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Cell type-specific bidirectional regulation of the glucocorticoid-induced leucine zipper (GILZ) gene by estrogen

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Abstract

Estrogen has numerous beneficial physiological actions; however, by acting as a mitogen, it plays a significant role in the induction and maintenance of breast cancer. Although the positive effects of estrogen on gene expression are well described, negative gene regulation is not. Using microarray analysis, we identified 27 genes that were up-regulated and 20 that were down-regulated by estrogen in MCF-7 human breast cancer cells. One gene encoding GILZ (glucocorticoid-induced leucine zipper protein), a putative apoptosis-regulating transcription factor, is rapidly down-regulated by estrogen in these cells. Estrogen antagonists block the down-regulation. The region of the GILZ promoter between nucleotides −104 and −69 mediates both basal activity and estrogen-dependent down-regulation in MCF-7 cells. This region contains a functional Oct-1 binding site and a cyclic AMP response element binding protein (CREB) binding site. The same DNA region mediates up-regulation by estrogen in HeLa and HEK293 cells, indicating that cell-specific factors are involved in estrogen regulation of this gene. The estrogen receptor (ER) is present in GILZ promoter protein complexes, but it does not bind directly to the promoter itself, as the DNA-binding domain of the estrogen receptor is not required for down-regulation. Elimination of the CREB binding site blocks both basal activity and estrogen regulation. Our results suggest that ER action at the CRE may mediate estrogen-dependent, cell-specific regulation of this gene.

Keywords: Estrogen regulation; Negative regulation; GILZ; Steroid; Microarray

1. Introduction

The estrogen receptors (ERα and β) mediate most of the physiological effects of estrogen. These effects are mainly due to the role of ERs as transcription factors; however, there is increasing evidence that there are also non-genomic actions of estrogen that are mediated by the ERs or a related receptor [1]. The ER has multiple mechanisms by which it can influence global gene expression in a cell when ligand is bound. These include: (a) direct binding to promoters through an estrogen response element (ERE), where it serves to recruit other proteins involved in transcription [2], (b) indirect binding to DNA through interactions with other transcription factors at sites such as AP-1 or Sp1 [3–6], and (c) influencing the expression of downstream transcription factors, ultimately leading to changes in the expression of genes that are not direct targets of ER binding. Although estrogen can both turn on and turn off genes, the mechanisms of down-regulation are poorly understood compared to those underlying up-regulation.

One of the physiological targets of estrogen is the mammary gland. In the mammary gland, the balance between cell proliferation, differentiation, and apoptosis is important for normal tissue development [7]. Perturbation of the balance between proliferative and apoptotic states can lead to the accumulation of damaged cells, resulting in the development of cancer. Estrogen induces up-regulation of genes that induce

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cell proliferation and DNA replication [8], which can promote the growth of transformed cells. Normally, cells with unrepaired DNA damage are eliminated by mechanisms that trigger apoptosis; however, estrogen has been shown to suppress apoptosis of breast cells. It is associated with down-regulation of pro-apoptotic genes, such as Bax and Bad [9], and it directly induces expression of anti-apoptotic genes, such as Bcl-2 [10].

MCF-7 breast adenocarcinoma cells are a good model system for studying gene regulation by estrogen. It is one of the few cell lines available that expresses a significant amount of endogenous ERα, and shows an increase in proliferation in response to estrogen treatment. In this study, we have used microarray analysis to look at changes in gene expression in MCF-7 cells in response to 17β-estradiol. Many of the affected genes that we identified have not been previously shown to be estrogen-responsive. Here we describe a detailed analysis of the glucocorticoid induced leucine zipper (GILZ) gene, which is down-regulated by estrogen in MCF-7 breast cancer cells, but is up-regulated in HEK293 and HeLa cells.

2. Materials and methods

2.1. Cell culture

MCF-7 adenocarcinoma cells were maintained in RPMI 1640 (Gibco BRL, Rockville, MD) supplemented with 10% heat-inactivated defined fetal bovine serum (HyClone Laboratories, Logan, UT), 6 ng/mL bovine insulin, non-essential amino acids, penicillin/streptomycin, and L-glutamine (Gibco BRL). When cells were at approximately 50% confluence, they were washed twice and incubated for 24 h in MCF-7 assay medium (phenol red-free RPMI 1640, 10% heat-inactivated charcoal/dextran-treated serum, 6 ng/mL bovine insulin, non-essential amino acids, penicillin/streptomycin, and L-glutamine). The cells were then incubated for 24 h (unless otherwise noted) in assay medium plus vehicle (ethanol), or 100 nM 17β-estradiol (E2; Sigma). In studies to evaluate the inhibition of protein synthesis, cycloheximide (Calbiochem, San Diego, CA) was also added to the culture media 24 h prior to cell lysis. Following the incubation RNA was isolated from the cells.

HEK293 human embryonic kidney cells were maintained in phenol red-free DMEM/F-12 (Sigma) supplemented with 10% heat-inactivated defined fetal bovine serum (HyClone Laboratories, Logan, UT), and penicillin/streptomycin. Cells were trypsinized and transferred to HEK293 assay medium (phenol red-free DMEM/F-12, 10% charcoal/dextran treated fetal bovine serum, penicillin/streptomycin) and aliquoted into 96-well culture dishes at 10,000 cells per well for subsequent assays. HeLa human cervical carcinoma cells were grown in DMEM (GibcoBRL) supplemented with 10% heat-inactivated defined fetal bovine serum (HyClone), penicillin/streptomycin, and sodium pyruvate (GibcoBRL). Cells were trypsinized and transferred to HeLa assay medium (phenol-red-free DMEM (GibcoBRL), 10% charcoal–dextran treated fetal bovine serum (HyClone), penicillin/streptomycin, and sodium pyruvate (GibcoBRL)), and aliquoted into 96-well culture dishes at 8000 cells per well for subsequent assays.

2.2. RNA purification

Total RNA was prepared from cells with the RNeasy mini kit (QIAGEN, Valencia, CA), using one QIAshredder (QIAGEN) per 100 mm dish of cells for homogenization. All steps were performed as described by the manufacturer. RNA was quantitated using a GeneQuant DNA/RNA Calculator (Amersham Pharmacia Biotech, Piscataway, NJ).

2.3. DNA microarray hybridization

Chips were generated and hybridized by the Microarray Group, Johnson & Johnson Pharmaceutical Research and Development, LaJolla, CA, as follows. The cDNAs printed on the microarrays were from the IMAGE consortium (Integrated Molecular Analysis of Genome and their Expression) and Incyte libraries. A total of 22,447 were printed, ranging in size from 200 to 1000 bp. They consisted of random expressed sequence tags, covering about 10,000 separate human genes. All clones were sequence-verified before PCR amplification. The IMAGE clones were purchased from the Human UniGene Library (Research Genetics, Huntsville, AL). Complementary DNAs (cDNAs) were printed in duplicate on GAPs II amino silane-coated slides (Corning, Corning, NY) using a Generation III Microarray Spotter (Amersham Biosciences). The cDNAs were amplified by PCR and purified with a Qiagen 96 PCR purification kit, then were mixed 1:1 with a 10 M NaSCN printing buffer. The cDNA spots were ≈250 μm in diameter with a 280 μm center-to-center spacing. Each microarray included 30-plant genes for the determination of non-specific hybridization (gift from Mark Schena, Stanford University). Prior to hybridization, printed microarrays were incubated in isopropanol at room temperature for 10 min, then in distilled water for 2 min and were centrifuged to dry.

To make the probe from the sample RNA, one round of T7 polymerase-based linear RNA amplification was performed by reverse transcription of RNA with a T7 promoter oligo(dT) primer and Cy3-dCTP-labeled fluorescent cDNA probes synthesized from the amplified RNA as described [11], except that to degrade the amplified RNA template, RNaseA (10 units) was added and incubated at 37 °C for 20 min. Then the probes were purified with a PCR purification kit (Qiagen, Valencia, CA), vacuum-dried, and resuspended in 50 μL of Version 2 hybridization buffer (Amersham Biosciences, Piscataway, NJ) with 50% formamide and containing human C_ot1 DNA (Invitrogen, Carlsbad, CA).

The probes were heated to 95 °C for 2 min, cooled to room temperature for 5 min, and applied to the slides. The slides were covered with glass cover slips, sealed with DPX (Fluka),

and hybridized at 42 °C overnight. Microarrays were scanned with a Generation III Array Scanner (Molecular Dynamics), or an Agilent G2565AA Microarray Scanner (Agilent Technologies, Palo Alto, CA). Fluorescence intensity for each feature of the array was obtained using Autogene 3.0 software (BioDiscovery, Los Angeles, CA) or Imagene 4.2 (BioDiscovery) software. Raw intensity data from the same experiment were normalized to the 75th percentile across all chips. Each gene identifier was spotted twice on each chip, and duplicate chips were hybridized to each labeled sample. Thus, quadruplicate data points were generated for each gene identifier ($n = 4$).

The average of all estrogen-treated samples was compared to the average of all control samples. All genes that showed a two-fold or greater difference between the two groups were analyzed by "electronic northern", a graphical representation of normalized spot intensity data from multiple samples for a specific gene. Only genes in which all samples within a group showed consistent results with a distinct difference between groups were considered to be estrogen-regulated.

2.4. Quantitative RT-PCR

Real time reverse transcription-coupled polymerase chain reaction (RT-PCR) was performed using a LightCycler instrument (Roche Molecular Biochemicals, Mannheim, Germany) with a SYBR Green I RNA Amplification Kit and RNA Control Kit (Roche Molecular Biochemicals). The procedure was carried out essentially as described by the manufacturer, using 60 ng total RNA per 20 μL reaction. The reverse transcription was performed at 55 °C for 20 min, followed by 45 cycles of PCR using an annealing temperature of 61 °C. A standard curve was created from the control reactions, and the relative expression levels of the experimental samples were determined using the LightCycler software (Version 3; Roche Molecular Biochemicals). All samples were compared to their respective controls and reported as percent expression, with controls set to 100%. LightCycler-generated melting curves were analyzed to verify the specificity of the PCR reactions and products.

2.5. Plasmid constructs

GILZ1198: The 5′ flanking region of GILZ (nucleotides −1198 to −1) was amplified from human genomic DNA (BD Biosciences Clontech, Palo Alto, CA) by PCR. The PCR fragment was cloned directly into the pGEM-T vector (Promega, Madison, WI). GILZ1198/pGEM was digested with *Kpn*I and *Hind*III (Promega) and ligated to digested pGL3basic luciferase reporter vector (Promega).

Truncated GILZ reporters: GILZ751, GILZ104, GILZ88, GILZ81, GILZ75, and GILZ69 were made by PCR using the GILZ1198 construct as a template. The 5′ primers were designed to give the appropriate 5′ ends, and added a *Kpn*I site for cloning. The 3′ primer amplified the *Hin*dIII site from the GILZ1198 construct. The PCR products were digested with *Kpn*I and *Hin*dIII and ligated directly to digested pGL3basic.

ERE: An oligonucleotide containing two tandem estrogen response elements (EREs) from the *Xenopus* vitellogenin gene [12] and flanking *Nhe*I and *Bgl*II sites (oligonucleotide sequence 5′-GGAGCTAGCTAGAGGTCACAGTGACCTA-CGAGTCCCTAGAGGTCACAGTGACCTACGAGATCT-GGA-3′) was subcloned into the vector pTA-Luc (BD Biosciences Clontech).

ERα/pCI-neo and ERα/pcDNA3.1: A full-length (2.0 kb) human estrogen receptor α (ERα) cDNA was cloned from testis RNA by RT-PCR. The amplified cDNA was cloned into the TA cloning vector pCR2.1 (Invitrogen Life Technologies, Carlsbad, CA). It was subcloned into *Eco*RI-digested pCI-neo (Promega) or *Eco*RI-digested pcDNA3.1(+) (Invitrogen).

ERβ/pcDNA3.1: A 1.5 kb fragment of the human estrogen receptor β (ERβ) cDNA, lacking about 100 bp of coding sequence from the amino-terminus of the receptor was cloned from testis RNA using RT-PCR. The amplified cDNA was cloned into the TA cloning vector pCR2.1. It was then excised using *Spe*I and *Xba*I and subcloned into *Xba*I-digested pcDNA3.1. Next, a 0.54 kb fragment of the receptor amino-terminus, containing the missing 100 bp of coding sequence, was amplified from human prostate RNA and cloned into the TA vector pGEM-T Easy (Promega). The insert was then subcloned into the *Eco*RV-digested pcDNA3.1 partial recombinant to generate the full-length coding sequence.

Mutant ER expression constructs: The mutants were based on HE 19 (Δ*N*-term), HE 11 (ΔDBD) and HE 15 (ΔLBD) described in Chambraud et al. [13]. High-fidelity PCR was performed using ERα/pcDNA3.1 as a template. PCR fragments were cloned into pGEM-T Easy, excised, gel-purified and inserted into pcDNA3.1(+) as described below. All PCR-generated clones were sequenced.

Δ*N*-term: The PCR fragment consisted of the coding sequence for amino acids 180–595 of the receptor. An initiator methionine was added to the 5′ end of the open reading frame. Restriction sites for *Hin*dIII and *Bam*HI were inclu at the 5′ and 3′ ends of the amplimer, respectively. The fragment was excised from pGEM-T Easy using *Hin*dIII and *Bam*HI digestion, which released two fragments: a *Hin*dIII–*Hin*dIII fragment encoding amino acids 180–340, and a *Hin*dIII–*Bam*HI fragment encoding amino acids 341–595. The latter was sub-cloned into pcDNA3.1(+) first. The former was then inserted into the new sub-clone to generate the final mutant.

ΔDBD: Two PCR fragments were generated. One consisted of coding sequences for amino acids 1–184 of the receptor, with *Hin*dIII and *Eco*RI sites at the 5′ and 3′ ends, respectively. The other consisted of amino acids 253–595, with *Eco*RI and *Bam*HI sites at the 5′ and 3′ ends, respectively. These sites were used to generate fragments for simultaneous insertion into *Hin*dIII- and *Bam*HI-digested pcDNA3.1(+). The linking *Eco*RI site introduced two novel amino acids (phenylalanine followed by glutamic acid) in place of the DNA binding domain.

ΔLBD: The PCR fragment consisted of the coding sequence for amino acids 1–281 of the receptor. A stop codon was added to the 3′ end of the open reading frame. Restriction sites for *Hin*dIII and *Bam*HI were included at the 5′ and 3′ ends of the amplimer, respectively, for sub-cloning purposes.

2.6. Luciferase assays

Sixteen to twenty-four hours after transfer to the assay plates, HEK293 and HeLa cells were transfected with 10 ng/well reporter plasmid and 55 ng/well receptor plasmid using 1 or 0.8 μL/well Lipofectamine 2000 (Gibco BRL, Grand Island, NY). Transfection proceeded for approximately 16 h, after which the transfection mixture was replaced with assay medium containing vehicle or test compound at the appropriate concentration. After a 24-h incubation, Steady-Glo luciferase reagent (Promega) was added and luciferase activity was determined on an MLX Microtiter Plate Luminometer (Dynex Technologies, Chantilly, VA).

MCF-7 cells were plated at 10,000 cells per well on 96-well plates in MCF-7 assay medium (see above). After 16–24 h, cells were transfected with 100 ng/well reporter plasmid and 0–100 ng/well receptor plasmid, using 0.8 μL/well LipoTAXI reagent (Stratagene, La Jolla, CA) in a volume of 20 μL. After a 4–6-h incubation, 20 μL of MCF-7 assay medium containing 20% charcoal/dextran-treated fetal bovine serum were added to each well. Approximately 20 h later, medium was replaced with MCF-7 assay medium containing appropriate amounts of vehicle or test compound. Luciferase activity was determined 24 h later as described above for HEK293 and HeLa cells. All results are reported as percent expression, normalized to no reporter, no receptor, empty reporter plasmid, empty receptor plasmid, and/or no estrogen controls, as indicated in figure legends.

4-Hydroxy-tamoxifen was purchased from Sigma (St. Louis, MO), and raloxifene was obtained from Organix, Inc. (Woburn, MA).

2.7. Electrophoretic mobility shift assays

Electrophoretic mobility shift assays (EMSAs) were carried out as previously described [14], except that 100 ng (Fig. 7a and Fig. 8) or 400 ng (Fig. 7a) of poly(dI-dC)/(dI-dC) were used. Pre-incubations contained 1 μg MCF-7 cell nuclear extract (Oncogene, San Diego, CA), anti-Oct-1 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), anti-ER (Santa Cruz Biotechnology, Inc.), 6 pmol human recombinant ERα (PanVera, Madison, WI), and/or a 1:1000 molar excess of cold competitor oligonucleotides:

Oct-1: 5′-GATCTTCTAGTGATTTGCATTCGACA-3′;

Octmut: 5′-GATCTTCTAGTGGCTTGCATTCGACA-3′;

ERE: 5′-GATCTTACAGGTCACCTTGACCTTACT-3′;

GILZ: 5′-GATCCATGGCGTCAGGGGCCATGCAAATGATGAG-3′;

CREBcon (CRE consensus): 5′-GATCAGAGATTGCCTGACGTCAGAGAGCTAG-3′;

GILZ/CREB (CRE consensus oligonucleotide with the GILZ putative CREB site): 5′-GATCAGAGATTGCCTGACGCCAGAGAGCTAG-3′;

mutCREB: 5′-GATCAGAGATTGCCTGTGGTCAGAGAGCTAG-3′.

Oct-1, CREBcon, Octmut and mutCREB were based on the sequences of oligonucleotides available from Santa Cruz Biotechnologies. Pre-incubations were performed at room temperature for 20 min or on ice for 2 h, after which ^{32}P-labelled GILZ oligonucleotide was added, and incubations continued for 20 min at room temperature. Reactions were separated on 6% TBE gels (Invitrogen). The gels were dried and exposed to a phosphor screen for 1–16 h, and imaged on a STORM840 (Molecular Dynamics).

3. Results

3.1. Microarray analysis of gene expression

Microarrays containing 22,447 cDNAs were used to analyze gene expression in MCF-7 cells after a 24 h treatment with vehicle or 100 nM E2. Five samples from each treatment were hybridized to the microarrays; however, only samples that were processed together are directly comparable, resulting in multiple, smaller comparison groups for each chip.

Using normalized hybridization intensity data, we directly compared the estrogen-treated samples to controls, followed by electronic northern analysis. Electronic northern analysis compares the normalized spot intensities of a given gene on a microarray chip across treatment conditions. Table 1 shows a compilation of the genes that showed consistent estrogen-dependent regulation across all comparable samples. Several of these genes have been previously reported to be estrogen-regulated, including: HMG-1 [15], *myc* [16], nucleophosmin [17], cathepsin D [18] and TGFβ3 [19]. Recently, ephrin A1 and Id3 were also identified as estrogen-regulated genes using microarrays [20].

3.2. Confirmation of microarray results

In order to confirm the results for selected genes from the microarrays, quantitative RT-PCR was performed using two of the original sample sets. All 18 of the genes that were tested were confirmed using this approach (see Table 1, right column), which validates the rigorousness of the method used to select estrogen-regulated genes. The estrogen responsiveness of nine of the genes from Table 1 was further verified by quantitative RT-PCR using three additional RNA sample sets that had not been used for the microarrays (Fig. 1a and b). All nine genes were verified as being estrogen-regulated in all sample sets.

Table 1
Estrogen-regulated genes in MCF-7 cells

GenBank Accession #	Gene product name	Function/location	Confirmed
Up-regulated genes			
NM_002128	HMG-1	Transcription co-factor	Y
NM_000269	Nm23a	Transcription factor	
Y00396	Myc	Transcription factor	
NM_006392	Nop56	Nucleolar	Y
NM_005381	Nucleolin	Nucleolar	Y
AA669758	Nucleophosmin	Nucleolar	Y
AA063624	LYAR-related protein	Nucleolar	Y
AK021577	P120	Nucleolar	
AF104032	LAT1	Amino acid transporter	
U45448	P2X1 receptor	ATP gated channel	
M88461	NPYY	Neuronal receptor	
NM_001408	EGFL2	G-protein receptor	
NM_001526	HCRTR2	G-protein receptor	
NM_000706	Vasopressin receptor 1A	G-protein receptor	
NM_001877	Cd3 receptor 2	Complement receptor	
X00474	pS2	Cancer marker	Y
NM_001909	Cathepsin D	Protease	
H23124	Neuronal olfactomedin-1	Extracellular matrix	
NM_003090	SNRPA1	RNA processing	
NM_006868	RAB31	Vesicle trafficking	
NM_006597	HSPA8	Heat shock protein	
M80254	Cyclophilin 3	Mitochondrial	
NM_005729	Cyclophilin F	Mitochondrial	
AA449334	IMAGE clone 785703	Unknown	
AA418828	IMAGE clone 767991	Unknown	
N75569	Novel	Unknown	
AA449333	Novel	Unknown	
Down-regulated genes			
J03241	TGFb-3	Growth factor	Y
AA496452	Granulin	Growth factor	Y
NM_004428	Ephrin A1	Growth factor	Y
AA775091	GILZ	Transcription factor	Y
AA433851	ESX	Transcription factor	Y
AA664389	TSC-22	Transcription factor	Y
AA074535	HPIP	Transcription co-factor	Y
AA547158	Id1	Transcription co-factor	Y
T65736	SP56	Transcription co-factor	Y
AF036892	ACTR	Transcription co-factor	Y
AA48219	Id3	Transcription co-factor	Y
Y12692	Wnt11	Signal transduction	
I03203	Peripheral myelin protein 22	Intercellular junctions	
AA188256	Multidrug resistance protein 5	Cellular export	
XM_006951	ENAC alpha	Sodium channel	
L78207	SUR1	Potassium channel	
AA453898	Sialyltransferase	Posttranslational modifications	Y
AA459364	p53DINP1	p53 Regulator	
H92504	Hypothetical protein	Unknown	
W84612	IMAGE 256835	Unknown	

Genes that were found by microarray hybridization to be consistently regulated by estrogen in all E2-treated MCF-7 cell samples are listed with corresponding GenBank accession numbers. All of the genes that were tested by RT-PCR were confirmed as estrogen-regulated, and are indicated at the right with Y. Confirmation data are shown in Fig. 1

The results from the microarrays showed an approximately 5.5-fold increase for pS2 RNA and a 50% decrease for both granulin and GILZ RNAs following estrogen treatment. However, real time PCR, which provides a more accurate quantitation of expression changes, revealed a greater than 30-fold increase for pS2, and 55% and 75% decreases in granulin and GILZ RNA levels, respectively (Fig. 1).

3.3. Time course of estrogen treatment

Since all microarray and confirmatory RNA samples were purified from MCF-7 cells after 24 h treatment with E2, we examined pS2 and GILZ expression at several additional time points (Fig. 2a). The level of pS2 RNA is almost three times higher in the estrogen sample than in the corresponding

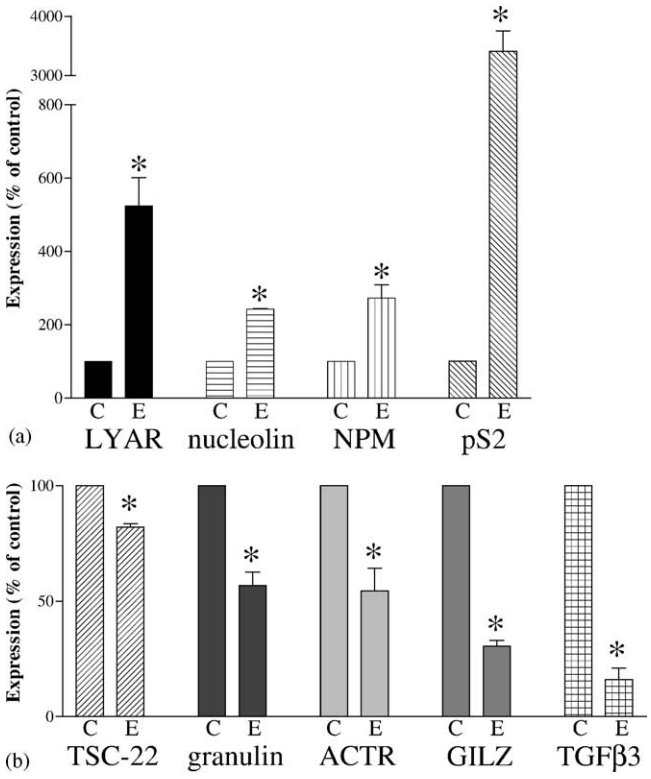

Fig. 1. Quantitative RT-PCR verification of microarray results. Three additional RNA sample sets were generated for RT-PCR verification of selected genes identified by microarray hybridization. (a) RT-PCR verification of four of the up-regulated genes. (b) RT-PCR verification of five down-regulated genes. All results are normalized to vehicle control samples. Data plotted are the average of two or more determinations with each of the three sample sets. Error bars indicate the standard deviation between samples. (*) Indicates that sample differs from control with $P < 0.05$ by a two-tailed paired *t*-test. *E* = 100 nM E2; C = ethanol control.

untreated control at 10 h, with expression continuing to increase to approximately 30-fold at 50 h. GILZ, however, which is down-regulated by estrogen, showed a maximal decrease in RNA levels of nearly 75% at 10 h, after which the expression level increases.

Given these results, we re-examined expression of some other genes at various times of E2 treatment by microarray analysis (Fig. 2b). These results clearly demonstrate differential kinetics of gene expression following estrogen treatment.

Rapid down-regulation of GILZ suggested that it is directly regulated by estrogen, and does not require intermediate protein synthesis. Cycloheximide treatment did not significantly affect estrogen-dependent down-regulation of GILZ, as assessed by RT-PCR and chip analysis (data not shown), supporting a direct mechanism.

3.4. Analysis of the GILZ promoter region

In order to locate regions that mediate estrogenic regulation, we initially cloned a genomic fragment spanning nucleotides −1 though −1199 of the human GILZ gene promoter. This DNA fragment was inserted into pGL3basic to make a luciferase reporter construct (GILZ1198).

To test for estrogen-dependent down-regulation of the GILZ1198 reporter, we co-transfected MCF-7 cells with GILZ1198 and ERα/pcDNA3.1 or pcDNA3.1 empty vector (Fig. 3a). The GILZ1198 reporter was down-regulated by estrogen in a dose-dependent manner in cells co-transfected with the empty pcDNA3.1 vector; however, cells overexpressing ERα gave a more robust and consistent down-regulation. Because of this, overexpression of ER was used in all subsequent experiments. As a positive control, the ERE construct was transfected; luciferase activity was significantly induced with or without ERα overexpression. Co-transfection of ERβ with GILZ1198 also demonstrated greater estrogen-dependent down-regulation than with co-transfection of the empty expression vector (data not shown), indicating that GILZ is regulated by both ERs.

To examine estrogen regulation of the GILZ reporter construct in other cell types, GILZ1198 was co-transfected with an ERα expression vector into HeLa human cervical adenocarcinoma cells and human embryonic kidney (HEK293) cells (Fig. 3b), neither of which express endogenous ER. Surprisingly, the GILZ reporter was up-regulated by E2 nearly ten-fold in HeLa cells and three- to four-fold in HEK293 cells.

3.5. Estrogen-dependent down-regulation of GILZ is antagonized by SERMs

To verify that ER mediates the estrogen response of the GILZ promoter in MCF-7 cells, we tested for antagonism by tamoxifen and raloxifene. MCF-7 cells were cotransfected with GILZ1198 or a shorter promoter construct, GILZ751, and with ERα/pcDNA3.1. The transfected cells were treated with 1 nM E2 or 1 nM E2 and 100 nM of either antagonist (Fig. 4). Down-regulation of the two GILZ reporters was antagonized by both raloxifene and tamoxifen.

3.6. Identification of an estrogen-responsive sequence in the GILZ promoter

The GILZ1198 promoter fragment supported a dose-dependent change in luciferase activity by estrogen in MCF-7, HeLa, and HEK293 cells; therefore, this clone must contain at least one estrogen-responsive region. Additional GILZ luciferase reporter constructs were made that truncated GILZ1198 from the 5′ end (Fig. 5a). Truncations to GILZ104 (nucleotides −1 to −105 of the GILZ gene) gave results identical to GILZ1198 for both basal activity and estrogen response, indicating that the 104 nucleotides immediately 5′ to the transcription start site contain the regulatory elements for both functions.

Further truncation of GILZ104 to GILZ88 and GILZ81 both reduced basal activity to similar levels, approximately 65% of GILZ1198. Truncation to GILZ75 further decreased basal activity to 10% that of GILZ1198. GILZ88, 81, and

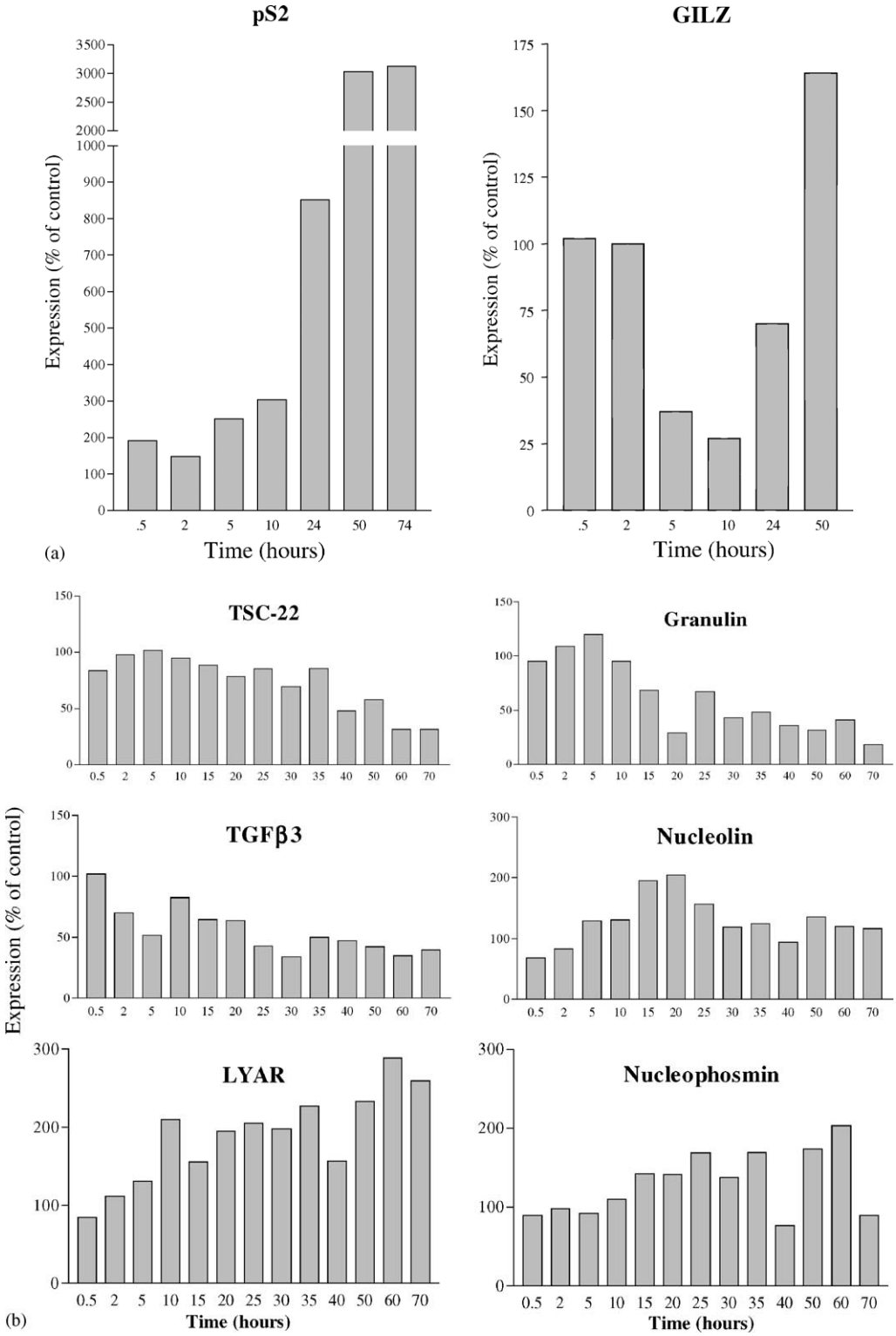

Fig. 2. Time course of estrogen treatment. MCF-7 cells were treated with 100 nM E2 for the indicated times, and the expression levels of specific genes were examined in each sample. (a) Quantitative RT-PCR analysis of expression levels over time for pS2 and GILZ. (b) Microarray analysis of expression level changes over time for several genes. In all cases, each sample was normalized to a vehicle control sample.

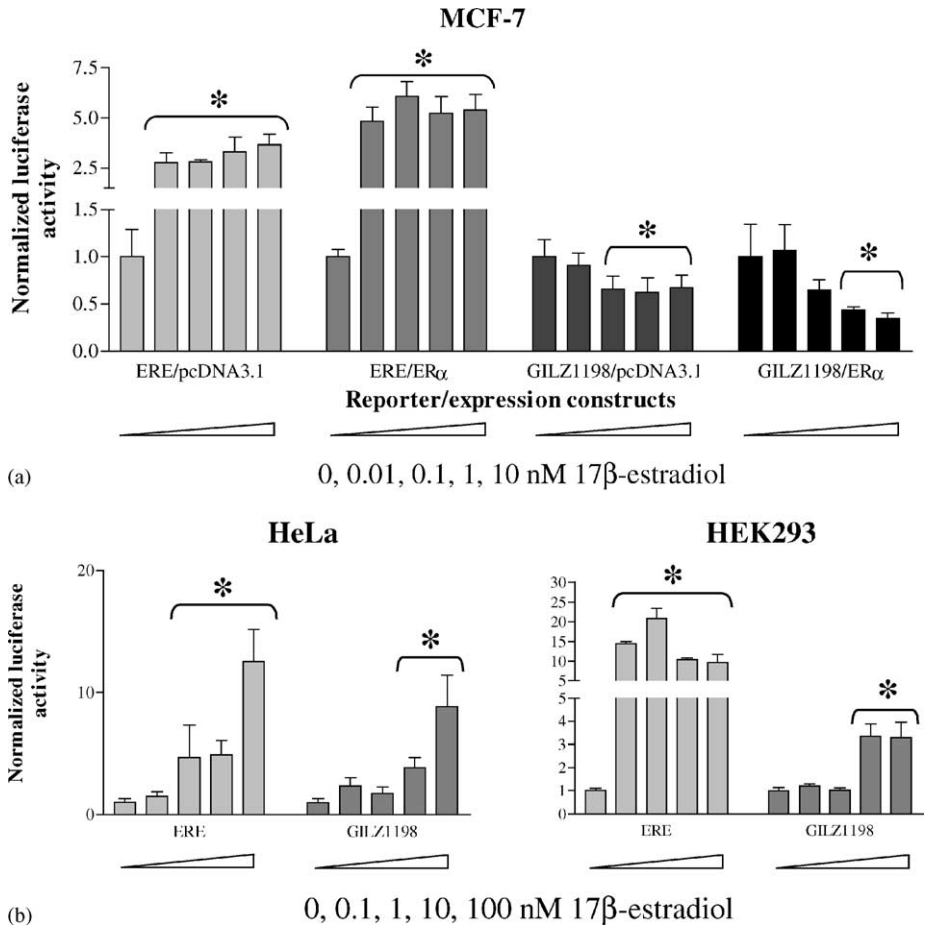

Fig. 3. The GILZ promoter demonstrates a cell type-specific response to estrogen. (a) MCF-7 cells were transfected with reporter constructs GILZ1198 and ERE along with either ERα/pcDNA3.1 or empty pcDNA3.1 vector. After transfection, cells were treated with various concentrations of E2 for 24 h; then luciferase activity was determined. Luciferase activity was normalized to a no estrogen control. (b) HeLa and HEK293 cells were transfected with the reporter constructs GILZ1198/pGL3basic and ERE/pTAbasic, along with ERα/pCIneo (HEK293), or ERα/pcDNA3.1 (HeLa). After 24 h of treatment with E2, luciferase activity was determined. For both (a) and (b), values are the mean of four replicates, with error bars representing standard deviations. (*) Indicates that the value differs from the no-estrogen control with $P < 0.05$, using one-way ANOVA with the Dunnett post-test.

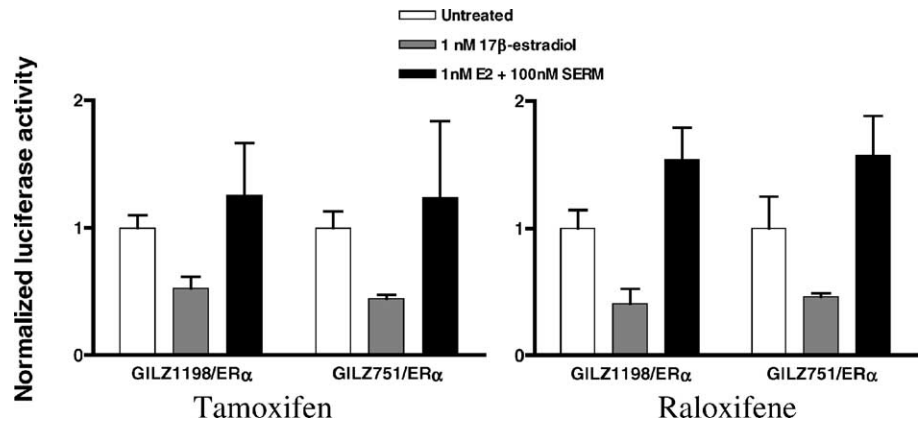

Fig. 4. Estrogen antagonists inhibit estrogen-mediated down-regulation of GILZ. MCF-7 cells were transfected with the indicated reporter constructs along with ERα/pcDNA3.1. After transfection, cells were treated with 1 nM E2 or 1 nM E2 + 100 nM antagonist (raloxifene or tamoxifen) for 24 h; then luciferase activity was determined. Luciferase activity was normalized to empty reporter and no estrogen controls. Values are the mean of four replicates, with error bars representing standard deviations. (*) indicates that sample differs from control with $P < 0.05$ by a two-tailed paired *t*-test. *E* = 100 nM E2; C = ethanol control.

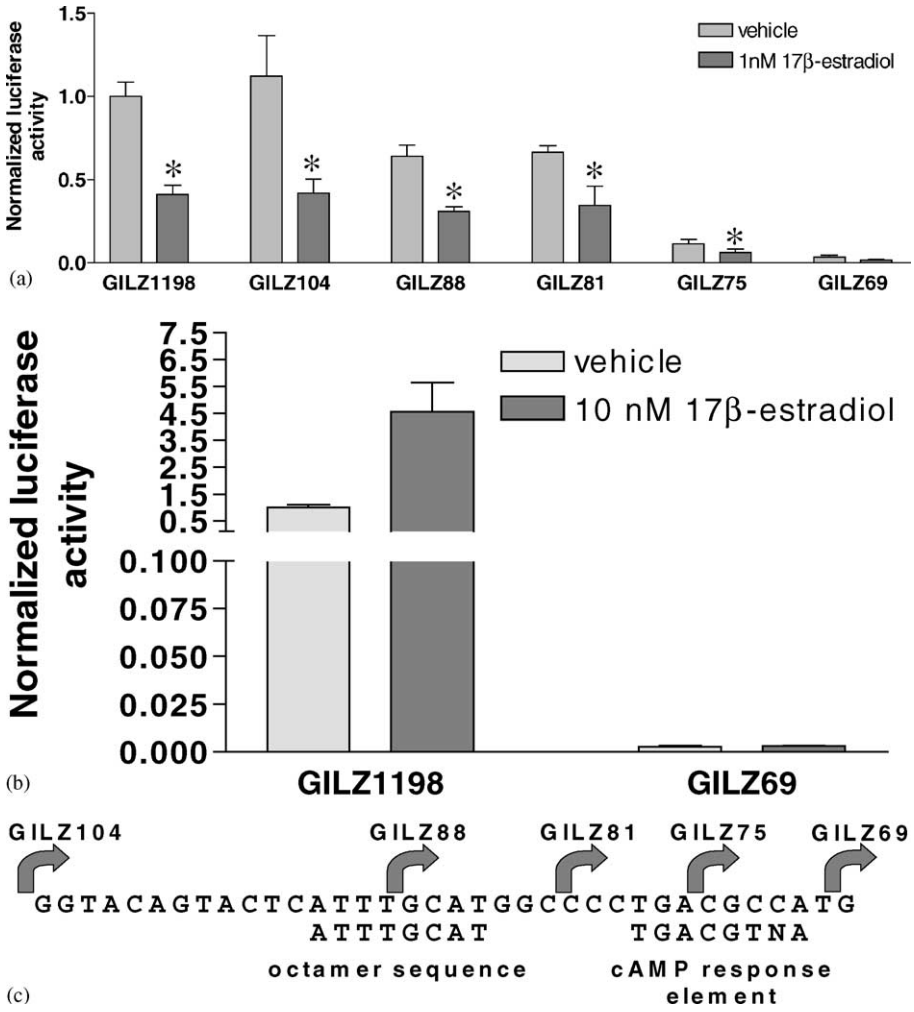

Fig. 5. Basal promoter activity and estrogen response of the GILZ gene are closely linked. (a) Truncations of GILZ1198 were constructed and used for luciferase reporter assays. Cells were transfected with the reporter constructs and ERα/pcDNA3.1. After 24 h of treatment with E2, luciferase activity was determined. Luciferase activity for each construct was normalized to the vehicle-treated samples for the GILZ1198 construct, so that basal activity is indicated by the "no estrogen" samples. Values are the mean of four replicates, with error bars representing standard deviations. (*) Indicates that the estrogen-treated sample differs from the vehicle control with $P < 0.05$ by a two-tailed unpaired *t*-test. (b) HEK cells were cotransfected with the reporter constructs and ERα/pcDNA3.1. Cells were treated with E2 and luciferase activity was determined as described above. (c) The sequence of the GILZ promoter between nucleotides −105 and −68. The 5′ ends of the truncation clones are indicated. The octamer and CRE consensus sequences are indicated below the potential corresponding motifs in the GILZ sequence.

75 all maintained an estrogen-dependent decrease in expression of about 50% in MCF-7 cells. GILZ69 eliminated basal activity entirely (Fig. 5a). Since there is no basal activity, it is not possible to determine whether this construct will be down-regulated. GILZ104 and GILZ69 were also tested in HEK293 cells: GILZ104 showed basal activity and estrogen-dependent up-regulation identical to GILZ1198, while GILZ69 had no basal activity and no up-regulation with estrogen (Fig. 5b). These results demonstrate that both the basal activity and estrogen responsiveness of the GILZ gene require promoter elements between −105 and −70.

Sequence analysis of the region between nucleotides −105 and −70 (Fig. 5c) showed that there are no consensus ERE, Sp1 or AP-1 sites, all of which are known to mediate estrogen regulation. There is, however, a perfect octamer sequence, which is a binding site for POU-domain transcription factors. Additionally, there is a close match to a CRE (cAMP response element), which is bound by members of the cyclic AMP response element (CREB) family of transcription factors.

The lack of an ERE suggests that the association between ERα and the GILZ promoter is indirect. Electrophoretic mobility shift assays showed that recombinant ERα does not directly bind to GILZ104 (data not shown). To provide additional evidence that the ERα does not bind directly to the GILZ promoter, we co-transfected mutant ERα expression vectors with the GILZ luciferase reporters (Fig. 6). In both HEK and MCF-7 cells, the DNA binding domain (DBD) was not required for estrogen-dependent regulation of the GILZ promoter, although the ligand binding domain (LBD) and N-terminal region were both necessary. Protein levels of the overexpressed receptor and receptor fragments were found to

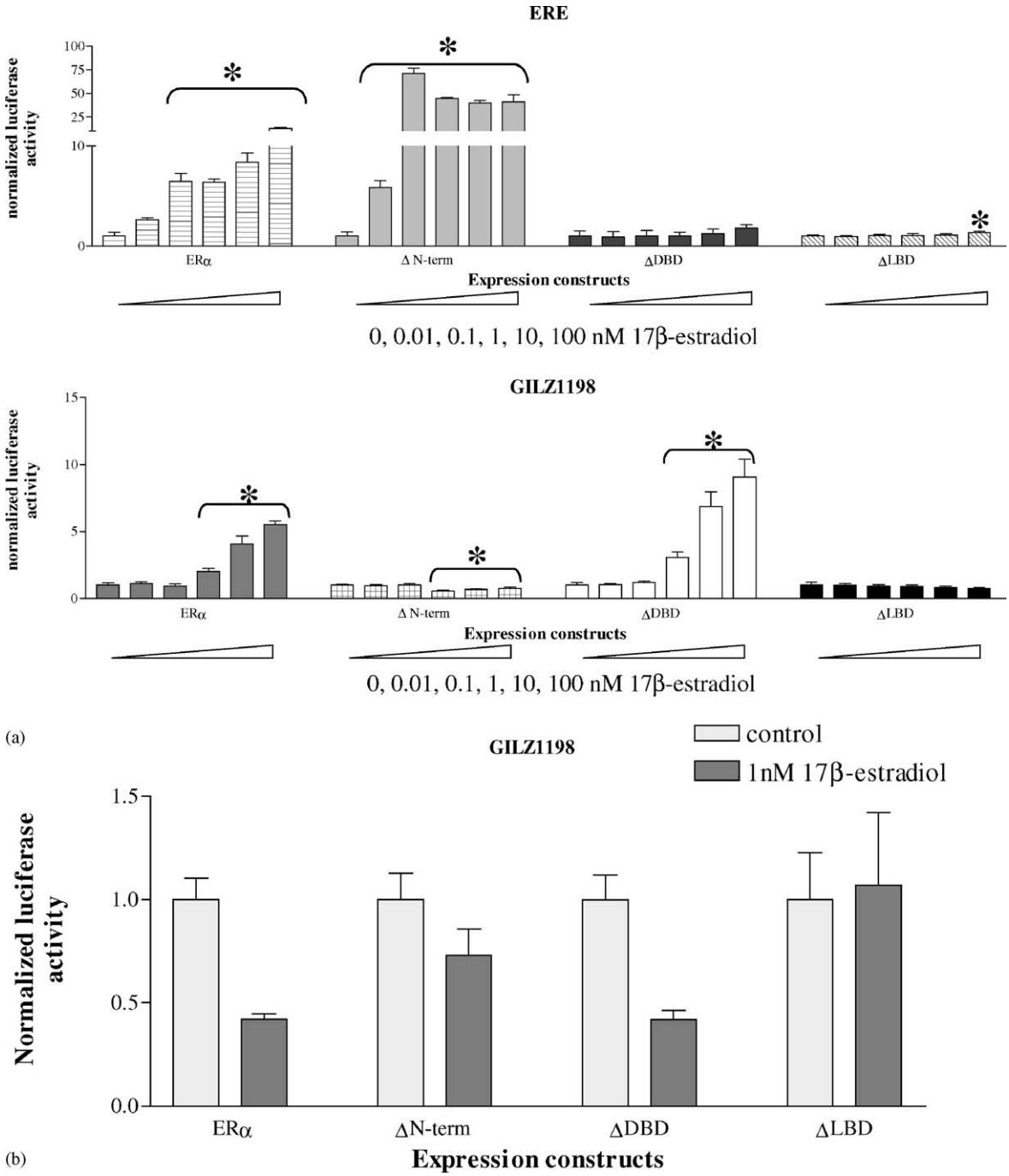

Fig. 6. GILZ regulation by estrogen does not require the ER DNA binding domain. (a) HEK293 cells were transfected with reporter constructs GILZ1198/pGL3basic and ERE/pTAbasic along with ERα/pcDNA3.1 or ERα mutants. After transfection, cells were treated with varying concentrations of E2 for 24 h; then luciferase activity was determined. Luciferase activity was normalized to a no estrogen control. Values are the mean of four replicates, with error bars representing standard deviations. (*) Indicates that the value differs from the no-estrogen control with $P < 0.001$, using one-way ANOVA with the Dunnett post-test. (b) MCF-7 cells were transfected with GILZ1198/pGL3basic, along with ERα/pcDNA3.1 or ERα mutants. After 24 h of treatment with E2, luciferase activity was determined. Luciferase activity was normalized to no estrogen controls. Values are the mean of four replicates, with error bars representing standard deviations. (*) Indicates that the value differs from the ERα no-estrogen control with $P < 0.001$, using one-way ANOVA with the Dunnett post-test.

be similar by immunoblotting (data not shown). The control ERE behaved as expected, requiring the DBD and LBD, but not the N-terminus for up-regulation.

3.7. Oct-and CREB bind to the GILZ promoter

Electrophoretic mobility shift (EMSA) experiments were used to look at the ability of a POU-domain transcription factor to bind to the octamer consensus in the GILZ promoter (Fig. 7a). Using MCF-7 nuclear extracts, Oct-1 was found to bind to a GILZ promoter fragment of nucleotides −96 through −70, producing at least two complexes, both of which are competed with an excess of competitor octamer (Oct-1) or GILZ oligonucleotide, but not by a mutant octamer sequence (Octmut). Addition of anti-Oct-1 antibodies resulted in a supershift of the Oct-1-containing bands, confirming that the POU-domain transcription factor bound is Oct-1. Identical results were obtained using HeLa cell nuclear extracts (data not shown).

When a labeled CREB consensus oligonucleotide was used in gel shifts with an MCF-7 cell nuclear extract, a specific complex was formed (Fig. 7b, arrow), which was supershifted by anti-CREB antibodies (Fig. 7b, arrowhead). The CREB-containing complex and supershifted complex were both competed by a 1000-fold molar excess of cold CREB consensus oligonucleotide, the GILZ oligonucleotide (nucleotides −96 through −70), and the GILZ/CREB oligonucleotide, which is the CREB consensus oligonucleotide containing the GILZ CREB sequence (Fig. 7b, lanes 3–5). A mutant CREB consensus oligonucleotide and a mutant Oct-1 (non-specific control) oligonucleotide were unable to compete CREB from the labeled CREB consensus oligonucleotide (Fig. 7b, lanes 6 and 7). Although the GILZ oligonucleotide was able to specifically compete CREB from a consensus CREB sequence, we were unable to identify a CREB-containing complex on the labeled GILZ oligonucleotide; it is possible that the interaction between CREB and the GILZ oligonucleotide is disrupted during electrophoresis.

Since ERα does not directly bind to the GILZ promoter, ERα could be functioning by sequestering factors required for GILZ expression, or by indirectly associating with the DNA. To distinguish between these possibilities, we looked for an association of the receptor with GILZ promoter complexes in gel shift assays (Fig. 8). In MCF-7 cell extracts, we were not able to detect a complex that was competed by unlabeled ERE or supershifted with anti-ER antibody. However, when 6 pmoles of recombinant human ERα were added to the extract, one complex was enhanced and specifically competed with ERE oligonucleotides, but not by a non-specific oligonucleotide, and was supershifted with anti-ER.

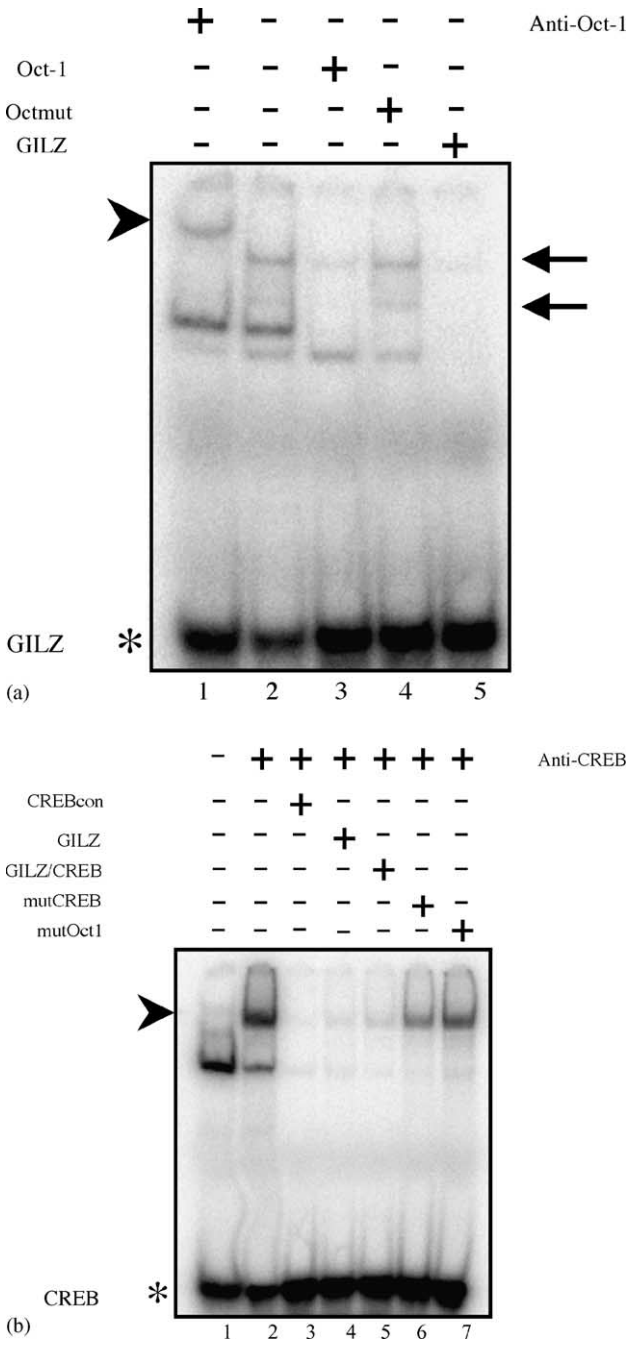

Fig. 7. Oct-1 and CREB bind specifically to the GILZ promoter. (a) MCF-7 cell nuclear extracts were used in gel shifts with a GILZ promoter fragment (nucleotides −96 through −70). Lane 1: no cold competitor, anti-Oct-1 added; lane 2: no cold competitor or antibody; lane 3: 1000-fold molar excess of cold Oct-1 oligonucleotide; lane 4: 1000-fold excess of Octmut oligonucleotide; lane 5: 1000-fold excess of GILZ oligonucleotide. The arrowhead at the top left indicates a supershifted complex. (*) Indicates the unbound, labeled GILZ oligonucleotide. The two arrows at the right side show Oct-1-containing complexes. (b) MCF-7 cell nuclear extracts were used in gel shifts with a consensus CREB site oligonucleotide. Lane 1: no cold competitor or antibody; lane 2: no cold competitor, anti-CREB added; lanes 3–7: 1000-fold molar excess of the indicated cold competitor oligonucleotides, plus anti-CREB antibody. The arrowhead at the top left indicates a supershifted complex. (*) Indicates the unbound, labeled CREB site containing oligonucleotide.

4. Discussion

In this study we have used microarrays to analyze changes in gene expression in MCF-7 cells in response to estrogen

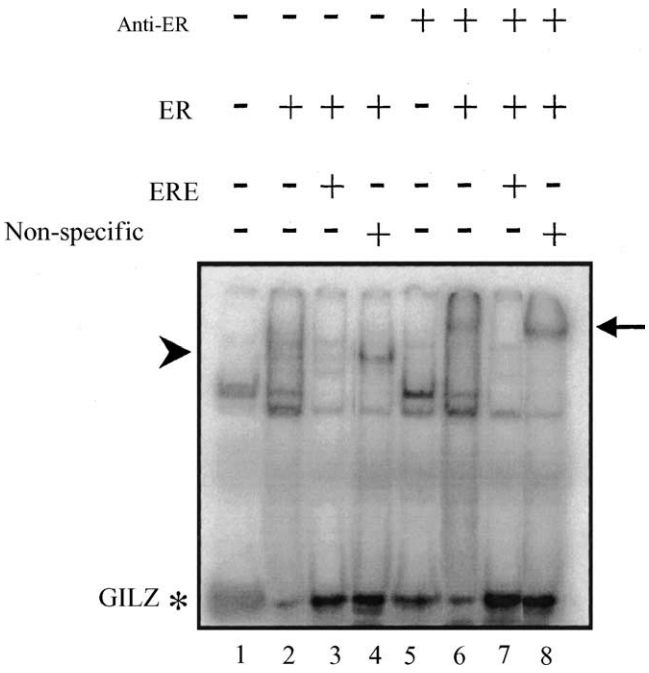

Fig. 8. The ER associates with the GILZ promoter. Six picomoles of recombinant human ERα were added to 1 μg of MCF-7 cell nuclear extract for use in gel shifts with a GILZ promoter fragment. Lanes 1 and 5: no added ER; lanes 2 and 6: no added cold competitor oligonucleotide; lanes 3 and 7: 1000-fold molar excess of ERE competitor oligonucleotide; lanes 4 and 8: 1000-fold excess of non-specific oligonucleotide. Lanes 5–8 also contain anti-ER antibody. The arrow on the top right indicates the supershifted band. The arrowhead on the top left indicates the complex enhanced by added ER. (*) Indicates the unbound labeled GILZ oligonucleotide.

treatment. We have identified a number of novel estrogen-regulated genes, including several that encode nucleolar proteins and many that are involved in transcriptional control. All of the genes that we chose for testing by quantitative RT-PCR were confirmed as being up or down-regulated by E2. A time-course of estrogen treatment was used to examine temporal changes in gene expression. Additionally, we have further characterized the regulation of one of the down-regulated genes using luciferase reporter analysis of the promoter region.

Normalized data comparisons combined with in silico electronic northern analyses were used to identify specific genes that were estrogen-regulated. This method of analysis proved to be extremely effective; 100% of the genes further tested by RT-PCR were confirmed as being estrogen-responsive. Using such stringent selection criteria may have caused some estrogen-responsive genes to be omitted from our compilation (Table 1); however, there is a strong likelihood that the genes identified using this method are genuinely regulated by estrogen.

From the results of time course experiments, it is clear that estrogens regulate these genes with differential kinetics. Some mRNAs show a continuous increase or decrease in expression over the entire 70 h (for example, TGFβ-3 and LYAR, Fig. 2), while others show a transient change (GILZ and nucleolin, Fig. 2). The biphasic regulation seen for GILZ and nucleolin indicates that there are other factors involved in their regulation that may also be estrogen regulated. Direct, rapid regulation of GILZ by estrogen is subsequently ablated; there may be newly transcribed factors that alter the effect of ER at the GILZ promoter or block binding of ER. Alternatively, factors required for the down-regulation of GILZ might themselves be down-regulated, and no longer available.

A large number of the estrogen-regulated genes that we identified, particularly those that were down-regulated, are involved in transcriptional control. Surprisingly, a number of the up-regulated genes encode nucleolar proteins. A search of the chips for additional nucleolar protein genes revealed that not all are up-regulated; specifically, NOF1/NOP2, HNP36, and MSP58 were not affected by estrogen. This suggests a functional significance for those nucleolar genes that are up-regulated. Up-regulation of these nucleolar proteins has been implicated in aspects of cell proliferation; therefore, they may be involved in mediating the proliferative effects of estrogen on breast cells. Further work is necessary to evaluate the contribution of each of these proteins to estrogen-induced cell proliferation.

Many genes were found to be down-regulated by E2. While many of the responses were not large (two-fold or less as judged by quantitative PCR), small changes in the levels of transcription-related proteins could induce major changes in the cell. Each factor that is directly estrogen-responsive has the potential to influence the expression of dozens of other genes, leading to an amplification of estrogenic signaling.

p53DINP1 (p53-dependent damage-inducible nuclear protein-1), one of the genes down-regulated by 17β-estradiol (Table 1), has been shown to be a regulator of p53 action [21]. This protein was suggested to mediate p53-dependent apoptosis in response to double-stranded DNA breaks. Down-regulation of p53DINP1 by estrogen may contribute to the survival of cells with significant DNA damage, which can lead to oncogenesis.

TSC-22 (transforming growth factor-β-stimulated clone-22) and GILZ are small leucine zipper proteins that have 64% amino acid identity to each other [22] and have been implicated in transcriptional regulation. They lack the amino-terminal basic DNA binding region that is found in most leucine zipper proteins; therefore, they may act as transcription co-factors. TSC-22 has been shown to translocate to the nucleus concomitant with apoptosis induced by X-ray irradiation, and overexpression sensitizes human salivary gland cancer cells (HSG) to irradiation [23–25].

Functional analysis of GILZ has primarily been carried out in T-cells, although it is widely expressed [26]. In T-cells, GILZ can inhibit activation-induced cell death (AICD), an apoptosis pathway that is frequently initiated when the T-cell receptor/CD3 complex is bound to an antigen. GILZ is up-regulated by dexamethasone (dex) and has been suggested to be a transcriptional regulator that mediates the anti-apoptotic effect of dex on AICD [22]. GILZ cannot inhibit apoptosis induced by other stimuli, such as UV irradiation, starvation,

and anti-Fas monoclonal antibody [27]. AICD is a cell-type-specific event; the function of GILZ in other cell types is unknown, although it is likely to be involved in some aspect of apoptosis.

Genes that are down-regulated are of interest because the mechanisms of down-regulation by estrogen are not well understood. Ligand-bound ER has not been demonstrated to bind directly to DNA to repress gene expression. Insulin-like growth factor receptor 1 (IGFR-1) gene expression is down-regulated in rat aortic smooth muscle cells by estrogen [28]; in this case, Sp1 is prevented from binding to the DNA by ER, in the absence of a demonstrable interaction of ER with DNA. Similarly, direct DNA binding is not required for inhibition of GnRH expression in JEG-3 human choriocarcinoma cells following estrogen treatment [29].

We identified a region of the 5′-flanking sequence of the GILZ gene that is estrogen-responsive in HEK293, HeLa, and MCF-7 cells. Interestingly, the GILZ1198 reporter clone was down-regulated by E2 in MCF-7 cells and up-regulated in HEK293 and HeLa cells. TGFβ3 has been shown to be down-regulated in breast cells [30,31] and up-regulated in rat bone [19]; therefore, it is possible that the HEK293 and HeLa results are representative of estrogenic up-regulation in other cell types. However, HEK293 cells are not derived from an estrogen-responsive tissue, and it is not clear if up-regulation by estrogen in these cells is physiologically relevant.

In MCF-7 cells, GILZ reporter constructs were down-regulated by estrogen, consistent with microarray and RT-PCR results for the endogenous GILZ gene. Down-regulation produced by endogenous ER was variable and somewhat weak; co-transfection with ERα or β greatly enhanced the estrogenic effect. The variability of the down-regulation by endogenous ER was experiment-dependent, not sample dependent, suggesting that transfection efficiency may be an important factor. In some cases, the reporter plasmid may titrate the endogenous ER in the transfected cells, resulting in a decreased response to estrogen treatment. Several genes have been reported to require co-transfection of ERα to demonstrate estrogen-dependent regulation in MCF-7 cells [32–35,36]. We did not look beyond nucleotide −1198 or into the coding sequence of the gene for additional elements; therefore, we cannot rule out the possibility that there are additional regulatory sequences that contribute to the estrogen response of the GILZ gene. Indeed, the weakness of down-regulation in the absence of co-transfected receptor, and the requirement for high levels of purified receptor to see an ER complex in gel shifts, suggests that additional elements beyond those between −1198 and −1 of the promoter may be involved in regulation. Nevertheless, we have identified at least one important element for estrogenic regulation of the gene. The fact that GILZ regulation is reproduced by fusion of the promoter to a luciferase reporter eliminates the possibility that estrogen is regulating the GILZ gene post-transcriptionally.

The activities of the two reporter plasmids, GILZ1198/pGL3basic and GILZ104/pGL3basic, were virtually indistinguishable from each other in terms of both basal activity and estrogenic regulation. Truncation to GILZ88 and GILZ81 eliminated half and the entire Oct-1 binding site, respectively. Both of these constructs reduced basal activity, but maintained the estrogen response, indicating that Oct-1 contributes to basal activity, but is not required for down-regulation by estrogen. Further truncation to GILZ75 removed part of the CRE sequence. Basal activity was dramatically decreased, but estrogen regulation was maintained. GILZ69, which removed the entire CRE, eliminated both basal activity and estrogen regulation. Together, these results suggest that the CRE is important for basal activity and estrogen response. Truncation of the CRE by GILZ75 was insufficient to completely inactivate the site. Replacement of the first three nucleotides in the consensus sequence with a *Kpn*I site significantly disrupted the sequence; however, the sequence could still be acting as a weak binding site in the reverse orientation.

At the GILZ promoter, ER can be incorporated into DNA-bound protein complexes, suggesting that down-regulation is due to protein-protein interactions on the DNA rather than inhibition of DNA binding. The ability of the GILZ CRE sequence to compete CREB from a CRE consensus sequence, along with the finding that truncation of the GILZ promoter to remove the CRE eliminates estrogen regulation, suggests that one or more members of the CREB/ATF family bind to this sequence and participate in regulation of the GILZ gene. ER and CREB/ATF proteins have been shown to physically interact at both a CRE in the cyclin D1 gene promoter [37] and at an ERE in an artificial 2ERE-pS2-CAT reporter [38], supporting this hypothesis. CREB and ER share at least one coactivator, CBP (CREB binding protein), which may participate in association of the two transcription factors. Furthermore, there are many members of the CREB/ATF family that have different expression profiles. The repertoire of CRE-binding proteins in a given cell type might be important in determining the direction of estrogen-dependent regulation of GILZ. More work will be required to work out a detailed molecular mechanism of the regulation of GILZ by estrogen.

This study has demonstrated the usefulness of gene chip analysis for identifying new estrogen-regulated genes. The GILZ gene is down-regulated in MCF-7 cells, and up-regulated in other cell types. The only known function for GILZ is to inhibit activation-induced cell death in T-cells, suggesting that it may play an estrogen-dependent role in apoptosis in some cell types. Future work will focus on determining the function of GILZ in MCF-7 cells and on any relationship between GILZ expression levels and the proliferation of breast cancer cells.

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