2,3,4,7-TETRACHLORODIBENZO-*p*-DIOXIN INHIBITS TRANSCRIPTIONAL ACTIVATION OF CREATINE KINASE B GENE PROMOTER CONSTRUCTS BY 17β-ESTRADIOL IN MCF-7 CELLS

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Abstract

Creatine kinase B (CKB) is inducible by 17β-estradiol (E2) in the rodent mammary and uterus and E2 also induces reporter gene activity with constructs (pCKB) containing CKB gene promoter inserts linked to the chloramphenicol acetyltransferase (CAT) gene. E2-responsivess is primarily associated with two regions of the CKB promoter (-568 to -523; -195 to +5) and two potential core pentanucleotide inhibitory dioxin response elements (iDREs) are present at -1074 to -1070 and -819 to -815. Results of extensive mutation and deletion analysis of the CKB gene promoter show that inhibitory aryl hydrocarbon receptor (AhR)-estrogen receptor (ER) interactions are iDRE-independent and mechanistic studies on other interacting pathways are ongoing.

Introduction

Creatine kinase B (CKB) catalyzes phosphorylation of creatine and this enzyme is highly inducible by estrogens in the rodent reproductive tract and mammary gland and in human breast cancer cells lines[1-3]. Transient transfection studies with CKB gene promoter constructs (pCKB) in breast, endometrial and other cancer cells lines show that E2 induces chloramphenicol acetyltransferase (CAT) reporter gene activity and the -568 to -523 region of the promoter is responsible, in part, for induction by 17β-estradiol (E2)[3]. Research in this laboratory has focused on determining the mechanisms of inhibitory aryl hydrocarbon receptor (AhR)-estrogen receptor (ER) crosstalk in breast cancer cell lines and development of selective AhR modulators (SAhRMs) for clinical treatment of breast cancer in women[5,6]. Results of some studies show that inhibition of E2-induced cathepsin D, pS2 and c-*fos* gene expression requires interaction of the AhR complex with an inhibitory pentanucleotide (GCGTG) core dioxin responsive element (iDRE) in their respective gene promoters[7-9]. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) inhibits induction of reporter gene activity (CAT) by E2 in cells transfected with several pCKB1 constructs containing several different CKB gene promoter inserts. The upstream region of the CKB gene promoter contains core DRE motifs at -1074/-1070 and -819/-815; however, mutation of these sites did not affect inhibitory AhR-ER crosstalk suggesting that other interacting pathways may be important.

Materials and Methods

Cells, culture and oligonucleotide

MCF-7 cells were maintained in MEM with phenol red and supplemented with 10% FCS plus 0.2X antibiotic/antimycotic solution, 0.035% sodium bicarbonate, 0.011% sodium pyruvate, 0.1% glucose, 0.238% Hepes, and 6×10^{-7}% insulin. SL2 cells were grown at room temperature in T-150

flasks in Schneider's medium (Gibco BRL, Gaithersburg, MD) supplemented with 10% fetal calf serum (heat inactivated at 56°C for 30 min) and 0.5X antibiotic/antimycotic solution.

The CKB8 oligonucleotide structure (sense strand) and its descriptor are given below. The Sp1 and nonconcensus palindromic estrogen response element (ERE) sequences are *underlined*, the mutated bases are indicated with an *asterisk* (*), and the Hind III and Bam HI linker sequences are *italicized*. The oligonucleotide was cloned into pBLTATA-CAT vector to give pCKB8.

CKB8 (-568 to -523) oligonucleotide:
5' - *AGC TT*G GGC CCG CCC AAG GTC AGA ACA CCC TGG GTG CTT CCG GGC GGG ACC *G* 3'

Transient transfection and CAT assays

Cultured MCF-7 cells were transiently transfected by the calcium phosphate method with 10 μg reporter plasmids and 5 μg of hER. Three hours prior to transfection, the medium was replaced with 5 ml of the charcoal stripped DME F-12 medium. Cells in each petri dish were transfected with 1 ml transfection cocktail containing appropriate plasmids, 30 μl of 2.5 M $CaCl_2$, and 500 μl 2X HBS (pH 7.05). After incubation for 14 to 16 hr at 37°C and air:CO_2 (95%:5%), the cells were washed once with 5 ml of PBS and dosed with 2 nM E2 and/or 10 nM TCDD in 10 ml of the charcoal stripped DME F-12 medium. Two days later, the cells were washed once with 5 ml of PBS and harvested by scraping. Cells were lysed in 200 μl of 0.25 M Tris-Cl (pH 7.6) by three repeated cycles of freezing in liquid nitrogen for 1.5 min, thawing at 37°C for 1.5 min, sonication for 1.5 min, and vortexing for 3 seconds. The cell debris was pelleted and the protein concentration was measured by the method of Bradford using bovine serum albumin as standard. Cell lysate was incubated with 1 μl [^{14}C]chloramphenicol (53 mCi/mmol) and 42 μl of 4 mM acetyl CoA for 3 hr at 37°C. The reaction was stopped by vortexing with 700 μl of ethylacetate. After vortexing for 30 sec and centrifuging at 16,000 *g* for 1 min at room temperature, 600 μl of ethyl acetate was collected, dried in vacuum, resuspended in 30 μl ethyl acetate, spotted on a thin-layer chromatography (TLC) plate (Whatman Lab Sales, NY), and developed using a 95:5 chloroform:methanol solvent mixture in order to separate the acetylated products. The density of the acetylated chloramphenicol was quantitated using the counts/min obtained from the Packard Instant Imager (Meriden, CT).

Construction of CKB promoter deletion constructs

pCKB1 was digested with Bgl II to release a fragment containing CKB promoter fragment from -1434 to +5. After gel electrophoresis and purification, the Bgl II fragment was ligated into the promoterless vector pBLCAT3 to give pCKB3. For construction of pCDB4, pCKB5, and pCKB6, unidirectional nested deletions into the CKB promoter insert was carried out using Exo III/Mung Bean Nuclease Deletion Kit (Stratagene, La Jolla, CA). PCKB 7 was kindly provided by Dr. Benfield (DuPont Merck Pharmaceutical Co., Wilmington, DE).

Site-directed mutagenesis

Mutations of the core DRE sequences (5'-GCGTG-3') at -1070/-1074 and -819/-815 in the CKB gene promoter were performed using Transformer Site-Directed Mutagenesis Kit (Clontech Laboratories, Pal Alto, CA). pCKB1 was digested with Hind III and Kpn I, and the Hind III-Kpn I fragment containing the 2.9 kb CKB promoter insert was ligated into pBluescript-KS

(Stratagene, La Jolla, CA) to give pBS/CKB. After core DRE sequences were mutated in the pBS/CKB vector, the Hind III-Kpn I fragment was ligated into pCKB1 vector in which the original Hind III-Kpn I fragment containing wild-type core DRE had been removed. The core DRE mutations were confirmed by DNA sequencing.

Results and Discussion

Deletion analysis of the CKB gene promoter (Fig. 1) shows that E2 induces CAT activity with multiple constructs containing both upstream and downstream promoter inserts. TCDD inhibits E2-mediated induction of all constructs and the inhibitory AhR-ER crosstalk was not dependent on the presence of iDREs. The results in Figure 2 summarize results obtained using single and double iDRE mutants for pCKB1 (-2897- to +5) and pCKB3 (-1434 to +5). Wild-type and mutant constructs were inducible by E2 in transient transfection studies in MCF-7 cells and all of these induced responses were inhibited by TCDD. Thus, in contrast to other genes such as c-*fos*, cathepsin D, heat shock protein 27 and pS2 that contain functional iDREs[7-9], inhibition of hormone-induced CKB expression by TCDD does not depend on AhR complex interactions with iDREs. The mechanisms of inhibitory AhR-ER crosstalk on the CKB gene promoter are unknown and are currently being investigated.

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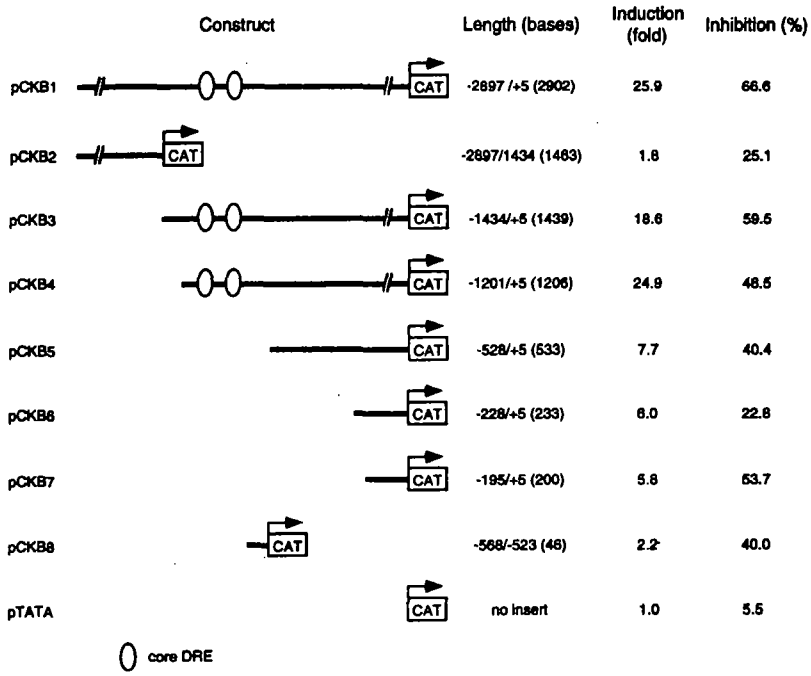

Figure 1. Deletion analysis of the CKB gene promoter. Constructs were transfected into MCF-7 cells and the induction by E2 and inhibition of the E2-induced responses by TCDD was also observed.

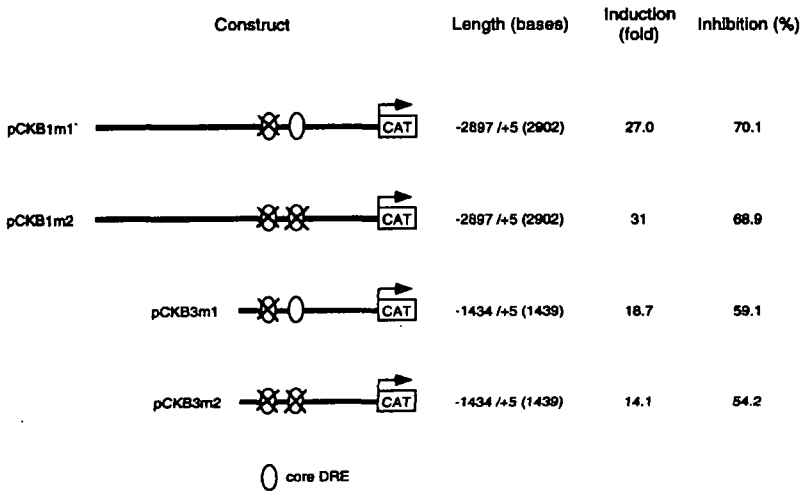

Figure 2. Mutation analysis of pCKB1 and pCKB3. Mutations of putative core iDRE motifs did not affect inhibitory AhR-ER crosstalk in MCF-7 cells.