Effect of ligand structure on the proteolytic digestion of the transformed and nuclear AhR complexes

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Abstract

The effect of ligand-structure on the transformed and nuclear Ah receptor (AhR)-dioxin repsonive elemtents (DREs) complexes was analyzed by the proteolytic clipping band shift assay. Analysis of the digestion patterns of the AhR:DRE complexes indicated that trypsin proteolysis of AhR:DRE complexes was concentration-dependent and ligand-structure independent. In contrast, UV cross-linking SDS-PAGE analysis of nuclear extracts from TCDD- or MCDF-treated Hepa 1c1c7 cells showed that formation of AhR:DRE complexes were ligand-structure-dependent. Moreover, proteolytic digestion patternes of UV cross-linked nuclear AhR:DRE complexes from TCDD- or MCDF-treated cells resulted in a tryptic digestion pattern that was ligand structure specific.

Introduction

Ligand-dependent differences in formation of steroid hormone receptor complexes with genomic DNA-recognition sites have been reported[1,2]. For example, subtle differences were apparent in association and dissociation of ER complexes with genomic EREs bound to ligands with varying potencies as agonists[2]. However, in another study[3] binding of mouse uterine ER with DNA sequences appeared to be relatively independent of ligand structure. For example, the dissociation rates and equilibrium dissociation constants of the ER-ERE complexes formed by estradiol- or estriol-bound ER showed no discernible ligand-dependent differences despite their different potencies as estrogens. Moreover, estradiol stimulates the formation of an ER:ERE complex with increased mobility in native gels compared to the complex formed in the absence of hormone or with tamoxifen[1]. Therefore, the major objectives of this study was to investigate the ligand-induced conformational properties and domain organization of the transformed and nuclear AhR complexes utilizing the proteolytic clipping band shift assay[4].

Materials and Methods

Proteolytic clipping band shift assay

Transformed cytosol from Long Evans rats and nuclear extracts from Hepa 1c1c7 cells were treated with various ligands and prepared as described[5,6]. Both ligand-treated cytosol and nuclear extracts were then subjected to the proteolytic clipping band shift assay as described[4].

Preparation of BrdU-substituted DNA

For cross-linking studies, 10 pmol of the synthetic oligonucleotide, 5'-GATCTCCGGTCCTTCTCACGCAACGCCTGGGC-3', was annealed to a 10 pmol 7 bp complementary primer, 5'-GCCCAGG-3'. The annealed template was end-filled with the Klenow fragment of DNA polymerase in the presence of 0.1 μM dGTP, dATP, BrdU and 1

μM [^{32}P]dCTP as described[7], and was designated as the BrdU-substituted DRE oligonucleotide.

UV cross-linking

Nuclear extracts (10 μg) and ligand transformed cytosolic extracts (80 μg) were incubated with the BrdU-substituted ^{32}P-labeled DRE for 15 min at 20°C following a 15 min incubation at 20°C with 400 ng of poly[d(I-C)] in HEGD buffer. The gel mobility shift assay products were UV irradiated directly on a FOTODYNE UV transilluminator, > 205 nm for 30 min at 20°C. Samples were then mixed with 20 μl of an SDS-loading buffer, heated to 95°C for 5 min and then subjected to electrophoresis on 12% SDS-polyacrylamide gels. Ligand:AhR:DRE complexes were resolved by autoradiography of the dried gel. Molecular weights of UV cross-linked nuclear and transformed ligand:AhR:DRE complexes were calculated from [^{14}C]methylated protein standards obtained from Amersham Corp.(Arlington Heights, IL).

Results

The effects of ligand structure on the proteolytic digestion of the transformed rat hepatic cytosolic AhR:DRE complexes were also analyzed by the proteolytic clipping band-shift assay. Rat cytosol was transformed with TCDD, 1,2,7,8-TCDF and 2,3,7,8-TCDF and subjected to the proteolytic cliiping band shift assay with trypsin. Figure 1 shows that all of the ligands exhibited a concentration-dependent loss of liganded:AhR:DRE complex formation when subjected to limited proteolysis with trypsin.

The effect of ligand structure on the structural and molecular properties of the UV cross-linked nuclear AhR:DNA complexes from TCDD-, MCDF- or αNF-treated Hepa1c1c7 cells was investigated by utilizing a consensus DRE (BrdU-DRE) in which BrdU residues were incorporated (Figure 2). Comparisons of the relative mobilities of the cross-linked nuclear AhR:BrdU-DRE complexes from TCDD-, MCDF and αNF-treated Hepa 1c1c7 to those of known molecular weight standards revealed the formation of a 200-kDa AhR:BrdU-DRE cross-linked complex from both the TCDD- and αNF-treated Hepa 1c1c7 cells (Figure 2, lanes 1 and 3). In contrast, treatment of Hepa 1c1c7 cells with 1 μM MCDF resulted in formation of two AhR:BrdU-DRE complexes with the apparent molecular masses of 200- and 112-kDa (Figure 2, lane 2). Moreover, all of the AhR:BrdU-DRE complexes were removed in competition experiments with the addition of a 200-fold excess of unlabeled DRE probe (data not shown).

The differences in the molecular and structural properties of the UV cross-linked nuclear AhR:BrdU-DRE complexes from TCDD- of MCDF-treated Hepa 1c1c7 cells (Figure 2, lanes 1 and 2) were also investigated by the proteolytic clipping band-shift assay. Figure 3 illustrates the concentration-dependent effects of trypsin on the UV cross-linked AhR:BrdU-DRE complexes from Hepa 1c1c7 cells treated with 10 nM TCDD or 1 μM MCDF. Treatment of nuclear AhR:BrdU-DRE complexes from TCDD-treated Hepa 1c1c7 cells with trypsin, prior to UV cross-linking and SDS-PAGE analysis, results in the concentration-dependent loss of the 200-kDa complex and formation of a 112-kDa complex. In contrast, treatment of nuclear AhR:BrdU-DRE complexes from MCDF-treated Hepa 1c1c7 cells with trypsin, prior to UV cross-linking and SDS-PAGE analysis, resulted in a concentration-dependent loss of only the minor 200-kDa AhR:BrdU-DRE complex.

Discussion

The effects of ligand structure on the molecular properties of the transformed rat hepatic cytosolic AhR:DRE complexes were analyzed using the proteolytic clipping band shift assay with cytosol transformed with ligands of varying potencies. The results show that with this assay the molecular and structural properties of the AhR:DRE complexes liganded with TCDD, 1,2,7,8-TCDF and 2,3,7,8-TCDF were comparable (Figure 1).

UV cross-linking experiments have confirmed that the nuclear and transformed cytosolic AhR:DRE complexes are heterodimeric, consisting of a non-ligand-binding (Arnt) protein and a ligand-binding subunit, namely the Ah receptor[8-10]. In contrast, guinea pig hepatic transformed cytosolic TCDD:AhR:BrdU-DRE complexes consist of one ligand-binding subunit (105-kDa) and at least two distinct non-ligand binding subunits of 97- and 115-kDa[11].

However, the effect of ligand structure on the molecular and structural properties of the UV cross-linked nuclear ligand:AhR:BrdU-DRE complexes has yet to be determined. Therefore, the objective of the second phase of this study was to investigate the molecular and structural properties of nuclear extracts form Hepa 1c1c7 cells treated with TCDD, αNF or MCDF. Cross-linking of nuclear extracts from Hepa 1c1c7 cells treated with MCDF results in formation of two UV cross-linked nuclear AhR:BrdU-DRE complexes with apparent molecular masses of 200- and 112-kDa (Figure 2). In contrast, only the 200-kDa UV cross-linked nuclear AhR:BrdU-DRE complex was observed with nuclear extracts from TCDD-,.or αNF-treated cells. Previous studies have shown that the 200-kDa AhR:BrdU-DRE complex is heterodimeric, composed of the Ah receptor and Arnt proteins[8,9,11] and that the lower molecular weight complex corresponded to the ligand-binding subunit[11]. Analysis of the UV cross-linked nuclear AhR:BrdU-DRE complexes from TCDD- or MCDF-treated Hepa 1c1c7 cells indicates that their proteolytic digestion maps are also ligand-dependent (Figure3). These data suggest that the MCDF-induced nuclear Ah receptor complexes are monomeric or a heterodimer in which one of the partners is unable to form a DNA cross-link. In contrast, nuclear extracts from cells treated with TCDD only form the 200-kDa heterodimer.

In summary, UV cross-linking experiments of nuclear extracts from TCDD-, αNF- and MCDF-treated Hepa 1c1c7 cells results in the ligand-dependent formation of AhR:BrdU-DRE complexes and that the altered conformation of nuclear AhR:BrdU-DRE complexes from MCDF-treated cells may be responsible for the differential effects of MCDF on gene expression. For example, analysis of nuclear extracts from cells treated with 10 μM αNF or 1 nM TCDD results in the induction of CYP1A1 gene expression and formation of a 200-kDa complex. However, in Hepa 1c1c7 cells treated with 100 or 1000 nM MCDF, only minimal induction of CYP1A1 gene expression is observed, a 112-kDa complex is preferentially formed and only low levels of a 200-kDa complex are observed.

References

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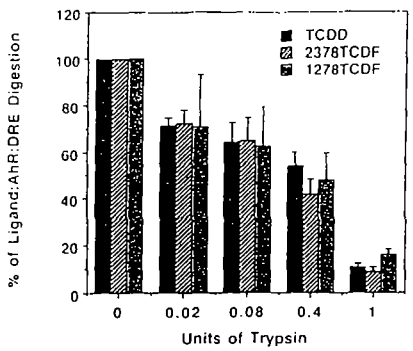
Figure 1.



Proteolytic clipping band-shift assay of the concentration-dependent tryptic digestion of transformed rat hepatic cytosolic ligand-AhR:DRE complexes

Figure 2.

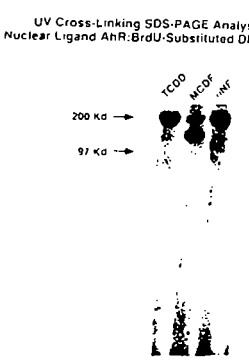
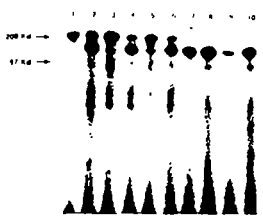


Figure 3.



Autoradiograph of the proteolytic clipping band-shift assay of the concentration-dependent tryptic digestion of the nuclear AhR:BrdU-DRE complexes from Hepa 1c1c7 cells treated with TCDD or MCDF. Nuclear extracts from Hepa 1c1c7 cells treated with 10 nM TCDD, or 1 μM MCDF were prepared and AhR:BrdU-DRE complexes were subjected to the proteolytic clipping band-shift assay by trypsin, UV irradiated and analyzed by SDS-PAGE as described in the Materials and Methods section. Lanes 1,3,5,7,9 and lanes 2,4,6,8 and 10 correspond to AhR:BrdU-DRE complexes from TCDD- or MCDF-treated Hepa 1c1c7 cells, respectively. The following concentrations of trypsin were added to the AhR:BrdU-DRE complexes as described in the Materials and Methods section: 0 units (lanes 1and 2) 0.02 units (lanes 3 and 4), 0.2 units (lanes 5 and 6), 2.0 units (lanes 7 and 8) and 10.0 units (lanes 9 and 10)