Ligand-dependent Interaction of Estrogen Receptor-α with Members of the Forkhead Transcription Factor Family*

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Estrogen acting through the estrogen receptor (ER) is able to regulate cell growth and differentiation of a variety of normal tissues and hormone-responsive tumors. Ligand-activated ER binds DNA and transactivates the promoters of estrogen target genes. In addition, ligand-activated ER can interact with other factors to alter the physiology and growth of cells. Using a yeast two-hybrid screen, we have identified an interaction between ERα and the proapoptotic forkhead transcription factor FKHR. The ERα-FKHR interaction depends on β-estradiol and is reduced significantly in the absence of hormone or the presence of Tamoxifen. A glutathione S-transferase pull-down assay was used to confirm the interaction and localized two interaction sites, one in the forkhead domain and a second in the carboxyl terminus. The FKHR interaction was specific to ERα and was not detected with other ligand-activated steroid receptors. The related family members, FKHR1 and AFX, also bound to ERα in the presence of β-estradiol. FKHR augmented ERα transactivation through an estrogen response element. Conversely, ERα repressed FKHR-mediated transactivation through an insulin response sequence, and cell cycle arrest induced by FKHR1 in MCF7 cells was abrogated by estradiol. These results suggest a novel mechanism of estrogen action that involves regulation of the proapoptotic forkhead transcription factors.

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1 The abbreviations used are: ER, estrogen receptor; ERE, estrogen response element; AR, androgen receptor; FKHR, forkhead family transcription factor; GR, glucocorticoid receptor; PR, progesterone receptor; VDR, vitamin D receptor; IRS, insulin-response sequence; GSTF, glutathione S-transferase; PCR, polymerase chain reaction; GFP, green fluorescent protein; MEM, minimum essential medium; EGFP, enhanced GFP.

β-estradiol induces homodimerization of ER, which is able to bind specific regulatory sequences in the promoters of ER target genes called estrogen response elements (EREs) (6). It is through this classic model of steroid hormone action that ERs alter the expression of a set of target genes. Several target genes for ERα in hormone-responsive breast tumors have been described including progesterone receptor (PR) (7), pS2 (8), TGF-α (9), cathepsin D (10), HSP27 (11), and GREB1 (12). These genes are directly activated by ERα, and the induction of gene expression depends on the ability for ERα to bind to the promoters of each target gene.

Increasingly, it has been reported that estrogen acting through ERs can have profound effects on cells. Hormones acting through mechanisms independent of DNA binding. One mechanism that has been proposed is through the ability of ERs to regulate the activity of other nuclear transcription factors by mechanisms involving direct protein-protein interactions. In many cases the interactions between ERs and other nuclear factors have been shown to be ligand-dependent. One example of this alternate mechanism of gene regulation is the effect of ERs on the expression of AP1-regulated genes (13). ERα and ERβ have been shown to interact with AP1 but with different ligand activation. In experiments in HeLa cells, ERα stimulated an AP1 reporter plasmid in the presence of estrogen or antiestrogens. However, ERβ demonstrated activation of AP1-mediated transcription only in the presence of antiestrogens. At the functional level, the classic model of steroid action and this alternate mechanism of ER action are indistinguishable; in both cases estradiol induces alterations in the pattern of gene expression. Through interaction with other nuclear factors, ERs are able to modulate the expression of genes normally controlled by factors that are not thought to be influenced by steroid hormones. More recently there has been an important finding that ERs can regulate the cell cycle and apoptosis through a small fraction of receptors that may be present in the cell membrane (14). In this model of regulation, the ligand-activated ERα, ERβ, or androgen receptor (AR) was able to attenuate apoptosis through the activation of the Src/Shc/ERK pathway.

Understanding the mechanisms that regulate cell physiology in response to hormone is an important step toward developing new therapies for diseases of hormone-responsive tissues. We have identified additional proteins that interact with ligand-activated ERα using a yeast two-hybrid screen. One of these was a ligand-dependent interaction between ERα and the forkhead family transcription factor FKHR. This interaction was specific for ligand-activated ERα; no significant interaction could be detected between FKHR and AR, glucocorticoid receptor (GR), progesterone receptor (PR), or vitamin D receptor (VDR). A ligand-specific interaction between ERα and the re-
lated proteins FKHL1 and AFX was also confirmed. The interaction between ERα and FKHR augmented transactivation through EREs but repressed the ability of FKHR to transactivate through an insulin-response sequence (IRS). Expression of FKHL1 induced alterations in the cell cycle in MCF7 cells that were reversed with estrogen. These findings establish an important link between ERα and transcriptional regulation of the proapoptotic family of forkhead transcription factors and provide a novel mechanism of estrogen action in hormone-responsive cells.

**EXPERIMENTAL PROCEDURES**

**Cell Lines**—The cell lines COS-1 and MCF7 were obtained from ATCC and maintained as described previously (15).

**Two-hybrid Screen**—The full-length ERα cDNA was amplified using the oligos 5'-E9InDe (5'-GGGAAATTCGATATCACATTTCTGCTCCACACAAAAGTACAGGGCTT-3') and 3'-EBam (5'-GGCGAGGGATCTCTTGAGACTGGGAAACCCCTC-3') and was cloned into the NdeI and BamHI site of pGBK7T (CLONTECH). This vector encoding the ER-Gal4 DNA binding domain fusion was co-transfected with the human mammary gland MATCHMAKER cDNA library (CLONTECH) into AH109 yeast. The yeast were selected following the instructions of the manufacturer except the plates were watered with carrier (water), β-estradiol (final concentration on the plate 100 nM), or Tamoxifen (final concentration 1 μM).

**GST Pull-down Assay**—The GST-fusion proteins including the ligand-binding domains (LBDs) of AR- (629950, 6299-10510), ERα (83950, 839-10510), and VDR- (88950, 88-10510) were generated by PCR amplification of the coding region of pFB-12A2 with the primers 5'-CAAGCTTCAAGGTGTCTTGTGCTGGGGACAGC; and FKHR108, 5'-GAAACCATGGGCCAGGAGG-CTGGAGAGATGCTTT; FKHR106, 5'-GAAACCATGGGCCAGGAGGCTT-CACTGGCCAGA-CCCTGACACCCAGCTATGTG; FKHR104, 5'-CAAGCTTTA-CTTTATTG-GGCTCTACAGCGGAGAGGCTG; FKHR102, 5'-CAAGCTTCACTGGCCAGA-CCCTGACACCCAGCTATGTG; and FKHR108, 5'-CAAGCTTTAACAGGAGGCTT-CACCTACACTGCGACACCCAGCTATGTGTCGT) containing a HI site of pGBKT7 (CLONTECH). This vector encoding the ERβ1-HI site of pGBK7T (CLONTECH) into AH109 yeast. The yeast were selected following the instructions of the manufacturer except the plates were watered with carrier (water), β-estradiol (final concentration on the plate 100 nM), or Tamoxifen (final concentration 1 μM).

**DNA Constructs**—The full-length ERα cDNA was amplified using the oligos 5'-E9InDe (5'-GGGAAATTCGATATCACATTTCTGCTCCACACAAAAGTACAGGGCTT-3') and 3'-EBam (5'-GGCGAGGGATCTCTTGAGACTGGGAAACCCCTC-3') and was cloned into the NdeI and BamHI site of pGBK7T (CLONTECH). This vector encoding the ER-Gal4 DNA binding domain fusion was co-transfected with the human mammary gland MATCHMAKER cDNA library (CLONTECH) into AH109 yeast. The yeast were selected following the instructions of the manufacturer except the plates were watered with carrier (water), β-estradiol (final concentration on the plate 100 nM), or Tamoxifen (final concentration 1 μM).

**Transactivation Assays**—DNA was introduced into either MCF7 or COS-1 cells by lipid-mediated transfection using Fugene-6 (Roche Molecular Biochemicals) according to manufacturer instructions. For COS-1 cells, the cells were plated at 2.5 x 10⁶ cells/well in 6-well plates 24 h prior to transfection and were 80% confluent at the time of transfection. Each transfection contained 250 ng of Gal4 reporter plasmid (p5GEnbluc), 100 ng of pGal-Control vector, and various amounts of pCMVGal50/FKFR109-110, pCMVGal50/FKFR111-110, or pCMVGal50 as indicated. All transfections were performed in triplicate, and the cells were harvested 48 h post-transfection. Cell extracts for luciferase or β-galactosidase assays were prepared using a luciferase assay system (Promega). Luciferase assays were performed with 5 μl of cell extract and 100 μl of luciferase assay buffer. The enzyme activity was measured for 2 s in a luminometer (Analytical Luminescence Laboratory, San Diego, CA). β-galactosidase activity in cell extracts was assayed using the Galacto-Light system (Applied Biosystems, Bedford, MA).

For MCF7 cell transfections, cells were seeded at 5 x 10⁵/well in 6-well plates in growth medium the day before transfection. On the day of transfection the medium was replaced with fresh growth medium. DNA-lipid complexes were formed in serum-free minimum essential medium (MEM) and then placed on the cells. Generally, 1 μg each of reporter plasmid and transactivator plasmid and 0.4 μg of pβgal-control vector were used per 18 h of transfection. The presence of the DNA-lipid complexes the transfection mixture was removed, and the cells were washed twice with phosphate-buffered saline. For hormone response assays, phenol red-free MEM containing 10% charcoal dextran-treated fetal bovine serum with or without the appropriate hormone was added as described previously (12). For assays not requiring hormone-controlled conditions the transfection mixture was replaced with MEM plus 10% fetal bovine serum. The cells were replaced at 37 °C/5% CO₂ until harvest. The luciferase activity was assayed using 10 μl of lysis and 100 μl of luciferase assay reagents and luminescence was measured as described above. β-galactosidase activity in 10 μl of the lysates was measured as described above. For each sample the luciferase relative light units were normalized with the β-galactosidase relative light units.

**Cell Cycle Analysis**—MCF7 cells were transfected with the expression plasmids pCMs-EGFP, pCMs-FKHL1-WT, or pCMs-FKHL1-TM by electroporation. For each sample, 4 x 10⁶ cells were washed with cold MEM and then resuspended at 5 x 10⁶ cells/ml in cold MEM. Ten micrograms of DNA was added, and the cells were electro- porated in 0.4-cm cuvettes using a Bio-Rad Gene Pulser set at 260V, 1 μF, 960 μF, and 200 μF. Growth medium was added to bring the volume to 12 ml, and the cells were plated in four 6-cm dishes at 37 °C/5% CO₂. After 18 h, the medium was removed, and the cells were rinsed twice with PBS. The appropriate medium was added to each plate, and the cells were replaced at 37 °C for 24 h.

Samples were processed for cell cycle analysis by flow cytometry as described (18). Briefly, the cells were harvested by brief trypsinization and placed on ice. All steps were performed at 4 °C. The cells were washed once in PBS with 1% fetal bovine serum, 1% formaldehyde in PBS for 1 h. After two additional washes the cells were fixed in 70% ethanol/30% PBS overnight at 4 °C. The cells were washed twice before resuspension in PBS + 1% fetal bovine serum, RNase A (100 μg/ml), and propidium iodide (40 μg/ml). After incubation at 37 °C for 1 h, 20,000 cells from each sample were analyzed on a FACStar flow cytometer for GFP and propidium iodide fluorescence. The data were analyzed using Flowjo software (Treestar Software). The GFP-positive single cell population was plotted as a histogram of propidium iodide fluorescence, and the Watson Pragmatic model (19) was used to perform cell cycle analysis.
There was a binding domain fusion protein (DNA-BD/ER). As seen in Fig. 1, note the growth of DNA-BD/ER and AD/FKHR only in the presence of Gal4 activation domain fusion (AD/T) was not able to interact these yeast transformants to grow. As controls, a T antigen/H9251 into yeast with the plasmid expressing the ER protein in-frame with the Gal4 activation domain. Transformation was subsequently plated on Leu/Trp/His/Ade medium with β-estriadiol (E2), Tamoxifen (Tam), or no ER ligand (None) as shown. The growth of DNA-BD/ER and AD/FKHR only in the presence of β-estriadiol.

**RESULTS**

**Ligand-dependent Interaction of ERα and FKHR**—The yeast two-hybrid system was used to identify proteins with ligand-dependent interaction with ERα. Full-length ERα was cloned as a fusion protein with the DNA binding domain of Gal4. A normal human mammary epithelial cell cDNA library cloned to create a fusion protein with the Gal4 activation domain was used in yeast co-transfection. Yeast transformants were screened first on Leu/Trp medium to select for double transformants. To identify proteins that interact with ERα in a ligand-specific fashion, yeast colonies were selected on Leu/Trp/His/Ade medium supplemented with β-estriadiol, tamoxifen, or no ER ligand. Approximately 300 yeast colonies were identified that demonstrated a ligand-dependent growth phenotype, and four colonies were isolated that demonstrated growth only with β-estriadiol (data not shown). The plasmids encoding the activation domain fusion proteins were recovered from these four yeast transformants, and the inserts were sequenced. One of these inserts was found to encode FKHR from amino acid 211 through the stop codon, which had been cloned as a fusion protein in-frame with the Gal4 activation domain.

The plasmid encoding FKHR (AD/FKHR) was co-transfected into yeast with the plasmid expressing the ERα-Gal4 DNA binding domain fusion protein (DNA-BD/ER). As seen in Fig. 1, there was β-estriadiol-dependent growth on Leu/Trp/His/Ade medium supplemented with the fusion plasmids containing ERα and FKHR. By contrast, tamoxifen failed to allow these yeast transformants to grow. As controls, a T antigen-Gal4 activation domain fusion (AD/T) was not able to interact with ERα as evidenced by the lack of growth on His/Ade medium, whereas DNA-BD/p53 and AD/T co-transfection generated yeast capable of growth independent of ERα ligand. These results indicate an estradiol-dependent interaction between ERα and FKHR.

A GST pull-down assay was used to confirm the ligand-dependent interaction between ERα and FKHR. The full-length FKHR protein was synthesized by in vitro transcription/translation from the cloned cDNA. The labeled FKHR protein was incubated with a panel of GST-fusion proteins encoding a variety of nuclear hormone receptor ligand binding domains. The ligand binding domains of ERα, AR, GR, PR, and VDR were each bound in parallel in the presence and absence of their respective ligands. As seen in Fig. 2, only the ERα-GST fusion protein in the presence of β-estriadiol was able to bind FKHR. There was no significant interaction detected between FKHR and the ligand binding domains of the other nuclear hormone receptors.

**Domains of FKHR Involved in ERα Binding**—To determine the domain of FKHR involved with the interaction with ERα, subregions of FKHR were expressed individually using in vitro transcription/translation and tested for ligand-dependent interaction with ERα using the GST pull-down assay (Fig. 3). The region of FKHR from amino acid 211 to 655 (fragment I) interacted with ERα-GST, and the interaction was enhanced greatly with β-estriadiol. The region of FKHR from amino acids 211 to 466 (fragment II) and 211 to 561 (fragment VI) behaved in a similar fashion; both interacted with ERα, and the binding was enhanced by β-estriadiol. However, FKHR amino acids 445–655 (fragment III) and 280–655 (fragment IV) bound to ERα-GST only in the presence of β-estriadiol. Neither fragment V (amino acids 280–446) nor fragment VII (amino acids 280–561) interacted with ERα-GST. These results demonstrate that two regions of FKHR interact with ERα. The carboxyl-terminal region from amino acids 561 to 655 binds tightly to ERα, and binding completely depends on β-estriadiol. A second region from amino acids 211 to 280, which includes part of the forkhead domain, interacts weakly with ERα in the absence of ligand; however, binding is enhanced significantly in the presence of β-estriadiol.

The forkhead domain of FKHR is homologous with the two other family members, FKHR1 and AFX. A BLASTp search of the carboxyl-terminal 95 amino acids of FKHR also demonstrated strong homology between these three proteins in this region. FKHR1 has 39% identity and 55% homology with FKHR in the carboxyl-terminal 95 amino acids. As seen in Fig. 4, a comparison of the region of FKHR from amino acids 592 to 633 demonstrates 65% identity and 76% homology with FKHR1 amino acids 601–643. AFX contains a region from amino acids 462 to 501 with 56% identity and 82% homology with this region of FKHR. This finding suggests that FKHR1 and AFX may also bind to ERα in a ligand-dependent fashion. To test this possibility, in vitro synthesized FKHR1 and AFX were tested for ERα binding using the GST pull-down assay. As seen in Fig. 5, both AFX and FKHR1 bound to ERα only in the presence of β-estriadiol. In addition, a FKHR1 protein with mutations of the three regulatory phosphorylation sites (FKHRL1-TM) (1) also bound to ERα in a ligand-dependent fashion.

The conserved nature of the regions of FKHR that interact with ERα suggests that these are important functional domains. In addition, the highly acidic pI of the carboxyl terminus suggests that this region might function as an activation domain. To test this possibility, the region of FKHR from 211 to 655 and 534 to 655 were cloned into a eukaryotic expression vector as fusion proteins with the DNA binding domain of Gal4. The ability of these fusion proteins to transactivate transcription was assayed by co-transfection with a Gal4 luciferase reporter. As seen in Fig. 6, the region of FKHR from 211 to 655 provides trans-activating function with a nearly linear increase of induction with increased expression. The activity from the FKHR fusion protein is approximately one-third of the activity.
of a VP16-Gal4 fusion (data not shown). The terminal 122 amino acids (534–655) contained all the transactivation function of the larger insert at every concentration tested (Fig. 6). These data are in agreement with earlier studies that localized the activation domain of FKHR to amino acids 596–655 (20).

FKHR Augments ERα/H9251 Transactivation at an ERE—Because the carboxyl-terminal region of FKHR demonstrated strong ligand-dependent interaction with ERα/GST fusion protein or GST protein alone in the presence (+) or absence (−) of β-estradiol (E2). Bound proteins were analyzed by SDS-polyacrylamide gel electrophoresis and detected by autoradiography. Ligand-dependent interaction between GST-ERα and AFX and between GST-ERα and FKHR1 or FKHR1-TM is indicated by an arrow.

The functional effects of the ERα-FKHR interaction on the transcriptional activation of an ERE by ERα was examined using MCF7 cells (an ERα-positive hormone-responsive breast carcinoma cell line) that has been shown to have negligible expression of FKHR (21). The activity of the Vit-ERE-LUC reporter plasmid was assayed in MCF7 cells in which an FKHR expression plasmid was co-transfected in the absence or presence of ERα ligands. As seen in Fig. 7A, β-estradiol treatment resulted in the expected transactivation of expression from the Vit-ERE-LUC plasmid. Also as expected, Tamoxifen blocked
the activity of β-estradiol. In the absence of ligand, FKHR had no effect on expression; however, in the presence of β-estradiol, FKHR resulted in a reproducible augmentation of ERα-mediated transactivation. In the presence of FKHR, Tamoxifen again reduced expression to baseline levels. These results indicated that FKHR can augment ERα transactivation through ERα sequences in a ligand-dependent fashion.

**ERα Represses FKHR Transactivation at an IRS**—The effect of the ERα–FKHR interaction was examined for alterations in the ability of FKHR to transactivate through the IRS element from the IGFBP-1 promoter. FKHR-mediated transactivation of the 3XIRS-LUC reporter plasmid was assayed in MCF7 cells co-transfected with an FKHR expression plasmid in the presence or absence of ERα ligands. As seen in Fig. 7B, β-estradiol had negligible effects on expression from the 3XIRS-LUC reporter, whereas FKHR expression augmented luciferase expression in the absence of ERα ligand. In contrast, β-estradiol completely repressed FKHR-mediated transactivation. Tamoxifen partially repressed FKHR-mediated expression but had no effect on luciferase expression in the absence of FKHR. These results suggest that the ligand-dependent interaction of ERα and FKHR inhibits FKHR-mediated transcriptional activation.

**FKHRL1-induced Cell Cycle Alteration Is Abrogated by Estradiol**—FKHR and related family members are known to induce cell cycle arrest or apoptosis in tumor cells (17, 22, 23). The above experiments demonstrated that the interaction between ligand-activated ERα and FKHR repressed transactivation by FKHR while augmenting ERα-mediated transactivation. To examine the physiologic consequences of this interaction, the effect of estrogen treatment on FKHR-induced cell cycle alterations was examined in MCF7 cells. FKHRL1 or the triple mutant FKHRL1-TM was cloned into the CMS-EGFP vector, which constitutively expresses EGFP. The plasmid pCMS-EGFP, pCMS-FKHRL1, or pCSM-FKHRL1-TM was introduced into MCF7 cells, and the cells were cultured in the presence or absence of β-estradiol for 24 h. The cells were analyzed by flow cytometry for EGFP expression to identify transfected cells and for DNA content indicative for cell cycle stage or apoptosis. Histograms of DNA content for EGFP-positive cells are shown in Fig. 8A. In the absence of β-estradiol, FKHRL1 expression reduced the percentage of cells in G1 from 46% (pCSM-EGFP-transfected) to 39%. FKHRL1-TM contains three mutations in which all three phosphorylation sites have been mutated to alanine (17). Hence, the triple mutant cannot be inactivated by Akt-mediated phosphorylation. Expression of FKHRL1-TM resulted in a progressive decrease in the percentage of cells in G1 to 30%. In the presence of β-estradiol, transfection with vector alone, FKHRL1, or FKHRL1-TM had no significant effect on cell cycle. As seen in Fig. 8, the percentage of cells in G1 transfected with FKHRL1 or FKHRL1-TM in the presence of estrogen was 50.2 and 48.5%, respectively. These values were not statistically different from vector-transfected cells. In FKHRL1-TM-transfected cells, there was a statistically significant difference in the percentage of cells in G1 comparing cells grown in the presence and absence of estrogen (p < 0.001). These data, particularly when viewed in combination with the alterations in the percentage of cells in G1 and the sub-G1 (apoptotic) regions, demonstrate that FKHRL1, and to a greater extent FKHRL1-TM, induce changes in cell cycle in MCF7 cells that are reversed with β-estradiol.

**DISCUSSION**

The forkhead or “winged helix” transcription factors have been shown to play important roles in cell differentiation, embryogenesis, and oncogenesis (24, 25). FKHR was first identified as a transcription factor that was involved in a translocation with PAX3 in alveolar rhabdomyosarcoma (26–28). Based on homology in the forkhead domain, FKHR is most closely related to two other human genes, FKHRL1 (16) and AFX (29), and the *Caenorhabditis elegans* homolog Daf-16 (30). Studies of the functional regulation and physiologic effect of this gene family have confirmed the similarity initially based on their structural homology. The activity of FKHR and the related proteins are all regulated through phosphorylation by Akt (17, 31–34). In HepG2 cells, FKHR has been reported to transactivate the IGFBP-1 promoter through the IRS (21, 34–36). Insulin inactivation of FKHR is mediated through phosphorylation at three residues of FKHR (31, 37). Phosphorylated FKHR is retained in the cytoplasm, and its exclusion from the nucleus is associated with a loss of target gene expression (17, 31, 32).

A number of studies have shown that FKHR and the related proteins can regulate apoptosis and cell cycle arrest. Cytokine stimulation in lymphocytes is mediated through phosphatidylinositol 3-kinase, and down-regulation of p27kip1 is a common pathway leading to cell cycle stimulation. It has been shown in B cells that the inactivation of FKHRL1 by phosphorylation results in decreased p27kip1 expression and reduced apoptosis (22). Similarly in neuronal tissue, FKHRL1 has been shown to
induce apoptosis through transcriptional mechanisms that induce a number of genes including Fas ligand (17). Akt-mediated phosphorylation of FKHR is induced by survival factors and is associated with retention of FKHR1 in the cytoplasm. In addition, the PTEN tumor suppressor gene is known to antagonize the activity of phosphatidylinositol 3-kinase (38). In PTEN-deficient cells, FKHR and FKHRL1 are retained in the cytoplasm, whereas restoring PTEN induces the translocation of FKHR to the nucleus and transcriptional activity (23). In cells that demonstrate PTEN-mediated cell cycle arrest or apoptosis, FKHR activation is able to induce similar changes in cell cycle or apoptosis. AFX has also been shown to induce cell cycle arrest through the activation of p27kip1 (39). Finally, these pathways have also been shown to be functional in breast cells. In MDA-MB-231 breast carcinoma cells, epidermal growth factor treatment activates phosphatidylinositol 3-kinase and Akt and has been shown to induce FKHR phosphorylation and nuclear exclusion (40).

Our studies have demonstrated that FKHR, FKHRL1, and AFX bind to ERα in a ligand-dependent fashion. One of the regions of FKHR involved in the interaction corresponds to the transactivation domain in the carboxyl terminus. The interaction of ERα with this region of FKHR was strongly dependent on β-estradiol. The data from reporter assays indicated that ERα repressed FKHR transactivation, and the repression by ERα was dependent on β-estradiol and partially reversed by Tamoxifen. The reporter assay data were supported by data showing that FKHRL1 and FKHRL1-TM induced alterations of the cell cycle in MCF7 cells that were reversed by estrogen. One interpretation is that under the conditions used here, FKHRL1 is inducing a cell cycle block in G2/M that is removed by estrogen. These results are consistent with a recent study showing that FKHR expression reduced the colony formation of MCF7 cells (41).

Estrogen is known to act as a mitogen in hormone-responsive breast tumors, and the interaction between ERα and forkhead transcription factors may explain the cell cycle arrest induced by estrogen withdrawal or Tamoxifen treatment. It has also been reported that estrogen replacement in postmenopausal women is protective for Alzheimer’s disease (42, 43). Because FKHRL1 has been shown to induce cell death by apoptosis in neuronal cells (17), it is plausible that ERα interaction with forkhead proteins in neuronal tissue may support cell survival.

The interaction between ERα and FKHR augmented ERα-dependent transactivation through EREs. This result was in contrast to a recent report by Zhao et al. (41), who reported that FKHR repressed ERα transactivation through EREs. However, those experiments were performed in HepG2 cells that constitutively express high levels of FKHR, which was in contrast to our experiments that were performed in hormone-responsive
MC7 cells that express negligible levels of FKHR. It may be that the differences in the physiologic effects of the ERα-FKHR interaction observed depend on the cell line and the promoter being studied. Nevertheless, the finding that the ERα-FKHR interaction augments ERα transactivation at an ERE while repressing FKHR-mediated transactivation makes physiologic sense in light of the role of estrogen as a mitogen. Our data suggest that in cells in which both factors are expressed, the presence of estrogen stimulates expression of ERα-responsive target genes while repressing FKHR target genes, resulting in progression of the cell cycle. Estrogen withdrawal (or treatment with an antiestrogen) promotes FKHR activation, expression of FKHR target genes, loss of ERα target genes, and cell cycle arrest.

A variety of cofactors have been described for ERα, and the repertoire of cofactors that are expressed will vary from cell to cell and within the same cell type depending on the physiologic conditions. This consideration was also evident in our examination of the effect of ERα on FKHR-mediated transactivation through an IRS element. Repression by ERα was maximal at 60–72 h after β-estradiol treatment but was negligible at 24 h and intermediate at 48 h (data not shown). This means that the physiologic effect of the ERα-FKHR interaction in MCF7 cells is likely to be influenced by factors induced by β-estradiol and may not be present in cells that do not express the receptor.

In summary, we have demonstrated a ligand-dependent interaction between ERα and the FKHR family of forkhead transcription factors. These data provide a novel mechanism of estrogen regulation through the modulation of forkhead transcription factors. This finding describes an important link between cell surface signaling mechanisms that act through the phosphatidylinositol 3-kinase pathway and nuclear hormone receptors. The physiologic consequences of this interaction will likely depend on the cell type and growth state of the cell. However, ERα and the forkhead proteins are found in a plethora of cell types, and this interaction is likely to influence either cell cycle arrest or apoptosis in a variety of tissues. The identification of crosstalk between these two signaling mechanisms also provides an important mechanism whereby steroid hormones can influence the action of cell surface receptors and cell surface ligands can influence the actions of steroid hormones.

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Ligand-dependent Interaction of Estrogen Receptor-α with Members of the Forkhead Transcription Factor Family

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