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Characterization of Polyclonal Antibodies to the *Ah* Receptor Prepared by Immunization with a Synthetic Peptide Hapten

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SUMMARY

A synthetic peptide based on the N-terminal amino acid sequence of the *Ah* receptor purified from C57BL/6J mice, linked to keyhole limpet hemocyanin, proved a remarkably good immunogen. All six rabbits that were immunized produced polyclonal antiserum that reacted with the synthetic peptide and the denatured and undenatured *Ah* receptor. Western blots were especially useful for antibody characterization; hepatic cytosol from C57BL/6J mice, in which the *Ah* receptor was photoaffinity labeled with 2-azido-3-[^{125}I]iodo-7,8-dibromodibenzo-*p*-dioxin, was resolved by gel electrophoresis and electrotransferred to nitrocellulose. Coincidence of the major band immunochemically stained with immunoaffinity-purified antibodies (with apparent M_r = 95,000) and the radiolabeled band on the autoradiograph indicated the specificity of the antibody. The estimated sensitivity of detection of the *Ah* receptor on a blot is 60 to 120 pg/200 μg of protein/gel lane. On Western blots, the antipeptide antibodies stained the photoaffinity-labeled *Ah* receptor from all four murine variants and all vertebrate forms examined (chicken, rodents, monkey, human), indicating conservation of these N-terminal epitopes. The immunoaffinity-purified antibodies also immunoprecipitated undenatured photoaffinity-labeled *Ah* receptor from diluted cytosol.

The *Ah* receptor is a soluble protein found in a variety of tissues of vertebrate species that, upon ligand binding, binds to a specific DNA enhancer sequence and initiates transcription of the mRNA for the cytochrome P-450 (CYPIA1) gene (1). The known ligands for the *Ah* receptor are foreign planar aromatic compounds, such as polycyclic aromatic compounds (e.g., 3-methylcholanthrene) and halogenated aromatic compounds (e.g., TCDD); at present, there is no known endogenous physiological ligand (2). All of the effects of TCDD and related halogenated aromatic hydrocarbons (e.g., induction of cytochrome P-450IA1, immune suppression, wasting syndrome, proliferation and/or differentiation of various epithelial tissues, and tumor promotion) are thought to be mediated by virtue of their binding to the *Ah* receptor (3). This hypothesis is supported by two lines of evidence, 1) for a wide variety of TCDD analogues there is a correlation between their binding affinity for the receptor and their potency to produce various biological responses and 2), among inbred strains of mice that carry a genetic polymorphism at the locus that encodes the *Ah* receptor, TCDD-induced toxicity segregates with the allele that determines the high affinity form of the receptor (3, 4).

The *Ah* receptor is thought to be a member of the erb-A superfamily of proteins, which includes the steroid hormone, vitamin D, retinoic acid, and thyroid hormone receptors (5). Like the steroid hormone receptors, the unliganded *Ah* receptor is associated with the 90-kDa heat shock protein (6, 7) and, upon ligand binding, undergoes a conformational change, displays increased binding for DNA, and shows a selective affinity for the specific transcriptional enhancer sequence (8, 9). The *Ah* receptor appears to differ from the steroid hormone receptors in two notable ways; 1) as mentioned above, there is no known physiological ligand for this receptor and 2) there is a remarkable structural diversity of this protein (as determined by apparent molecular weight) among vertebrate species and within various strains of the same species (10, 11). Further understanding of the *Ah* receptor and confirmation that it is a member of the erb-A family must await the cloning and sequence analysis of the gene.

In the accompanying paper (12), we report the purification to homogeneity of the *Ah* receptor from B6 mice. From the sequence analysis of the N-terminal amino acids of this protein, we have prepared a synthetic peptide, which upon linkage to KLH was used as an immunogen. In this report, we examine the polyclonal antisera to this peptide, their reactivity with the *Ah* receptor from B6 mice, and their cross-reactivity with the *Ah* receptors from other mammalian species.

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ABBREVIATIONS: TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; KLH, keyhole limpet hemocyanin; B6, C57BL/6; MOPS, 3-(*N*-morpholino)propanesulfonic acid; SAC, formalin-fixed *Staphylococcus aureus*, Cowan strain; EGTA, ethylene glycol bis (β-aminoethyl ether)-*N,N,N'N'*-tetraacetic acid; NBT, nitro blue tetrazolium; BCIP, 5-bromo-4-chloro-3-indolyl phosphate; PBS, phosphate-buffered saline.

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Experimental Procedures

Materials. Glycine, Tris·HCl, Nonidet P-40, hydrogen peroxide (30%) solution, MOPS, NBT, BCIP, *o*-phenylene diamine, and Freund's complete and incomplete adjuvants were purchased from Sigma Chemical Co. (St. Louis, MO). Polyvinylidene difluoride (Immobilon) membranes were obtained from Millipore Corp. (Bedford, MA). Protein A-Sepharose CL-4B and tresyl-activated Sepharose 4B were bought from Pharmacia-LKB-Biotechnology, Inc. (Piscataway, NJ). Nitrocellulose membranes (0.45 μm) were obtained from Schleicher and Schuell, Inc. (Keene, NH) or Hoefer Scientific Instruments (San Francisco, CA). Goat anti-rabbit IgG (no. 111-005-003), goat anti-rabbit IgG linked to alkaline phosphatase (no. 111-055-003), and rabbit peroxidase anti-peroxidase (no. 323-005-025) were purchased from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA). SAC (no. 9321 SB) was obtained from Bethesda Research Laboratories (Gaithersburg, MD).

Animals. Male New Zealand white rabbits (12 weeks old) were obtained from New Franken Research Rabbits (New Franken, WI) and maintained on Wayne Rabbits Ration plus occasional pelleted alfalfa. All mice were obtained from The Jackson Laboratory and/or maintained, bred, and phenotyped as previously described (11).

Buffers. MEN is 25 mM MOPS, 1 mM EDTA, 0.02% sodium azide, pH 7.5; MENG is MEN plus 10% glycerol; PBS is 0.8% NaCl, 0.02% KCl, 0.14% Na_2HPO_4, 0.02% KH_2PO_4, pH 7.2; PBST consists of PBS with 0.1% Tween-100; TBST is 50 mM Tris, 150 mM NaCl, 0.2% Tween 20, pH 7.5; and BLOTTO is 5% nonfat dry milk in TBST.

***Ah* receptor purification and N-terminal amino acid sequence.** The *Ah* receptor from the hepatic cytosol fraction of B6 mice was purified to homogeneity (10^5-fold), and the following consensus sequence was obtained for the N-terminal amino acids, as described in the accompanying paper (12): (ala/asp)-ser-Arg-Lys-arg-arg-Lys-Pro-Val-Gly-Lys-Thr-Val-Lys-Pro-Ile-Pro-Ala-Glu-Gly-Ile-Lys-ser-Asn-Pro-ser-Lys. (The uncapitalized amino acids indicate uncertainty, Ala versus Asp at position 1, Ser at positions 2, 23, and 26, and Arg at positions 5 and 6.)

Synthetic peptide synthesis. A synthetic peptide corresponding to N-terminal sequence residues 3 to 22 (Arg_3–Lys_{22}), with the addition of a C-terminal cysteine, was prepared by Applied Biosystems. The peptide was characterized by mass spectrometry, high performance liquid chromatography, and amino acid analysis. A portion of the peptide was coupled through the terminal cysteine thiol to KLH with the heterobifunctional cross-linking agent *N*-succinimidyl bromoacetate (13), in a ratio of 3 parts peptide to 5 parts KLH (w/w), by Applied Biosystems.

Preparation of the cytosolic fraction, photoaffinity labeling, and partial purification of the *Ah* receptor. The livers of mice, and when possible other species, were perfused *in situ* with MEN buffer containing 150 mM NaCl plus 5 mM EGTA, to chelate Ca^{2+} and minimize Ca^{2+}-dependent proteolysis of the receptor (14). The livers and other organs (see Fig. 6) were homogenized at 0° in 9 volumes (w/v) of MENG buffer containing 5 mM EGTA, and often 20 mM sodium molybdate (to stabilize to *Ah* receptor), and the 100,000 × *g* supernatant was prepared as previously described (15). The cytosolic fraction of the human lymphoblastoid cell line 721 and the murine Hepa 1 cell line were similarly prepared. The cytosolic fraction, diluted to a protein concentration of 0.2–1.0 mg/ml, was photoaffinity labeled with 2-azido-3-[^{125}I]iodo-7,8-dibromo-dibenzo-*p*-dioxin (16), as previously described (15). A partially purified preparation of photoaffinity-labeled *Ah* receptor was prepared (100-fold enrichment) by chromatography over phosphocellulose and DEAE-cellulose columns, as described in the accompanying paper (12). The enriched preparation was used on Western blots to screen antisera early in the course of immunization.

Gel electrophoresis, electrophoretic transfer, and autoradiography. Protein samples were resolved by denaturing electrophoresis on discontinuous polyacrylamide slab gels (acrylamide to bisacrylamide, 37.5:1.0; 3% stacking gel, 7.5% separating gel), at approximately ~10 mA current for 16 hr at 4°, on a 1.5-mm-thick gel (16). The proteins were electrotransferred from the gel to nitrocellulose in a Hoefer semi-dry transfer unit (Hoefer Scientific Instruments, San Francisco, CA), using a buffer of 48 mM Tris, 39 mM glycine, 0.37% sodium dodecyl sulfate, 20% methanol, at 0.8 mA/cm^2 for 1.5 hr (~120 mA for a standard 11- × 14-cm gel) (17). The nitrocellulose blot was rinsed three times with PBS, incubated with BLOTTO buffer for 1.5 hr at 20°, washed again three times with PBST, dried, and stored wrapped in cellophane at −20° until use.

Immunization, bleeding, serum preparation, and storage. The peptide-KLH conjugate was suspended in saline (2.0 mg/ml), emulsified by mixing with an equal volume of Freund's adjuvant, and injected into two intramuscular sites in the rear legs (2 × 0.2 ml) and four subcutaneous dorsal sites over the shoulder region (4 × 0.1 ml), for a total of 0.8 ml (0.8 mg of conjugate, ~0.3 mg of peptide) per immunization. The initial immunization used Freund's complete adjuvant and subsequent immunization, at 3- to 4-week intervals, used Incomplete adjuvant (18). The animals were bled 10 days after each booster immunization from the ear vein, using negative pressure applied to a vessel that cupped the ear. After the addition of sodium azide (final concentration, 0.01–0.05%, w/v), the blood was heated at 37° for 1 hr, chilled at 0° for at least 1 hr, and centrifuged. The serum was stored at 4° or −20° or immediately processed by immunoaffinity purification.

Immunoaffinity gel and antibody purification: Protein A gel and immunoglobulin isolation. The synthetic peptide was coupled to tresyl-activated Sepharose 4B in 0.1 M sodium bicarbonate/0.5 M sodium chloride buffer, pH 8.0, according to the manufacturer's instructions. The coupling efficiency was always >90% (0.5 μmol of peptide bound/ml of gel), as estimated by analysis of unbound peptide in the reaction supernatant. After blocking with ethanolamine and thorough washing, the gel was equilibrated and stored in 10 mM Tris, pH 7.5, plus 0.02% NaN_3, at 0°.

Serum was diluted 1:9 with 10 mM Tris buffer, pH 7.5, filtered through a disposable syringe filter (Uniflow plus glass microfiber prefilter, 0.45-μm cellulose acetate membrane; Schleicher & Schuell), and slowly loaded on the immunoaffinity column at 4° (20 ml of serum diluted to 200 ml with buffer, applied to a 4-ml column over 3–4 hr). After washing with 10 mM Tris buffer, pH 7.5, and the same buffer containing 500 mM NaCl, the adsorbed protein was eluted with 100 mM glycine·HCl, pH 2.5, and collected in a tube containing a small volume of 1 M Tris buffer, pH 8.0 (19). The immunoaffinity-purified antibody was stored at 4° as collected, with the addition of 1 mg/ml bovine serum albumin and 0.05% sodium azide. The average yield of immunoaffinity-purified antibodies was 40 to 100 μg protein/ml of immune serum.

The immunoglobulin fraction of preimmune serum was prepared by adsorption to a Protein A-Sepharose column and elution with 100 mM glycine, in a manner similar to that described above for specific antibodies.

Enzyme-linked immunosorbent assay. The synthetic peptide (2 μg/ml) was adsorbed to 96-well flat-bottomed plates (50 μl/well). After blocking with excess protein, the antiserum was assayed with successive incubations with primary rabbit antiserum (50 μl of various dilutions), goat anti-rabbit IgG diluted 1:1000, and peroxidase-rabbit antiperoxidase complex diluted 1:5000 and was visualized with freshly prepared *o*-phenylenediamine/H_2O_2 in 50 mM citrate buffer, pH 5 (20).

Immunochemical staining. Two different modes of immunochemical staining were employed. 1) To compare different batches of antiserum or immunoaffinity-purified antibodies, i.e., screening situations requiring a common antigen, 3–4-mm-wide vertical strips of blot were stained in individual wells (Accutran disposable incubation trays; Schleicher and Schuell). 2) For comparison of different quantities or sources of antigen (stained with the same antibodies), the whole blots were sealed in plastic bags for exposure to the primary antibody.

Two standard immunochemical stains were used, horseradish peroxidase-antiperoxidase and alkaline phosphatase, but the latter was used routinely because of its 2- to 4-fold greater sensitivity. The blots were blocked with BLOTTO buffer, all antibodies were dissolved in

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BLOTTO buffer, and the blots were washed with TBST buffer between each incubation. The primary antibody was most commonly immunoaffinity-purified antibodies from rabbit 3, at 2.5 μg/ml, incubated for 1 hr. The blot was then incubated with a 1:5000 dilution of immunoaffinity-purified goat anti-rabbit IgG linked to alkaline phosphatase, with subsequent color development with NBT/BCIP as a reagent (17).

Immunoprecipitation. The photoaffinity-labeled cytosolic fractions of B6 liver and other tissues, at 200–500 μg of protein/ml in MEN buffer plus 10% glycerol buffer, 150 mM NaCl, and 1% Nonidet P-40, were incubated with immunoaffinity-purified antisera (4 μg) for 1 hr at 0°, followed by 100 μl of a 10% suspension of SAC for 30 min at 0° (22). The precipitate was collected by sedimentation, washed with 150 mM NaCl containing 1% Nonidet P-40, resuspend in electrophoresis sample buffer, and heated at 85° for 10 min before being loaded on a gel. An aliquot of labeled cytosol that was acetone precipitated was used as the control for recovery.

Results

A synthetic peptide, based on the N-terminal amino acid sequence of the *Ah* receptor of B6 mice (Ala_3–Lys_{22}), with an added C-terminal cysteine, and coupled to KLH, proved to be remarkably immunogenic. All six rabbits that were immunized developed antibodies that reacted with the synthetic peptide by enzyme-linked immunosorbent assay, reacted with the denatured *Ah* receptor on Western blot, and precipitated the undenatured *Ah* receptor.

Specificity. Immunochemical staining of Western blots proved especially useful in characterizing the antibodies. The *Ah* receptor in hepatic cytosol from B6 mice was photoaffinity labeled with 2-azido-3-[^{125}I]iodo-7,8-dibromodibenzo-*p*-dioxin, cytosol proteins were resolved by gel electrophoresis and transferred to nitrocellulose, and the bands were visualized by immunochemical staining and autoradiography. As seen in Fig. 1A, preimmune serum (1:100 dilution) stains a few bands (Fig. 1A, *lane 1*), whereas immune serum (1:100 dilution) from the same rabbit stains several additional bands (Fig. 1A, *lane 2*). The multiplicity of bands makes it difficult to identify anti-*Ah* receptor antibodies and characterize their specificity. Several techniques simplified this task. 1) Because the *Ah* receptor was specifically labeled with the ^{125}I-photoaffinity ligand, identification of the receptor is made easy. Fig. 1B is an autoradiograph of the blots in Fig. 1A. 2) In screening antiserum early in the course of immunization, a 100-fold-enriched preparation of *Ah* receptor was used (12). This reduces the complexity of proteins present and increases the concentration of *Ah* receptor, making it easier to detect low levels of antibody. 3) The antibodies were purified by an affinity column to which the synthetic peptide was attached. When the immune serum in Fig. 1A, *lane 2* was passed over an immunoaffinity column and the unadsorbed fraction was used Fig. 1A, *lane 3*, the staining was similar to that of whole serum. However, the immunoaffinity-purified fraction (adsorbed and eluted) gave selective staining of the 95-kDa band (Fig. 1A, *lane 4*). The purified immunoglobulin fraction of preimmune serum did not stain this band (Fig. 1A, *lane 5*). The relative specificity of antibodies from different rabbits immunized with the synthetic peptide coupled to KLH is seen in Fig. 1C. Immunoaffinity-purified antibodies at a relatively high concentration (15 μg of protein/ml versus the usual concentration of 2.5 μg/ml, e.g., Fig. 1A, *lane 4*) were used to stain blots of hepatic cytosol. In some rabbits, the antibodies are quite selective, whereas others produce antibodies that cross-react with many proteins (e.g., Fig. 1C, compare *lane 3* with *lane 2*).

Fig. 1. Immunochemical staining of the *Ah* receptor. Liver cytosol from B6 mice was ^{125}I-photoaffinity labeled and resolved by denaturing electrophoresis. The proteins were electrotransferred onto nitrocellulose and the blot was cut into 3–4-mm-wide strips. The strips were individually stained with different primary antibodies, as detailed below, and then all were developed alike with goat anti-rabbit IgG, linked to alkaline phosphatase, and substrate, as detailed in Experimental Procedures. A, *Lane 1*, preimmune serum (rabbit 3) (1:100 dilution); *lane 2*, immune serum (rabbit 3) (1:100 dilution; *lane 3*, flow-through fraction of immune serum passed over peptide affinity column; *lane 4*, immunoaffinity-purified antibodies of no. 3 serum at 2.5 μg/ml; *lane 5*, immunoglobulin fraction of preimmune serum at 2.5 μg/ml. B, Autoradiograph of strips in A. C, Immunochemical stain with immunoaffinity-purified antibodies from four different immunized rabbits. The concentration of antibodies is 15 μg/ml (versus 2.5 μg/ml in A, *lane 3*) to emphasize cross-reactivity with other proteins. *Lanes 1–4*, rabbits 1–4, respectively.

Sensitivity. The concentration of the *Ah* receptor in the liver cytosol fraction of B6 mice, estimated by saturation ligand binding, is ~100 fmol/mg of protein (21), or approximately 1 ng of 95-kDa protein in 100 μg of cytosol protein applied to a gel lane.

To determine the approximate sensitivity of immunochemical staining, 2-fold dilutions of photoaffinity-labeled B6 cytosol were subjected to electrophoresis and blotting, and the receptor was visualized by immunochemical staining with alkaline phosphatase (Fig. 2A) or by autoradiography (Fig. 2B). The *Ah* receptor is easily visualized in Fig. 2A, *lane 2* or *3* (6.3 and 12.5 μg of cytosol protein, equivalent to approximately 60 and 120 pg of receptor, respectively). An 8-day exposure of the autoradiograph (Fig. 2B) shows roughly the same sensitivity. The immunochemical stain with peroxidase-antiperoxidase was 2- to 4-fold less sensitive (data not shown).

Species cross-reactivity. One of the most interesting as-

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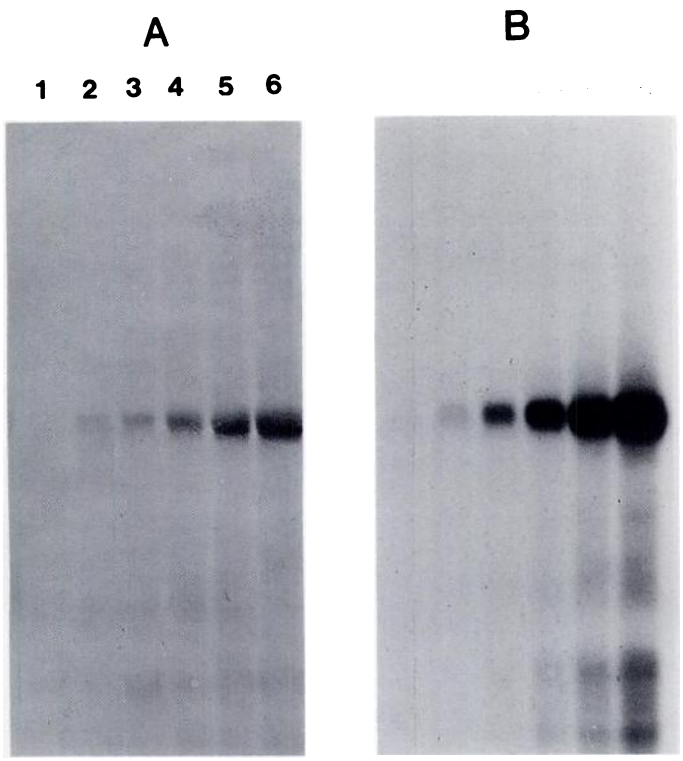

Fig. 2. Sensitivity of detection of *Ah* receptor with immunochemical staining and photoaffinity labeling. Two-fold dilutions of ^{125}I-photoaffinity-labeled liver cytosol from B6 mice were subjected to denaturing gel electrophoresis, blotted to nitrocellulose, incubated with immunoaffinity-purified antibodies (rabbit 3, 2.5 μg/ml) and goat anti-rabbit IgG linked to alkaline phosphatase, and stained as described in Experimental Procedures. A, Immunochemical stain. Protein concentration: *lane 1*, 3 μg; *lane 2*, 6.25 μg; *lane 3*, 12.5 μg; *lane 4*, 25 μg; *lane 5*, 50 μg; *lane 6*, 100 μg. B, Autoradiograph of the same blot, exposed for 8 days.

pects of the *Ah* receptor is the high frequency of structural variation, i.e., differences in apparent molecular weight of this protein among vertebrate species (10) and among strains of the same species (11). Four allelic variants of the *Ah* receptor have been identified among inbred strains of mice (15), and an additional variant was described in the more distant deer mouse (*Peromyscus maniculatus*). It was interesting to determine whether the epitopes in the N-terminal region of the *Ah* receptor from B6 mice (carrying the Ah^{b-1} allele) are preserved in other murine variants (Fig. 3) and more broadly in the receptor from other species (Fig. 4). In Fig. 3 is shown the immunochemical stain of a Western blot of photoaffinity-labeled hepatic cytosol from mouse strains expressing four alleles, as well as *Peromyscus*. The most intensely stained and highest molecular weight band in each lane of the blot was superimposable on the ^{125}I-photoaffinity-labeled band of the autoradiograph (not shown), confirming its identity as the *Ah* receptor. The antipeptide antibodies stain all the murine variants of the *Ah* receptor, indicating they share common epitopes, as follows: Fig. 3, *lane 1*, B6 (Ah^{b-1} allele), 95-kDa protein; *lane 2*, B6·C-H-38^c/By (Ah^{b-2} allele), 104-kDa receptor; *lane 3*, B6J·D2N(Ah^d) (Ah^d allele), 104-kDa low affinity receptor; *lane 4, Mus spretus* (Ah^{b-3} allele), 105-kDa receptor; *lane 5, Peromyscus maniculatus*, 130-kDa receptor; and *lane 6*, Hepa 1 cell line (derived from a hepatoma in C57L/J mice) (Ah^{b-1}), which overexpresses 95-kDa protein; a 70-kDa proteolytic product of the receptor is also present.

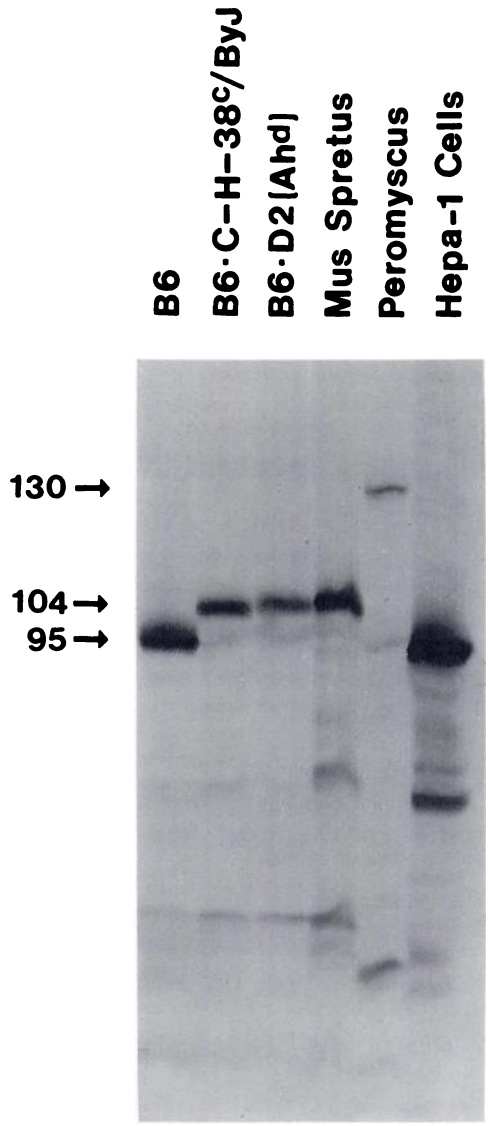

Fig. 3. Immunochemical staining of allelic variants of the *Ah* receptor among inbred strains of mice. The cytosolic fraction of liver from five strains of mice and Hepa 1 cells were ^{125}I-photoaffinity labeled; 150 μg of each were subjected to denaturing gel electrophoresis and transferred to nitrocellulose, and the proteins were visualized by immunochemical staining and autoradiography (latter not shown). *Lane 1*, B6 mice (Ah^{b-1} allele, 95 kDa); *lane 2*, B6·C-H-38^c/By (Ah^{b-1} allele, 104 kDa); *lane 3*, B6J·D2N (Ah^d) (Ah^d allele, low affinity 104 kDa); *lane 4, Mus spretus* (Ah^{b-3} allele, 105 kDa); *lane 5, Peromyscus maniculatus* (~130 kDa); *lane 6*, Hepa 1 cells (overexpression of Ah^{b-1} allele, 95-kDa protein and also 70-kDa and other proteolytic products of the receptor).

The conservation of these epitopes in the *Ah* receptor of other vertebrate species was examined (Fig. 4). The *Ah* receptor in each species is defined as the highest molecular weight photoaffinity-labeled band in the autoradiograph of the blot (Fig. 4B). The immunochemical stain of each species tested [mouse, rat (two molecular weight variants), guinea pig, hamster, monkey, human, and chicken] is shown. In each of the mammalian species tested, as well as in the chicken, the main immunochemically stained band is coincident with the radiolabeled band. Thus, the epitopes in the synthetic peptide are highly conserved in the *Ah* receptor in vertebrate evolution.

Immunoprecipitation. The capacity of the antipeptide antibodies to immunoprecipitate the *Ah* receptor was examined

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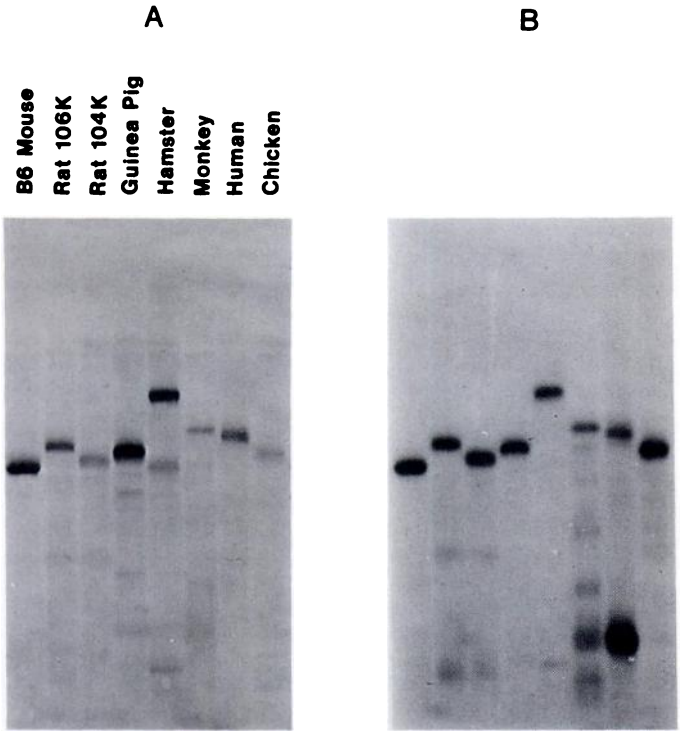

Fig. 4. Immunochemical stain with antipeptide antibodies of *Ah* receptor from selected vertebrate species. The cytosolic fraction of liver from various species and from the human lymphoblastoid cell line 721 were labeled, resolved on gel electrophoresis, and blotted. The origin and preparation of these samples was previously described (10). A, Immunochemical stain of blot; B, autoradiograph of blot.

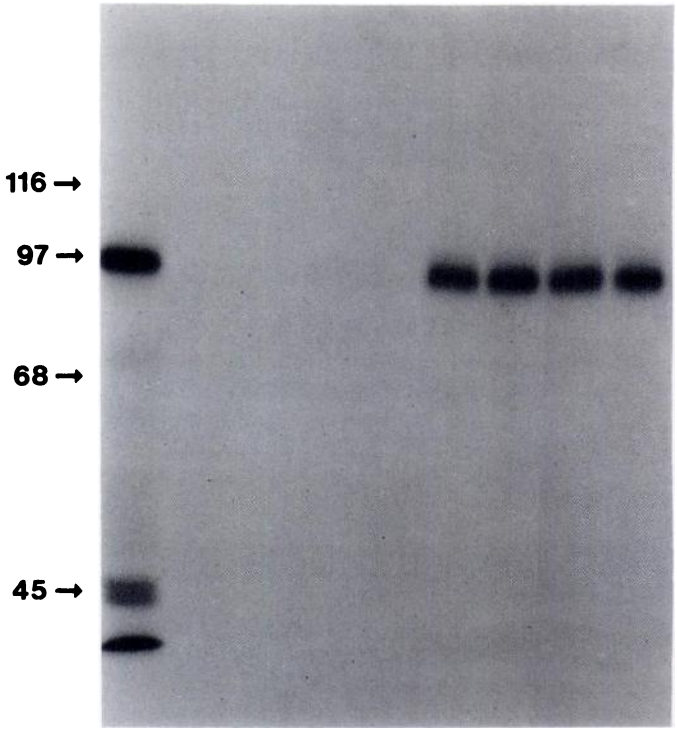

Fig. 5. Immunoprecipitation of ^{125}I-photoaffinity-labeled *Ah* receptor. Hepatic cytosol from B6 mice was ^{125}I-photoaffinity-labeled in MENG buffer. Aliquots (600 μl, 0.5 mg of protein/ml) were diluted with 0.1 volume of 10-fold concentrated immunoprecipitation buffer, to give a final concentration 1% Nonidet P-40 and 150 mM NaCl. The samples were then preincubated with 2 μg of the immunoglobulin fraction of preimmune serum (*lanes 2–5*) or 2 μg of the immunoaffinity-purified fraction of immune serum (*lanes 6–9*) from the same four rabbits, followed by immunoprecipitation with SAC. The precipitate was washed and boiled, and the supernatant was resolved on gel electrophoresis. *Lane 1*, an acetone precipitate of the cytosol protein, which was then solubilized and resolved by electrophoresis to give the total radioactivity in the 95-kDa band. The figure depicts an autoradiograph of the gel.

using ^{125}I-photoaffinity-labeled hepatic cytosol (Fig. 5). An aliquot of labeled cytosol, acetone precipitated and resolved by gel electrophoresis, contained ~4000 dpm in the 95-kDa band (Fig. 5, *lane 1*). In a similar aliquot incubated with 2 μg of the purified immunoglobulin fraction from preimmune sera of each of four rabbits, followed by precipitation with SAC (Fig. 5, *lanes 2–5*), there was virtually no 95-kDa protein precipitated (50–150 dpm), whereas incubation with immunoaffinity-purified antibodies (2 μg) from immune sera of the same rabbits and subsequent addition of SAC precipitated 50–60% of the total labeled 95-kDa protein present in the cytosol (Fig. 5, *lanes 6–9*).

Immunoprecipitation is especially useful in concentrating and identifying the *Ah* receptor in extracts of tissues where receptor concentration is low. The cytosolic fractions of various tissues from B6 mice were photoaffinity labeled and resolved by gel electrophoresis, and the labeled bands were visualized by autoradiography (Fig. 6, *Cytosols*). In addition to the 95-kDa band, each tissue shows other labeled bands, which represent nonspecifically labeled proteins or proteolytic products of the 95-kDa band. Immunoprecipitation of the same labeled tissue cytosols eliminates much of the labeling in bands other than the 95-kDa band and, hence, reduces the background (Fig. 6, *Immunoprecipitates*). In kidney, a tissue with a low concentration of *Ah* receptor (Fig. 6, *lane 4* in *Cytosols* and *Immunoprecipitates*), by immunoprecipitation of increasing aliquots of labeled cytosol the signal of the radiolabeled receptor is enhanced to the desired level of sensitivity (Fig. 6, *Kidney Immunoprecipitates*).

Discussion

In this report we have characterized antibodies to the *Ah* receptor, produced in response to immunization with a synthetic peptide hapten that corresponds to the N-terminal sequence of the *Ah* receptor. The synthetic peptide proved to be an extremely good antigen, producing high titers of antibodies in all rabbits immunized, antibodies that reacted with the denatured and undenatured *Ah* receptor. For synthetic peptide haptens to elicit antibodies that react with the undenatured protein, it is thought that the sequence should be exposed on the surface of the native protein, contain hydrophilic amino acids, often located at the C- or N-terminal, and, more uncertainly, contain proline residues [because β-turns often form portions of known epitopes (18)]. The synthetic 20-amino acid peptide chosen was from the N-terminal end of the *Ah* receptor (from B6 mice) and contained eight basic amino acids, a glutamic acid, and three prolines.

Perhaps as surprising as the immunogenicity of the synthetic peptide is the observation that the antibodies produced react with the *Ah* receptor from all species tested (chicken, rodents, monkey, human), because we have inferred that the *Ah* receptor is structurally diverse in different species and different strains within the same species (from the differences in apparent

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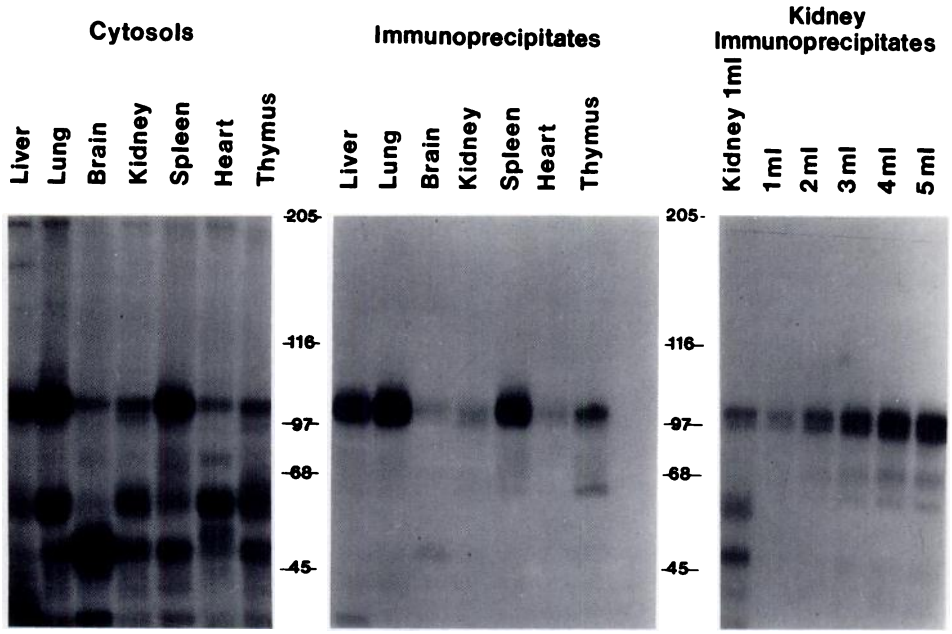

Fig. 6. Immunoprecipitation of ^{125}I-photoaffinity-labeled *Ah* receptor from the cytosolic fraction of various mouse tissues. The cytosolic fractions of various tissues from B6 mice (200 μg of protein/ml) were photoaffinity labeled and acetone precipitated and resolved by denaturing gel electrophoresis (A) or immunoprecipitated and subjected to electrophoresis (B and C) (see Experimental Procedures). The radiolabeled bands were visualized by autoradiography. A, Labeled tissue cytosols (200 μg of protein/lane). B, Immunoprecipitates of photoaffinity-labeled cytosols. One-milliliter aliquots of labeled cytosols (200 μg of protein) were diluted with 100 μl of a 10× concentrated immunoprecipitation buffer, incubated with 4 μg of immunoaffinity-purified antibodies (rabbit 4) for 1 hr at 4°, and then immunoprecipitated with SAC. C, Immunoprecipitates of varying volumes of labeled kidney cytosol. One to 5 ml of labeled cytosol were incubated with 4 μg of antibody/ml and subsequently precipitated with 100 to 200 μl of a 10% suspension of SAC.

molecular weight determined by mobility in denaturing gel electrophoresis).

The synthetic peptide contains highly antigenic epitope(s) and epitope(s) that are conserved in evolution. It is tempting to speculate and attribute both of these functions to the unusual sequence Arg-Lys-Arg-Arg-Lys-Pro. This sequence has five basic amino acids in a row and conforms to the tenets of a good antigen. This sequence also resembles the nuclear recognition sequence that is responsible for targeting some larger proteins to the nucleus through the nuclear pore, e.g., c-abl (23), SV40 large T antigen (24), c-myc (25), adenovirus E1a-gene product (26), and c-rel (27). The nuclear recognition sequences include a series of four or five basic amino acids and often an adjacent proline; however, there is no precise consensus sequences, and one can only define the function of such a sequence after gene cloning using an expression assay. The *Ah* receptor is believed to be a member of the erb-A family of genes; whether the unliganded *Ah* receptor is located in the cytoplasm or nucleus *in vivo* is not resolved (8–10). Interestingly, such a run of five basic amino acids is found in the estrogen receptor (28), glucocorticoid receptor (29), and the human retinoic acid receptor γ (30). Picard and Yamamoto (31) defined two nuclear localization sequences in the glucocorticoid receptor by expression assay and one of these contained the canonical proline and five-basic amino acid sequence.

This is the first report of antibodies to the *Ah* receptor. The specificity of antipeptide antibodies, their reactivity across vertebrate species, and their reactivity with denatured and undenatured receptors should make them useful reagents in several areas, such as immunoaffinity purification, immunohistochemical staining, and turnover studies using immunoprecipitation.

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