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INSENSITIVITY OF CATHEPSIN D GENE TO ESTRADIOL IN ENDOMETRIAL CELLS IS DETERMINED BY THE SEQUENCE OF ITS ESTROGEN RESPONSIVE ELEMENT

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In MCF7 cells, transcription of the lysosomal protease cathepsin D is stimulated by estrogens via a non-consensus estrogen responsive element (ERE). By contrast, in estrogen responsive Ishikawa endometrial cancer cells, the cathepsin D gene is unresponsive to estrogens. We now show that the transfected cathepsin D promoter, which can be induced by estrogens in several cell types, is insensitive in Ishikawa cells. The block is not due to a mutation in the cathepsin D promoter or estrogen receptor, but involves the cathepsin D ERE, and implies a C at position 3 of the ERE sequence. Our results suggest that in Ishikawa cells, cathepsin D insensitivity to estrogen most likely occurs through a specific interaction with the ER, or with an endometrial factor which may compete with the ER for binding to the cathepsin D ERE. © 1994 Academic Press, Inc.

In breast cancers, cathepsin D expression is markedly enhanced, and associated with increased risk of developing metastases (for review, see ref. 1). In estrogen receptor (ER) positive human breast cancer cells, cathepsin D gene transcription is directly stimulated by estrogens (2, 3). By contrast, *in vivo* in rat uterus (4) and in primary cultures of epithelial endometrial cells (5), cathepsin D gene expression was found to be increased by progestins but not by estradiol. Accordingly, in ER positive Ishikawa endometrial cancer cells, estradiol had no effect on cathepsin D mRNA and protein levels (6), whereas it stimulated cell proliferation (7) and expression of transforming growth factor α (8), progesterone receptor (6) and alkaline phosphatase (9). Moreover, transfected rat uteroglobin (10) or *Xenopus laevis* vitellogenin A2 (6) promoters were also induced by estradiol in these cells, indicating that they contain functional ER and are a good model to investigate insensitivity of the cathepsin D promoter to estradiol.

The human cathepsin D promoter contains an imperfect estrogen responsive element (ERE), 5'GGcCgggcTGACC3' located at -261 (ref. 11, and Augereau et al.,

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in press), which could stimulate transcription of heterologous Herpes simplex virus thymidine kinase (Tk) promoter. We now show that transfected cathepsin D recombinants containing this site can be induced by estrogens in several cell lines, but are unresponsive in endometrial Ishikawa cells. We show that this estradiol insensitivity is dependent on the ERE structure, and discuss models which could account for this tissue specific response.

MATERIALS AND METHODS

Cell culture

Cells were maintained in Dulbecco's Modified Eagle's Medium supplemented with 10% foetal calf serum (Gibco), (Ishikawa, MCF7, T47D, NIH3T3, COS-1 and BG1 cells) or 5% foetal calf serum (HeLa cells). One week before transfection, cells were switched to medium without phenol red supplemented with charcoal treated serum.

Recombinants

Cathepsin D gene recombinants (Fig. 1) were described in Augereau et al. (in press), and vERE-TkCAT in Philips et al. (12). vERE-CD112 was produced by inserting the vERE (5' CTAGTAGGTCACTGTGACCCCGGATC 3') in the Hind III site of CD112. E2m4CD112 was produced by insertion of oligonucleotide 5' CTGGGCCGGGC-TGACCCCAAAAG 3' (where underlined bases are mutated relative to CDE2) between the Hind III and Sph I sites of CD112. Oligonucleotide 5' TCGACCCAGAAGCTGGGCCGGGCTGACCCCGCGGCG 3', and its mutated forms at underlined positions as described in Fig. 3B, were inserted as monomers or dimers in the Sal I site of pBL-CAT8+, to produce WTCDE2, G1ACDE2, C3TCDE2, and G1AC3TCDE2. The HEO ER expression vector (13) was a gift of P. Chambon. pRSV ß gal plasmid contains the *E. coli* ß galactosidase gene and was used as an internal control for transfection.

DNA transfection and chloramphenicol acetyl transferase (CAT) assay

Transfections were performed as in Cavaillès et al. (11), with 1 µg of TkCAT recombinant, 200 ng of HEO, 2 µg pRSVß gal for a total of 5 µg. Standard CAT assays were normalized for ß galactosidase activity (14), and autoradiographies of chromatograms were quantitated using a Joyce Loebl chromoscan 3 densitometer.

RESULTS AND DISCUSSION

Estradiol unresponsiveness of cathepsin D is not due to promoter or estrogen receptor mutations

To eliminate any possibility that the estradiol insensitivity of endogenous cathepsin D genes in Ishikawa cells could be due to a mutation, we transiently transfected CD3542 (Fig. 1), an estrogen responsive cathepsin D promoter recombinant, together with the HEO ER expression vector (13). Fig. 2A shows that CD3542 was not induced by 10 nM estradiol in Ishikawa cells, whereas it was efficiently induced in MCF7 cells. Insensitivity is therefore not due to a mutation in the cathepsin D promoter or in the endogenous ER, since a functional ER was

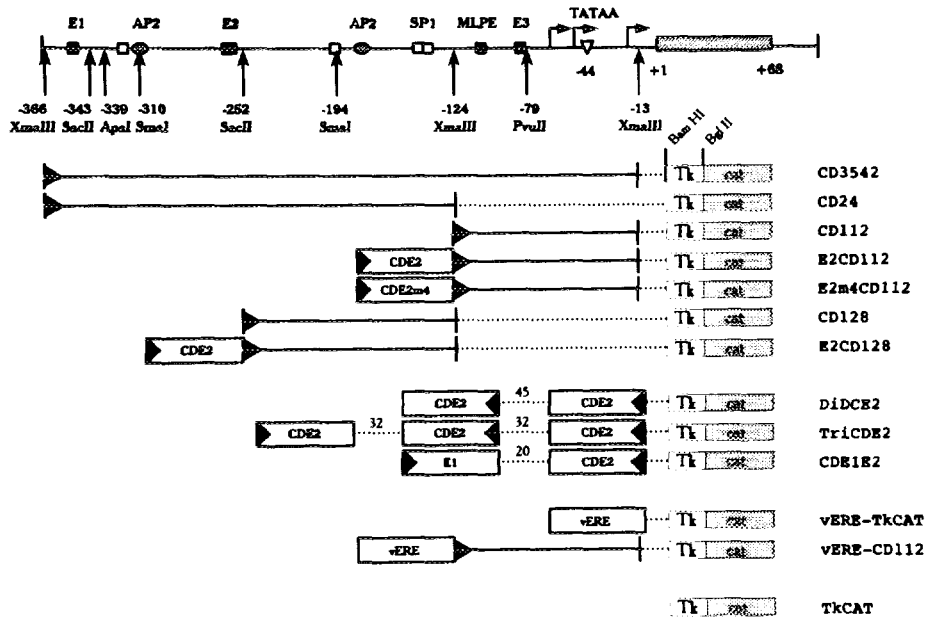


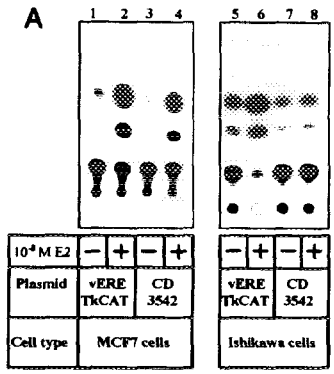
Figure 1. Structure of cathepsin D TkCAT recombinants. The cathepsin D proximal promoter restriction map is shown on the top. Coordinates are given relative to the A of the translation initiation codon. E1, E2 and E3 indicate three half palindromic ERE-like sequences; SP1, MLPE, and AP2 are general regulatory elements (24). The recombinants are shown below, with the corresponding cathepsin D promoter fragments. Large boxes with arrowheads show positions and orientations of inserted *oligonucleotides* (ERE: *consensus* ERE; CDE2, CDE2m4: *cathepsin D* ERE).

cotransfected, and similar results were obtained using HEO (glycine 400 to valine) or the wild-type HEGO (not shown) ER expression vector. It is also unlikely that there is a masking effect of promoter sites by nucleosomes, since transiently transfected plasmids do not seem to be packed into a chromatin structure (15).

We thus evaluated the cell specificity of estrogen induction of the cathepsin D promoter. CD3542 was efficiently induced in MCF7 cells, and to a lower extent in T47D (human breast cancer), BG1 (human ovarian cancer), and HeLa (human cervix cancer) cells (Fig. 2B). Similarly, at least one construct containing the cathepsin D ERE was induced by estradiol in MCF7, T47D, HeLa, and monkey kidney COS-1 cells, and in NIH-3T3 mouse fibroblasts (DiCDE2, TriCDE2, in Fig. 2B). We conclude that the cathepsin D promoter could be induced by estradiol in a wide range of mammalian cell types. Since vERE-TkCAT (Fig. 2, A and C) containing a consensus ERE and the rabbit uteroglobin (10) promoter containing an imperfect ERE were efficiently induced by estrogens in Ishikawa cells, it would seem that hindrance of estrogenic induction was restricted to the cathepsin D promoter, and specific to Ishikawa cells.

Although the mechanism of growth factor action on estrogen regulation is unknown, EGF was shown to increase ER phosphorylation and partially bypass

A



C

Ishikawa cells		
Names of Recombinants	Control	E2 fold Induction
TkCAT	100	-
CD3542	118	0.91 (12)
CD24	243	1.09 (11)
CD112	141	1.37 (10)
E2CD112	106	1.13 (3)
E2m4CD112	68	1.20 (3)
CD128	210	1.29 (3)
E2CD128	206	0.76 (3)
DiCDE2	87	1.61 (9)
TriCDE2	126	1.57 (12)
CDE1E2	95	1.63 (3)
vERE-TkCAT	146	3.24 (15)
vERE-CD112	-	4.92 (3)

B

Names of Recombinants	Estradiol Fold Induction					
	MCF7	T47D	HeLa	BG1	Cos1	NIH3T3
vERE-TkCAT	9.3	7.8	4.2	17.6	4.8	42.9
CD3542	12.8	2.4	2.3	3.9	-	-
CD24	4.7	2.5	2.0	-	-	2.9
CD112	0.5	0.7	0.6	-	-	0.5
DiCDE2	5.9	8.8	2.3	-	3.5	20.2
TriCDE2	18.9	16.1	14.1	6.3	5.3	35.1
CDE1E2	5.6	1.6	1.6	4.8	0.7	8.4

Figure 2. Estrogen regulation of cathepsin D promoter in various cell lines. A. Examples of CAT assay autoradiograms from Ishikawa and MCF7 cells transfected with the indicated recombinants, as described in the Materials and Methods. B. and C. Induction of recombinants (described in Fig. 1) was calculated as a ratio of mean CAT gene expression (normalized for ß gal expression) in 10nM estradiol versus control medium. Basal expression of recombinants was calculated as a percentage of that of the Tk promoter. The experiments were repeated at least 3 times in B, and as indicated in parentheses in C. (- : not determined).

estradiol requirements in ERE-dependent responses (16). We therefore tested whether treatments known to affect phosphorylation could relieve the block to estrogen induction of the cathepsin D promoter in Ishikawa cells. However, none of the tested combinations of estradiol with either 8 nM EGF, 1 µg/ml insulin, or both compounds, or 1 mM 8Br-cAMP, restored estrogen regulation of CD3542 (not shown).

Cathepsin D promoter insensitivity to estradiol is due to its ERE.

We then tested whether a repressor site could interfere with CDE2 ERE-mediated estrogen induction by deleting various parts of the cathepsin D proximal promoter (Fig. 1). E2CD112 was deleted of sequences upstream of -280 and between -250 and -124, notably removing the E1 site and the cluster of SP1 sites, whereas E2CD128 was deleted of sequences upstream of -280 and downstream of -124, removing the E1 site and the most proximal 124 bp sequences. Irrespective of the associated promoter sequences, the cathepsin D CDE2 ERE remained

unresponsive to estradiol (Fig. 2C, E2CD112 and E2CD128), suggesting that no specific site interfered with the estrogen response of the cathepsin D promoter; other constructs (not shown) gave similar results. This conclusion was confirmed using vERE-CD112, a hybrid construct containing the consensus ERE inserted upstream of CD112, which appeared to be efficiently induced by estradiol in Ishikawa cells (Fig. 2C). Moreover, except for CD24 and CD128, changes in the basal transcription levels following these deletions were limited (Fig. 2C), and did not reveal any inhibitory sequence. The twofold increase in basal transcription from CD24 and CD128 was likely due to a cathepsin D gene sequence position closer to the Tk promoter. This suggests that stimulatory sites located upstream of -124 are used for cathepsin D transcription in endometrial cells. By contrast, sites downstream of -124 appeared to be dispensable (CD112), whereas in MCF7 cells these sites are used since CD112 is constitutively stimulated (Augereau et al. in press).

In fact, recombinants containing the isolated cathepsin D ERE were unresponsive to estradiol in Ishikawa cells (CDE1E2, diCDE2 and TriCDE2 in figure 2C). Since CDE2 contains an associated GC-rich sequence (underlined in Fig. 3B), we tested whether it could hinder the cathepsin D ERE mediated estrogen response. Mutagenesis of this sequence (E2m4CD112 described in the Materials and Methods) did not restore estrogen sensitivity of the remaining cathepsin D ERE (Fig. 2C), suggesting that this site was not responsible for the insensitivity. In Ishikawa cells, insensitivity of the cathepsin D promoter to estradiol thus seemed to be

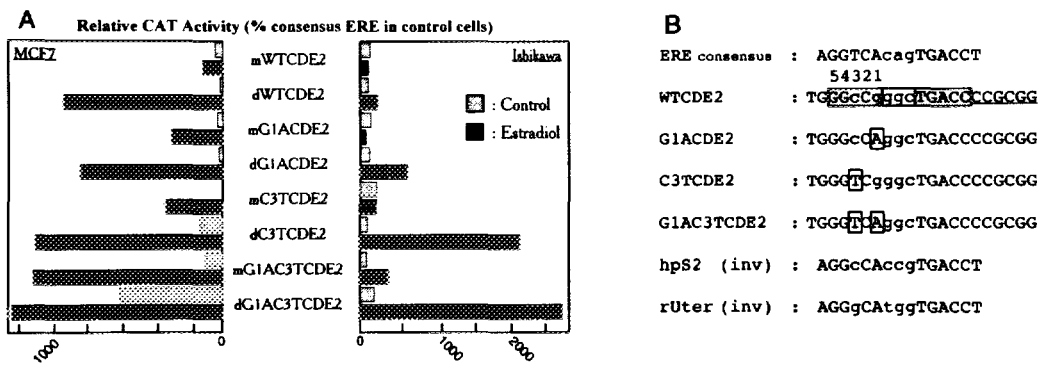


Figure 3. Estrogen induction in Ishikawa cells depends on the ERE structure. A. Mutants of WTCDE2 inserted as monomers (m) or dimers (d) upstream of the Tk promoter were cotransfected with the HE0 ER expression vector in MCF7 and Ishikawa cells. Expression was given as a percent of that of the consensus ERE (mG1AC3TCDE2) in control cells. Experiments were repeted 3 and 4 times in MCF7 and Ishikawa cells,respectively. B. Sequence of various EREs and derivatives of the CDE2 site. Numbering of positions in a half ERE is shown below the vERE. The CDE2 ERE is boxed and the GC-rich sequence is underlined. Positions differing from the consensus ERE are written in lower case letters. Nucleotides mutated in CDE2 are boxed. The hpS2 (17) and rUteroglobin (10) ERE sequences were written from the complementary strand in order to align their conserved halves with that of CDE2.

primarly due to its ERE. However, the fact that DiCDE2 and TriCDE2 were as efficiently or even more efficiently induced than the consensus ERE in MCF7 cells (Fig. 2B), whereas they were not induced at all in Ishikawa cells (Fig. 2C), strongly suggested that the lower affinity of the ER for CDE2 was not the primary cause of insensitivity.

Endometrial cell and ERE sequence specificities of estrogen insensitivity.

The cathepsin D CDE2 site is an imperfect ERE (Fig. 3B). To specify which base change was responsible for estradiol insensitivity, we therefore mutated CDE2 at positions 1 or 3 or both, to convert it to a site more closely related to a consensus ERE (Fig. 3B). The resulting sites were inserted as single or double copies (respectively labelled m and d in Fig. 3A) upstream of the Tk promoter.

In MCF7 cells, single mutant EREs, either as monomers (mG1ACDE2 and mC3TCDE2) or dimers (dG1ACDE2 and dC3TCDE2), were similarly induced by estradiol. The monomer and dimer of the double mutant (G1AC3TCDE2 corresponding to the consensus ERE in the CDE2 environment) were almost as efficiently induced, but the dimer had higher basal transcription (Fig. 3A). By contrast, in Ishikawa cells, single mutant monomers were unresponsive, whereas the double mutant produced 3-fold induction. However, the dimer of C3TCDE2 was almost as efficiently induced as the ERE-like double mutant, whereas estradiol induction of the G1ACDE2 dimer was about 4-fold lower (Fig. 3A), showing that a specific nucleotide change is able to relieve estrogen insensitivity of cathepsin D ERE in these cells.

In Ishikawa cells, inhibition of ERE-mediated estrogen induction is thus dependent on the ERE structure, and the 3rd position of an ERE half site appeared to be important for control of the gene-specific estrogen response. In agreement with this model, pS2, another estrogen regulated gene in mammary cells (17) which has an ERE whose structure is identical to the G1ACDE2 'revertant' (Fig. 3B), is poorly induced by estrogens in Ishikawa cells (FM, unpublished results). By contrast, the rat uteroglobin ERE (Fig. 3B), which has a G in the 3rd position, is efficiently induced in these cells (10). Since estrogen regulation is seen with a T (consensus) or a G (uteroglobin) at this position, but not with a C (cathepsin D, and pS2) (according to Truss et al. (18), the ER cannot tolerate an A at this position), differential sensitivity of uteroglobin, pS2, and CDE2 EREs to estradiol suggests that inhibition of the estrogen response is dependent on a specific interaction at this position. An endometrial factor might, for instance hinder ER binding to specific EREs. It was recently reported that a human endometrial cell line contained high levels of COUP-TF (19), which inhibited ER mediated activation of the mouse lactoferrin gene by competing with ER for overlapping promoter elements. However, such a mechanism seems unlikely since no consensus COUP-TF binding site was

recognized in the vicinity of the cathepsin D ERE, and Ishikawa cells do not seem to contain detectable levels of COUP-TF (Alison Butler and Malcolm Parker, personal communication). Competition for ER binding by RxRβ was also reported by Segars et al. (20), but experiments are needed to determine whether this factor could be involved in insensitivity of the cathepsin D promoter.

Alternatively, since the ER from Ishikawa cells, but not MCF7 cells, differentiates between G1ACDE2 and C3TCDE2, this cell specificity might suggest that the ER bound to specific EREs might not efficiently transactivate in Ishikawa cells due to post translational (or absence of) modifications. Finally, binding of the ER to imperfect EREs could require an additional factor which might be absent from Ishikawa cells. Such a requirement was recently demonstrated for progesterone (21), androgen and glucocorticoid receptors (22), and it was previously shown (23) that highly purified ER also needed a cofactor for efficient binding to an ERE. Gel shift experiments will help to analyze interactions at this site, and discriminate between these hypotheses.

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