

UNIT VII: Nuclear Receptors: PAS Proteins and their roles in Tumor Promotion (continued).

Reading: Hypoxia-inducible factor-1 modulates gene expression in solid tumors and influences both angiogenesis and tumor growth (1997). PNAS, 94:8104-8109.

General comments about PAS proteins.

A new superfamily of conditional transcription factors has recently been uncovered. They are collectively referred to as PAS proteins. The term PAS is derived from the founding members of this superfamily, namely Per (“period”, regulator of circadian rhythms), Arn’t (“Ah receptor nuclear translocator”) and Sim (“single minded,” regulator of midline cell differentiation). Today there are 15 known members, at least five of these proteins have important roles in carcinogenesis. At the present time, only one of these proteins is known to be a bona fide “receptor”. That is, only the Ah receptor has been shown to bind a low molecular weight ligand and transduce its signal. At the present time, only about 1/3 of the PAS superfamily have any significant characterization. It seems likely that some of these proteins will also be true receptors and play roles in carcinogenesis.

The Ah receptor

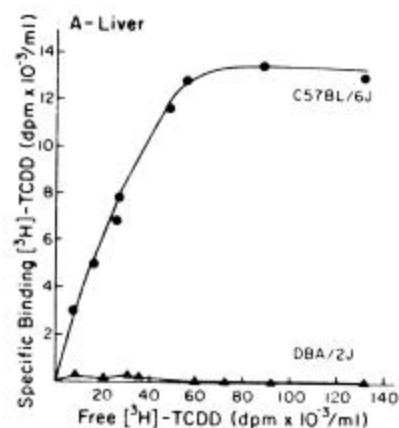
The Ah receptor (AHR) is a PAS protein that lies at the center of two important paradigms in chemical carcinogenesis. First, this protein mediates the upregulation of batteries of P450s and phase II enzymes that occurs in response to a number of coal tar carcinogens, like benzo(a)pyrene (BaP), dimethylbenzanthracene (DMBA) and 3-methylcholanthrene (3MC). Second, potent agonists of this receptor (compounds that are extremely active at inducing P450 expression) like the halogenated-dioxins (e.g., 2,3,7,8-tetrachlorodibenzo-p-dioxin or “TCDD”) and -biphenyls (e.g., “PCBs and PBBs”) are also potent tumor promoters and this activity is also mediated by the AHR. Before cloning and PCR, a series of elegant experiments provided the proof for the existence and biological importance of the AHR in carcinogenesis. Like all good proofs, this one had multiple components.

Genetic aspect of proof

We have already talked extensively about polymorphisms at loci encoding enzymes that metabolize chemical carcinogens. In addition, we have discussed the idea that many of these enzymatic activities are often inducible by substrate. The coal tar carcinogens provided an early example that the mechanisms of induction are also highly polymorphic. Early on, it was observed that batteries of P450s were upregulated in response to exposure to certain coal tar carcinogens like BaP or 3MC. More importantly, this inducible response was highly polymorphic across various mouse strains. In some mouse strains, a marked induction was observed, in others, no response was seen at all. Simple crosses and backcrosses revealed that a single autosomal dominant trait was responsible for this phenotype. This locus was named *Ah*, for aryl hydrocarbon responsiveness. The locus encoding the “responsive strains” was denoted *Ah^b* and the locus encoding the nonresponsive strain was denoted *Ah^d* (named after the prototype mouse strains (C57BL/6J and DBA/2J, respectively).

Pharmacological Proof

Biological processes that are reversible, show structure-activity, and can be induced at low concentrations of a compound (in the absence of any obvious pathology) show potential to be receptor mediated processes. In any receptor hunt, one must be aware that receptors can reside in almost any cellular compartment. Most receptors for low molecular weight ligands are found in the plasma membrane. Fewer are soluble, being found either in the cytosolic fraction or nuclear fraction of cells (e.g. Zn Finger receptors). Characteristics of most receptors are that they display low abundance and high affinity for their ligands (KDs in the range of 10^{-9} - 10^{-12} M, the KD is the concentration at which the receptor is half saturated). Another way to state this is that they are saturable at low concentrations of ligand. To identify and characterize a receptor you must have a way to demonstrate these properties. This usually requires a high affinity ligand that is radiolabeled. In the search for the putative receptor encoded by the AHR, ^3H -TCDD was used in saturation binding studies of various cell fractions. The results of these experiments indicated that responsive strains had a high affinity, saturable TCDD binding site and that nonresponsive strains had a much lower affinity site. In fact, in early experiments it was difficult to demonstrate that the nonresponsive strains had any binding sites at all (right).



Given that inbred mouse strains like C57 and DBA are homozygous at all loci, what genetic experiments would you perform to prove that the Ah locus responsive allele is autosomal dominant (hint, what outcomes would you predict from a crosses and backcrosses)? How would you use a similar approach to prove that the Ah locus encoded the TCDD binding site?

Once the binding site was in hand, the proof had to be extended to show that it was in fact responsible for the induction phenomenon. To do this, structure activity studies were employed. These are accomplished by comparing the ability of various congeners to compete for the binding site with the radiolabeled agonist. The figure on the left shows raw data from such an experiment using unlabeled polycyclic aromatic hydrocarbons as competitors. The data set on the right shows the correlation of the affinity for receptor binding and the biological potency (measured by induction of CYP1A1). **This correlation is almost perfect. Under what conditions would you expect to see deviation from this correlation, while still being consistent with an AHR receptor mediated process.**

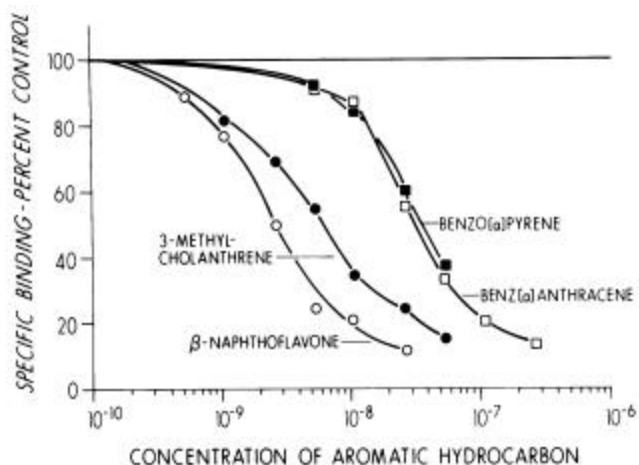













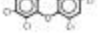


Table 2
The Cytosol Binding Affinity and Biological Potency of Dibenzo-*p*-dioxin Congeners Relative to TCDD

| | Relative binding affinity | Relative biological potency | | Relative binding affinity | Relative biological potency |
|---|---------------------------|-----------------------------|---|---|---|
|  | 100 ^a | 100 ^b |  | inactive (5.4 × 10 ⁻⁷) ^c | inactive (9.4 × 10 ⁻⁸) ^d |
|  | 167 | 100 |  | inactive (2.7 × 10 ⁻⁸) | inactive (9.4 × 10 ⁻⁸) |
|  | 43 | 43 |  | inactive (5.4 × 10 ⁻⁹) | inactive (9.4 × 10 ⁻⁸) |
|  | 20 | 22 |  | inactive (5.4 × 10 ⁻⁹) | inactive (4.7 × 10 ⁻⁷) |
|  | 16 | 8 |  | inactive (5.4 × 10 ⁻⁹) | inactive (9.4 × 10 ⁻⁸) |
|  | 13 | 3 |  | inactive (2.7 × 10 ⁻⁸) | inactive (9.4 × 10 ⁻⁸) |
|  | 14 | 0.06 |  | inactive (1.1 × 10 ⁻⁸) | inactive (9.4 × 10 ⁻⁸) |

These aspects of the AHR proof are now routinely used to demonstrate that any given response is AHR mediated (i.e., it must segregate with the *Ahr* locus and must show the appropriate SAR). A number of TCDD responses meet these criteria, birth defects, immune suppression, porphyria, chloracne and induction of P450s, GST, and glucuronyl transferases.

Does TCDD tumor promotion of HCC segregate with the Ah locus

TCDD is one of the most carcinogenic compounds known. It induces HCC in rats at 0.1 ug/Kg. This activity as a complete carcinogen is believed to be due to its potent tumor promoting capacity. In the rat, using diethylnitrosamine as an initiator, TCDD was shown to be the most potent tumor promoters for HCC ever described. Given that TCDD is also quite potent at producing HCC without an initiator, it is thought that TCDD might be promoting spontaneously arising mutations very efficiently. This is a difficult idea to prove. In support of it are the observations that TCDD is not mutagenic in the Ames assay and does not covalently bind to DNA, even in the presence of induced microsomes. Figure is from Cancer Research, 40:316).

Table 1
Promoting effect of TCDD on hepatocarcinogenesis by a single dose of DEN and PH^a
Female rats (200 g) were incubated with DEN where shown. Seven days later, TCDD (injected s.c.) or phenobarbital (0.05% in the diet) administration was begun and was continued for 26 weeks, at which time the animals were sacrificed, and the livers were examined. The low and high doses of TCDD were 0.14 and 1.4 μg/kg/2 weeks, respectively, administered s.c. DEN was given at a dose of 10 mg/kg. See text for further details.

| Group | Treatment | No. of animals | No. of enzyme-altered foci/cu cm of liver | Mean vol of enzyme-altered foci (cu mm) | % liver vol occupied by foci | N. of rats with carcinoma |
|-------|-----------------------------|----------------|---|---|------------------------------|---------------------------|
| 1 | PH + DEN | 4 | 309 ± 96 ^b | 0.02 | 0.7 | 0 |
| 2 | PH + TCDD (low dose) | 4 | 34 ± 17 | 0.05 | 0.2 | 0 |
| 3 | PH + TCDD (high dose) | 5 | 25 ± 7 | 0.04 | 0.1 | 0 |
| 4 | PH + phenobarbital | 4 | 56 ± 13 | 0.01 | 0.1 | 0 |
| 5 | PH + DEN + TCDD (low dose) | 5 | 1068 ± 166 | 0.08 | 9.0 | 0 ^c |
| 6 | PH + DEN + TCDD (high dose) | 7 | 871 ± 65 | 0.49 | 43.0 | 5 ^d |
| 7 | PH + DEN + phenobarbital | 10 | 533 ± 103 | 0.15 | 6.0 | 8 |

^a PH, partial hepatectomy.

^b Mean ± S.D.

^c Three rats exhibited neoplastic nodules in the liver.

^d One rat exhibited neoplastic nodules in the liver.

Interestingly, segregation analysis of HCC tumor promotion has never been conclusively shown to segregate with the *Ahr* locus. This may seem surprising but may be related to a number of factors; 1) the C57Bl/6J and DBA/2J strains used in cancer studies also differ at a number of other loci that can influence HCC. 2) Given that DBA mice still respond to TCDD at ten fold higher doses, it may be difficult to choose a dose of TCDD that is active in responsive strains and not in nonresponsive strains. This is especially true in chronic exposure studies

like two stage tumor promotion models. **Using modern techniques in mouse husbandry and embryo manipulation, how could you prove a role for Ahr in HCC? How would you design such an experiment to conclusively demonstrate TCDD's activity as a tumor promoter or complete carcinogen?**

The effects of TCDD on AHF

Unlike Phenobarbital or peroxisome proliferators, TCDD is not a very potent mitogen in hepatocytes. In addition, it does not appear to increase labeling indices in AHF during two-stage protocols. One thing TCDD does do is lead to a marked decrease in apoptosis in AHF. **What two mechanisms might decreased rates of apoptosis play in tumor promotion?**

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Table 2

Effect of chronic TCDD treatment on cell division (BrdU-labeling) and apoptosis in GST-P-positive liver foci and normal hepatocytes

| Treatment ^a | 31 days | | 73 days | | 115 days | |
|--------------------------------------|---------------|--------------|--------------------------|--------------|---------------|--------------------------|
| | Normal tissue | Foci tissue | Normal tissue | Foci tissue | Normal tissue | Foci tissue |
| BrdU-labeling index (%) ^b | | | | | | |
| Control | 0.73 ± 0.36 | 13.94 ± 1.84 | 0.56 ± 0.20 | 10.25 ± 5.36 | 0.33 ± 0.22 | 6.79 ± 4.46 |
| TCDD | 0.41 ± 0.13 | 19.19 ± 6.62 | 0.26 ± 0.08 ^c | 11.50 ± 3.13 | 0.40 ± 0.43 | 6.79 ± 4.46 |
| Apoptotic index (%) ^b | | | | | | |
| Control | 0.14 ± 0.08 | 1.64 ± 1.33 | 0.29 ± 0.20 | 6.14 ± 5.79 | 0.32 ± 0.23 | 6.19 ± 3.93 |
| TCDD | 0.10 ± 0.09 | 1.14 ± 0.97 | 0.07 ± 0.03 ^c | 1.38 ± 0.87 | 0.20 ± 0.08 | 0.80 ± 0.75 ^c |

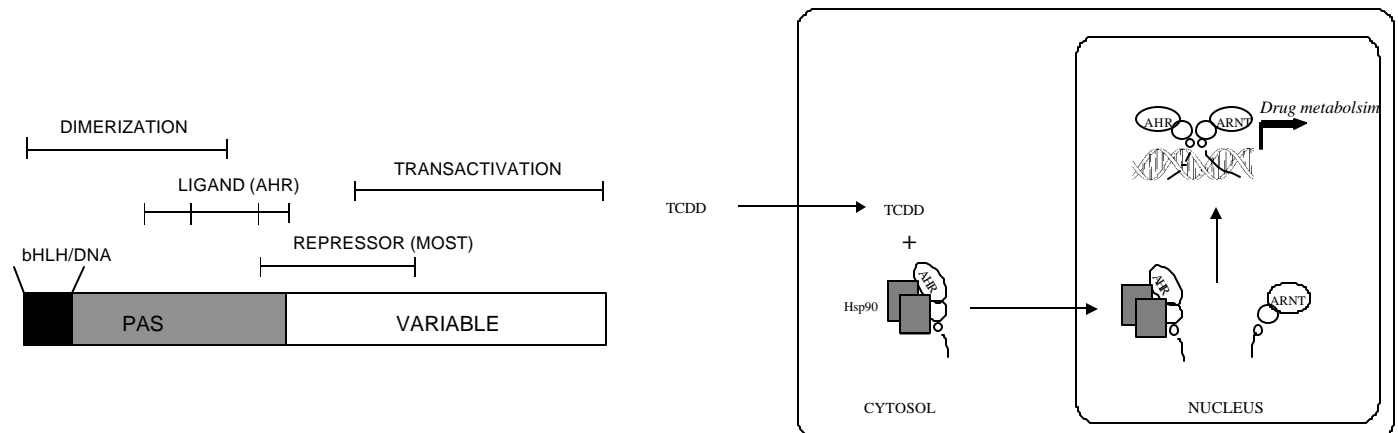
^a Female Wistar rats were given DEN (10 mg/kg body wt.) by stomach tube for 10 days. After a recovery period of 8 weeks, animals were either treated with TCDD (1.4 µg/kg body wt.; s.c. injections at bi-weekly intervals) for the time periods indicated or served as vehicle controls (corn oil). Groups of animals were killed at 31, 73 or 115 days after start of TCDD treatment.

^b BrdU-labeling and apoptotic indices were determined as described previously [7]. Values represent means ± S.D. from 4-5 animals.

^c Significantly different from control ($P < 0.05$; Wilcoxon rank sum test).

Can knowledge of the TCDD signal transduction pathway shed any light?

Molecular cloning studies have demonstrated that the AHR is a member of the PAS superfamily of regulatory proteins. Like the Zn Finger receptor superfamily, PAS proteins also function as dimers that bind cognate enhancer sequences upstream of target promoters to upregulate batteries of genes. All PAS protein have the following domain structure and appear to signal in a manner similar to the AHR (see below). Note the similarities and differences with Zn Finger receptors. Also like Zn Finger receptors, none of the genes directly upregulated by their cognate response elements explains their effects on cell proliferation or tumor promotion.



Armed with the knowledge the ARNT is now also called Hif1? and that c4 mutant hepatoma cell lines are completely lacking this protein, provide your prediction of the signal transduction pathway for hypoxia-responsive angiogenesis as described by Maxwell et al. (your assigned reading). What genes are being upregulated and how?

Additional Reading:

Ah Receptor Signaling Pathways (1996). Ann. Rev. Cell Dev. Biol. 12:55-89.