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The specific DNA binding activity of the dioxin receptor is modulated by the 90 kd heat shock protein

Anna Wilhelmsson, Scott Cuthill, Marc Denis, Ann-Charlotte Wikström, Jan-Åke Gustafsson and Lorenz Poellinger

Department of Medical Nutrition, Karolinska Institute, Huddinge University Hospital F-60, Novum, S-141 86 Huddinge, Sweden

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The dioxin receptor is a gene regulatory protein which exhibits many structural and functional similarities to steroid hormone receptors. In this study we compare the subunit composition of two forms of the dioxin receptor, sedimenting at ~9S and ~6S respectively, which are present in nuclear extract from wild-type Hepa 1c1c7 mouse hepatoma cells following treatment *in vivo* with dioxin. The nuclear ~9S receptor form contained the 90 kd heat shock protein, hsp90. As assessed by a gel mobility shift assay, this receptor form did not bind to the xenobiotic response element (XRE) of the target gene cytochrome P-450 IA1. In contrast, the smaller ~6S receptor form did not contain any immunochemically detectable hsp90. Moreover, this receptor form specifically bound to the XRE recognition sequence. Thus, the specific DNA binding activity of the dioxin receptor was inhibited by association with hsp90, and the ~9S dioxin receptor species could be regarded as a nonactive receptor form. Neither the ~9S nor the ~6S receptor forms were detected in nuclear extract from a dioxin treated mutant clone of Hepa 1 that expresses a nuclear translocation deficient receptor phenotype. We conclude that activation of the dioxin receptor is, at least, a two step process involving binding of the ligand and dissociation of hsp90 from the ligand-binding receptor protein. Inhibition of the DNA binding activity of transcription factors by protein–protein interaction has also been described for several steroid hormone receptors and for the NFκB factor. Finally, the presence of hsp90 in the nuclear extract suggests that hsp90 may play a role in modulation of the DNA binding activity of the receptor within the nucleus.

Key words: dioxin receptor/hsp90/protein–DNA interaction/protein–protein interaction/transcription factors

Introduction

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) is a model compound for a large group of halogenated aromatic hydrocarbons producing a variety of toxic effects in experimental animals (Poland and Knutson, 1982). The effects of TCDD and its congeners are mediated by the intracellular, soluble dioxin receptor protein that binds TCDD saturably and with high affinity (Poland *et al.*, 1976). At the biochemical level the best characterized cellular response to TCDD is the induction of cytochrome P-450IA1 (for discussion of P-450 nomenclature, see Nebert *et al.*, 1989). This induction is caused by an increased transcription rate of the P-450IA1 gene and requires the presence of functional dioxin receptor (Gonzalez *et al.*, 1984; Israel and Whitlock, 1984). More recent studies have shown that short *cis*-acting enhancer-like regulatory sequences, termed xenobiotic response elements (XREs, Fujisawa-Sehara *et al.*, 1986, 1987) confer dioxin inducibility on target genes and are recognized by the dioxin receptor *in vitro* (Denison *et al.*, 1988; Fujisawa-Sehara *et al.*, 1988; Hapgood *et al.*, 1989).

The mechanism of action of dioxin, as outlined above, is strikingly similar to the mechanism of action of steroid hormones. Steroid hormones also exert their effects by first interacting with soluble, intracellular receptor proteins. The steroid hormone receptors then undergo a process, termed activation or transformation, that enables them to interact with steroid response elements, thereby altering the transcription rate of target genes (reviewed in Yamamoto, 1985). Apart from the mechanistic similarities between the dioxin and steroid hormone receptor systems, several physicochemical properties of the dioxin receptor are similar to those of steroid hormone receptors, most notably the glucocorticoid receptor (Denison *et al.*, 1986; Wilhelmsson *et al.*, 1986; Cuthill *et al.*, 1987). Both receptors can be detected as large, M_r ~300 000 non-DNA binding complexes in cytosolic preparations (Sherman and Stevens, 1984; Wilhelmsson *et al.*, 1986). The large form of the glucocorticoid receptor has been shown to be a heteromeric complex containing one steroid binding M_r ~94 000 subunit and two molecules of the 90 kd heat shock protein hsp90 (Joab *et al.*, 1984; Denis *et al.*, 1987). During the activation process hsp90 dissociates from the steroid binding receptor subunit (Tienrungroj *et al.*, 1987 and references therein; Denis *et al.*, 1988b) thus exposing the DNA binding surface of the protein. We have investigated the role of hsp90 in modulating the DNA binding activity of the dioxin receptor in wild-type Hepa 1c1c7 mouse hepatoma cells and a mutant clone (c4; Legraverend *et al.*, 1982; Hankinson, 1983) which expresses similar levels of dioxin receptor compared with parental Hepa 1c1c7 cells but is deficient in nuclear accumulation of the receptor. To this end, we have developed a rapid procedure to separate a heteromeric, hsp90-containing dioxin receptor complex from an hsp90-free dioxin receptor form. Specific binding of the receptor to the XRE sequence can only be demonstrated following dissociation of hsp90 from the ligand binding receptor subunit. Thus, hsp90 appears to generally inhibit receptor action, and activation of the dioxin receptor represents a complex process involving ligand binding and dissociation of hsp90 prior to association with target DNA sequences.

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Results

FPLC anion exchange chromatography of cytosolic and nuclear dioxin-receptor complexes

To determine biochemical properties of cytosolic and nuclear forms of the dioxin receptor, cytosolic and nuclear extracts from Hepa 1c1c7 cells were analyzed by FPLC anion exchange chromatography. Following labeling of cytosol from untreated cells with [^{3}H]TCDD the dioxin receptor was recovered as a single peak of radioactivity from Mono Q anion exchange columns. The cytosolic dioxin receptor appeared to have a relatively high affinity for the resin reflected in elution at 40 mS/cm (~0.35 M KCl) (Figure 1A). Analysis of the Mono Q peak fraction on sucrose density gradients containing 0.4 M KCl revealed an ~9S peak with a small plateau at ~5–6S (Figure 1B). The sedimentation properties of the cytosolic dioxin receptor eluted from Mono Q were identical to those seen when dioxin receptor in crude cytosol was analyzed under the same conditions (Figure 1B). When crude cytosolic receptor was analyzed on gradients containing 0.15 M KCl, only the ~9S peak was seen (data not shown). However, the KCl concentration in the sucrose density gradient (0.15 or 0.4 M respectively) did not influence the sedimentation position of cytosolic dioxin receptor eluted from Mono Q. In conclusion, prolonged manipulation of the receptor by FPLC chromatography and sucrose density gradient centrifugation only induced a very small amount of receptor sedimenting as a plateau at ~5–6S. Thus, unlike steroid receptors in general (Sherman and Stevens, 1984) the large ~9S dioxin receptor form was relatively stable to elevated ionic strength.

Dioxin receptor present in nuclear extract from [^{3}H]-TCDD-treated Hepa 1c1c7 cells was also analyzed by FPLC anion exchange chromatography. This resulted in a different elution profile of nuclear receptor compared with the cytosolic species. The nuclear receptor was recovered in two peaks from Mono Q columns (Figure 2A), one eluting at 27 mS/cm (~0.23 M KCl), and the other eluting at 40 mS/cm (~0.35 M KCl). Each peak contained ~50% of totally recovered receptor. To ensure minimal contamination of the nuclear extract with cytosolic dioxin receptor, nuclei were extensively washed prior to extraction. As a control for the level of cytosolic contamination, nuclear extract from the c4 nuclear translocation deficient cells was subjected to anion exchange chromatography. In contrast to the elution profile of wild type receptor, no protein bound [^{3}H]TCDD could be detected in Mono Q fractionated nuclear extract from *in vivo* treated c4 cells (data not shown). A heterogeneous population of ~6S and ~9S receptor forms were also detected by sedimentation analysis at low ionic strength of crude nuclear extract from wild type cells (Figure 2B). At higher ionic strength (0.15–0.4 M KCl), only the ~6S species was observed following the prolonged sucrose gradient centrifugation (Hapgood *et al.*, 1989; data not shown). Our initial attempts at sedimentation analysis of nuclear material were hampered by extensive receptor aggregation in sucrose gradients prepared in low ionic strength. We found, however, that omission of dialysis of the nuclear extract prior to sucrose gradient centrifugation efficiently prevented receptor aggregation and facilitated detection of both the ~6S and ~9S receptor forms.

Sucrose density gradient analysis showed that the sedi-

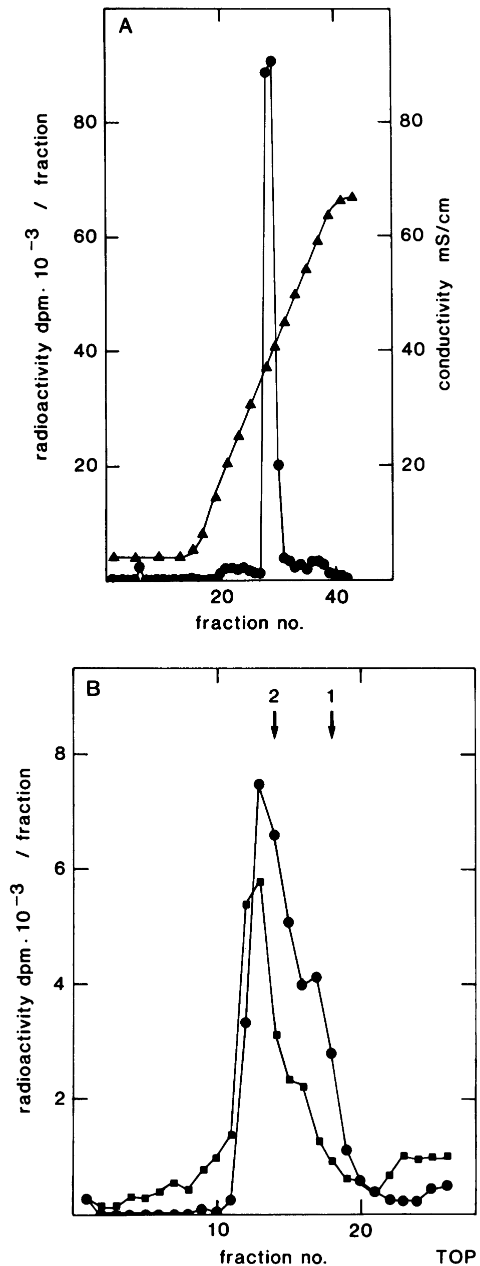

Fig. 1. Anion exchange chromatography and sedimentation properties of Hepa 1c1c7 cytosol labeled with [^{3}H]TCDD. Cytosol from untreated wild type Hepa 1c1c7 cells (~12 mg protein), was labeled with 3 nM [^{3}H]TCDD for 2 h on ice. Unbound ligand was removed by dextran coated charcoal treatment. (**A**) Labeled material was applied on a Mono Q column equilibrated in EPM buffer. Retained material was eluted with a linear 0–0.6 M KCl in EPM gradient at a flow rate of 0.5 ml/min. 1 ml fractions were collected and assayed for radioactivity (●) and conductivity (▲). (**B**) Sedimentation properties of 50 μl of crude Hepa 1c1c7 cytosol (4 mg of protein/ml) labeled with [^{3}H]TCDD and diluted to 200 μl with buffer EPGM (■) and 200 μl of [^{3}H]TCDD labeled cytosolic material eluted from Mono Q (●). Samples were analyzed on 10–40% (w/v) sucrose gradients prepared in EPGM buffer containing 0.4 M KCl. Sedimentation markers, 1 (bovine serum albumin 4.4S) and 2 (aldolase 7.9S), were simultaneously analyzed on separate gradients. Due to the very low level of nonspecific (non-receptor) binding of [^{3}H]TCDD in Hepa 1c1c7 cells (Cuthill *et al.*, 1987) control experiments performed in the presence of excess non-radiolabeled ligand have not been included in the figures.

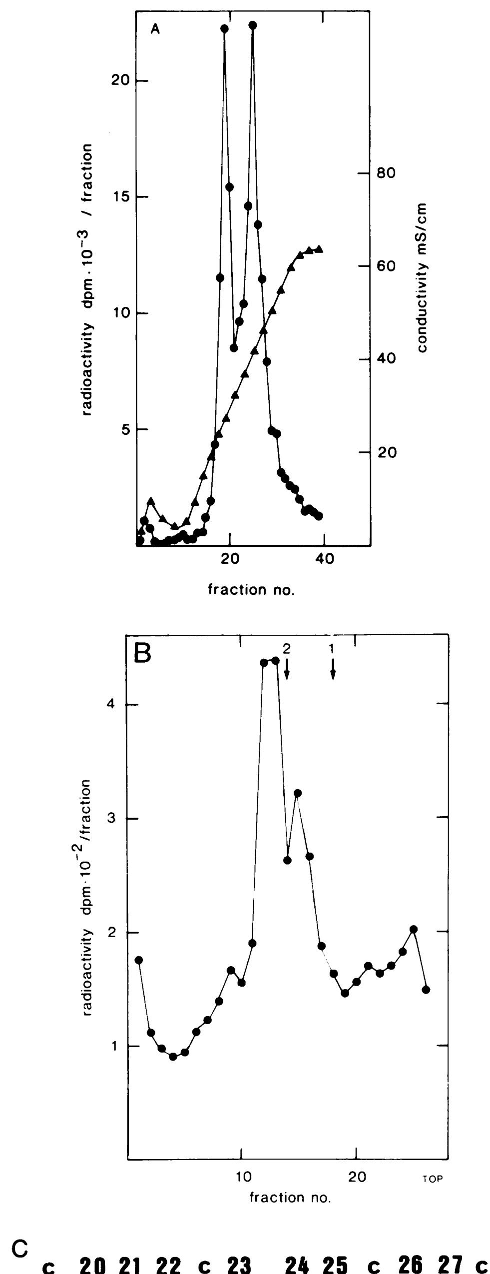

Fig. 2. Anion exchange chromatography and sedimentation analysis of nuclear extract prepared from [^{3}H]TCDD induced wild type Hepa 1c1c7 cells. (**A**) Nuclear extract was prepared as described in Materials and methods. 2 ml (~6 mg protein) was applied on a Mono Q column equilibrated in EPM buffer. Retained material was eluted with a 0–0.6 M KCl gradient in buffer EPM at a flow rate of 0.5 ml/min. 1 ml fractions were collected and assayed for radioactivity (●) and conductivity (▲). (**B**) Sedimentation properties of 200 μl of nondialyzed crude nuclear extract (●) on 10–40% (w/v) sucrose gradients prepared in EPGM buffer containing 50 mM KCl. The same sedimentation markers as in Figure 1 were used. (**C**) Analysis of hsp90 content of Mono Q fractionated nuclear extract. Aliquots (100 μl) of the indicated fractions were analyzed on SDS–polyacrylamide gels, transferred to nitrocellulose membranes and assayed for hsp90 content with an immunoblot procedure described in Materials and methods. Lanes marked C contain 100 μg of rat liver cytosolic protein.

mentation properties of the nuclear receptor form present in treated wild type cells and eluting at 40 mS/cm from Mono Q were identical to those of the cytosolic dioxin receptor species (cf. Figures 1B and 3A). In contrast, the wild-type nuclear receptor form eluting at 27 mS/cm from Mono Q sedimented in the ~6S region of the gradient (Figure 3B).

Association of dioxin receptor with hsp90

A large, ~9S receptor has previously only been demonstrated in cytosolic preparations. The presence of an ~9S dioxin receptor species in fractionated wild type nuclear extract prompted us to examine a possible heteromeric composition of this complex. Since an ~9S cytosolic form of the dioxin receptor has been shown to be associated with hsp90 (Denis *et al.*, 1988c; Perdew, 1988), the ~9S nuclear receptor was analyzed for hsp90 content. Nuclear extract was chromatographed on Mono Q and aliquots of the obtained fractions were characterized with immunoblots (Figure 2C). In this assay, polyclonal hsp90 specific antibodies raised against purified rat hsp90 (Denis, 1988) were used. To avoid saturation of the anti-hsp90 antibodies with possible non-receptor associated hsp90 in the subsequent experiments, a Mono Q fraction (no. 25 in Figure 2A and C) rich in ~9S dioxin receptor but separated from the majority of the hsp90 immunoreactivity was selected for further characterization. Aliquots of this material were incubated with purified, lyophilized anit-hsp90 antibodies or control antibodies (rabbit anti-rat immunoglobulins) and analyzed on separate sucrose density gradients. Under these conditions, an anti-hsp90 antibody induced shift of the dioxin receptor to the bottom of the gradient tube was observed (Figure 3A). In contrast, when the dioxin receptor species in Mono Q fraction 19 (corresponding to the ~6S receptor species) (Figure 2A) was analyzed under identical conditions, no antibody-dependent shift in sedimentation of the nuclear receptor could be detected (Figure 3B). Thus, hsp90 was associated with the receptor form eluting at higher salt concentration (~0.35 M KCl) but not with the receptor form eluting at a lower concentration of KCl (~0.23 M) from Mono Q.

DNA binding properties of nuclear dioxin receptor eluted from Mono Q anion exchange columns

The two forms of nuclear dioxin receptor described above were assayed for specific DNA binding activity employing a gel mobility shift assay. As a specific DNA target sequence, a synthetic dioxin responsive element, XRE1 (Fujisawa-Sehara *et al.*, 1987; Hapgood *et al.*, 1989) derived from the rat cytochrome P-45OIA1 gene was used. Nuclear extract from wild type cells treated *in vivo* with [^{3}H]TCDD was separated on Mono Q columns to produce the two distinct receptor forms with maximal ligand binding activities in fractions 19 and 25 respectively (see Figure 2A). Gel mobility shift analysis of the obtained fractions showed a plethora of protein–DNA complexes. However, only fractions 18–20 containing the ~6S, hsp90-free receptor species showed the previously described (Fujisawa-Sehara *et al.*, 1988; Hapgood *et al.*, 1989) *bona fide* dioxin inducible protein–DNA complex (Figure 4A, complex indicated by an arrow). This complex was not detected by an identical analysis of nuclear extract from [^{3}H]TCDD

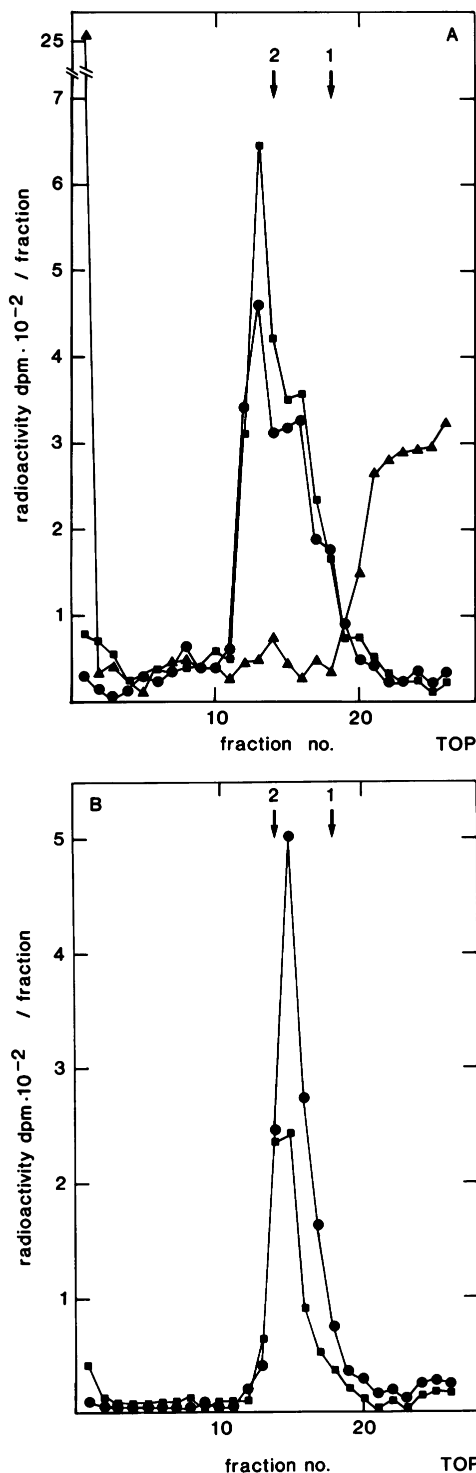

Fig. 3. Assay for dioxin receptor associated hsp90 in fractionated nuclear extract from [^{3}H]TCDD treated wild type Hepa 1c1c7 cells. Nuclear extract from [^{3}H]TCDD treated cells was prepared and analyzed on Mono Q as described in Materials and methods and in the legend to Figure 2. (**A**) Aliqots (250 μl, ~10 μg of protein) of [^{3}H]TCDD labeled material eluting at 40 mS/cm (~0.35 M KCl) were incubated for 45 min on ice in the presence of ~50 μg lyophilized anti-hsp90 antibodies (▲) or ~50 μg of rabbit anti-mouse immunoglobulins (●) or in the absence of antibody (■). (**B**) Aliquots (250 μl, ~10 μg of protein) of [^{3}H]TCDD labeled material eluting at 27 mS/cm (~0.23 M KCl) from Mono Q were incubated for 45 min on ice in the absence (●) or presence (■) of ~40 μg lyophilized anti-hsp90 antibodies. Samples were analyzed on 10–40% (w/v) sucrose gradients prepared in EPGM buffer containing 0.05 M KCl. The same sedimentation markers as in Figure 1 were used.

treated mutant c4 cells (Figure 4B), whereas the elution patterns of the residual DNA binding activities were very similar to those observed in wild type nuclear extract (Figure 4, cf. panels A and B). Formation of the dioxin induced protein–DNA complex in fraction 19 of wild type nuclear extract (see Figure 4A) was greatly reduced or inhibited by an excess of the unlabeled XRE1 oligonucleotide (Figure 4C, lanes 3 and 4), whereas an excess of an equally long, unrelated sequence containing the octamer element of the BCL1 immunoglobulin heavy chain promoter (Poellinger and Roeder, 1989) showed little or no competition in the concentration range tested (Figure 4C, lanes 5 and 6). Addition of anti-hsp90 antibodies to the reaction mixture did not change the mobility of the XRE1 specific complex (data not shown). Interestingly, gel mobility shift analysis of aliquots from fractions 24–26 of Mono Q separated wild type nuclear extract (containing the ~9S, hsp90 associated wild type receptor form; see Figures 2 and 3) did not indicate the presence of the XRE specific protein–DNA complex (Figure 4A). After prolonged exposure of the film, a very faint band with the same mobility and binding specificity as the retarded band indicated by an arrow in Figure 4A could be detected (data not shown). It was estimated that this complex constituted <5% of the corresponding complex generated with the same amount of protein from fraction 19 of Mono Q separated wild type nuclear extract. Thus, several lines of evidence suggest that the XRE specific complex contains the dioxin receptor: (i) it is dioxin inducible (Fujisawa-Sehara *et al.*, 1988; Hapgood *et al.*, 1989); (ii) it is not detectable in nuclear extract of dioxin resistant, nuclear translocation deficient c4 cells; (iii) it co-chromatographs with the ~6S form of nuclear dioxin receptor; and (iv) it comigrates with the specific [^{3}H]TCDD binding activity during gel mobility shift analysis (Fujisawa-Sehara *et al.*, 1988; Nemoto *et al.*, 1989).

Crosslinking of proteins to XRE1

The M_r of the component(s) involved in formation of the specific XRE1–protein complex observed on gel shifts was estimated by chemical crosslinking analysis. Heat treated, [^{3}H]TCDD labeled Hepa 1c1c7 cytosol was used as dioxin receptor source since this allowed a larger scale up of the gel mobility shift incubations than fractionated nuclear extract. Heat treatment of wild type cytosol has previously been shown to induce an XRE specific protein–DNA complex with the same relative mobility as the XRE specific complex obtained with nuclear extract (see Discussion and Figure 5A). The specific protein–DNA complex (indicated by an arrow in Figure 5A) was excised subsequent to gel mobility shift analysis and incubated with formaldehyde. Analysis of the crosslinked material by SDS–PAGE and autoradiography revealed a single band migrating to a position indicative of an M_r of ~135 000 (Figure 5B).

Discussion

We (Denis *et al.*, 1988c) and others (Perdew, 1988) have recently shown that a large ~9S cytosolic form of the dioxin receptor contains hsp90. In this paper we show that an ~9S, hsp90 associated form of the receptor is also present in fractionated nuclear extracts of dioxin treated wild type cells. This receptor form represents a nonactive species incapable of interaction with the XRE1 target DNA sequence. How-

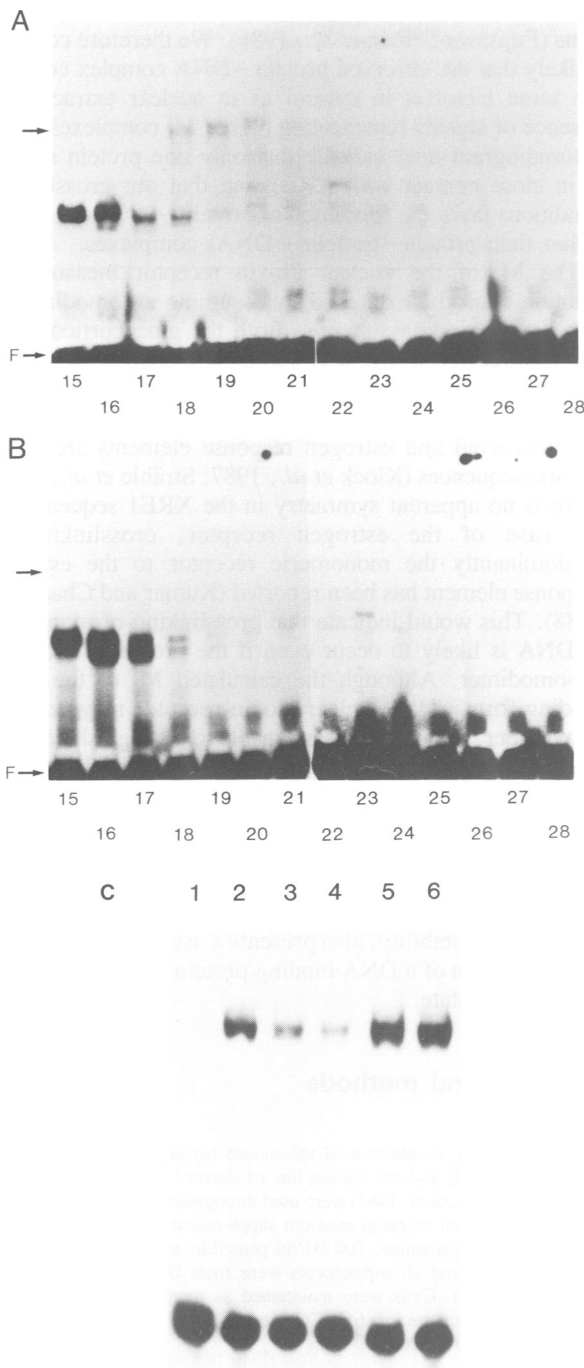

Fig. 4. Gel mobility shift analysis of fractionated nuclear extract from [^{3}H]TCDD treated wild type Hepa 1c1c7 and nuclear translocation deficient mutant Hepa c4 cells. Nuclear extracts were prepared and analyzed on Mono Q as described in Materials and methods and in the legend to Figure 2. Aliquots (10 μl) from each of Mono Q fractions 15 to 28 (see Figure 2A) were incubated with ^{32}P labeled XRE1 and analyzed on native polyacrylamide gels as described in Materials and methods. Results were visualized by autoradiography. Gel mobility shift analysis of Mono Q fractionated nuclear extract from (**A**) wild type or (**B**) mutant, translocation deficient cells. The arrow indicates the position of the dioxin induced, wild type cell specific protein−DNA complex. F indicates the position of the free probe. (**C**) Binding competition analysis of protein (3.7 μg) from fraction 19 of Mono Q fractionated wild type nuclear extract. **Lane 1**, no protein added; **lane 2**, binding reaction in the absence of any competitor DNA; **lanes 3** and **4**, binding reactions in the presence of 3- and 6-fold molar excess of unlabeled XRE1 respectively; **lanes 5** and **6**, binding reactions in the presence of 3- and 6-fold molar excess of unlabeled octamer element from the immunoglobulin heavy chain promoter.

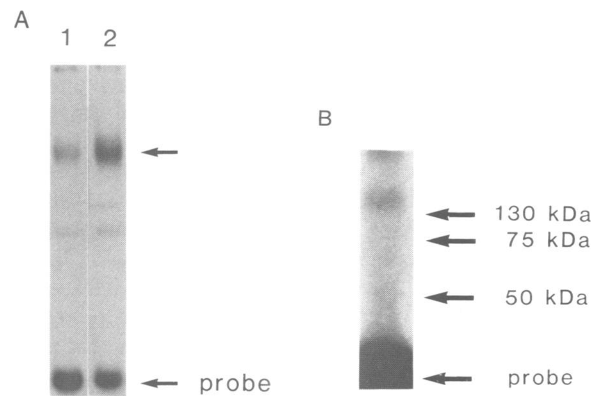

Fig. 5. Chemical crosslinking of protein to XRE1. Heat treated, [^{3}H]TCDD labeled wild type Hepa 1c1c7 cytosol was subjected to gel mobility shift analysis as described in Materials and methods. The XRE1 specific retarded band was excised and protein−DNA complexes crosslinked with 1% formaldehyde. Following electroelution and precipitation protein−DNA complexes were resolved on SDS−polyacrylamide gels. Probe denotes unbound XRE1 DNA. (**A**) Gel mobility shift analysis of [^{3}H]TCDD labeled, heat treated cytosol and ~6S nuclear dioxin receptor. **Lane 1**, ~6S nuclear dioxin receptor eluted from Mono Q (~2 μg of protein), **lane 2**, [^{3}H]TCDD labeled, heat treated cytosol (~14 μg of protein). Arrow indicates the XRE1 specific protein−DNA complex. (**B**) SDS−PAGE and autoradiography of formaldehyde crosslinked protein−XRE complexes. Prestained mol. wt makers (Bio Rad) were: 130 kd, phosphorylase b; 75 kd, bovine serum albumin and 50 kd, ovalbumin.

ever, it is also possible to detect an additional, hsp90 free dioxin receptor form sedimenting at ~6S, in nuclear extract of treated cells. This species, in turn, represents a DNA binding form of the receptor, specifically recognizing the XRE1 target DNA sequence. Therefore, release of hsp90 from the ligand binding receptor molecule seems to result in the unmasking of a cryptic DNA binding surface on the receptor. Alternatively, it is possible that a surface on the receptor is exposed which allows interaction with factor(s) that mediate the binding of receptor to XRE1. In any case, we can ascribe a functional significance to dioxin receptor−hsp90 interaction. The activation process of the dioxin receptor appears to be similar to the activation of the glucocorticoid receptor (Tienrungroj *et al.*, 1987; Denis *et al.*, 1988b and references therein) in that it (i) requires binding of ligand (Hapgood *et al.*, 1989; Fujisawa-Sehara *et al.*, 1988) and (ii) involves dissociation of hsp90. Taken together, these data, as well as similarities in crude biochemical and functional properties (Cuthill *et al.*, 1987; Cuthill and Poellinger, 1988), support the hypothesis that the dioxin receptor is related to the superfamily of steroid and thyroid hormone receptors.

Recently it has been shown that hsp90 interacts with the glucocorticoid receptor in the C-terminal, hormone binding domain of the receptor (Denis *et al.*, 1988a; Pratt *et al.*, 1988). Consistent with this finding, deletions of amino acids corresponding to the ligand binding domain yields receptor derivatives that constitutively modulate transcription (Godowski *et al.*, 1987). Thus, the C-terminal part of the glucocorticoid receptor carries not only the hormone binding function but is also, in its unliganded state, capable of repressing receptor action, presumably in an indirect manner via interaction with hsp90. Interestingly, the hormone binding domain retains its repressive function both in rearranged receptor derivatives and when fused to the completely unrelated adenovirus E1A transactivating protein

(Picard *et al.*, 1988). A similar mechanism for inactivation of the DNA binding function of a transcription factor has also been described for the immunoglobulin κ light chain enhancer binding protein NFκB (Baeuerle and Baltimore, 1988a,b). DNA binding of NFκB is not evident in non-lymphoid cells unless NFκB is first dissociated from the inhibitory protein IκB (Baeuerle and Baltimore, 1988b).

The role of hsp90, if any, in controlling nuclear translocation of steroid receptors is at present not known. In view of the role of hsp70 and related proteins in intracellular transport processes of unfolded precursor forms of proteins (Chirico *et al.*, 1988; Deshaies *et al.*, 1988), it is conceivable that hsp90 may have a similar function in the intracellular transport of receptors. Therefore, the nuclear translocation of a soluble receptor might not necessarily imply a preceding release of the receptor from hsp90 as has been postulated (Picard *et al.*, 1988). Instead, it possible that hsp90 modulates the DNA binding activity of the glucocorticoid and dioxin receptors within the nucleus. Hsp90 has generally been considered a cytosolic protein. In the case of the dioxin receptor, both hsp90 associated and hsp90 free forms were found in fractionated nuclear extract. Though this phenomenon might be explained by cytosolic contamination we regard this possibility unlikely since the nuclei were extensively washed prior to extraction and were shown by electron microscopy analysis to be essentially intact and contain maximally an ~20% contamination with extracellular debris (predominantly mitochondrial and microsomal material). More importantly, control experiments with nuclear extract from treated nuclear translocation deficient cells did not show any detectable cytosolic contamination. Moreover, immuno-cytochemical experiments employing optical sectioning by confocal laser microscopy also reveal the presence of a small but detectable pool of hsp90 in the cell nucleus (G.Akner, K.-G.Sundqvist, M.Denis, A.-C. Wikström and J.-Å.Gustafsson, unpublished). Finally, with regard to subcellular localization of both the dioxin and glucocorticoid receptors, recent evidence suggests a nuclear localization also for non-liganded receptor forms and that occupied and non-occupied receptors only differ in their affinity to nuclear structures (Whitlock and Galeazzi, 1984; Gasc *et al.*, 1989).

Formaldehyde crosslinking of the XRE1 specific protein–DNA complex generated a complex with an apparent M_r of ~135 000 (Figure 5B). The contribution of the DNA (calculated mol. wt ~25 000) to the electrophoretic mobility of the complex is difficult to assess but the crosslinked protein has an M_r lower than the 192 000–216 000 calculated for the nuclear dioxin receptor in solution (Hapgood *et al.*, 1989). The M_r of the crosslinked species is in the same range as that of the ligand binding M_r ~100 000 dioxin receptor monomer (Poellinger *et al.*, 1983; Poland and Glover, 1987; Perdew, 1988). The heat treated cytosol used in the crosslinking studies caused a gel shift with the same relative mobility as the gel shift seen with fractionated nuclear extract. It has previously been shown that specifically receptor bound [^{3}H]TCDD co-chromatographs/co-migrates with the XRE1 specific protein–DNA complex generated by both nuclear and cytosolic extracts (Hapgood *et al.*, 1989; Fujisawa-Sehara *et al.*, 1988). Furthermore, appearance of the XRE1 specific complex in nuclear extracts correlates with the depletion of the corresponding complex from cytosolic preparations (Fujisawa-Sehara *et al.*, 1988). We therefore consider it likely that the observed protein–DNA complex contains the same factor(s) in cytosol as in nuclear extract. The absence of signals representing higher M_r complexes in the autoradiogram may indicate that only one protein species is in close contact with XRE1 and that our crosslinking conditions favor the formation of protein–DNA complexes rather than protein–protein(–DNA) complexes.

The M_r of the nuclear dioxin receptor measured in solution could be explained by assuming a homodimer of two ligand binding subunits. Both the glucocorticoid and estrogen receptors have been reported to interact with DNA as homodimers (Kumar and Chambon, 1988; Tsai *et al.*, 1988; Wrange *et al.*, 1989). However, whereas the glucocorticoid and estrogen response elements are palindromic sequences (Klock *et al.*, 1987; Strähle *et al.*, 1987) there is no apparent symmetry in the XRE1 sequence. In the case of the estrogen receptor, crosslinking of predominantly the monomeric receptor to the estrogen response element has been reported (Kumar and Chambon, 1988). This would indicate that crosslinking of monomers to DNA is likely to occur even if the protein interacts as a homodimer. Although the calculated M_r of the DNA binding form of the nuclear dioxin receptor may reflect a homodimer, we are at present unable to distinguish between the possibilities that the receptor interacts with DNA as a monomer or a homo- or heterologous multimer.

The finding that hsp90 by protein–protein interaction efficiently inhibits the specific DNA binding activity of the dioxin receptor not only emphasizes the significant analogies between the dioxin and steroid receptor systems but, in view of its relative stability, also presents a useful model to study the conversion of a DNA binding protein from a non-active to an active state.

Materials and methods

Cells

Hepa 1c1c7 cells, a subclone of the mouse hepatoma cell line Hepa 1 (Hankinson, 1979) and the mutant line c4 derived from it (Legraverend *et al.*, 1982; Hankinson, 1983) were used throughout this study. Cells were grown in minimum essential medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 IU/ml penicillin and 100 μg/ml streptomycin. Medium and all supplements were from Gibco (Gibco Limited, Paisley, Scotland). Cells were maintained as monolayers at 37°C in a humidified atmosphere of 6% CO_2 in air and were grown to near confluence before harvest.

Preparation of cytosol and labeling conditions

Near confluent wild type cells were rinsed twice with cold phosphate buffered saline (pH 7.4) and harvested by scraping. The cells were suspended in one volume of cold buffer EPGM (1 mM EDTA, 20 mM potassium phosphate, 10% (w/v) glycerol, 2 mM 2-mercaptoethanol, pH 7.2), and homogenized in a Kontes all glass Dounce homogenizer (B type pestle). The homogenate was then centrifuged at 120 000 *g* for 45 min. The resulting clear supernatant was used as cytosol. Cytosol was immediately frozen and stored at −70°C until use. Cytosol was labeled with [^{3}H]TCDD (Chemsyn Science Laboratories, Lenexa, KS, USA) as previously described (Cuthill *et al.*, 1987). For gel mobility shift analyses cytosol was labeled with 10 nm [^{3}H]TCDD at 25°C for 2 h.

Preparation of nuclear extract

Wild type or mutant cells were treated with 1 nM [^{3}H]TCDD for 1 h at 37°C. Nuclear extract was prepared as described by Dignam *et al.* (1983) with the following modifications: (i) nuclei were washed with at least an additional 10 ml buffer A/ml cells prior to extraction; (ii) protein was extracted from nuclei for 45 min instead of 30 min and usually dialyzed to a final concentration of 0.15 M KCl; and (iii) the centrifugation after

dialysis was replaced with filtration through a filter of 0.2 μm pore size (Millipore, Molsheim, France). The nuclear extract was used immediately following preparation.

Sucrose density gradient centrifugation

200–300 μl of sample was layered onto 10–40% (w/v) linear sucrose gradients prepared in EPGM buffer containing 0.05 or 0.4 M KCl, as indicated. Gradients were centrifuged in an SW60Ti rotor in a Beckman L-8 55 M centrifuge at 260 000–300 000 g_{av} for 14–16 h to a preset cumulative centrifugal effect ($\omega^2 t$) of 1.70×10^2 rad^2/s. Fractions of ~150 μl were collected from the bottom of the tube by gravity flow. Following fractionation, the bottom of the tube was cut off and assayed for radioactivity together with the first fraction. ^{14}C-Labeled bovine serum albumin (4.4S) and aldolase (7.9S) were used as external sedimentation markers.

FPLC anion exchange chromatography

Anion exchange chromatography of labeled samples was performed on prepacked Mono Q HR 5/5 columns connected to two pumps P-500 via a mixing chamber (5 mPa). All buffers and samples were filtered through filters of 0.2 μm pore size (Millipore) before use. Samples were applied to the column equilibrated in EPM buffer (1 mM EDTA, 20 mM potassium phosphate, 2 mM 2-mercaptoethanol, pH 7.2) at a flow rate of 0.5 ml/min. The column was washed with 5 ml of EPM buffer followed by elution of retained material with 0–0.6 M KCl gradients in EPM buffer generated by a gradient programmer (GP-250). 1 ml fractions were collected and assayed for radioactivity and conductivity. All FPLC equipment was from Pharmacia (Uppsala, Sweden).

Affinity purification and monospecificity test of anti-hsp90 antibodies

The antibodies against the rat liver glucocorticoid receptor associated hsp90 described by Denis (1988) were used in this study. Specific anti-hsp90 antibodies were selected for by affinity chromatography on hsp90–Sepharose columns (Denis *et al.*, 1988a). To assess the specificity of the affinity purified antibodies 100 μg of rat liver cytosolic protein was run in parallel with 100 μg of Hepa 1c1c7 cytosolic protein on 7.5% SDS–polyacrylamide gels. The resolved proteins were transferred to nitrocellulose membranes which were then incubated with affinity purified anti-hsp90 antibodies. Adsorbed antibodies were detected with horseradish peroxidase conjugated goat anti-rabbit immunoglobulins (Denis, 1988). The antibody preparations thus shown to be monospecific both for rat and mouse hsp90 were transferred to 50 mM ammonium carbonate buffer (pH 7.4) using Sephadex G-25 PD-10 columns) Pharmacia Biotechnology, Uppsala, Sweden) and lyophilized before use in sucrose density gradient or gel mobility shift experiments.

Gel mobility shift assay

The gel mobility shift assay was performed essentially as described (Hapgood *et al.*, 1989). Wild type cytosol was labeled with 10 nM [^3H]TCDD for 2 h at 25°C. Nuclear extract from wild type or mutant cells was fractionated on a Mono Q FPLC anion exchange column. Aliquots of nuclear dioxin receptor from the indicated Mono Q fractions or from [^3H]TCDD labeled crude cytosol were incubated for 15 min on ice in the presence of 10 mM HEPES pH 7.9, 4 mM potassium phosphate pH 7.2, 10% glycerol (v/v), 0.12 mM EDTA, 0.5 mM DTT, 0.4 mM 2-mercaptoethanol, 3 mM $MgCl_2$, 100 mM KCl, 4 mM spermidine, 10 ng tRNA/μl, 0.5 ng sonicated salmon sperm DNA/μl, and 4 ng poly(dI.dC)/μl in a final volume of 49 μl. 2 fmol ^{32}P end labeled probe XRE1 was added in the absence or presence of unlabeled competitor oligonucleotide as indicated in Figure 4, and incubated for an additional 20 min at room temperature. Protein–DNA complexes were immediately analyzed on 4% polyacrylamide gels.

Crosslinking experiments

[^3H]TCDD labeled, heat treated Hepa 1c1c7 cytosol (40 μg protein) was incubated with 10 fmol ^{32}P labeled XRE1 as described above, and protein–DNA complexes were separated from free DNA by gel mobility shift assay. Complexed DNA was excised and immersed in 1% formaldehyde for 1 h at 4°C. Following this treatment gel pieces were quickly rinsed with distilled water. Protein–DNA complexes were electroeluted in a Bio Rad model 422 electroeluter according to instructions from the manufacturer. Eluted material was precipitated with trichloroacetic acid (final concentration 20%), and subjected to SDS–PAGE. The gel was dried and radiolabeled XRE1 was visualized by autradiography.

Safety precautions

Since TCDD is extremely toxic (Poland and Knutson, 1982) its use requires special handling procedures as outlined by Poland *et al.* (1976). Contaminated disposable materials were sent away for high temperature incineration.

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