Characterization of the molecular and structural properties of the transformed and nuclear aryl hydrocarbon (Ah) receptor complexes by proteolytic digestion

Santostefano, M. and Safe, S.
Department of Veterinary Physiology and Pharmacology
Texas A&M University
College Station, TX 77843-4466

Abstract

Proteolytic clipping band shift assay analysis of Ah receptor (AhR):dioxin responsive element (DRE) complexes revealed that the molecular and structural properties of the transformed and nuclear TCDD:AhR:DRE complexes were comparable. For example, both transformed and nuclear TCDD:AhR:DRE complexes exhibited a concentration-dependent decrease in molecular weight and loss of DRE binding when subjected to limited proteolysis with trypsin. In addition, limited proteolysis of TCDD:AhR:DRE complexes with V8 protease resulted in a concentration-dependent increase in mobility of TCDD:AhR:DRE complexes; however, no significant loss of formation of TCDD:AhR:DRE complexes was observed. Moreover, UV cross-linking SDS-PAGE analysis of the transformed and nuclear TCDD:AhR:DRE complexes resulted in the formation of a specific 200-kDa complex. These results suggest that the molecular and structural properties of the transformed and nuclear TCDD:AhR:DRE complexes are indistinguishable.

Introduction

Previous studies have demonstrated that the physical properties of the transformed cytosolic and nuclear AhR complexes are comparable[1,2]. For example, the nuclear and transformed cytosolic AhR exhibit similar sedimentation coefficients, Stokes radii and frictional/axial ratios. In addition, photoaffinity labeling of the salt transformed cytosolic and nuclear AhR complexes revealed that these complexes were indistinguishable[2]. However, some variation between the molecular masses of the transformed cytosolic and nuclear AhR complexes has been reported suggesting that the molecular properties of these complexes may be different[1]. Therefore, the current study investigated the molecular and structural properties of the transformed and nuclear ligand:AhR:DRE complexes using the proteolytic clipping band shift assay[3].

Materials and Methods

Proteolytic clipping band shift assay

Transformed cytosol from Long Evans rats and nuclear extracts from Hepa 1c1c7 cells were treated with TCDD and prepared as described[4,5]. Both ligand-treated cytosol and nuclear extracts were then subjected to the proteolytic clipping band shift assay as described[3]. Eighty μg of ligand-

treated cytosol and 10 µg of nuclear extracts were incubated with 400 ng of poly[d(I-C)] in HEGD buffer for 15 min for 20°C. Following the addition of a [^{32}P]-labeled DRE oligonucleotide (0.2 - 1.0 ng, 100,000 cpm), the mixture was incubated for an additional 15 min at 20°C. One µl of trypsin (0 - 10 units) or protease V8 (0 - 10 µg) was added and incubated at 20°C for 10 min. Protein-DNA complexes were resolved on a 6% non-denaturing polyacrylamide gel (acrylamide:bisacrylamide, 30:0.8) and electrophoresed at 120 V for approximately 3 hr in 0.9 M Tris borate and 2 mM EDTA, pH 8.0. The gels were dried and the results are expressed as a percentage of specific binding of a non-protease-treated sample as determined by imaging on a Betagen Betascope 603 blot analyzer. Each data point for the DRE binding studies is expressed as a mean ± standard errors for at least four separate determinations.

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 pb 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[6], 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 20 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 Corporation (Arlington Heights, IL).

Results

Previous studies have demonstrated that some of the molecular and structural properties of the TCDD-induced transformed and nuclear AhR complexes are similar[7-9]. However, the structural similarities of the *in vitro* and *in vivo* AhR complexes bound to DNA have yet to be determined. Thus, the DRE-binding similarities of the transformed and nuclear TCDD:AhR complexes were investigated using the proteolytic clipping band shift assay. Figure 1 shows that the tryptic digestion map of rat hepatic cytosol transformed with TCDD is indistinguishable from the tryptic digestion map of nuclear extracts prepared from TCDD-treated Hepa 1c1c7 cells. In addition, proteolytic digestion of the DRE-bound transformed and nuclear AhR complex with trypsin resulted in a concentration-dependent loss in binding of the AhR:DRE complexes (Fig. 2), and this was accompanied by an increase in the mobility of the degraded AhR complexes within the gel (Fig. 1). In contrast, treatment with V8 protease did not significantly result in loss of DNA-bound transformed and nuclear AhR complexes (Fig. 3); however, the V8 protease digestion resulted in an increased mobility of the degraded complex within the gel (data not shown). Furthermore, UV cross-linking experiments of nuclear extracts from TCDD-treated Hepa 1c1c7 cells to a BrdU-DRE revealed the formation of a 200-kDa AhR:BrdU-DRE complex that was similar to the 200-kDa AhR:BrdU-DRE complex

observed in rat cytosol transformed with TCDD (Fig. 4). These results suggest that the molecular and structural properties of the nuclear and transformed AhR complexes were indistinguishable using the proteolytic clipping band shift assay.

Discussion

Previous studies have demonstrated that the physical properties of the transformed cytosolic and nuclear AhR complexes are comparable[1,2]. For example, the nuclear and transformed cytosolic AhR exhibit similar sedimentation coefficients, Stokes radii and frictional/axial ratios. In addition, photoaffinity labeling of the salt transformed cytosolic and nuclear AhR complexes revealed that these complexes were indistinguishable[2]. However, some variation between the molecular masses of the transformed cytosolic and nuclear AhR complexes has been reported[1]. Thus, the current study investigated the molecular and structural properties of the transformed and nuclear ligand:AhR:DRE complexes using the proteolytic clipping band shift assay[3]. Treatment of transformed cytosolic and nuclear TCDD:AhR:DRE complexes with trypsin, which cleaves at the C-terminal side of the basic chains of lysine and arginine residues, resulted in the concentration-dependent increase in the mobility of the ligand:AhR:DRE complexes (i.e. loss in molecular masses) and loss of the formation of ligand-inducible AhR:DRE complexes (Fig. 1). Thus, it is possible that the decreased DNA-binding of the transformed TCDD:AhR:DRE complexes is due to proteolysis of sites within the heterodimerization domains. In addition, analysis of the trypsin digestion patterns of the nuclear TCDD:AhR:DRE complexes are indistinguishable from tryptic digestion patterns of the nuclear TCDD:AhR:DRE complexes (Fig. 2). Moreover, analysis of the UV cross-linked transformed cytosolic or nuclear AhR:BrdU-DRE complexes (Fig. 4) revealed the presence of a 200-kDa AhR:BrdU-DRE complex. These results provide additional support that the DNA-bound molecular and structural properties of the nuclear and transformed cytosolic AhR complexes are comparable.

The differential effects of proteases on the molecular and structural properties of the transformed and nuclear AhR was also investigated using V8 protease, which cleaves at the C-terminal side of glutamic acid residues. Treatment of transformed cytosolic and nuclear TCDD:AhR:DRE complexes with V8 protease results in the concentration-dependent increase in the mobility of the proteolytic digestion products of the TCDD:AhR:DRE complexes (data not shown); however, no significant overall loss (based on radioactivity) in TCDD-induced transformation of the AhR to a DRE-binding form occurs (Fig. 3). Thus, the increase in mobility of the transformed and nuclear TCDD:AhR:DRE complexes appears to be due to cleavage of sites outside region required for heterodimerization and subsequent DRE-binding formation. In contrast, the lack of significant loss of transformed TCDD:AhR:DRE complexes may be due to the conformations of the basic helix-loop-helix domains of the ligand:AhR-Arnt:DRE complexes in which V8 protease cleavage sties are relatively inaccessible.

References

1. Prokipcak, R.D. and Okey, A.B. 1988. Physicochemical characterization of the nuclear form of Ah receptor from mouse hepatoma cells exposed in culture to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. *Arch. Biochem. Biophys.* 267:811-828.

2. Prokipcak, R.D. and Okey, A.B. 1990. Equivalent molecular mass of cytosolic and nuclear forms of Ah receptor from Hepa-1 cells determined by photoaffinity labeling with 2,3,7,8-[^{3}H]tetrachlorodibenzo-*p*-dioxin. *Biochem. Biophys. Res. Comm.* 172:698-704.

3. Schreiber, E., Matthias, P., Muller, M.M. and Schaffner, W. 1988. Identification of a novel lymphoid specific octomer binding protein (OTF-2B) by proteolytic clipping band shift assay (PCBA). *EMBO J.* 7:4221-4229.

4. Bandiera, S., Safe, S. and Okey, A.B. 1982. Binding of polychlorinated biphenyls classified as either phenobarbitone-, 3-methylcholanthrene-, or mixed-type inducers to cytosolic Ah receptor. *Chem. -Biol. Interact.* 39:259-277.

5. Merchant, M., Morrison, V., Santostefano, M. and Safe, S. 1992. Mechanism of action of aryl hydrocarbon receptor antagonists: inhibition of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin-induced *CYP1A1* gene expression. *Arch. Biochem. Biophys.* 298:389-394.

6. Sambrook, J., Fritsch, E. and Maniatis, T. 1989. *Molecular Cloning: A Laboratory Manual.* Cold Spring Harbor Laboratory Press, Cold Spring Harbor.

7. Denison, M.S. and Yao, E.F. 1991. Characterization of the interaction of transformed rat hepatic cytosolic Ah receptor with a dioxin responsive transcriptional enhancer. *Arch. Biochem. Biophys.* 284:158-166.

8. Shen, E.S. and Whitlock, J.P., Jr. 1992. Protein-DNA interactions at a dioxin-responsive enhancer -mutational analysis of the DNA-binding site for the liganded Ah receptor. *J. Biol. Chem.* 267:6815-6819.

9. Yao, E.F. and Denison, M.S. 1992. DNA sequence determinants for binding of transformed Ah-receptor to a dioxin-responsive enhancer. *Biochemistry* 31:5060-5067.

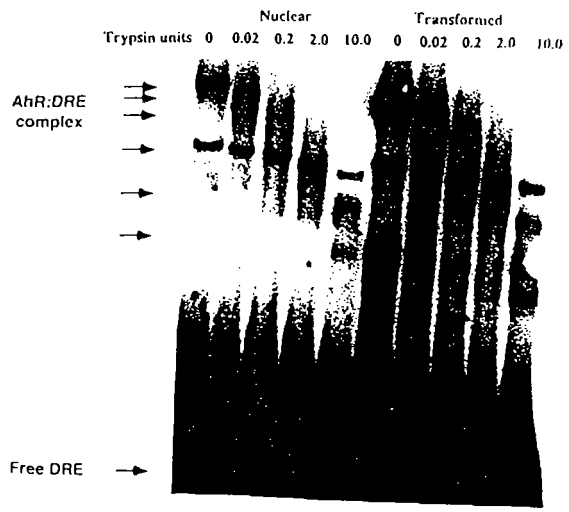

Figure 1. Autoradiography of the concentration-dependent digestion of transformed and nuclear TCDD:AhR:DRE complexes by trypsin.

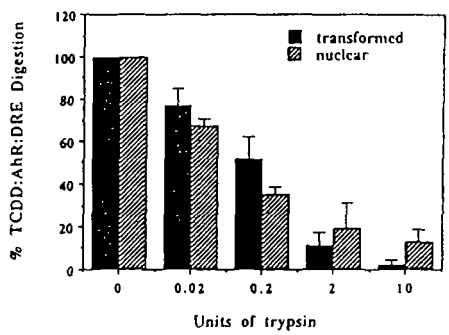

Figure 2. Concentration-dependent digestion of transformed and nuclear TCDD:AhR: DRE complexes by trypsin.

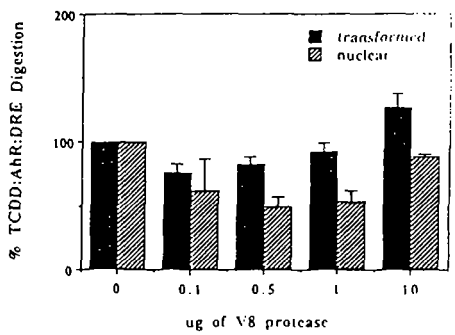

Figure 3. Concentration-dependent digestion of transformed and nuclear TCDD:AhR: DRE complexes by V8 protease.

Figure 4. UV cross-linking analysis of transformed and nuclear AhR:DRE complexes.