8. Heintz, N.H., Tierney, B., Bresnick, E. and Schaeffer, W.I. (1981) Cancer Res. 41, 1794-1802.
9. Dixit, R., Bickers, D.R., and Mukhtar, H. (1982) Biochem. Biophys. Res. Commun. 104, 1093-1101.
10. Hanson-Painton, O., Griffin, M.J., and Tang, J. (1983) Cancer Res. 43, 4198-4206.
11. Hollenberg, M.D. and Cuatrecasas, P., in: The Receptors (1979), O'Brien, R.D. (ed), pp. 193-214, Plenum Press, New York.
12. Bradford, M. (1976) Anal. Biochem. 72, 248-254.
13. Udenfriend, S., Stein, S., Bohlem, P. Dairman, W., Leimgruber, W., and Weigele, M. (1972) Science 178, 871-872.
14. Sica, V., Parikh, I., Nola, E., Puca, G.A., and Cuatrecasas, P. (1973) J. Biol. Chem. 248, 6543-6558.
15. Work, T.S. and Work, E. (1979) Laboratory Techniques in Biochemistry and Molecular Biology, Vol. 7, p.395, North Holland Publishing Co., Amsterdam.
16. Grandics, P., Puri, R.K., and Toft, D.O. (1982) Endocrinology 110, 1055-1057.
17. Laemmli, U.K. (1970) Nature (Lond.) 227,680-685.
18. Fairbanks, G., Steck, T.L., and Wallach, D.F.H. (1971) Biochemistry 10, 2606-2617.
19. Morrissey, J. (1981) Anal. Biochem. 117, 307-310.
20. Tierney, B., Munzer, S., and Bresnick, E. (1983) Arch. Biochem. Biophys. 225, 826-835.
21. Sica, V., and Bresciani, F. (1979) Biochemistry 18, 2369-2378.
22. Bresciani, F., Sica, V., Weizs, A., Buonaguro, F.M., Bova, R., Puca, G.A., Molinari, A.M., and Endrenyi, L. in: Perspectives in Steroid Receptor Research (1980), Bresciani, F. (ed.), pp. 273-298, Raven Press, New York.