Estrogenically regulated LRP16 interacts with estrogen receptor α and enhances the receptor's transcriptional activity

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Abstract

Previous studies have shown that leukemia related protein 16 (LRP16) is estrogenically regulated and that it can stimulate the proliferation of MCF-7 breast cancer cells, but there are no data on the mechanism of this pathway. Here, we demonstrate that the LRP16 expression is estrogen dependent in several epithelium-derived tumor cells. In addition, the suppression of the endogenous LRP16 in estrogen receptor α (ER α)-positive MCF-7 cells not only inhibits cells growth, but also significantly attenuates the cell line's estrogen-responsive proliferation ability. However, ectopic expression of LRP16 in ER α -negative MDA-MB-231 cells has no effect on proliferation. These data suggest the involvement of LRP16 in estrogen signaling. We also provide novel evidence by both ectopic expression and small interfering RNA knockdown approaches that LRP16 enhances ER α -mediated transcription activity. In stably LRP16-inhibitory MCF-7 cells, the estrogen-induced upregulation of several well-known ER α target genes including *cyclin D1* and *c-myc* is obviously impaired. Results from glutathione S-transferase pull-down and coimmunoprecipitation assays revealed that LRP16 physically interacts with ER α in a manner that is estrogen independent but is enhanced by estrogen. Furthermore, a mammalian two-hybrid assay indicated that the binding region of LRP16 localizes to the A/B activation function 1 domain of ER α . Taken together, these results present new data supporting a role for estrogenically regulated LRP16 as an ER α coactivator, providing a positive feedback regulatory loop for ER α signal transduction.

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Introduction

Estrogen plays a crucial role in the control of development, sexual behavior, and reproductive functions. Its effects have been linked to the progression of the majority of human breast cancers and acts as a potent mitogen for many breast cancer cell lines (Masood 1992, Prall *et al.* 1998a). Mechanistic studies on estrogen-induced mitogenesis have revealed multiple cell cycle regulatory pathways activated by estrogen (Foster & Wimalasena 1996, Planas-Silva & Weinberg 1997, Prall *et al.* 1997). The biological actions of estrogen are manifest through the transcriptional activation of the ligand-dependent estrogen receptor α and β

(ER α and β ; Mangelsdorf *et al.* 1998, McDonnell & Norris 2002). The activation of an ER results in an altered expression of its direct transcriptional targets, thereby affecting downstream secondary biological activities. It is believed that 75% of carcinoma *in situ* breast lesions and similar percentages of invasive breast cancers express elevated levels of ER α protein; whereas in normal breast epithelial cells, only 10% of cells express ER α and only at low levels (Allred *et al.* 2001). Patients expressing elevated levels of ER α are therefore suitable candidates for hormonal therapy, which aims to block estrogen stimulation of breast cancer cells (Deroo & Korach 2006).

ER α contains six domains named A through F. The structure of ER α is similar to that of other nuclear receptors (NRs), having three separate functional domains; a variable activation function 1 domain (AF-1) in the N-terminal A/B domain, a central DNA-binding domain (LBD, ligand-binding domain) located in the C domain and a C-terminal LBD, domain E that contains AF-2 (Nilsson *et al.* 2001). The AF-1 domain is regulated by growth factors and its activity depends on the cellular and promoter context, while the AF-2 domain is dependent on ligand binding (Lees *et al.* 1989, Shao & Brown 2004). The two activating domains act synergistically to generate maximum transcriptional activity of ER α (Danielian *et al.* 1992, Métivier *et al.* 2001). ER α -mediated transcription is a highly complex process involving multiple of coregulatory factors (Hall *et al.* 2001, Hall & McDonnell 2005). The cofactors can be broadly divided into subgroups of either coactivator which augments the function of an activated receptor or corepressors which sustain the inactivated state of a receptor. Transcriptional activation of ER α involves interactions with coactivator molecules and the basal transcription machinery in a sequential and cyclic fashion at the promoter region of responsive genes (Glass & Rosenfeld 2000, Shang *et al.* 2000, McKenna & O'Malley 2002, Métivier *et al.* 2003). The balance of ER α cofactors in target cells, coupled with different concentrations of ER α and its ligand, allows fine-tuning of target gene transcription in response to estrogen. Over the past decade, more than 20 coactivator molecules have been identified using biochemical approaches as well as yeast two-hybrid screens (Klinge 2000, Shao & Brown 2004). Most of the coactivators bind to the ER α AF-2 domain in a ligand-dependent fashion. Only two related coactivators specific to ER α AF-1 have been identified to date, p68 and p72 RNA helicases (Endoh *et al.* 1999, Watanabe *et al.* 2001). The *LRP16* gene was originally isolated from human lymphocyte cells, and was identified as an estrogen-responsive gene by our group (Han *et al.* 2003). We have previously documented that 17 β -estradiol (E₂) upregulated the leukemia related protein 16 (LRP16) mRNA level in an ER α -dependent manner in MCF-7 human breast cancer cells (Han *et al.* 2003). One-half of the estrogen response element (ERE)/GC-rich Sp1-binding site and several GC-rich Sp1-binding sites within the promoter region of *LRP16* gene were identified as the main contributors to the E₂-responsive regulatory mechanism (Zhao *et al.* 2005, Si *et al.* 2006). Overexpression of LRP16 significantly stimulated MCF-7 cell proliferation by promoting the transition

of G₁ to S (Han *et al.* 2003). In primary breast cancer samples, our data indicated that LRP16 mRNA was overexpressed in nearly 40% of all samples. The clinicopathologic characteristics, including ER α /PR status, tumor diameter, and the involvement of axillary lymphoid nodes were tightly linked with LRP16 mRNA overexpression (Liao *et al.* 2006). Taken together, these data implied that LRP16 may play an important role in carcinogenesis and/or progression of hormone-dependent breast cancer.

The goal of this study was to gain an understanding of the functional role of LRP16 in hormone-sensitive breast cancer cells. To do this, we investigated the expression of LRP16, its interaction with ER α and its effect on ER α -mediated signaling. First, we showed that both mRNA and protein expression levels of LRP16 are positively linked with estrogen action in several breast cancer cell lines. Next, the proliferation rate of ER α -positive MCF-7 cells, but not ER α -negative MDA-MB-231 cells, was shown to be related to LRP16 expression levels. Furthermore, we showed that LRP16 enhanced the transcriptional activity of ER α . This provided evidence that *LRP16* is not only a target gene of ER α but also a novel ER α coactivator, thus creating a positive regulatory feedback loop. Therefore, LRP16 may play an important role in the proliferation of ER α -positive breast cancer cells by amplifying the function of ER α .

Materials and methods

Cell culture

All cell lines were purchased from American Type Culture Collection (ATCC, Rockville, MD, USA) and cultured according to the instructions. Pure estrogen antagonist ICI 182 780 was provided by Dr Qinong Ye (College of Military Medicine Science of China). E₂ was purchased from Sigma. Phenol red-free DMEM and estrogen-deprived fetal calf serum (FCS) were prepared as described previously (Zhao *et al.* 2005).

Cell proliferation assay

Cells were seeded at 1×10^4 per well in 24-well plates. After cell attachment, the medium was replaced with 1 ml fresh DMEM supplemented with 1% FCS. For each cell line, cells from three dishes were counted daily to generate proliferation curves. For the E₂-stimulated proliferation experiment, a total of 5000 cells were seeded and cultured in each well of a 24-well plate in phenol red-free DMEM supplemented with charcoal-stripped FCS (5%). After 12 h, cells were treated with E₂ in the same fresh medium for 24 h. Cell proliferation rate

was quantified using CellTiter 96 Aqueous One Solution Cell Proliferation Assay (Promega). Each experiment was performed in triplicate and repeated on three occasions.

Plasmids

The mU6pro vector containing the mouse U6 snRNA promoter was kindly provided by Dr Turner from University of Michigan (Yu *et al.* 2002). Mammalian expression plasmid for ER α (pS5G-hER α) was provided by Prof. Hajime Nawata at Kyushu University, and the reporter 3 $\times$ ERE-TATA-*Luc* was provided by Prof. Donald P McDonnell at Duke University Medical Center (Norris *et al.* 1998). The fragment of –101 to –24 bp from the LRP16 upstream regulatory region containing multiple GC-rich sites was cloned into pGL3 basic vector as described (Si *et al.* 2006).

Two siRNA-coding oligonucleotides against human LRP16 were designed and verified to be specific to LRP16 by a Blast search against the human genome. The LRP16-siRNA-374-targeting sequence is AAGCAGCGGGAG GAACATTCA; the LRP16-siRNA-668-targeting sequence is AAGACTGGCAAGGCCAAGATC; the green fluorescent protein (GFP)-siRNA, which does not match any known human cDNA was used as control as described previously (Yu *et al.* 2002). The hairpin LRP16-siRNA-374, LRP16-siRNA-668 and GFP-siRNA oligonucleotides were chemically synthesized, annealed, and inserted into the mU6pro vector under the control of U6 snRNA promoter by BbsI and EcoRI sites. To construct the pLPC-based and siRNA-expressing retrovirus system, the elements including U6 snRNA promoter and the downstream hairpin siRNA insert from mU6 plasmid were subcloned into P_{CMVIE} element-deleted pLPC vector by HindIII and EcoRI sites.

The human LRP16 full-length cDNA was cloned and inserted into the pcDNA3.1 expression vector as described previously (Han *et al.* 2003). The LRP16 cDNA with the Kozak sequence was also subcloned into the pcDNA3.1 expression vector to construct the pc3.1-K16. LRP16 cDNA was cloned into pGEX-6P-1 by EcoRI and HindIII sites to construct the glutathione S-transferase (GST)-LRP16 fusion. The truncated LRP16 fragment (coding 83–324 amino acids) was cloned into the pRSET-C vector by KpnI and BamHI sites for prokaryotic expression. The full-length LRP16, ER α cDNA, and its truncated fragments coding for AB, ABC, and DEF domains were cloned into either pBIND or pACT vectors by BamHI and KpnI sites to construct pBIND- and pACT-LRP16, pACT-ER α , pACT-ER α -AB, pACT-ER α -ABC, and

pACT-ER α -DEF vectors. All the constructions were confirmed by restriction enzyme mapping and DNA sequencing.

Viral production and infection of target cells

293GP2 cells were plated into 10 cm dishes and cultured until reaching 80–90% confluency. pLPC-based plasmids (7.5 μ g/well) and VSVG expression vector (2.5 μ g/well) were transiently cotransfected into 293GP2 cells using Superfect transfection reagent (Qiagen) according to manufacturer's protocol. After 2 days, the packaged pseudo-retrovirus in the supernatant was harvested by centrifugation at 8000 *g* for 10 min and was diluted 1:2 in DMEM. The retrovirus was then used to infect MCF-7 cells for an additional 48 h. Subsequently, MCF-7 cells were selected with 2 μ g/ml puromycin (Invitrogen) for 2 weeks. All of the MCF-7 parental cells were killed by puromycin within this period. The selected cells were maintained as stably siRNA-expressing cells in DMEM supplemented with 1 μ g/ml puromycin.

RNA extraction and northern blot analysis

Total RNA was extracted by TRIZOL reagent (Invitrogen) and analyzed by northern blot as described previously (Han *et al.* 2003). The membranes were probed to a 550 bp fragment of LRP16, a 1.6 kb fragment of ER α , 438 bp of c-fos, 744 bp of cathepsin D, 317 bp of RAR α , 780 bp of MTA3, 440 bp of pS2, and a 515 bp of β -actin fragment labeled with [α -³²P]dCTP by random priming.

Generation of LRP16 rabbit polyclonal antibody

The pRSET-C-LRP16 plasmid was transformed into the competent BL21 cells. Cells were cultured and 1 mM isopropyl- β -thiogalactoside (IPTG) was added to induce the synthesis of recombinant protein. The bacteria were harvested and were lysed by sonication and the supernatant was collected by centrifugation. Proteins samples were run on SDS-PAGE and negatively stained with KCl (250 mM). The gel segments containing human truncated LRP16 recombinant protein (28 kDa) were cut out. The recombinant protein was extracted from frozen gels by dialysis and dissolved in PBS. The protein was diluted 1:1 in complete Freund's adjuvant for the first injection and incomplete Freund's adjuvant for subsequent injections. ELISA was done to measure the titer of serum from immunized rabbits. Pooled serum from days 120, 135, and 147 was purified using the ImmunoPure

(A/G) IgG purification kit (Pierce Chemical Co., Rockford, IL, USA).

Antibodies and immunoblotting

Antibodies against β -actin, cyclin D1, ER α (Santa Cruz, Biotechnology, Santa Cruz, CA, USA), and c-Myc (Invitrogen) were used in this study. All immunoblotting procedures were performed as described (Han et al. 2003).

Protein–protein interaction assays in cell-free (GST pull-down) and cell systems (coimmunoprecipitation, CoIP)

BL-21 bacteria were transformed with pGEX-6P-1 (GST alone) or GST-LRP16 and cultured in an incubator at 37 °C; 1 mM IPTG was added to the culture to induce expression of GST or GST-LRP16. Bacteria were pelleted and lysed in buffer (40 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1 mM dithiothreitol, 1 mM EDTA, 0.5% NP40, 10% glycerol, 0.4 mM phenylmethylsulfonyl fluoride, 2 U/ml leupeptin, and 2 U/ml aprotinin) along with lysozyme (5 mg/l) and a bacterial protease inhibitor cocktail (Sigma). Suspensions were vortexed to resuspend the pellet, incubated on ice for 30 min, and sonicated for 5 min. The insoluble fraction was removed by centrifugation, and the supernatants incubated with glutathione-Sepharose beads (Amersham Bioscience) for 30 min at room temperature. For the pull-down assays, 30 μ l of the 50% GST or GST-LRP16 bead slurry were incubated with 100 fmol of recombinant human ER α proteins (Invitrogen) supplemented with 10 nM E₂ or DMSO at 4 °C for 3 h. To detect protein that bound specifically, the beads were washed six times with lysis buffer, boiled in SDS-PAGE samples buffer, and subjected to SDS-PAGE and analyzed by immunoblotting.

CoIP studies were performed using transfected cell lines. Briefly, MCF-7 cells were plated in 10 cm dishes and cultured using phenol red-free DMEM supplemented with 5% charcoal-stripped FCS until reaching 50–60% confluency. ER α and LRP16 expression vectors were cotransfected using Superfect transfection reagent (Qiagen). After 36 h, cells were treated with DMSO or 10 nM E₂ for an additional 10 h and then lysed (20 mM Tris (pH 7.4), 50 mM NaCl, 1 mM EDTA, 0.5% NP-40, 0.5% SDS, 0.5% deoxycholate, and protease inhibitors). Five hundred nanograms of lysate were precleared with 50 μ l protein A-Sepharose beads (Upstate Biotechnology, Lake Placid, NY, USA) and rabbit IgG for 2 h at 4 °C. Either anti-ER α (Santa Cruz, rabbit anti-hER α) or anti-LRP16 antibody (rabbit anti-human LRP16) was then added and

incubated overnight at 4 °C. One hundred microliters of protein A agarose were then added to the antibody/lysate mixture for another 2 h at 4 °C, and the beads were pelleted and washed thrice with lysis buffer. Bound proteins were eluted in SDS sample buffer, subjected to SDS-PAGE, and analyzed by immunoblotting.

Luciferase reporter assays

Cells that had reached a 50% confluency rate in 35 mm dishes were cotransfected using Superfect (Qiagen). 3 $\times$ ERE-TATA-Z_{wc} (0.25 ng) or LRP16 promoter gene construct, 0.25 ng ER α expression vector, and the LRP16 expression vector (pc3.1-K16) with the indicated amounts were used to cotransfect cells. SiRNA duplexes against the LRP16 gene (synthesized by JiKai Co., Shanghai, China), reporter genes, and ER α expression vector (1:1:0.5 μ g) were cotransfected using LipofectAMIEEN 2000 according to manufacturer's recommendations (Invitrogen). pRL-SV40 was also cotransfected (1 ng/per well) and used as the internal control. Cell extracts were prepared 42 h after transfection, and the luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega). All experiments were performed in triplicate and repeated thrice.

Mammalian two-hybrid assays

Mammalian two-hybrid assays (Promega) were performed according to manufacturer's protocol with minor modifications. Briefly, MCF-7 cells were cultured in phenol red-free and steroid-deprived medium for at least 3 days, and then plated in 3.5 cm dishes. MCF-7 or 293T cells were transiently cotransfected with the indicated vectors using Superfect transfection reagent. Luciferase activities were assayed as described previously.

Statistical analysis

Experiments were performed in triplicate, and the results were expressed as the mean $\pm$ s.e.m. Statistical analysis was performed using Statview software. All data were evaluated for paired variables to compare two groups. $P < 0.05$ was considered to be statistically significant.

Results

Upregulation of LRP16 expression is dependent on estrogen action

Previously published work from our laboratory has demonstrated that E₂ upregulated mRNA levels of the

human *LRP16* gene in MCF-7 cells (Han *et al.* 2003). To assess the effects of anti-estrogens on the expression level of LRP16, a more selective steroidal estrogen antagonist, ICI 182 780 (10 nM), was used to treat proliferating MCF-7 cells. Total RNA over a 24 h time course was extracted, and northern blot analysis was used to determine the mRNA level of LRP16. Figure 1A demonstrated that the level of LRP16 mRNA decreased within 3 h of treatment. These results, combined with the induced effect of LRP16 expression by E₂ as previously reported (Han *et al.* 2003), suggest that the LRP16 expression is dependent on estrogen action. To confirm this finding, we treated the endometrial cell line Ishikawa with E₂ (10 nM) or estrogen antagonist ICI 182 780 (10 nM). The significant increase in LRP16 mRNA levels was observed after E₂ stimulation; whereas, inhibition of ER α activity led to a decline in the expression of LRP16, but not β -actin (Fig. 1A). Next, we overexpressed ER α in Ishikawa cells cultured in normal medium, and observed an increase of LRP16 mRNA level (Fig. 1B). We also followed the expression patterns of LRP16 mRNA in ER α -positive and ER α -negative human breast cancer cell lines by northern blot. The LRP16 transcript was differentially expressed and was only abundant in the ER α -positive MCF-7 and T47D cell lines (Fig. 1C).

To confirm that the LRP16 mRNA levels were indicative of protein levels, we generated LRP16-specific antibodies using a truncated amino acid sequence highly specific for LRP16. Antibody

specificity was confirmed through immunoblotting of protein extracts from MDA-MB-231 cells transfected with vector only or LRP16 cDNA with the Kozak sequence modified. As expected, high protein level of LRP16 in MDA-MB-231 cells transfected with the LRP16 expression vector was detected using this antibody (Fig. 1D). These data clearly demonstrate that estrogen regulates the expression of LRP16 and establish the dependence of LRP16 expression on estrogen action in breast cancer cell lines.

Suppression of LRP16 expression reduces estrogen-mediated proliferation response of MCF-7 cells

The dependence of LRP16 expression on estrogen activity led us to study different LRP16 expression levels and the proliferative properties of breast cancer. To do so, we chose several sequences in the coding region of the human *LRP16* gene and designed short interfering RNAs (siRNA) targeted against each sequence. To test whether these siRNAs could suppress LRP16 expression in MCF-7 cells, we infected these cells with the retroviral vector transducing a DNA segment specifying such siRNA sequences and then selected the cells stably expressing the siRNA. An siRNA against GFP, whose sequence has previously been used as a control-siRNA in human cells (Yu *et al.* 2002), was also introduced into MCF-7 cells. As shown in Fig. 2A, siRNA-374 and siRNA-668 caused a specific reduction in *LRP16* gene expression level,

Figure 1 LRP16 biosynthesis requires ligand-bound ER α . (A) MCF-7 cells and Ishikawa cells were grown in normal growth medium supplemented with 10 nM ICI 182 780 for the indicated time. Ishikawa cells were grown in media stripped of steroids for at least 3 days and were treated with E₂ for the indicated time. Northern blots were probed for LRP16 and β -actin. (B) Ishikawa cells were cultured with normal medium and then transfected with ER α cDNA. Northern blots of total RNA were hybridized with probes for LRP16, ER α , and β -actin. The growth media for these experiments (DMEM with 10% FBS) contain sufficient steroids to provide normal ER α function. (C) Northern blot of total RNA from the indicated cell lines was hybridized with probe for LRP16. Total RNA was used as a loading control. Immunoblots were probed for ER α and β -actin. (D) MDA-MB-231 cells were stably transfected with empty vector or LRP16 cDNA. Immunoblots were probed for LRP16 using polyclonal anti-LRP16 antibodies in the indicated cell lines. These results shown in A, B, C, and D are representatives of two independent experiments.

Figure 2 Suppression of LRP16 expression reduced the growth rate and estrogen response of MCF-7 cells. (A) LRP16 and β -actin expression of both mRNA and protein was examined by northern blot and immunoblotting in MCF-7 cells stably expressing LRP16-siRNA374, LRP16-siRNA668, or control-siRNA. These are representative pictures of two independent experiments. (B) Logarithmic population of the MCF-7 cells expressing either control-siRNA or LRP16-siRNAs. Each data point represents the mean $\pm$ S.E.M. number of cells counted in triplicate dishes (* P < 0.05, # P < 0.01; statistical analyses with control-siRNA cells). This experiment was repeated thrice. (C) MCF-7 cells with different LRP16 levels were cultured for 5 days in phenol red-free DMEM supplemented with steroid-stripped FBS (5%) and then treated with E₂ at different concentrations for 24 h. Cell proliferation rate was quantified using CellTiter 96 Aqueous assay. Each data point represents the mean $\pm$ S.E.M. number of cells counted in triplicate dishes (* P < 0.05, # P < 0.01; statistical analyses with control-siRNA cells). This experiment was repeated thrice.

leading to a 90 and 60% decrease respectively, of LRP16 mRNA relative to the control-siRNA. Consistent with the change in LRP16 mRNA, LRP16 protein was dramatically blocked by siRNA-374, and was partially silenced by siRNA-668.

We further examined the loss of LRP16 expression on the proliferation rate of MCF-7 cells. Growth rates of MCF-7 cells carrying either LRP16-siRNA-374

or LRP16-siRNA-668 were markedly decreased in contrast to the control cells carrying GFP-siRNA on the third day (Fig. 2B). This proliferation curve demonstrates a linear association between LRP16 expression level and the proliferation activity of MCF-7 cells, indicating that LRP16 is required for the proliferation of MCF-7 cells.

Next, we stably transfected LRP16 cDNA with a modified Kozak sequence into MDA-MB-231 cells and observed that MDA-MB-231 cells carrying either pcDNA3.1-KLRP16 or empty vector grew at a similar rate (data not shown), indicating that LRP16 is not required for the proliferation of MDA-MB-231 cells.

Differential requirement of MCF-7 and MDA-MB-231 proliferation for LRP16 expression level as demonstrated above led us to speculate that the inhibition of MCF-7 proliferation by LRP16-siRNA resulted from the attenuated responsiveness of MCF-7 cells to estrogen. To test this hypothesis, the established stably transfected cell lines with different LRP16 expression levels were used to examine whether suppression of LRP16 has an anti-estrogenic activity on MCF-7 cell proliferation. E₂ could stimulate proliferation of MCF-7 cells carrying either LRP16-siRNAs or control-siRNA in a dose-dependent manner, but the degree of stimulation of E₂ on the cell proliferation of MCF-7 carrying LRP16-siRNAs was much less than that on the cell proliferation of MCF-7 carrying control-siRNA beginning from 10⁻¹¹ to 10⁻⁷ M/l of E₂ treatment (Fig. 2C). This indicated that LRP16 is required for the E₂-stimulated proliferation of MCF-7 cells.

LRP16 modulates ER α -mediated transactivation

The effect of LRP16 on the proliferative response of MCF-7 to estrogen suggested that LRP16 participates in the genetic program initiated by ligand-bound ER α . We reasoned that LRP16 might directly regulate the ER α transcriptional activity. To address this question, the transcriptional activity of a 3 $\times$ ERE-TATA-*Luc* reporter gene was first assayed. In the presence of estrogen, LRP16 enhanced ER α -mediated transactivation of the reporter gene in a dose-dependent manner (Fig. 3A). However, this effect was abolished when estrogen was stripped from the culture media. In contrast to MCF-7 cells, the weak intrinsic activation of LRP16 for ER α -mediated transcription was observed in a LRP16 dose-dependent manner in HeLa cells (Fig. 3A). To further test the requirement for LRP16 of ER α -mediated transactivation in MCF-7 cells, we utilized RNA interference in the cotransfection system. Analysis of 3 $\times$ ERE-TATA-*Luc* reporter

Figure 3 LRP16 is required for ER α -mediated transactivation. (A and B) MCF-7 and HeLa cells were grown in media stripped of steroids for at least 3 days, then cotransfected with the indicated luciferase reporter and the effector molecules ER α (0.25 ng) and the indicated LRP16 cDNA. After 36 h, cells were treated with E₂ (10 nM) or dimethyl sulfoxide (DMSO). The relative luciferase activity levels were normalized in all cases by mock effector transfection for LRP16 and arbitrarily assigned a value of 1. The experiment was repeated thrice. (C) MCF-7 cells were grown in media stripped of steroids for at least 3 days, then cotransfected with the indicated luciferase reporter and the effector molecule ER α and LRP16-siRNAs or control-siRNA oligonucleotides. The relative normalized luciferase activity level for control-siRNA transfections was arbitrarily assigned a value of 1. This experiment was repeated thrice.

gene activities in this system revealed that the inhibition of LRP16 led to the decreased ER α -mediated transactivation in the presence of E₂ (Fig. 3C). In the absence of E₂, inhibition of the endogenous LRP16 in MCF-7 cells did not decrease the reporter gene activities, confirming that LRP16 has no intrinsic activity for ER α -mediated transactivation in MCF-7 cells.

To exclude selection for the promoter of ER α -mediated transactivation enhanced by LRP16, a promoter that lacked a classical ERE sequence but had E₂ responsiveness was used. Several reports have demonstrated that the GC-rich region in the upstream regulatory region of ER α target genes is the main region to confer the E₂ responsiveness (Watanabe *et al.* 1998, Safe 2001). The fragment from -101 to -24 bp

of the *LRP16* gene 5'-proximal region was identified to confer the E₂-stimulated transcriptional activities through GC-rich sites, and we also confirmed that the transcriptional activity of *LRP16* gene by E₂ through this region is mediated through ER α -Sp1 interaction (Si *et al.* 2006). To test whether LRP16 is required for transcriptional activation of genes through ER α -Sp1 interaction, we utilized the LRP16 promoter (-101 to -24 bp)-luciferase fusion as a model promoter for this system. We observed the similar results by a GC-rich promoter and using the classical ERE promoter model in both MCF-7 and HeLa cells (Fig. 3B and C).

To address the necessity for LRP16 in estrogen induction of ER α target genes, we chose several ER α target genes including *c-fos*, *MTA3*, *pS2*, *RAR α* , and *cathepsin D* to detect their estrogen

response. All of the chosen targets genes are well-documented E₂-induced genes in MCF-7 cells and their promoters contain either functional EREs or GC-rich Sp1-binding sites (Jeltsch *et al.* 1987, Weisz & Rosales 1990, Augereau *et al.* 1994, Sun *et al.* 1998, Fujita *et al.* 2004). Northern blot analyses demonstrated that the suppression of *LRP16* gene expression in MCF-7 cells at least partially impaired the estrogen-mediated upregulation of all ER α target genes except cathepsin D at different culture conditions and time points (Fig. 4A). Cyclin D1 and c-myc are two well-known cell cycle-related genes (Weisz & Bresciani 1988, Sabbah *et al.* 1999, Vastro-Rivera *et al.* 2001). The protein levels of both cyclin D1 and c-myc were measured in LRP16-inhibitory MCF-7 cells by immunoblotting. There was an observed parallel depletion of growth recovery with estrogen after synchronization with ICI 182 780 (Fig. 4B). In addition, we also detected the ER α expression by immunoblotting, and the results confirmed that the suppression of LRP16 in MCF-7 cells did not change the ER α level (data not shown). These data excluded the fact that the attenuation of ER α -mediated transcription in LRP16-inhibitory cells could have been caused by the alteration of ER α expression itself. Finally, we concluded that LRP16 is required for ER α -mediated transactivation.

LRP16 physically interacts with ER α

The evidence that LRP16 enhances the function of ER α transactivation prompted us to examine whether LRP16 protein binds to ER α . As shown in Fig. 5A, LRP16 can bind to ER α in a GST pull-down assay, suggesting a direct interaction independent of estrogen stimulation in a cell-free system. By comparison of the intensity of the target bands in Fig. 5A, it was observed that the interaction of LRP16 and ER α proteins was enhanced in the presence of E₂. Subsequently, MCF-7 cells were cultured with phenol red-free DMEM supplemented with steroid-stripped serum for at least 3 days and cotransfected with LRP16 and ER α cDNA to determine whether LRP16 and ER α interact in a cell system. We used a rabbit anti-human ER α antibody to immunoprecipitate LRP16 from cell lysates, and immunoblotting with an anti-LRP16 antibody confirmed the interaction between ER α and LRP16 in the cell system irrespective of the presence of estrogen (Fig. 5B). Next, we performed the same experiment in the reciprocal manner (i.e. immunoprecipitation with a LRP16 antibody and immunoblotting with a rabbit anti-human ER α antibody). Again we detected an interaction between LRP16 and ER α (Fig. 5B). A comparison of the intensity of the target bands in Fig. 5B confirmed that E₂ enhanced the interaction of LRP16 and ER α since the cross-reaction of the IgG heavy chain with the secondary antibody serves as a loading control.

Figure 4 Inhibition of LRP16 attenuates the estrogen-stimulated upregulation of ER α -induced target genes. (A) MCF-7 cells with stable LRP16-siRNAs or control-siRNA expression were grown in media stripped of steroids for at least 3 days, then treated with E₂ (10 nM) for the indicated time. Northern blot analyses were performed to quantify the mRNA levels of *MTA3* (24 h), *pS2* (24 h), retinoic acid receptor α (*RAR* α ; 6 h), *cathepsin D* (6 h), and *c-fos* (3 h) genes. Total RNA loading was used as a loading control. These results are representatives of two independent experiments. (B) MCF-7 cells with different LRP16 expressions were first cultured in steroid-deprived medium for at least 3 days and subsequently treated with ICI 182 780 (10 nM) for 2 days for cells synchronization, as described previously (Sabbah *et al.* 1999). The cells were then released from the G₀/G₁ block by replacing the culture medium containing 10 nM E₂. After 3 h, exposure of E₂, cyclin D1, c-myc, and β -actin proteins was analyzed by immunoblotting. This experiment was repeated twice.

Figure 5 Interaction between LRP16 and ER α in cell-free and cell systems. (A) Beads alone, GST alone, or GST-LRP16 fusion protein, were incubated with recombinant ER α as described in Materials and methods. Samples were subjected to SDS-PAGE and ER α was analyzed by immunoblotting. (B) MCF-7 cells were cultured in phenol red-free and steroid-stripped media for at least 3 days and were subsequently cotransfected with LRP16 and ER α expression vectors, stimulated with E₂ or DMSO as described in Materials and methods. Immunoprecipitations were performed with protein extracts using ER α or LRP16 antibodies. Immunoblots were probed with LRP16 (left panel) or ER α antibodies (right panel). The cross-reaction of IgG heavy chain was used to determine similar loading levels between lines. Except the major LRP16 band at 35 kDa size site, multiple non-specific bands were also observed in the input lane of the left panel, which were formed by non-specific reactions between the rabbit anti-LRP16 antibody and the protein extracted from MCF-7 cells. Results shown in A and B are representatives of two independent trials.

Involvement of A/B AF-1 functional domain in the interaction of ER α with LRP16

To define the individual domains of the ER α involved in binding to LRP16, mammalian two-hybrid assays were performed in MCF-7 and 293T cells. The ER α constructs were composed of amino acids 1–595 (pBIND-ER α), 1–263 (pBIND-ER α -ABC), 1–180 (pBIND-ER α -AB), and 263–595 (pBIND-ER α -DEF) fused to the DBD of GAL4; whereas, full-length LRP16 was fused to the VP16 activation domain (pACT-LRP16; Fig. 6A). As shown in Fig. 6B, the pG5-*Luc* reporter gene was induced in MCF-7 cells when pACT-LRP16 was cotransfected with pBIND-ER α , pBIND-ER α -ABC, or pBIND-ER α -AB construct but not with pBIND-ER α -DEF. The addition of E₂ (10 nM) further enhanced the transcription by about 1.5-fold in the case of pBIND-ER α , but not with pBIND-ER α -ABC or pBIND-ER α -AB. In addition, results observed in 293T cells were similar to those described in MCF-7 cells (data not shown). To exclude possible vector-dependent transactivation (Finkel *et al.* 1993), we exchanged the vectors between LRP16 and the individual ER α domains and repeated the experiments. The results were consistent with the

previous experiment (data not shown). These data indicated that LRP16 binds to the N-terminal A/B AF-1 but not AF-2 domain of ER α . Even though binding is not dependent on the presence of estrogens, E₂ enhances LRP16 binding to the full-length ER α molecule, which is consistent with the results as shown in Fig. 5.

Discussion

ER α , a member of the NR family, has an established role in promoting breast cancer. ER α regulates the transcription of genes involved in breast cancer cell proliferation, invasion, and metastasis. In the complex regulation of ER α signal transduction, emerging evidence indicates that an auto-regulation loop could be an important model for ER α functional activity. For example, cyclin D1 is a well-documented estrogen target gene in many ER α breast cancer cell lines (Vastro-Rivera *et al.* 2001). Surprisingly, cyclin D1 has been shown to have a novel role in the growth regulation of estrogen-responsive tissues by activating ER α -mediated transcription in the absence of estrogen and enhancing transcription in its presence (Zwijsen *et al.* 1997). Several lines of evidence suggest that most

Figure 6 LRP16 specifically binds to the A/B AF-1 domain of ERα. (A) Schematic of VP16-fused LRP16, Gal4-fused ERα, and its deletion constructs. (B) Mammalian two-hybrid assays were carried out to test the interaction between LRP16 and certain ERα functional regions in mammalian cells. MCF-7 cells were cultured with steroid-stripped media for at least 3 days and cotransfected with a DNA mixture containing 0.5 μg pG5-LUC, 0.75 μg pACT or pACT-LRP16, 0.75 μg pBIND or pBIND-ERα, or its deletion constructs. Approximately 30 h after transfection, 10 nM E₂ were added to the medium. Cells were harvested after 42 h of transfection and the luciferase activity was measured. The relative luciferase activity levels (normalized) for pACT and pBIND parent vector transfections were arbitrarily assigned a value of 1. This experiment was repeated thrice.

of the ERα cofactor expression levels are not hormonally regulated (Misiti *et al.* 1998, Thenot *et al.* 1999, Vienonen *et al.* 2003). Yet, there is evidence that a number of cofactors themselves are regulated by E₂. For example, the expression of the corepressor SHARP (SMART/HDAC1 associated repressor protein) is increased by E₂ (Shi *et al.* 2001) and expression of the coactivator AIB1 is decreased (Lauritsen *et al.* 2002). This self-regulatory model can also be extended to other NR members. For example, the expression of the SWI3-related gene product (SRG3), which is developmentally regulated in the prostate, is induced by androgen through the androgen receptor (AR). SRG3 in turn enhances the transactivation of AR, providing a positive feedback loop in a NR signaling pathway (Hong *et al.* 2005). Importantly and interestingly, our results now document one feedback-enhancing pathway regulating ERα-mediated transcriptional activity by an estrogen-responsive gene, *LRP16*. This pathway may be involved in the progression of ERα-positive breast cancer and may reflect the self-maintaining nature of ERα signaling in estrogen-sensitive target cells.

Previous studies have demonstrated that E₂ induced the upregulation of *LRP16* mRNA and the reporter gene activity in ERα-positive MCF-7 cells (Han *et al.* 2003). However, the expression pattern of *LRP16* gene has been shown to be ubiquitous in multiple human

tissues, not exclusively in hormone-dependent tissues (Han *et al.* 2001). In this study, using several breast cancer cell lines and ERα modulation, we provide evidence to confirm the positive relationship between *LRP16* expression and estrogen action. We have demonstrated consistent changes in *LRP16* expression at both mRNA and protein levels (Fig. 1). This link, initially derived from cell culture systems, is also supported by data from primary breast carcinomas. In clinical samples, *LRP16* overexpression was observed in about 30–40% of primary breast carcinomas by northern blot and semi-quantitative RT-PCR analyses, and the positive status of ERα and PR were significantly linked to *LRP16* expression level (Liao *et al.* 2006).

This study provides the first evidence for a functional role of *LRP16* gene in strengthening the ERα-responsive gene activation and estrogen-dependent proliferation of breast cancer cells. Previously, we demonstrated that the ectopic expression of *LRP16* promoted the proliferation of MCF-7 cells as well as a G₁/S transition, meanwhile stimulating cyclin E expression but not p53, p21, and Rb expression (Han *et al.* 2003). In contrast, herein we show by siRNA knockdown approaches that inhibition of the endogenous *LRP16* gene in MCF-7 cells suppresses cell growth. The experimental results including the fact that inhibition of *LRP16* attenuated the E₂-stimulated

responsiveness of MCF-7 cells and that the enforced expression of LRP16 in ER α -negative MDA-MB-231 human breast cancer cells did not stimulate cell proliferation strongly suggest that LRP16 participates in ER α signaling. This prompted us to investigate the primary cell cycle checkpoint proteins that confer the mitogenic role of estrogen. Cyclin D1 and c-myc, two well-known ER α target genes, are believed to be sufficient to recapitulate the effects of estrogen on cell cycle progression (Prall *et al.* 1998b, Carrol *et al.* 2002). We show herein that the inhibition of LRP16 attenuated the induction of cyclin D1 and c-myc expression by E₂ in MCF-7 cells (Fig. 4B). In addition, the response defect of several ER α -induced genes for estrogen stimulation was also observed in LRP16-inhibitory MCF-7 cells (Fig. 4A). In addition, we show by both ectopic expression and siRNA knockdown approaches that LRP16 enhances the transactivation of ER α -targeting promoters (Fig. 3). In general, results from the cell growth study point to the possibility of LRP16-mediated modulation of the ER α -responsive transcription in cancer development.

The ligand-dependent activation of ER α requires the ligand-dependent association of coactivator complexes (Beato *et al.* 1995, Horwitz *et al.* 1996, McKenna *et al.* 1999, Glass & Rosenfeld 2000). Presently, most transcriptional mediators and cofactors have been identified as interacting with and activating the AF-2 activity of ER α in a ligand-dependent fashion (Klinge 2000, Shao & Brown 2004). Although it has previously been reported that the full activation of the AF-1 domain containing in ER α can stimulate the proliferation of breast cancer cells (Fujita *et al.* 2003), less is known about the coactivators for N-terminal A/B AF-1 domain. The enhancement of ER α -mediated transactivation by LRP16 can be attributed to the formation of an LRP16/ER α functional complex. By cell-free and cell system assays, we first demonstrated that LRP16 physically interacts with ER α in a manner that is estrogen independent but appears to be enhanced by E₂ (Fig. 5). Further analyses showed that the region interacting with LRP16 was specifically located in the A/B region that contained the AF-1 domain of ER α . In addition, E₂ enhanced the binding of LRP16 to the full-length ER α molecule but not to the AF-1 domain alone (Fig. 6), suggesting that the interaction of LRP16 and certain cofactors was recruited to the AF-2 domain upon E₂ stimulation. These results emphasized the possible mechanism of LRP16 modulating the co-operative interaction of AF-1 and AF-2 by interacting with other cofactors, since the maximally transcriptional activity of ER α was exhibited. The coactivator complex recruited for AF-2 that may interact with

LRP16 after E₂ treatment is being investigated in our laboratory. However, the differing impact of LRP16 on ER α -mediated transcriptional activity in the absence of E₂ between MCF-7 and HeLa cells was observed in this study (Fig. 3A), indicating cell-specific intrinsic activity of ER α by functional interaction of LRP16/AF-1 domain.

Over the past decade, a large number of proteins have been identified that interact with and regulate transcriptional activity of ER α (Klinge 2000, Dobrzycka *et al.* 2003, Shao & Brown 2004). To our knowledge, there has been no recognition of an ER α cofactor regulated directly by ER α where the expression level is dependent on estrogen activity. ER α directly activates transcription of LRP16 in response to estrogen stimulation. Interestingly, LRP16, in turn, binds to ER α and enhances its transcriptional activity in the presence of a ligand signal. Ultimately, one biological output of estrogen signaling through the interaction of LRP16 and ER α manifests in the magnification of the ER α -mediated transactivation signal and the expression modulation of ER α target genes. The ability of ER α target gene *LRP16* to functionally modulate ER α -mediated transcriptional activity contributes both to the complexity and the plasticity of the regulatory circuit.

Our findings could potentially have clinical benefits since an understanding of the activation linked to the expression of ER α may be important for endocrine therapy of ER α -positive breast cancer. Based on the mechanism of the ER α /LRP16 regulatory loop, we would like to propose that patients with high LRP16 expression might benefit from LRP16 targeting alongside with anti-estrogen therapy. LRP16 targeting could potentially improve the initial efficacy of therapy or delay the emergence of therapeutic resistance.

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