Ribonuclease inhibits Ah receptor transformation *in vitro*

Ellen C. HENRY,* Kathleen A. HAYDEN, Patricia A. BAUMAN and Thomas A. GASIEWICZ
Environmental Health Sciences Center, University of Rochester Medical Center, Rochester, NY 14642, U.S.A.

The aryl hydrocarbon (Ah) receptor undergoes a ligand-dependent transformation to a heteromeric structure which has the ability to bind DNA sequence-specifically with high affinity. By this mechanism, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and related xenobiotics modify gene expression. We observed that transformation was inhibited *in vitro* by the presence of ribonuclease A (RNAase) during incubation of rat hepatic cytosol with TCDD. This effect was detected as a decreased ability of the TCDD–receptor complex to bind to calf thymus DNA covalently linked to Sepharose, and to a dioxin-responsive enhancer which is upstream of the cytochrome *P450IA1* structural gene. RNAase had no effect on previously transformed TCDD–receptor complexes. These observations indicated that RNAase acted during ligand binding and/or transformation of the Ah receptor. Saturation binding analyses demonstrated that RNAase decreased the receptor affinity for TCDD without changing the total number of binding sites. RNAase also inhibited transformation of the TCDD-bound, partially purified, untransformed, receptor. Thus RNAase does not interfere with ligand binding, but inhibits the subsequent transformation of the receptor monomer to the heteromeric, transcriptionally active, form.

INTRODUCTION

The aryl hydrocarbon (Ah) receptor is an intracellular gene regulatory protein which mediates the biochemical and toxic effects of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and related compounds (Poland & Knutson, 1982; Whitlock, 1987). Two *in vitro* assays have proved invaluable in characterizing Ah receptor functionality: (1) calf-thymus-DNA–Sepharose chromatography (Gasiewicz & Bauman, 1987), and (2) the gel mobility assay (Denison *et al.*, 1988a), using an oligonucleotide containing a specific dioxin-responsive element (DRE) located upstream from the structural gene for cytochrome *P450IA1*. Using these techniques we have distinguished several forms of the cytosolic receptor, differing in molecular mass, surface charge and affinity for DNA (Gasiewicz & Bauman, 1987; Henry *et al.*, 1989; Gasiewicz *et al.*, 1991). The unoccupied receptor is a large complex (~300 kDa in the rat) which does not bind DNA; two ligand-bound forms are detectable on DNA–Sepharose, but only the higher-affinity form binds to the specific DRE. It is this latter form which is also found in nuclei of TCDD-treated animals/cells and is presumed to be the transcriptionally active form (which we refer to as the transformed receptor). We have also determined that the ligand-bound monomeric receptor must associate tightly with at least one additional protein, forming a heteromeric structure which specifically recognizes the DRE (Gasiewicz *et al.*, 1991).

Structurally and functionally, the Ah receptor has many similarities with the receptors for steroid hormones, especially the glucocorticoids (Wilhelmsson *et al.*, 1986; Cuthill *et al.*, 1987), although no endogenous ligand for the Ah receptor is yet known. Numerous studies indicate that additional molecules associate with the ligand-binding subunit of the glucocorticoid receptor to mediate transformation and/or subsequent DNA binding of the receptor complex (reviewed by Bodine & Litwack, 1990). Results from several laboratories suggest that RNA may be one such component involved in the transformation of steroid hormone receptors (Kovacic-Milivojevic *et al.*, 1985; Kovacic-Milivojevic & Vedeckis, 1986; Rowley *et al.*, 1986; Kasayama *et al.*, 1987; Ali & Vedeckis, 1987a,b; Sablonniere *et al.*, 1988).

Thus it was of interest to determine whether there may be an analogous involvement of RNA with the Ah receptor. Furthermore, the presence of substantial TCDD specific binding in the DNA–Sepharose flow-through fractions during analysis of rat hepatic cytosol (Henry *et al.*, 1989) prompted us to examine methods to enhance Ah receptor binding to DNA. Since it was possible that RNA in the cytosol could compete non-specifically with the receptor for DNA-binding sites, one approach was to treat the cytosol with RNAase prior to chromatography. Whereas RNAase treatment of the activated glucocorticoid receptor enhances its binding to DNA–cellulose (Tymoczko & Phillips, 1983; Schmidt *et al.*, 1986), it has only marginal effects on Ah receptor binding to DNA–Sepharose (Henry *et al.*, 1989). However, we subsequently observed that RNAase inhibited transformation when it was present during TCDD binding. The goal of the studies reported here was therefore to further characterize the effects of RNAase on the Ah receptor.

EXPERIMENTAL

Chemicals

[1,6-³H]TCDD (38 Ci/mmol) was generously provided by Dr. Stephen Safe (Texas A&M University, College Station, TX, U.S.A.). In some experiments, [1,6-³H]TCDD (36 Ci/mmol) obtained from ChemSyn (Lenexa, KS, U.S.A.) was used. 2,3,7,8-Tetrachlorodibenzofuran (TCDF) was obtained from the National Institute of Environmental Health Sciences (Research Triangle Park, NC, U.S.A.). RNAase A (bovine pancreas, type IIIA, 70–98 units/mg), oxidized RNAase, RNAase S-protein, RNAase S-peptide, DNAase I (type IV), trypsin, soybean trypsin inhibitor, cytochrome *c*, lysozyme, calf thymus DNA and CNBr-activated Sepharose 4B were purchased from Sigma (St. Louis, MO, U.S.A.). The RNAase inhibitor Inhibit-Ace was purchased from 5 Prime–3 Prime, Inc. (West Chester, PA, U.S.A.).

Animals and tissue preparation

Male Sprague–Dawley rats (175–200 g) from Charles River (Wilmington, MA, U.S.A.) and DBA/2J mice from Jackson (Bar Harbor, ME, U.S.A.) were housed under laboratory con-

Abbreviations used: DRE, dioxin-responsive enhancer; K_D , apparent dissociation rate constant; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; TCDF, 2,3,7,8-tetrachlorodibenzofuran; TCDD ± TCDF, [³H]TCDD ± 100-fold excess of TCDF.

* To whom correspondence should be addressed.

ditions for at least 3 days prior to use, with a 12 h-light/12 h-dark cycle and free access to food and water. Hepatic cytosol (15–20 mg of protein/ml) was prepared as described previously (Gasiewicz & Bauman, 1987) and frozen at -80°C until use.

Incubations and determination of specific binding

Procedures for cytosol incubation with $[^3\text{H}]\text{TCDD} \pm$ a 100-fold excess of TCDF ($\text{TCDD} \pm \text{TCDF}$) were as described by Gasiewicz & Bauman (1987). All incubations were performed at 20°C . RNAase concentrations are indicated as units/ml; S-protein, S-peptide and oxidized RNAase were added at the molar equivalent of these RNAase concentrations. The activity of various RNAase preparations was determined essentially as described by Kunitz (1946). Specific binding was determined using hydroxyapatite (Gasiewicz & Neal, 1982). The method of Scatchard (1949) was used to estimate B_{max} and K_D values.

Assessment of DNA binding

Two *in vitro* methods were used to measure the ability of the TCDD–receptor complex to bind to DNA: (1) DNA–Sepharose chromatography, which can distinguish the different DNA-binding forms of the receptor (Henry *et al.*, 1989; Gasiewicz *et al.*, 1991), and (2) an electrophoretic gel mobility assay, using an oligonucleotide containing the DRE located upstream of the *P450IA1* gene, which can therefore detect sequence-specific receptor binding to DNA (Denison *et al.*, 1988a,b). DNA–Sepharose preparation, chromatographic conditions and analysis of eluted fractions were as described by Gasiewicz & Bauman (1987). Chromatography of crude cytosols incubated with $[^3\text{H}]\text{TCDD}$ and with $[^3\text{H}]\text{TCDD} + \text{TCDF}$ was performed concurrently on matched columns to obtain profiles of total and non-specific binding respectively. Specific binding, calculated as the difference between total and non-specific binding, is shown in all Figures. For some experiments, as indicated, a reconstituted system was used, consisting of partially purified untransformed receptor, isolated from rat hepatic cytosol by DNA–Sepharose chromatography, plus hepatic cytosol from DBA/2J mice, as described by Henry & Gasiewicz (1991). Transformation was evaluated by DNA–Sepharose chromatography following incubation of these mixtures for 2 h at 20°C . In this case a DNA–Sepharose column was attached to an f.p.l.c. system (Pharmacia–LKB), as described by Henry & Gasiewicz (1991). The gel retardation assay was performed using a synthetic oligonucleotide containing a single consensus sequence: 5'-TTGCGTG-3'. The complete sequences of the oligonucleotides are given in Gasiewicz *et al.* (1991). Cytosol ($\sim 90 \mu\text{g}$ of protein) was mixed with poly(dI,dC), as indicated in the Figures, plus $(3-4) \times 10^4$ c.p.m. of ^{32}P -labelled oligonucleotide (and adjusted to 0.07 M-NaCl). Samples were then analysed by non-denaturing PAGE and autoradiography, as described by Denison *et al.* (1988a).

RESULTS AND DISCUSSION

Effect of RNAase on the DNA–Sepharose profile

As we have previously shown, $[^3\text{H}]\text{TCDD}$ specific binding in rat hepatic cytosol after a 2 h incubation at 20°C is eluted principally at 0.28–0.33 M-NaCl as peak 2, the higher-affinity DNA-binding form of the receptor (see Fig. 1a). In contrast, after 2 h at 4°C , the major peak of specific binding is peak 1, which has a lower DNA affinity and is eluted at ~ 0.15 M-NaCl (Gasiewicz & Bauman, 1987; Henry *et al.*, 1989). We additionally have qualitative evidence that the transformed peak (peak 2) also has greater stability and higher affinity for TCDD than has the

untransformed receptor (peak 1) (Kester, 1987; Henry *et al.*, 1988; Rucci & Gasiewicz, 1988). When RNAase was present during incubation with $\text{TCDD} \pm \text{TCDF}$, peak 2 was dramatically decreased (Fig. 1b). A similar profile was observed when RNAase was added to cytosol 2 h before addition of TCDD (results not shown). Addition of RNAase after TCDD binding and transformation were complete elicited no such effect on the DNA–Sepharose profiles of specific binding (Fig. 1c), although there was a slight increase in non-specific binding in the region of peak 1 (results not shown). Thus the Ah receptor appeared to be sensitive to RNAase only before and/or during ligand binding and transformation; furthermore, the profile in Fig. 1(c) confirms that the presence of RNAase during chromatography cannot account for the alterations seen in Fig. 1(b).

RNAase inhibits binding to the DRE

Co-incubation of rat hepatic cytosol with RNAase and TCDD also inhibited the binding of the TCDD–receptor complex to its specific DRE, as determined using a gel retardation assay (Fig. 2). In five separate experiments it was observed that RNAase

Fig. 1. Effect of RNAase on the DNA–Sepharose profile

(a) Rat hepatic cytosol (~ 15 mg of protein/ml) was incubated for 2 h at 20°C with 3 nM- $[^3\text{H}]\text{TCDD} \pm 300$ nM-TCDF (control). (b) RNAase (0.2 unit/ml) was added simultaneously with $\text{TCDD} \pm \text{TCDF}$ as above for 2 h, or (c) RNAase was added after the 2 h incubation with $\text{TCDD} \pm \text{TCDF}$ for a further 2 h at 20°C . All cytosols were analysed by DNA–Sepharose chromatography. Only specific binding is shown ($\blacksquare$). The gradient of NaCl is also shown ($\triangle$). The effects of RNAase at 2 and 20 units/ml were essentially the same as indicated in (b) or (c). When cytosol was incubated for 2 h with RNAase prior to adding $\text{TCDD} \pm \text{TCDF}$ for a further 2 h incubation, the profile was the same as shown in (b).

Fig. 2. RNAase inhibits the sequence-specific DNA binding of the Ah receptor

Rat hepatic cytosol was incubated with TCDD in the absence (lane 2) or the presence (lanes 3–8) of RNAase at the indicated concentrations (units/ml) for 2 h prior to mixing with 480 ng of poly(dI,dC) and 32 P-labelled oligonucleotide for analysis by gel retardation. Lane 1, cytosol without TCDD or RNAase; lane 9, RNAase was preincubated with Inhibit-Ace before addition to cytosol at a final concentration of 0.5 unit/ml; lane 10, cytosol incubated with TCDD for 2 h prior to addition of RNAase (0.5 unit/ml) for a further 2 h incubation; lane 11, cytosol incubated 2 h with RNAase (0.5 unit/ml) without TCDD. The upper arrow indicates the position of the TCDD-dependent band, and F indicates the 32 P-labelled probe.

concentrations below 0.05 unit/ml were essentially without effect; a maximal or near-maximal response occurred at 0.2 unit/ml ($\sim 0.13 \mu\text{M}$) and above. This effect was abolished by the RNAase inhibitor Inhibit-Ace (Fig. 2, lane 9). The faster-migrating band showing enhanced intensity in lanes which contained effective concentrations of RNAase was not TCDD-dependent, since the same phenomenon was observed in RNAase-treated cytosol that was not exposed to TCDD (Fig. 2, lane 11). RNAase (0.01–2 units/ml) added to previously transformed cytosol was without effect on receptor binding to its specific sequence (shown at 0.5 unit/ml in Fig 2, lane 10), consistent with the observations on DNA–Sepharose (Fig. 1c).

Specificity for RNAase

RNAase S-protein is a proteolytic fragment (RNAase amino acids 21–124) which contains an RNA-binding site, but lacks RNAase catalytic activity (Richards & Vithayathil, 1959; Richards & Wyckoff, 1971). When S-protein (13 μM) was present during incubation of cytosol with TCDD $\pm$ TCDF, the DNA–Sepharose profile lacked peak 2 (Fig. 3a). S-protein at the molar equivalent of RNAase of 20 units/ml ($\sim 13 \mu\text{M}$), but not at lower concentrations, also inhibited TCDD–receptor binding to the DRE (Fig. 4, lanes 5–9). S-peptide, a proteolytic RNAase fragment (amino acids 1–20), and oxidized RNAase had no effect on the DNA–Sepharose profile (Figs. 3b and 3c) or gel retardation (results not shown). The failure of lysozyme (Fig. 3d)

and cytochrome *c* (results not shown), low molecular mass basic proteins, to alter DNA–Sepharose profiles suggests that the action of RNAase is not likely to be a non-specific effect due to its size or overall surface charge. The addition of DNAase (10 $\mu\text{g}/\text{ml}$) to the incubation also did not alter the DNA–Sepharose profile of specific binding (results not shown). The effect of RNAase on the DNA–Sepharose profile was blocked by the RNAase inhibitor Inhibit-Ace (results not shown). Boiling RNAase for 10 min did not substantially affect RNAase catalytic activity; boiled and unboiled preparations had similar effects on DNA–Sepharose and gel retardation (compare Fig. 1b and Fig. 4, lanes 3 and 4), suggesting that contaminating protease activity is not responsible for the observed effects. RNAase treated with trypsin followed by trypsin inhibitor prior to the incubation with cytosol and TCDD also inhibited DRE binding (Fig. 4, lane 10), consistent with the reported insensitivity of RNAase to trypsin (Richards & Vithayathil, 1959; Richards & Wyckoff, 1971). Although S-protein is reported to be trypsin-sensitive (Richards & Vithayathil, 1959; Richards & Wyckoff, 1971), trypsin did not destroy its inhibitory effect (Fig. 4, lane 11). We conclude that the observed effects of RNAase on the Ah receptor are specific for this enzyme. Since S-protein was effective only at much higher concentrations, and retained activity even after trypsin treatment (Fig. 4), we suggest that contaminating RNAase activity (0.5–1.6 units/mg of S-protein) may account for its transformation inhibitory effect, or that it acts by a mechanism unrelated to RNA binding.

Effect of RNAase on Ah receptor affinity (K_D) and number (B_{max})

Specific binding of [^3H]TCDD (3 nM) in crude cytosol as determined by the hydroxyapatite assay was decreased by approx. 35–40% in those samples co-incubated with RNAase (results not shown). Some, but not all, of the decrease in specific binding was attributable to increased non-specific binding that was measurable by both hydroxyapatite and DNA–Sepharose. However, if RNAase were only blocking TCDD binding to the receptor, one would expect a quantitative effect on specific binding without necessarily a qualitative change in the DNA–Sepharose profile. The finding that both were altered suggests that RNAase prevents transformation of the receptor to the form which has higher affinity for TCDD and DNA.

An analysis of specific binding at various concentrations of TCDD indicated that RNAase (2 and 0.2 units/ml, but not 0.02 unit/ml) and S-protein, but not oxidized RNAase, increased the K_D value without affecting B_{max} (Fig. 5). Three repetitions of this protocol, using the same cytosol preparation, gave mean values ($\pm$ S.D.) of K_D (nM) as follows: control, 0.11 ± 0.01 ; RNAase, 0.22 ± 0.01 ; S-protein, 0.17 ± 0.01 ; oxidized RNAase, 0.12 ± 0.01 . While estimates of K_D values for ligand binding to the Ah receptor determined by Scatchard analysis are directly related to the protein concentrations used (Farrell & Safe, 1987; Bradfield *et al.*, 1988), the effect of RNAase was qualitatively similar at all protein concentrations tested: the K_D value was increased while B_{max} was unaltered (results not shown). Although the change in K_D was not large, it was statistically significant ($P < 0.001$), and in several cytosol preparations it was consistent, as was the lack of effect of oxidized RNAase. The effect of S-protein (0.13 μM) was intermediate. RNAase therefore decreases the affinity of the Ah receptor for TCDD without affecting the total receptor number; this is consistent with the observed inhibition of formation of the higher-affinity form of the receptor (peak 2), as well as with the decreased specific binding detectable by hydroxyapatite at a non-saturating concentration of TCDD, as noted above.

Fig. 3. Effects of S-protein (a), S-peptide (b), lysozyme (c) and oxidized RNAase (d) on the DNA-Sepharose profile

Cytosol was incubated with TCDD $\pm$ TCDF plus the indicated protein, added at 13 μ M, equivalent to RNAase at 20 units/ml. ■, Specific binding; △, NaCl concentration.

Fig. 4. Gel retardation assay of Ah receptor exposed to S-protein, boiled RNAase or trypsin-treated RNAase

Cytosol was co-incubated for 2 h with TCDD and RNAase (R) at 20 units/ml (lane 3), boiled RNAase (BR) (lane 4), S-protein (lanes 5–9, at molar equivalent concentration of indicated units/ml), or RNAase or S-protein (lanes 10 and 11 respectively) pretreated with trypsin followed by trypsin inhibitor. Lane 1, cytosol without TCDD; lane 2, cytosol incubated with TCDD alone. All samples were mixed with 200 ng of poly(dI,dC) and 32 P-oligonucleotide and were analysed by electrophoresis. The upper arrow indicates the position of the TCDD-dependent band, and F indicates the 32 P-labelled probe.

Effect of RNAase on reconstituted transformation

We have recently demonstrated that the process of Ah receptor transformation is separable from ligand binding (Henry & Gasiewicz, 1991). When untransformed receptor is partially purified (as peak 1 on DNA-Sepharose), it can be subsequently transformed in the presence of an additional cytosolic protein. The most convenient source of this factor is DBA/2J mouse liver, since this cytosol contains undetectable TCDD specific binding under these conditions. Similarly, transformation is inhibited in rat hepatic cytosol by pretreatment with activated

charcoal, and can be regenerated by DBA cytosol, as above (Henry & Gasiewicz, 1991). When RNAase was added at the time of mixing of DBA cytosol with peak 1 (Fig. 6) or charcoal-treated cytosol (results not shown), this reconstituted transformation was inhibited. The RNAase inhibitor Inhibit-Ace prevented this effect (Fig. 6c). Thus RNAase inhibits transformation even after the ligand is bound to the receptor monomer, indicating that it acts during the transformation process and not during ligand binding. Furthermore, since the transformed receptor has higher affinity than the untransformed form for TCDD, this result is consistent with the observed decrease in affinity, without a change in B_{max} , in crude cytosol treated with RNAase (Fig. 5).

These studies constitute the first report of inhibition of Ah receptor transformation by RNAase. Both DNA-Sepharose chromatography and binding to the specific DRE indicated that RNAase prevented formation of the transcriptionally active form of the receptor. This effect was not seen with a number of other proteins of similar charge and size. Additionally, based on experiments using S-protein as well as RNAase, we conclude that the transformation-inhibitory effect of RNAase is probably not simply due to the RNA-binding capability of this protein. These studies suggest that the effects of RNAase on the Ah receptor and steroid receptors may not be analogous. Notably, RNAase and S-protein have been reported to enhance DNA-cellulose binding of preactivated glucocorticoid receptors by up to several-fold (Tymoczko & Phillips, 1983; Schmidt *et al.*, 1986). Based on differential sensitivities to RNAase, as well as the effects of adding back RNA to different forms of the glucocorticoid receptor, it has been suggested that RNA becomes associated with an intermediate form of this receptor (Kovacic-Milivojevic *et al.*, 1985; Kovacic-Milivojevic & Vedeckis, 1986; Tymoczko *et al.*, 1987; Kasayama *et al.*, 1987; Ali & Vedeckis, 1987a). Although our results do not directly answer the question of whether RNA is associated with the Ah receptor itself, they do indicate that: (1) ligand binding is not RNAase-sensitive, (2) DNA binding of the transformed receptor is not RNAase-sensitive, and (3) transformation of the ligand-bound monomeric intermediate form to the high-affinity transcriptionally active

Fig. 5. Scatchard analysis of TCDD binding in the absence and the presence of RNAase, S-protein or oxidized RNAase

Cytosol (2.6 mg of protein/ml) was incubated with 0.1–3 nM [3 H]TCDD $\pm$ TCDF in the absence (a) or the presence of RNAase (0.2 unit/ml) (b), or S-protein (c) or oxidized RNAase (d) at the molar equivalent concentration (0.13 μ M). Specific binding was determined by hydroxyapatite assay. (a) $K_D = 0.11$ nM, $B_{max} = 118$ fmol/mg, $r = 0.99$; (b) $K_D = 0.23$ nM, $B_{max} = 114$ fmol/mg, $r = 0.96$; (c) $K_D = 0.16$ nM, $B_{max} = 114$ fmol/mg, $r = 0.97$; (d) $K_D = 0.13$ nM, $B_{max} = 116$ fmol/mg, $r = 0.97$.

form is inhibited by RNAase. We have previously shown that transformation requires formation of a heterodimer comprising the ligand-binding monomeric subunit (peak 1) and another cytosolic protein (Gasiewicz *et al.*, 1991; Henry & Gasiewicz, 1991). Our present studies imply that this step is RNAase-sensitive; however, it cannot be determined whether RNA is associated with either of the subunits or whether a third cytosolic component may be involved in transformation and may be RNAase-sensitive. This third component could even be RNA itself. The availability of purified receptor subunits will help to

Fig. 6. RNAase inhibits reconstituted transformation

Untransformed receptor (peak 1) was partially purified from TCDD-labelled rat hepatic cytosol by DNA-Sepharose chromatography (Henry & Gasiewicz, 1991). This preparation was mixed with DBA/2J mouse hepatic cytosol for 2 h in the absence (a) or the presence (b) of RNAase at 0.2 unit/ml prior to DNA-Sepharose chromatography. In (c), RNAase was preincubated (10 min) with Inhibit-Ace prior to mixing with peak 1 and DBA cytosol as for (b). $\blacksquare$, 3 H radioactivity; $\triangle$, NaCl concn. The 3 H appearing in the flow-through (fractions 1–15) is known from other experiments to represent non-specific binding; in the remainder of the profile, non-specific binding is very low compared with the total shown.

clarify the potential role of RNA/RNAase in Ah receptor regulation.

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