Evidence Suggesting ERAP 140 Interacts with the Aryl Hydrocarbon Receptor

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Abstract

Induction of CYP1A1 gene expression by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) in human breast cancer cells is mediated by the aryl hydrocarbon receptor (AhR). It has been suggested the human estrogen receptor (hER) may be required for Ah-responsiveness. Additionally, recent studies suggest coactivator proteins such as ERAP 140 modulate the transactivation of ER and other nuclear receptors. Studies were conducted to determine if ERAP 140 modulated the transactivation of the AhR in human MCF-7 breast cancer cells. Cotransfection of MCF-7 breast cancer cells with pRNH11c, containing the CYP1A1 (-1142 to +2434) regulatory region linked to a CAT (chloramphenicol acetyl transferase) reporter gene and ERAP 140 protein expression plasmid resulted in enhanced CAT activity after treatement with 10 *nM* TCDD compared to cells transfected with pRNH11c alone. Enhancement of Ah-responsiveness by ERAP 140 was diminished by cotransfection with a construct encoding hER. Consistent with this, a concentration-dependent increase in binding of the AhR to a [^{32}P]-dioxin responsive element (DRE) in electrophoretic mobility shift assays was observed in the presence of ERAP 140 compared to samples without ERAP 140. ERAP 140 can be immunoprecipitated with the AhR/Arnt complex using anti-AhR or anti-Arnt monoclonal antibodies. Collectively, these data suggest a protein-protein interaction between ERAP 140 and the AhR/Arnt complex which enhances transactivation of pRNH11c by the AhR in MCF-7 human breast cancer cells.

Introduction

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) initially binds to the cytosolic aryl hydrocarbon receptor (AhR) and then forms a transcriptionally active heterodimeric nuclear AhR complex containing the AhR and the AhR nuclear translocator (Arnt) proteins. Interaction of the nuclear AhR complex with dioxin responsive elements (DREs) (5'-CACGC-A-3') located in the 5'-promoter region of the CYP1A1 and other Ah-responsive genes results in transactivation of target genes [1].

Several studies have identified the AhR in both ER-negative (ER$^-$) and ER-positive (ER$^+$) breast cancer cell lines, however Vickers and coworkers [2] also observed that Ah-responsiveness, characterized by induction of CYP1A1, was observed only in ER$^+$ cell lines. Subsequent studies in this laboratory investigated the role of the ER in modulating Ah-responsiveness in ER$^-$ MDA-MB-231 breast cancer cells. This cell line expresses the AhR and after treatment with TCDD a nuclear AhR complex is formed but induction of CYP1A1 gene expression is not observed. Thomsen and coworkers [3] showed that chloramphenicol acetyl transferase (CAT) activity was induced by TCDD in MDA-MB-231 cells transiently transfected with a human ER expression plasmid (hER) and an Ah-responsive plasmid pRNH11c containing the 5'-regulatory region (-1140 to +2434) from the human CYP1A1 gene promoter. Ah-responsiveness was also restored in ER$^-$ Hs578T cells using pRNH11c and an ER variant which only expressed the C-terminal

region of the ER containing activating function-2 (AF-2) [4]. These studies suggest there is crosstalk between the AhR and ER signal transduction pathways.

Transcriptional activation of protein encoding genes involve the assembly of numerous transcription factors. Recent studies have identified several "coactivator" proteins which modulate the transcriptional activation of the steroid hormonal nuclear receptors [5] and ERAP 140 is a coactivator which interacts with the ER [6]. Given the relationship between the AhR and ER signal transduction pathways in human breast cancer cells and the observation that there may be crosstalk between the AhR and ER pathways, studies were conducted to determine if ERAP 140 modulates AhR-mediated transcriptional activation in MCF-7 breast cancer cells.

Experimental Methods

Chemicals and Biochemicals: 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) was prepared in this laboratory and was determined to be >99% pure by gas chromatography and mass spectroscopy. All other chemicals and biochemicals were of the highest quality available from commercial suppliers.

Cell Maintenance: The human adenocarcinoma MCF-7 cell line was obtained from the American Type Culture Collection (Rockville, MD). MCF-7 cells were grown in MEM (Gibco) medium supplemented with 10% fetal calf serum (FCS, Intergen, Newark, NJ), 10 Fg/L insulin, 2.38 g/L HEPES, 1.0 g/L glucose, 2.2 g/L Na_2HCO_3, 0.11 g/L sodium pyruvate and 2 ml/L antibiotic/antimycotic (Sigma) solution. Cells were grown in a 37°C incubator with a humidified mixture of 5% CO_2 and 95% air.

Expression Vectors: pRNH11c contains the 5'-regulatory region (-1140 to +2434) from the human CYP1A1 gene fused to the bacterial chloramphenicol acetyltransferase (CAT) reporter gene and was kindly provided by Dr. Ronald Hines (Wayne State University). An expression vector encoding for the ERAP 140 protein was generously provided by Myles Brown (Dana Farber Cancer Institute). The plasmid containing the human estrogen receptor (ER) cDNA was provided by Dr. Ming Jer Tsai (Baylor College of Medicine).

Determination of CAT activity: For transient transfection of protein encoding constructs, 3μg of chloramphenicol acetyltransferase (CAT)-reporter plasmid or vector only were transfected by calcium phosphate coprecipitation. Where indicated, cells were also transfected with selected concentrations of expression vector for ERAP 140 or the ER. Six hr later, cells were shocked with 25% DMSO for 4 min. Cells were treated with a noncytotoxic concentration of TCDD (10 nM) or DMSO vehicle (final concentration 0.2%) eighteen hr after the DMSO shock. Forty-eight hr after treatment the cells were harvested for analysis of CAT activity as described[2].

Electrophoretic Mobility Shift Assays Using In Vitro-Translated Proteins: Plasmids containing the AhR, Arnt and ERAP 140 cDNAs were used to *in vitro* transcribe and translate the corresponding protein in a rabbit reticulocyte lysate system (Promega). Equal volumes (3μl) of lysate containing the AhR and Arnt complex were transformed with 1 nM TCDD for 2 hours at 25°C. Selected volumes of lysate (1.5 and 3 μl) containing ERAP 140 were subsequently added, and incubated for 15 min at 25°C. The labeled DRE probe (60,000 cpm) was then added to the reaction mixtures in the presence of 1 μg of poly(dI-dC), and the mixtures were incubated for 15 min at 25°C. The reaction was carried out in 20 mM HEPES-5% glycerol, 100 mM KCl, 5 mM $MgCl_2$, 0.5 mM DTT, 1mM EDTA in a final volume of 25 μl. Reaction mixtures were loaded onto 5% polyacrylamide gel and run at 110 V in 0.09 M Tris, 0.09 M borate, 2 mM EDTA (pH 8.3). Gels were dried and protein-DNA binding was visualized by autoradiography.

Immunoprecipitation of ERAP 140-AhR/Arnt complex: For immunoprecipitation of ERAP 140 monoclonal antibodies against the AhR and Arnt were used. Briefly, the AhR, Arnt,

and ERAP 140 were *in vitro* transcribed/translated using the rabbit reticulocyte lysate system (Promega). Selected proteins were labeled using [^{35}S]methionine. Equal volumes (10 μl) of lysate containing AhR and Arnt were transformed with 1 nM TCDD for 2 hr at room temperature. Lysate (10 μl) containing ERAP 140 was added and the protein complex was incubated for an additional 1 hr at 25°C. To this mixture, anti-AhR or anti-Arnt antibodies were added and the samples were subsequently incubated for 2 hr at 25°C. Protein G-agarose beads (Santa Cruz) were added and incubated at 4°C for 5 hr. Immunoprecipitation of protein G-bound proteins was conducted according to the manufacturers recommendations. Proteins were subsequently separated by SDS-PAGE electrophoresis (5% gel) and visualized by autoradiography.

Results and Discussion

Transient transfection of MCF-7 cells with an Ah responsive gene and ERAP 140 was conducted to determine if ERAP 140 modulated the AhR-mediated transcriptional activation in human breast cancer cells (Figure 1a). MCF-7 cells were transfected with pRNH11c containing the 5'-regulatory region (-1140 to +2434) from the human CYP1A1 gene promoter fused to the chloramphenicol acetyl transferase (CAT) reporter gene and an ERAP 140 protein expression plasmid. After treatment with 10 nM TCDD there was increased CAT activity compared to DMSO treated cells (compare lanes 1 and 2). Treatment of MCF-7 cells with TCDD and cotransfected with pRNH11c and ERAP 140 resulted in a significantly increased induction of CAT activity (lanes 4and 5) compared to induction in the absence of ERAP 140 (lane 2). ERAP 140 had little effect on the basal transcription of pRNH11c (lane 3). Since ERAP 140 was initially identified as an ER associated protein, the squelching effect of ER on ERAP 140 - mediated enhancement of AhR transactivation was also investigated (Figure 1b). Transfection of hER into cells transfected with pRNH11c and ERAP 140 resulted in decreased CAT activity compared to cells cotransfected only with pRNH11c and ERAP 140 (compare lanes 3 and 4).

To determine if the enhanced transcriptional activation of pRNH11c by the AhR is accompanied by increased binding to DNA an electrophoretic mobility shift assay using *in vitro* transcribed/translated proteins was conducted (Figure 2). In the presence of TCDD, there was an increased intensity of a [^{32}P]-DRE-AhR retarded band compare the band in samples treated with DMSO (compare lanes 1 and 2). Inclusion of lysate containing a plasmid encoding for ERAP 140 resulted in enhanced binding of the AHR/Arnt complex to [^{32}P]DRE and this increase in binding was ligand and concentration dependent (compare lanes 4 and 5 with 3 and 2) .

It was of interest to determine if the enhanced binding and transactivation of the AhR by ERAP 140 was mediated by direct protein-protein interactions. Binding of [^{35}S]ERAP 140 to the AhR/Arnt complex was detected using monoclonal antibodies against the AhR and Arnt proteins (Figure 3). When the AhR and Arnt proteins were[^{35}S]-labeled (lanes 1 and 3 respectively) proteins of 110 kDa (AhR) and 97 kDa (Arnt) were detectable. A 140 kDa protein was detectable after immunoprecipitation of the protein complex using both the anti-AhR (lane 2) and anti-Arnt (lane 4) antibodies.

These studies were undertaken to determine if ERAP 140 modulated AhR transactivation in MCF-7 breast cancer cells. ERAP 140 enhanced TCDD-mediated transactivation of an Ah responsive gene and binding to DRE; moreover ERAP 140 directly interacted with the AhR and Arnt proteins and was immunoprecipitated using antibodies directed against AhR and Arnt. These data suggest ERAP 140 may also be a coactivator for the AhR in MCF-7 human breast cancer cells.

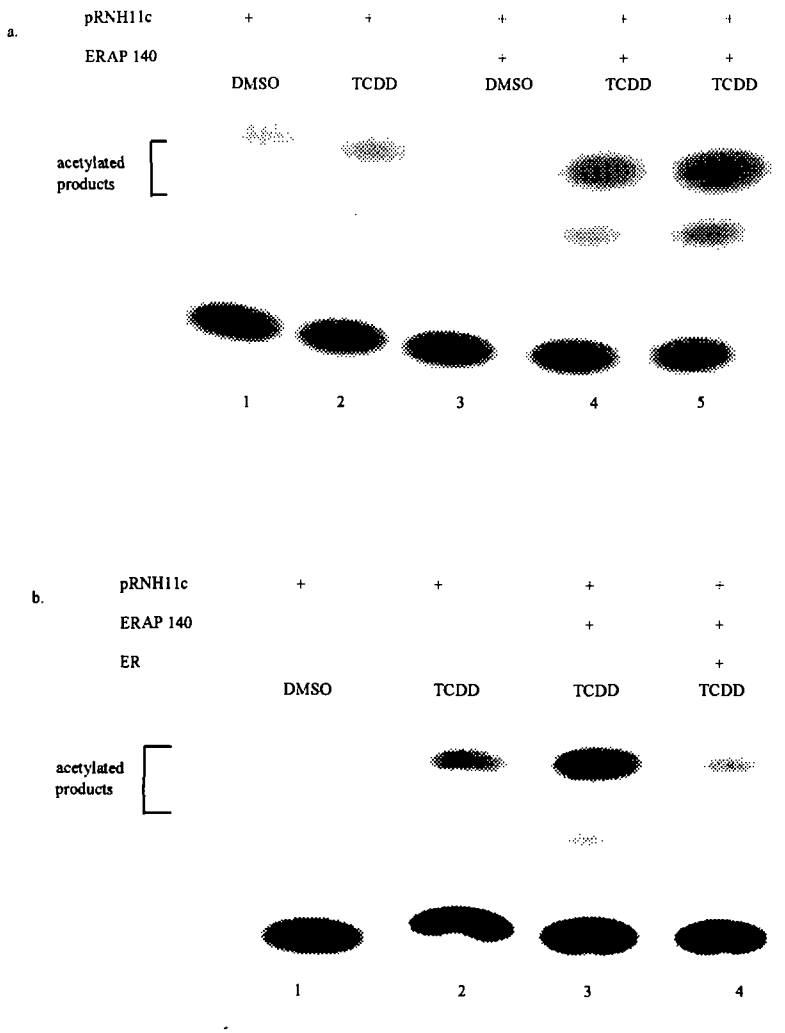

Figure 1. MCF-7 cells were transiently transfected with 3μg pRNH11c (lanes 1-5)and 10 (lane 4) or 50 ng (lanes 3,5) ERAP 140 and CAT activity was measured as described in the *Experimental Procedures* (panel a). To determine the effect of hER on CAT activity (panel b), MCF-7 cells were transfected with 3μg pRNH11c (lanes 1-4), 10 ng (lanes 3,4) ERAP 140 and 100 ng hER (lane 4).

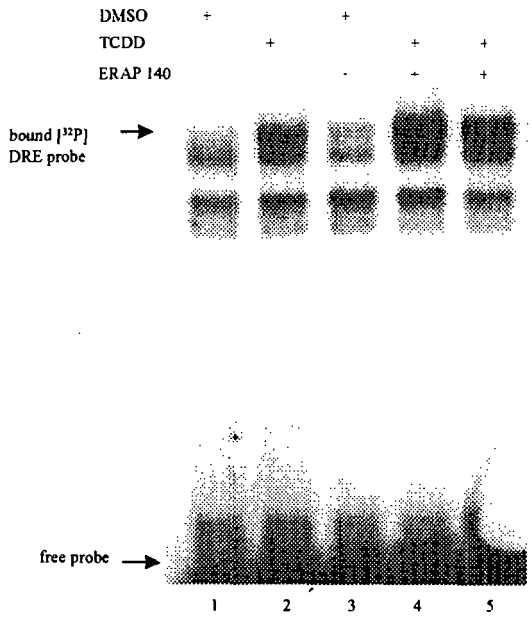

Figure 2. Electrophoretic mobility shift analysis of *in vitro* translated/transcribed proteins. ERAP 140, AhR and Arnt were expressed using the rabbit reticulocyte system as described in *Experimental Procedures*. Retention of a [^{32}P]-DRE probe by the AhR/Arnt complex in the presence of 3 (lane 3,4) and 1.5μl (lane 5) of lysate expressing ERAP 140 was measured.

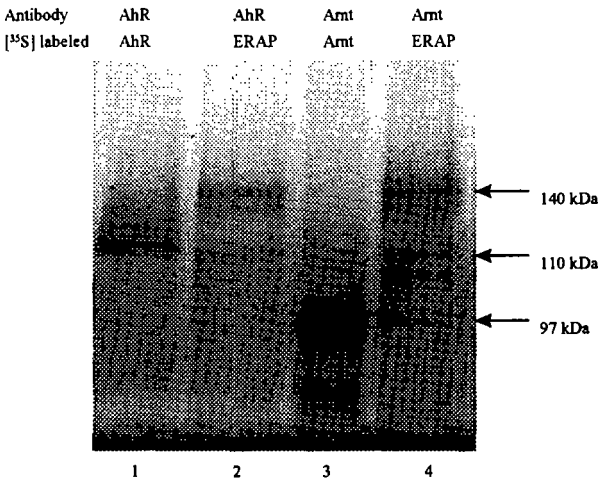

Figure 3. Immunoprecipitation of ERAP 140. AhR, Arnt and ERAP 140 were [^{35}S]-labeled using the rabbit reticulocyte lysate system as described in Experimental Procedures.

Acknowledgements

The financial assistance of the National Institutes of Health (ESO4917, and ESO5734) and the Texas Agricultural Experimental Station is gratefully acknowledged. S. Safe is a Sid Kyle Professor in Toxicology.

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