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STRUCTURAL DIFFERENCES BETWEEN THE GLUCOCORTICOID, DIOXIN AND OXYSTEROL RECEPTORS FROM RAT LIVER CYTOSOL

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The rat hepatic glucocorticoid, dioxin and oxysterol receptors were subjected to high performance liquid chromatography on size-exclusion and anion-exchange columns. Both the glucocorticoid receptor and the dioxin receptor had a Stokes radius R_S ~ 7.5 nm, expected value for heteromeric complexes containing a dimer of the M_r ~ 90,000 heat shock protein, hsp90 (R_S ~ 7.0 nm). The oxysterol receptor represented a much smaller entity (R_S ~ 6.0 nm). When analyzed on a Mono Q anion-exchange column, the molybdate-stabilized glucocorticoid receptor and dioxin receptor eluted as single peaks at ~0.30 M and 0.26-0.28 M NaCl, respectively, whereas the oxysterol receptor represented a less negatively charged species (0.11-0.14 M NaCl). Following washing of the Mono Q column with molybdate-free buffer, the activated monomeric glucocorticoid receptor was detected (0.10-0.12 M NaCl). In contrast, no modification in the elution pattern of the dioxin receptor and the oxysterol receptor was observed. These data demonstrate differences in the physico-chemical properties of the glucocorticoid, dioxin and oxysterol receptors, respectively, which might reflect structural differences.

Steroid hormones produce their biological effect, *i.e.* the modulation of expression of specific genes, through binding to specific soluble proteins, called receptors (1,2). The glucocorticoid, mineralocorticoid, progesterone, estrogen and androgen receptors have been thoroughly characterized (3-7). Recently, the cDNA's encoding these receptors have been isolated and sequenced, and comparison of the

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Abbreviations used: GR, glucocorticoid receptor; DR, dioxin receptor; OR, oxysterol receptor; hsp90, M_r ~ 90,000 heat shock protein; TA, triamcinolone acetonide, 9α-fluoro-11ß, 21-dihydroxy-16α, 17α[(1-methylidene)-bis(oxy)]pregna-1, 4-diene-3, 20-dione; TCDD, dioxin, 2, 3, 7, 8-tetrachlorodibenzo-*p*-dioxin; TCDF, 2, 3, 7, 8-tetrachlorodibenzofuran; HPSEC, high performance size exclusion chromatography; HPIEC, high performance ion exchange chromatography.

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deduced amino acid sequences revealed a high degree of similarity between these proteins (8). Like steroid hormones, the environmental contaminant 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD, dioxin) exerts its biochemical effects, *e.g.* the induction of specific isozymes of cytochrome P-450, *via* an interaction with a soluble intracellular receptor protein (9). Steroid receptors and dioxin receptor, although they do not share any common ligand-binding characteristics (10), have similar physico-chemical properties (3,11,12). Thus, they are present in cytosolic preparations associated with the M_r ~ 90,000 heat shock protein (13-15) in a large ~9S complex, stabilized by sodium molybdate, which is unable to bind to DNA (3,11,16). Following ligand-binding, the hsp90 dissociates from the large complex, and the receptor thus released is able to interact with DNA *in vitro*.

Like for steroid hormones and dioxin, the oxygenated sterols seem to repress expression of 3-hydroxy-3-methylglutaryl (HMG)-CoA reductase, and thereby cholesterol biosynthesis in mammalian cells (17-19) by binding to a protein, previously called oxysterol-binding protein, and now referred to as oxysterol receptor. This receptor has been identified and characterized in human lymphocytes (20,21) and in mouse fibroblasts (22-24).

The characterization of these receptors has mainly been performed using conventional liquid chromatographic techniques. More recently, high performance liquid chromatographic methods have been shown to have interesting preparative (25,26) and analytical (27-29) applications to study the glucocorticoid receptor. We report here on the use of HPSEC and HPIEC to investigate the structure of the receptors for dioxin and oxysterol, in comparison to the glucocorticoid receptor.

MATERIALS and METHODS

Chemicals - [6, 7-^{3}H]Triamcinolone acetonide (25.9 Ci/mmol) and 25-[26,27-^{3}H]hydroxycholesterol (87 Ci/mmol) were from New England Nuclear. [1, 6-^{3}H]TCDD (28 Ci/mmol) was a generous gift from Dr A. Poland (Madison, WI). TCDF was kindly provided by Dr C. Rappe (Umea, Sweden). Unlabeled triamcinolone acetonide, unlabeled hydroxycholesterol and all other analytical grade products were obtained from Sigma.

Cytosol Preparation and Labeling Conditions - Livers from male Sprague Dawley rats were perfused and homogenized in ETG buffer (20 mM Tris-HCl, 1 mM EDTA, 10% glycerol, 2 mM dithiothreitol, pH 7.4) or in ETGMo buffer (20 mM Na_2MoO_4 in ETG buffer) as previously described (30). The cytosolic fraction was incubated with 100 nM [^{3}H]triamcinolone acetonide, 10 nM [^{3}H]TCDD, or 30 nM [^{3}H]hydroxy-cholesterol for 3 h at 4°C, in the absence or presence of a 200-fold molar excess of [^{1}H]triamcinolone acetonide, [^{1}H]TCDF, or [^{1}H]hydroxycholesterol, respectively. The excess of free ligand was removed by dextran-coated charcoal treatment of the samples (11) prior to chromatography.

High Performance Size-Exclusion Chromatography - Proteins were separated with an automated FPLC™ system (Pharmacia Biotechnology, Uppsala, Sweden) consisting of two P-500 pumps controlled by a GP-250 solvent controller. Buffers and samples were filtered through a 0.45-µm filter before use. Labeled samples of rat liver cytosol (0.5 ml) were injected on a Superose™ 12 HR 10/30 column equilibrated with ETMo buffer (20 mM Tris-HCl, 1 mM EDTA, 20 mM Na_2MoO_4, pH 7.4) containing 150

mM NaCl. The flow rate was 0.5 ml/min. Fractions (1 min) were collected in a FRAC-100 fraction collector, and assayed for radioactivity content. The column was calibrated with blue dextran (V_o), ^3H_2O (V_t), and the following proteins: 1, ß-galactosidase (R_s ~ 6.9 nm), 2, ferritin (R_s ~ 6.15 nm), 3, aldolase (R_s ~ 4.8 nm), 4, bovine serum albumin (R_s ~ 3.55 nm) and 5, myoglobin (R_s ~ 2.0 nm). The distribution coefficients were calculated from $K_D^{1/3}$ *vs* R_s according to Porath (31).

High Performance Ion-Exchange Chromatography - Proteins were separated according to their charge by chromatography on a Mono Q™ HR 5/5 column connected to the FPLC system described above. The samples (1 ml) were loaded on the Mono Q column which was then washed with ET buffer (20 mM Tris-HCl, 1 mM EDTA, pH 7.4) or with ETMo buffer at a flow rate of 1 ml/min. Retained proteins were then eluted with a linear salt-gradient from 0 to 500 mM NaCl in ET or ETMo buffer in 25 min. One-minute fractions were collected, and 500-µl aliquots were counted.

General Methods and Safety Precautions - Radioactivity was measured in a 1216 Rackbeta II liquid scintillation counter (LKB-Wallac, Stockholm, Sweden) with an average counting efficiency of 40%. Since TCDD and TCDF are highly toxic compounds, their use necessitates special handling and disposal procedures (32). Contaminated disposable materials were sent away for high-temperature incineration. Salt concentration was determined by measuring the conductivity.

RESULTS

Although the exact subunit composition of the cytosolic non-activated glucocorticoid receptor is not known, there are indications that it might be composed of one M_r ~ 94,000 ligand- and DNA-binding unit (33) and a dimer of hsp90 (34,35). The glucocorticoid receptor was therefore used as a control in the present study.

We previously reported (36) that the molybdate-stabilized glucocorticoid receptor from rat liver has a Stokes radius R_s ~ 7.5 nm as determined by HPSEC (Figure 1A). The dioxin receptor present in rat liver cytosol, analyzed under identical experimental conditions, was found to have a similar R_s, *i.e.* ~ 7.5 nm (Figure 1B). Analysis of the fractions on slot blot using anti-hsp90 antibodies as a probe (37) showed that the "free" hsp90 was eluted in the ~7 nm region (data not shown) which is in complete agreement with the previously determined R_s of the purified hsp90 (30). When the cytosolic fraction was incubated with [^{3}H]hydroxycholesterol and analyzed on a Superose 12 column, a single peak of specifically bound radioactivity was eluted corresponding to an R_s ~ 6.0 nm, even in the presence of sodium molybdate (Figure 1C). This value was smaller than the R_s for hsp90 suggesting that the OR, in contrast to the GR and the DR (15), was not associated with hsp90 in rat liver cytosol.

In a second set of experiments, similar cytosolic preparations were analyzed on a Mono Q anion-exchange column in the presence of 20 mM Na_2MoO_4. The molybdate-stabilized GR eluted at ~ 0.30 M NaCl (Figure 2A) whereas the dioxin receptor was found to represent a slightly less negatively charged species, eluted with 0.26-0.28 M NaCl (Figure 2B). Again, these results were in agreement with the demonstration that both the GR and the DR associate with hsp90 (15), which has been proposed to be responsible for the behavior of the non-activated glucocorticoid receptor on anion-exchangers (38,39). In contrast, the OR was eluted with only 0.11-

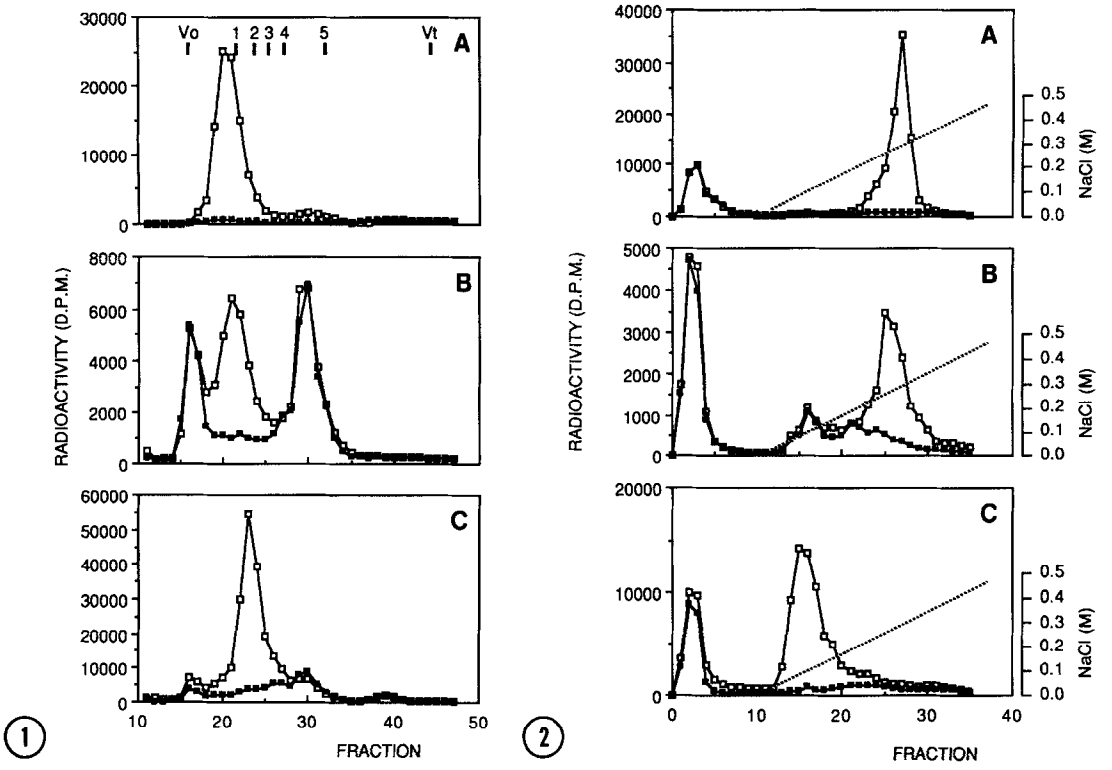

Fig. 1. **High performance size exclusion chromatography of the glucocorticoid, dioxin and oxysterol receptors from rat liver cytosol.** Rat liver cytosol prepared in ETGMo buffer was labeled with **A**, [^{3}H]triamcinolone acetonide; **B**, [^{3}H]dioxin and **C**, 25[^{3}H]hydroxycholesterol in the absence (□) or presence (■) of a 200-fold molar excess of unlabeled ligand. Labeled preparations were chromatographed on a Superose 12 column as described in "Materials and Methods".

Fig. 2. **High performance ion exchange chromatography of the glucocorticoid, dioxin and oxysterol receptors in the presence of sodium molybdate.** Cytosol prepared in ETGMo buffer and labeled with **A**, [^{3}H]triamcinolone acetonide; **B**, [^{3}H]dioxin and **C**, 25[^{3}H]hydroxycholesterol in the absence (□) or presence (■) of a 200-fold molar excess of unlabeled ligand was applied to a Mono Q column after removal of free ligand by charcoal treatment. The column was washed with 10 ml ETMo buffer and elution was carried out with a linear salt gradient (0-0.5 M NaCl) in ETMo buffer. Each 1-ml fraction was assayed for radioactivity and salt concentration (....).

0.14 M NaCl from the Mono Q column (Figure 2C), in the region of the salt gradient where the monomeric activated glucocorticoid receptor is eluted (29). Thus, the behavior of the OR on the Mono Q column was a second indication that it does not associate with hsp90 in rat liver cytosol.

The dissociation of the glucocorticoid receptor-hsp90 complex can be induced *in vitro* by a number of manipulations such as dilution, heating or increasing ionic strength (40). Chromatography on an anion-exchange column in the absence of molybdate has also been shown to induce GR activation (26,29,41). As shown on Figure 3A, more than 60% of rat glucocorticoid-receptor complexes were activated to a less negatively charged species (eluted with 0.10-0.12 M NaCl) only after a 30-

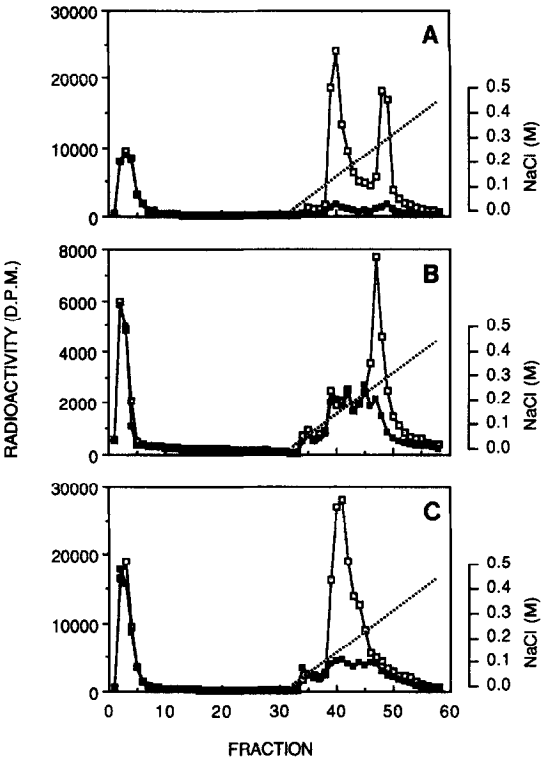

Fig. 3. **High performance ion exchange chromatography of the glucocorticoid, dioxin and oxysterol receptors in the absence of molybdate**. Cytosol was prepared in ETG buffer and labeled with **A**, [^{3}H]triamcinolone acetonide; **B**, [^{3}H]dioxin and **C**, 25[^{3}H]hydroxycholesterol in the absence (□) or presence (■) of a 200-fold molar excess of unlabeled ligand. Samples were applied to a Mono Q column, which was then washed for 30 min with ET buffer. Retained material was eluted with a linear salt gradient in ET buffer. Fractions were assayed for radioactivity and salt concentration (....).

minutes wash with ET buffer. When analyzed under the same conditions, the DR did not show any significant difference in behavior on the Mono Q column (Figure 3B). A similar elution profile was obtained even when the column was washed for up to 12 hours (not shown). Therefore, the conclusion was that the DR-hsp90 interaction was stronger than the GR-hsp90 interaction. Also in the case of the OR, no difference was seen when chromatography was performed in the absence of molybdate (Figure 3C).

In order to determine the DNA-binding ability of the different receptors eluted from the Mono Q column, labeled rat liver cytosol was chromatographed in the presence or absence of molybdate, and the fractions of interest were applied to DNA-cellulose columns. The percentages of receptor bound to DNA are presented in Figure 4. It is clear from this diagram that only the glucocorticoid-receptor complexes formed after washing of the column without molybdate are able to bind quantitatively to bulk DNA. None of the dioxin- and hydroxycholesterol-binding species were retained on the DNA-matrix.

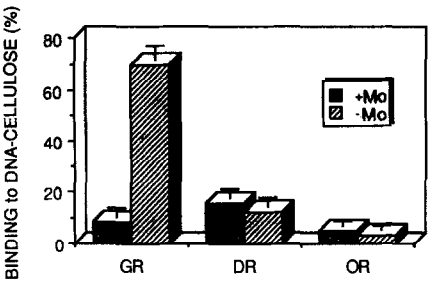

Fig. 4. **DNA-binding activity of the glucocorticoid, dioxin and oxysterol receptors eluted from the Mono Q anion-exchange column.** Samples labeled with [^{3}H]triamcinolone acetonide (GR), [^{3}H]dioxin (DR) or 25[^{3}H]hydroxy-cholesterol (OR) were chromatographed on a Mono Q column in the presence (+Mo) or absence (-Mo) of sodium molybdate as described in Fig. 2 and 3. Peaks of specifically bound radioactivity were collected, 5-fold diluted with ETGMo buffer, and applied to 1-ml DNA-cellulose columns. The first peak of glucocorticoid receptor eluted in the absence of molybdate was used in this assay. The columns were washed with 10 ml of ETGMo buffer, and eluted with 1 M NaCl in ETGMo buffer. Represented are the percentages of radioactivity retained on the DNA-cellulose column (mean ± SD, n=3).

DISCUSSION

The data presented here indicate that the GR and the DR have similar size, but slightly different charge, and that the DR is not susceptible to the subunit dissociation induced by the interaction with the Mono Q matrix in the absence of molybdate. Furthermore, since the OR was found to be smaller than the hsp90, and to be only slightly negatively charged, we would like to propose that, in contrast to the GR and the DR, the OR does not associate with hsp90. This conclusion is in agreement with previous reports indicating that molybdate has no effect on the OR (17,21).

It has recently been shown that hsp90 interacts with the ligand-binding domain of the GR (36,42). In addition, Pratt et al. (42) proposed that a 20-amino acid long site (residues 577-596 of the human GR) would be responsible for the interaction with hsp90. To our knowledge, cloning of the cDNA's encoding the DR and the OR has not been reported. If this hypothesis is true, the region that is responsible for the interaction with hsp90 should be present in the ligand-binding domain of the DR, but not of the OR.

In analogy to the steroid hormone receptors, the DR seems to specifically recognize a sequence motif in the 5' flank of a dioxin-regulated gene (43). Following chromatography both in the presence or absence of molybdate, the DR did not exhibit any significant DNA-binding activity. It is most likely that, in contrast to what we observed for the GR, the hsp90-DR complex was not dissociated during analysis on the Mono Q column, and thus, the DNA-binding ability of the DR was inhibited by hsp90.

The OR has also been proposed to act at the level of transcription by a mechanism analogous to that of steroid hormone receptors (17). The 25-hydroxy-cholesterol-receptor complexes obtained following anion-exchange chromatography

did not bind to bulk DNA. Since the OR does not seem to form a complex with hsp90, the DNA-binding activity of the receptor must be inhibited by another component. A candidate for masking the DNA-binding domain of the OR could be an RNA molecule since in order to demonstrate binding to DNA Taylor and Kandutsch must first treat their preparations with ribonuclease A (17). Alternatively, the protein we were investigating in rat liver cytosol might be a shuttle, transferring the oxysterol from the cytoplasm to its putative chromatin acceptor sites, resulting ultimately in modulation of transcription of specific genes, as in the case of retinol and retinoic acid (44). Clearly, a detailed dissection of the structure of the dioxin and oxysterol receptors is necessary for further elucidation of the mechanism of gene regulation by these ligands. It is likely that important information on these receptors will be derived from their cloned cDNA's.

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