Insulin-like growth factor I (IGF-I) displays estrogenic activity in breast cancer cells. This activity is strictly dependent on the presence of estrogen receptor α (ERα). However, the precise molecular mechanisms involved in this process are still unclear. IGF-I treatment induces phosphorylation of the AP1 domain of ERα and activation of estrogen regulated genes. These genes are characterized by important differences in promoter architecture and response element composition. We show that promoter structure is crucial for IGF-I-induced transcription activation. We demonstrate that on a complex promoter such as the pS2/TFF1 promoter, which contains binding sites for ERα and for the activating protein-1 (AP1) complex, transcriptional activation by IGF-I requires both ERα and the AP1 complex. IGF-I is unable to stimulate transcription of an estrogen-regulated gene under the control of a minimal promoter containing only a binding site for ERα. We propose a molecular mechanism with stepwise assembly of the AP1 complex and ERα during transcription activation of pS2/TFF1 by IGF-I. IGF-I stimulation induces rapid phosphorylation and an increase in protein levels of the AP1 complex. Binding of the phosphorylated AP1 complex to the pS2/TFF1 promoter allows recruitment of the chromatin remodeling factor Brg1 followed by binding of ERα via its interaction with c-Jun.

Control of breast cancer cell proliferation is a complex, multifactorial, and interactive process. Estradiol, a steroid hormone, which acts via its nuclear receptors (ERα and -β for estrogen receptor α and β) and growth factors such as insulin-like growth factor I (IGF-I) or IGF-II, epidermal growth factor, or transforming growth factor α, whose action is mediated by tyrosine kinase transmembrane receptors, controls gene expression and cellular proliferation of breast cancer cells (1). Although it was discovered more than 10 years ago that ERα could be activated in a ligand-independent fashion by growth factors like epidermal growth factor or IGF (2), the cross-talk between estrogens and growth factor pathways remains to be elucidated.

In the classical model of ER action, the receptor binds as a homodimer (3) or a heterodimer (4–7) to estrogen response elements (EREs) within the promoters of estrogen-responsive genes. Once present on the promoter of estrogen responsive genes, ER recruits an array of transcriptional cofactors (coactivators and corepressors) that bind to the receptor and interact with other transcription factors, including components of the general transcription machinery. Some of these cofactors possess chromatin-remodeling activities or histone modifying activities or recruit additional proteins to the complex to mediate transcription (for review, see Ref. 8). ERα may also modulate transcription without receptor-DNA interaction by functional interference with other transcription factors such as activating protein-1 (AP1) (for review, see Refs. 9–12).

The AP1 complex, which is implicated in diverse cellular processes, including differentiation, cell proliferation, and transformation (for review, see Ref. 13), predominantly consists of various combinations of Jun (c-Jun, JunB, JunD) and Fos (c-Fos, Fra-1, Fra-2, FosB) proteins. This complex can result from many different combinations of heterodimers and homodimers, and the combination determines the genes that are regulated by AP1 (14, 15). The AP1 complex formed of heterodimers or homodimers of Jun and Fos proteins has DNA binding activity via the TPA-responsive elements (TREs).

Furthermore transcriptional activity of the AP1 complex is post-translationally regulated by several kinases. Extracellular signal-regulated kinases 1 and 2 phosphorylate c-Fos on threonines 325 and 331. These phosphorylations regulate the transforming activity of c-Fos (16). Moreover, phosphorylation of c-Jun by c-Jun NH2-terminal kinases on residues serine 63 (17) and 73 potentiates its ability to activate transcription either as a homodimer (18, 19) or a heterodimer together with c-Fos.

Activation of the IGF-I receptor by IGF-I binding leads to a conformational change of its receptor, the auto phosphorylation of a tyrosine residue in the intracellular part of the receptor, and the subsequent activation of downstream signaling molecules. Then the IGF-I receptor is able to bind signaling...
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adaptor proteins like insulin receptor substrate 1 (IRS1) and Shc, linking the receptor to downstream signaling pathways like the phosphatidylinositol 3-kinase and the mitogen-activated protein pathways (for review, see Ref. 20). Growth factors such as IGF-I are able to elicit estrogenic responses in target tissues in the absence of estradiol, and they cooperate with the hormone in its presence (21). Several studies have demonstrated that estradiol, anti-estrogens, and growth factors induce a rapid hyperphosphorylation of ERα on serine 118, located at the N terminus within transcriptional activation function AF-1 (2, 22, 23). This phosphorylation is essential for activation of ERα bound or not to its ligand (24). However, this phosphorylation is not sufficient to elicit estrogenic responses in the absence of hormone, and there may be other ERα domains or partners that fulfill additional requirements for growth factor signaling through ERα (22). Supporting this, MCF7 breast cancer cells with a constitutively active MEK1 show increased interaction between ERα and its coactivators (SRC2 and SRC3) and hormone hypersensitivity (25). In addition, phosphatidylinositol 3-kinase-3 kinase, which is activated by IGF-I, has been shown to phosphorylate ERα on serine 167 within 1 h after treatment with growth factors. Active phosphatidylinositol 3-kinase increases the transcription-stimulating activity of both the AF-1 and AF-2 domains of ERα, whereas active protein kinases B (PKB), a downstream target of phosphatidylinositol 3-kinase, only increases the activity of AF-1, suggesting that phosphatidylinositol 3-kinase utilizes PKB-dependent and -independent pathways in activating ERα. In addition, LY294002, a specific inhibitor of the phosphatidylinositol 3-kinase/protein kinase B pathway, reduced phosphorylation of ERα in vivo (26).

The goal of the present study was to better understand the cross-talk between the IGF-I and estradiol pathways at the molecular level. We studied the pS2/TFF1 gene whose regulation serves as a paradigm for estrogen regulation of gene expression in breast cancer cells. The pS2/TFF1 gene has a composite promoter with an ERE (located between –405 and –393) and a TRE (AP1 response element, located between –338 and –332), and it has been reported that its activation can be achieved by estradiol or IGF-I (27). Our laboratory has shown that both estradiol and IGF-I treatments induce a rapid chromatin remodeling of the pS2/TFF1 promoter in a region encompassing the ERE and the TRE (28). The role of the AP1 element in the response of pS2/TFF1 to estradiol has been analyzed in detail by others in a cell line derived from the HepG2 hepatocarcinoma cell line, re-expressing ERα. They demonstrate that the ERE is essential for the response of pS2/TFF1 to estrogens and that the TRE is involved in both estrogen and phorbol-ester responses (29).

Here we demonstrate that activation of ERα induced by IGF-I is not sufficient to allow activation of a minimal estrogen-regulated promoter containing only an ERE sequence. We show that in response to IGF-I treatment, the AP1 complex is phosphorylated and rapidly recruited to the pS2/TFF1 promoter, whereas recruitment of ERα is delayed. On the pS2/TFF1 promoter, ERα and the AP1 complex cooperate during IGF-I-induced activation of the transcription, but have different functions. The AP1 complex is implicated in chromatin remodeling by recruiting chromatin-remodeling factors such as Brg1, whereas ERα is involved in later stages of transcription.

EXPERIMENTAL PROCEDURES

Chemicals—Estradiol was purchased from Sigma. ICI 184780 (Fulvestrant) was purchased from Zeneca Pharmaceuticals. IGF-I was purchased from Euromedex.

Antibodies—Anti-human ERα (HC20), anti-Brg1 (H-88), anti-phospho-c-Jun (KM-1), anti-c-Jun (sc-45), and anti-c-Fos (H-125) were purchased from Santa Cruz Biotechnology. Anti-c-Fos and anti-phospho-Thr-325 (ab27793) were purchased from Abcam. Anti-GAPDH was purchased from Chemicon International.

Cell Lines and Tissue Culture—MCF7 cells were grown in Dulbecco’s modified Eagle’s medium/F-12 containing 50 μg/ml gentamycin and 10% heat-inactivated fetal calf serum at 37 °C in a humidified atmosphere containing 10% CO2.

The stably transfected luciferase reporter MELN and MTLN cell lines were obtained as previously described (30). Briefly, to obtain MELN cells, ERα-positive breast cancer MCF-7 cells were transfected with the estrogen-responsive gene ERE-bGlob-Luc-SV-Neo (31). To obtain MTLN cells, MCF-7 cells were co-transfected with the AP1-responsive gene TRE3-Tk-Luc and the neomycin resistance gene pSV2-Neo (32). Selection of resistant clones by Geneticin was performed at 1 mg/ml. MELN and MTLN cells were grown in Dulbecco’s modified Eagle’s medium/F-12 containing 50 μg/ml gentamycin, 10% heat-inactivated fetal calf serum, and 1 mg/ml G418 at 37 °C in a humidified atmosphere containing 10% CO2.

To study the effects of estrogens and IGF-I, cells were grown for 2 days in media without phenol red without gentamycin and without serum. Cells were treated or not with 10 nM estradiol (E2), 5 nM IGF-I, or 100 nM Fulvestrant for the indicated times.

Luciferase Assays—Cells were grown in 3.5-cm diameter dishes and treated as indicated in the figure legends. Cells were then lysed in 200 μl or Promega lysis buffer and frozen. Luciferase activity was determined using the Luciferase assay reagent (Promega E397A) on a Centro LB 960 (Berthold). Luciferase activities were normalized to plated cell number or to total protein content measured by the Amido Schwarz technique (33). All experiments were performed at least in triplicate, and S.D. were calculated.

Western Blots—Western blots were performed as previously described (34). The quantity of extract loaded on the gels was normalized to total protein content, assayed by the Amido Schwarz technique. All antibodies were used at a 1/200 dilution, except for anti-phospho-c-Fos antibody, in which the dilution was 1/1000.

ChIP Experiments—Cells were grown to 95% confluence in Dulbecco’s modified Eagle’s medium/F-12 supplemented with 10% heat-inactivated fetal calf serum followed by 48 h in serum and phenol red-free media. After the addition of hormone or growth factor, cells were washed twice with phosphate-buffered saline (PBS) and cross-linked with 1.5% formaldehyde at 37 °C for 5 min. Cells were then rinsed twice with ice-cold PBS, collected into 100 mM Tris–HCl, pH 9.4, and 10 mM dithiothreitol, incubated for 15 min at 30 °C, and centrifuged for 5 min at 2000 × g. Cells were washed sequentially with 1 ml of ice-cold PBS, buffer I (0.25% Triton X-100, 10 mM EDTA, 0.5 mM EGTA,
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10 mM HEPES, pH 6.5), and buffer II (200 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, 10 mM HEPES, pH 6.5). Cells were then resuspended in 0.3 ml of lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl, pH 8.1, 1× protease inhibitor mixture (Roche Applied Science)) and sonicated three times for 15 s with a Branson Sonifier 250 (duty cycle 50%, output control 5) followed by centrifugation for 10 min. Supernatants were collected and diluted in buffer (1% Triton X-100, 2 mM EDTA, 150 mM NaCl, 20 mM Tris-HCl, pH 8.1) followed by immuno-clearing with 2 μg of preimmune serum, and protein A-Sepharose (45 μl of 50% slurry in 10 ml of Tris-HCl, pH 8.1, 1 mM EDTA) for 2 h at 4°C. Immuno-purification was performed overnight at 4°C with specific antibodies. After immunopurification, 100 μl of protein A-Sepharose and 2 μg of salmon sperm DNA were added, and the incubation was continued for another 1 h. Precipitates were washed sequentially for 10 min each in TSE I (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 10 mM Tris-HCl, pH 8.1, 150 mM NaCl), TSE II (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.1, 500 mM NaCl; except for Brg1, where SDS was removed from the buffer), and buffer III (0.25 mM LiCl, 1% Nonidet P-40, 1% deoxycholate, 1 mM EDTA, 10 mM Tris-HCl, pH 8.1). Precipitates were then washed 3 times with Tris-EDTA buffer and extracted with 1% SDS, 0.1 M NaHCO3 for 30 min at 65°C. Eluates were heated at 65°C overnight to reverse the formaldehyde cross-linking. After cross-link reversal, 1/3 of the DNA fragments were purified with a GenElute PCR clean-up kit (Sigma), and 1/20 of the purified DNA was quantified by quantitative PCR. Quantitative PCR were performed on an iCycler (Bio-Rad) using the oligonucleotides PS2 CH1 (5′-GGGCCATCTCTCCTATGATTACCTCTTCGTC-3′) and PS2 CH2 (5′-GGCCAGTTCTTTGCTTTAAGAAGC-3′) to amplify the pS2/TFF1 promoter fragment. Amplification conditions were 3 min at 95°C followed by 50 cycles (20 s at 95°C, 30 s at 62.5°C, 20 s at 72°C). For the purification of the inputs, 20 μl of extract was removed and heated overnight at 65°C, DNA was then purified as for immunoprecipitates, and 1/20 of the DNA was quantified by quantitative PCR.

Co-immunoprecipitations—For co-immunoprecipitations of cross-linked protein complexes, 1 × 106 cells were submitted to immunoprecipitation as in the ChIP procedure, except that after immunorecovery proteins were eluted by the addition of 30 μl of H2O and 10 μl of 4× electrophoresis loading buffer (200 mM Tris, pH 6.8, 8% SDS, 20% glycerol, 8 mM EDTA, 6% β-mercaptoethanol, 0.016% bromphenol blue). The totality of the immunoprecipitated material was electrophoresed on SDS gels and analyzed by Western blotting. For co-immunoprecipitations of native protein complexes, 1 × 106 MCF7 cells, grown for 3 days in steroid-free medium, were washed twice in PBS, resuspended in 500 μl of lysis buffer (50 mM Tris, pH 8, 0.4% Nonidet P-40, 300 mM NaCl, 10 mM MgCl2, anti-protease from Roche Applied Science (1 mini-tablet/10 ml)), phosphatase inhibitors from Sigma (P2850), and incubated for 15 min at 4°C. After centrifugation for 20 min at 16,000 × g, the lysate was adjusted to 1 ml with 500 μl of dilution buffer (50 mM Tris, pH 8, 0.4% Nonidet P-40, 2.5 μM CaCl2, 1 μl of DNase I). A total of 40 μl (v/v) of protein A/protein G beads were added, and the lysate was incubated for 45 min at 4°C on a rotating wheel. The supernatant was collected by centrifugation, 30 μl was withdrawn for input assays, and the remaining material was incubated with 1 μg of anti-c-Jun for 2 h at 4°C on a rotating wheel. A total of 20 μl (v/v) protein A/protein G beads were added again, and the incubation was continued for 2 more hours. Beads were washed 3 times with wash buffer (50 mM Tris, pH 8, 0.4% Nonidet P-40, 150 mM NaCl, 5 mM MgCl2), and proteins were eluted by the addition of 20 μl of H2O and 10 μl of 4× electrophoresis loading buffer (200 mM Tris, pH 6.8, 8% SDS, 20% glycerol, 8 mM EDTA, 6% β-mercaptoethanol, 0.016% bromphenol blue). The totality of the immunoprecipitated material was electrophoresed on SDS gels and analyzed by Western blotting.

RT-PCR Experiments—Cells were rinsed twice in ice-cold 1× PBS, RNA was then purified using the RNeasy mini kit (Qiagen). RNA was eluted in 100 μl of RNAse-free water, and 1/20 was submitted to quantitative RT-PCR (Platinum RT-PCR thermoscript one step, Invitrogen) with the following primers: pS2/TFF1, 5′-GTACACGGAGGCCACGAGAC- GTG(FAM)AC-3′ (forward) and 5′-AGGGCGTGACACCA- GGAAA-3′ (reverse); actin, 5′-CACAATGCCTCCTGGCCAC- CATTG(FAM)G (reverse) and 5′-GGTGAACAGCATGTGTT- TGGA-3′ (forward); GAPDH, 5′-GACCATTGCTGGGCTG- GTGGTC(FAM)C-3′ (reverse) and 5′-ACAGCAACAGGTT- GGGTGAC-3′ (forward). Amplification conditions were 30 min at 45°C and 3 min at 95°C followed by 50 cycles (20 s at 95°C, 30 s at 63°C, 20 s at 72°C).

Cell Electroporation and RNA Interference—Five million cells were electroporated by siRNA to a final concentration of 20 μM. Cells were electroporated on a gene pulser Xcell (Bio-Rad) at 250 V and 960 microfarads in 0.4-mm cuvettes. After electroporation, cells were plated and grown for 2 days in media without phenol red, without gentamycin, and without serum. Cells were treated or not with 5 ng IGF-I for 1 day.

siRNA-directed against c-Jun, c-Fos, and ERα were purchased from Dharmacon (siRNA, c-Jun M-003268–02, c-Fos M-003265–01, and ERα M-003401–02). Scramble siRNA, which does not recognize any human mRNA, was purchased from Eurogentec (CAUGUCAUGUGUCACAUUCdTdT) and used as a negative control.

RESULTS

IGF-I-induced Activation of the pS2/TFF1 Gene Requires Both ERα and the AP1 Complex—IGF-I is an activator of estrogen-regulated genes in breast cancer cells (21). It promotes chromatin remodeling at the promoter of the estrogen-regulated gene pS2/TFF1 (28). However, its mechanism of action is not elucidated, and it is not known whether the architecture of the promoter is important for this activation. This gene has a complex promoter that contains one binding site for the ERE and one binding site for the AP1 complex (Fig. 1A). We first determined the importance of these two transcription factors in IGF-I-induced activation of the pS2/TFF1 gene. As previously described (21) in MCF7 cells, IGF-I alone induced transcriptional activation of the estrogen-regulated gene pS2/TFF1 (Fig. 1B). To assess the importance of ERα in this activation, we treated cells both with IGF-I and the pure antiestrogen Fulvestrant (ICI 182 780). When bound to Fulvestrant, ERα is rapidly
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To evaluate the importance of c-Jun and c-Fos for the pS2/TFF1 activation mediated by IGF-I, we knocked down the two proteins using siRNAs directed against c-Jun or c-Fos mRNA. They were administrated to MCF7 cells separately or in combination. Cells were then stimulated or not by IGF-I, and RNA was purified. The effect of siRNA depletion of c-Jun and c-Fos was analyzed by Western blot (Fig. 2A). SiRNA directed against c-Fos was able to specifically lower c-Fos protein levels, whereas siRNA directed against c-Jun lowered both c-Jun and c-Fos protein levels. Lowering the protein level of a transcription factor involved in the formation of heterodimers often induces a decrease in partner protein levels. Moreover in the case of the AP1 complex, c-Jun is able to homodimerize, preventing its degradation when c-Fos protein levels are lowered. c-Jun and c-Fos siRNA have a clear effect on pS2/TFF1 activation mediated by IGF-I (Fig. 2B), dramatically impairing its activation compared with cells electroporated with the scramble siRNA. Treatment of cells with both siRNAs also abolished IGF-I-mediated activation of the pS2/TFF1 gene. These results demonstrate that both ERα c-Jun and c-Fos are essential for IGF-I action and that in the cell the absence of one of these proteins cannot be compensated by the presence of other members of their respective families.

Activation of Estrogen-regulated Genes by IGF-I Requires a Composite Promoter—We have shown that both ERα and the AP1 complex are required for IGF-I-mediated activation of the pS2/TFF1 gene. To better understand the role of each of the two promoter elements (ERE and TRE) in this transcriptional activation, we used the MELN cell line that has been derived from MCF-7 cells and engineered to express the reporter gene luciferase under the control of a synthetic promoter containing only an ERE upstream of a minimal –globin promoter (Fig. 3A). These cells were untreated or treated with estradiol and IGF-I alone or in combination, and induction of luciferase, GAPDH mRNA, and pS2/TFF1 mRNA were measured. Fig. 3B shows a robust induction of luciferase activity by estradiol, whereas IGF-I had no effect. The increase in GAPDH gene expression (a gene known to be induced by IGF-I (38)) after treatment of the MELN cells by IGF-I (Fig. 3C) demonstrates that the IGF-I pathway is not impaired in this cell line. This was further confirmed by the induction of the endogenous pS2/TFF1 gene by estradiol as well as by IGF-I in the MELN cell line (Fig. 3D). These results indicate that IGF-I treatment is not sufficient to promote the activation of a promoter containing a single ERE.

To investigate the importance of the TRE element in IGF-I-mediated activation of pS2/TFF1, we used a cell line engineered...
IGF-I-induced Activation of the pS2/TFF1 Gene

**A**

Construct integrated in MELN cells

**B**

Construct integrated in MTLN cells

FIGURE 3. IGF-I-activated ERα is unable to stimulate transcription of an estrogen-regulated gene with a minimal promoter containing only a unique ERE. *A*, schematic representation of the estrogen-regulated minimal promoter driving the luciferase gene integrated in MELN cells and showing the localization of the ERE. *B*, MELN cells were grown for 48 h in the presence of 100 nM Fulvestrant. Cells were rinsed and treated for 24 h with either vehicle (Ctl), E2, IGF-I, or a combination of E2 and IGF-I. Luciferase activity was measured, and the results were normalized to the number of cells. *C*, MELN cells were treated for 19 h with either vehicle (Ctl), E2, or IGF-I. RNA was purified, and GAPDH mRNA content was quantified by real-time RT-PCR. Results were normalized to actin mRNA expression levels. *D*, MELN cells were treated for 19 h with either vehicle (Ctl), E2, IGF-I, Fulvestrant, or a combination of IGF-I and E2. RNA was purified, and pS2/TFF1 mRNA content was quantified by real-time RT-PCR. Results were normalized to mRNA actin expression levels.

to express luciferase under the control of a promoter containing TREs, which are recognized by the AP1 complex (Fig. 4A). In this experiment we used TPA, which is a well known inducer of the AP1 complex (39, 40) as a positive control. Upon IGF-I treatment of the cells, luciferase activity was induced 3-fold when compared with untreated cells (Fig. 4B). We conclude that IGF-I can activate a minimal promoter containing TRE elements. Concomitant treatment of the cells with either IGF-I or TPA and Fulvestrant or estradiol did not change the level of activation of luciferase compared with cells treated with IGF-I or TPA alone (Fig. 4B, compare lanes + TPA with + TPA + 1CI and lanes + IGF-I with + E2 + IGF-I and with + ICI + IGF-I). This result demonstrates that the effect of Fulvestrant on the activation of the pS2/TFF1 gene by IGF-I (see Fig. 1B) is not due to a defect in the signal transduction pathway mediated by IGF-I in the presence of Fulvestrant. This also demonstrates that AP1-mediated activation of TRE-\(\alpha\)-Tk-Luc promoter is independent of ER\(\alpha\) action. Taken together, these results indicate that ER\(\alpha\) is able to cooperate with the AP1 complex to promote transcription on composite promoters such as pS2/TFF1 and that promoter architecture is crucial for the cooperation between the AP1 complex and ER\(\alpha\).

The AP1 Complex Binding Precedes the Association of ER\(\alpha\) on the pS2/TFF1 Promoter after IGF-I Stimulation—These data raise the question of the exact role of ER\(\alpha\) and the AP1 complex in pS2/TFF1 activation upon IGF-I stimulation. In the context of estrogen-stimulation, ER\(\alpha\) is recruited precociously to the pS2/TFF1 promoter, and it plays a central role in triggering transcription of the pS2/TFF1 gene (37). We wondered if in the case of IGF-I stimulation the situation was similar. Who triggers transcription, the AP1 complex or ER\(\alpha\)? To answer this question we performed ChIP experiments in which we measured the recruitment of c-Jun, c-Fos, and ER\(\alpha\) on the pS2/TFF1 promoter after various treatment times of the cells with IGF-I. Fig. 5 shows that after 1 h of treatment ER\(\alpha\) was not recruited to the pS2/TFF1 promoter. On the contrary c-Jun and c-Fos were present on pS2/TFF1. There was a 5-fold increase in c-Jun binding and a 2-fold increase in c-Fos binding to this promoter.
IGF-I-induced Activation of the pS2/TFF1 Gene

A

B

FIGURE 6. ERα interacts with both c-Jun and c-Fos upon IGF-I treatment. MCF7 cells were treated for the indicated times with IGF-I. A, nuclear extracts containing cross-linked proteins were prepared as for ChIP experiments and immunoprecipitated (IP) with anti-c-Jun or anti-c-Fos antibodies. The presence of ERα in the eluates was analyzed by Western blot using anti-ERα antibody. B, native protein complexes were prepared as indicated under "Experimental Procedures" and subjected to immunoprecipitation with anti-c-Jun antibody. The presence of ERα in the eluates was analyzed by Western blot using anti-ERα antibody.

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IGF-I induces phosphorylation of the AP1 complex and promotes its interaction with ERα—Results shown above indicate that both ERα and the AP1 complex are recruited to the pS2/TFF1 promoter but with different timing. AP1 complex being recruited earlier than ERα. Therefore, we investigated the mechanisms involved in the delayed interaction of ERα with the pS2/TFF1 promoter and if AP1 complex could be responsible for this recruitment. First, we studied the effect of IGF-I stimulation on the physical interaction between ERα and the AP1 complex. It has been previously shown that c-Jun and ERα interact directly in intact cells, forming a protein complex in a ligand-dependent manner (41). However, to date no interaction between ERα and c-Fos has been described. To assess if ERα and the c-Jun-c-Fos complex belong to the same protein complex after exposure to growth factors, we treated MCF7 cells for various times by IGF-I and cross-linked the proteins in whole cells to preserve the integrity of the complexes. This was followed by co-immunoprecipitation using anti-c-Fos and anti-c-Jun antibodies. Western blots using an antibody directed against ERα showed that both c-Jun and c-Fos co-immunoprecipitated with ERα upon stimulation by IGF-I (Fig. 6A). There was no detectable interaction between c-Jun and ERα after 1 h, a weak interaction after 2 h, and a strong interaction after 3 h of IGF-I stimulation. Co-immunoprecipitation of c-Fos and ERα was weaker than that of c-Jun and ERα, also maximal after 3 h of treatment with IGF-I. To get further insights on the interaction between ERα and the AP1 complex, we performed co-immunoprecipitations on native protein complexes under stringent conditions. After 3 h of IGF-I stimulation a clear interaction between ERα and c-Jun could be detected (Fig. 6B), confirming the results obtained on cross-linked complexes and suggesting a strong interaction between these two transcription factors. In contrast, we were unable to detect any interaction between ERα and c-Fos under IGF-I stimulation (data not shown), suggesting that these two proteins, even if they belong to the same complex, as suggested by the cross-linking experiments, probably did not interact directly or have a weak interaction.

IGF-I treatment causes the activation of the mitogen-activated protein kinases and phosphatidylinositol 3-kinase pathways, leading to the phosphorylation of many transcription factors and mediators. We then investigated if there was a correlation between the phosphorylation state of AP1 complex and the time course of interaction between AP1 complex and ERα. Fig. 7 shows the effects of IGF-I treatment on the two main proteins implicated in the proliferative action of the AP1 complex, c-Jun and c-Fos. First, the levels of the two proteins were increased (Fig. 7A). The c-Fos concentration increased rapidly, and within 30 min the protein level reached 50% that of its maximal level. The increase in c-Jun was slower, reaching 50% that of its maximal level after 80 min of IGF-I treatment. These results are in agreement with the fact that IGF-I treatment induces an increase of mRNA levels of both c-Jun and c-Fos (42). Second, c-Jun and c-Fos are phosphorylated upon IGF-I treatment of the cells (Fig. 7B). Phosphorylation of c-Fos was rapid, occurring within 15 min and strong on threonine 325 (Fig. 7B), a key residue in c-Fos activation known to be phosphorylated by extracellular signal-regulated kinases (16). This phosphorylation level was maintained over time. Phosphorylation of the c-Jun protein on serine 63 (Fig. 7B), which is located within its transactivation domain, was slower and reached its maximal level after 3 h of treatment with IGF-I.

The increase in unphosphorylated and phosphorylated c-Jun and c-Fos levels was quantified from the blots, and the ratio of phosphorylated c-Jun and c-Fos over total c-Jun and c-Fos proteins was calculated. Fig. 7C showed a constant increase, up to 12-fold of the phospho-c-Jun/c-Jun ratio. This indicates that IGF-I induces an increase in both c-Jun protein levels and c-Jun phosphorylation but that phosphorylation increased more than the amount of the protein. For the phospho-c-Jun/c-Fos ratio, a dramatic increase (more than 100-fold) occurred within 15 min after treatment with IGF-I and decreased rapidly. After 2 h of treatment the phospho-c-Fos/c-Fos ratio remained constant and was approximately three times the basal level. This reveals that IGF-I induces rapid phosphorylation of c-Fos more rapidly compared with the untreated control (Fig. 5). After 3 h of IGF-I stimulation, c-Jun and c-Fos were still present on the pS2/TFF1 promoter roughly to the same extent as after 1 h of treatment. Their binding to DNA was ~4-fold higher than that of the untreated controls. After 3 h of IGF-I stimulation, a strong recruitment of ERα to the pS2/TFF1 promoter was observed (16-fold more than in the untreated control, Fig. 5). Using the irrelevant anti-IgG antibodies, no enrichment of pS2/TFF1 DNA could be detected in between the different experimental conditions. These results taken together with the fact that IGF-I treatment is not sufficient to promote the activation of a promoter containing a single ERE suggests that the AP1 complex could be the factor recruiting ERα to the pS2/TFF1 promoter.
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FIGURE 7. IGF-I treatment induces the phosphorylation of both c-Jun and c-Fos. MCF7 cells were treated with IGF-I for the indicated times. 100 µg of total cell extract were resolved by electrophoresis, and blots were revealed with anti-c-Jun or anti-c-Fos antibodies (panel A) or anti-phospho-c-Jun, anti-phospho-c-Fos, and anti-GAPDH antibodies (panel B). A, quantification of total c-Jun and c-Fos levels from the Western blots. B, Western blot showing the phosphorylated forms of c-Jun and c-Fos. C, ratio of phosphorylated protein over total protein extract; the inset represents an enlargement of the phosphorylated c-Fos/c-Jun ratio.

FIGURE 8. Brg1 is recruited to the pS2/TFF1 promoter after IGF-I stimulation. MCF7 cells were treated for 1 h with either vehicle (Ctl), E2, IGF-I, Fulvestrant, or a combination of IGF-I and Fulvestrant. The amount of pS2/TFF1 promoter DNA co-immunoprecipitated (IP) with anti-Brg1 or anti-IgG antibodies was quantified by real time PCR. The quantification of the number of independent experiments is indicated (n). Results are expressed as in Fig. 5.

IGF-I to the pS2/TFF1 promoter upon IGF-I stimulation. However, the precise mechanism is still unknown. We have previously demonstrated in MCF-7 cells that IGF-I treatment induces chromatin remodeling of pS2/TFF1 promoter after 2 h of treatment (28). To assess if the chromatin remodeling of this promoter is due to the recruitment of ERα or of AP1, we analyzed by ChIP the presence of Brg1, a chromatin remodeling factor recruited precociously to the pS2/TFF1 promoter (37). MCF7 cells were treated for 1 h either with estradiol, IGF-I, or Fulvestrant alone or with a combination of IGF-I and Fulvestrant, and we measured the quantities of Brg1 associated to the promoter (Fig. 8). Upon estradiol stimulation, Brg1 was rapidly recruited to the pS2/TFF1 promoter, with a 10-fold enrichment compared with untreated cells. Upon IGF-I stimulation, Brg1 was also recruited to the pS2/TFF1 promoter (4-fold more than in the untreated control), suggesting that Brg1 may be responsible for IGF-I-induced chromatin remodeling of the pS2/TFF1 promoter. Furthermore co-treatment of the cells with IGF-I and Fulvestrant resulted in a recruitment of Brg1 similar to that obtained by treatment with IGF-I alone. This indicates an ERα-independent recruitment of Brg1 upon IGF-I stimulation. Using the irrelevant anti-IgG antibodies, no enrichment of pS2/TFF1 DNA could be detected in between the different experimental conditions (Fig. 8). These data support a direct role for AP1 via the recruitment of Brg1 in chromatin remodeling of the pS2/TFF1 promoter upon IGF-I stimulation.

DISCUSSION

Functional Cooperation between ERα and the AP1 Complex Is Dictated by Promoter Structure—We have investigated the cross-talk between estradiol and IGF-I signaling pathways in breast cancer cell lines. For this work we used three different kinds of promoters, a complex promoter containing both ERα and AP1 response elements and two minimal promoters, one containing one binding site for ERα and the other containing three binding sites for the AP1 complex. We show that cross-talk between ERα and the AP1 complex is greatly influenced by the structure of the promoter. First, our data, obtained in a cell line derived from MCF-7 cells that express luciferase under the control of a synthetic promoter containing an ERE, demonstrate that IGF-I is unable to stimulate transcription of a gene driven by a promoter containing only an estrogen response element. This result contrasts with a previous report that showed that IGF-I was able to induce the expression of a luciferase reporter transgene driven by a promoter containing two EREs in the uterus than its synthesis. c-Fos phosphorylation is sustained during its synthesis (Fig. 7, panel C, inset).

These data indicate that the IGF-I-dependent interaction between the c-Jun complex and ERα correlates with the time course of phosphorylation of c-Jun, suggesting a direct role of c-Jun phosphorylation in the interaction between ERα and the AP1 complex. Moreover this interaction of ERα with c-Jun after 3 h of IGF-I treatment coincides with the phosphorylated state of both c-Jun and c-Fos and a strong recruitment of ERα to the pS2/TFF1 promoter. We propose, therefore, that the AP1 complex is responsible for the recruitment of ERα to this promoter by IGF-I.

Chromatin Remodeling of the pS2/TFF1 Gene Activated by IGF-I Is Mediated by Direct Recruitment of Brg1 by AP1—Our results support a role for the AP1 complex in the recruitment of ERα to the pS2/TFF1 promoter upon IGF-I stimulation. However, the precise mechanism is still unknown. We have previously demonstrated in MCF-7 cells that IGF-I treatment induces chromatin remodeling of pS2/TFF1 promoter after 2 h of treatment (28). To assess if the chromatin remodeling of this promoter is due to the recruitment of ERα or of AP1, we analyzed by ChIP the presence of Brg1, a chromatin remodeling factor recruited precociously to the pS2/TFF1 promoter (37). MCF7 cells were treated for 1 h either with estradiol, IGF-I, or Fulvestrant alone or with a combination of IGF-I and Fulvestrant, and we measured the quantities of Brg1 associated to the promoter (Fig. 8). Upon estradiol stimulation, Brg1 was rapidly recruited to the pS2/TFF1 promoter, with a 10-fold enrichment compared with untreated cells. Upon IGF-I stimulation, Brg1 was also recruited to the pS2/TFF1 promoter (4-fold more than in the untreated control), suggesting that Brg1 may be responsible for IGF-I-induced chromatin remodeling of the pS2/TFF1 promoter. Furthermore co-treatment of the cells with IGF-I and Fulvestrant resulted in a recruitment of Brg1 similar to that obtained by treatment with IGF-I alone. This indicates an ERα-independent recruitment of Brg1 upon IGF-I stimulation. Using the irrelevant anti-IgG antibodies, no enrichment of pS2/TFF1 DNA could be detected in between the different experimental conditions (Fig. 8). These data support a direct role for AP1 via the recruitment of Brg1 in chromatin remodeling of the pS2/TFF1 promoter upon IGF-I stimulation.

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IGF-I-induced Activation of the pS2/TFF1 Gene

Numbers of publications assume that growth factors induce a change in ERα phosphorylation directly via the activation of mitogen-activated protein kinase and/or AKT pathways. However, whether or not the post-translational modifications of ERα induce its binding to the ERE in the absence of hormone remains unclear. Our results rule out the possibility that phosphorylation of ERα induced by IGF-I is sufficient to directly induce ERα binding to the ERE in the absence of hormone. First, IGF-I does not activate transcription of a gene under the control of a promoter containing only an ERE, and secondly, we see a strong induction of the ERα binding to the pS2/TFF1 promoter after 1 h of estradiol treatment (data not shown) but not after 1 h of IGF-I treatment. AP1 is a target of the IGF-I signaling pathway. We observed, as previously described, an increase in the protein levels of the two subunits of AP1, c-Fos and c-Jun, after treatment with IGF-I c-Fos, increasing more rapidly than c-Jun. Activation of both AP1 subunits by phosphorylation occurred at different rates, c-Fos being very rapidly phosphorylated, whereas c-Jun phosphorylation was delayed. It has been described that estradiol induces an interaction between ERα and c-Jun, but not c-Fos (46). We observed that IGF-I treatment induced interaction of ERα with c-Jun, with a time course corresponding to c-Jun phosphorylation. Furthermore, the co-immunoprecipitation studies on native protein complexes show a strong interaction between ERα and c-Jun upon IGF-I stimulation. IGF-I treatment also induced ERα interaction with c-Fos but with a time course that did not follow c-Fos phosphorylation. However, we were able to co-immunoprecipitate ERα and c-Fos in cross-linked protein complexes but not in native ones. This suggests that ERα and c-Fos belong to the same protein complex, but that their interaction is either indirect or weak. Furthermore, the extent of interaction between ERα and c-Jun corresponds to the level of phosphorylation of c-Jun observed over time, suggesting that this interaction may be mediated by c-Jun. Because we did not see an interaction between ERα and c-Jun after 1 h of treatment with IGF-I, whereas there was a clear interaction after a 3-h treatment, we analyzed ERα recruitment to the pS2/TFF1 promoter. Contrasting with what was seen after 1-h of treatment, after 3 h IGF-I-induced recruitment of ERα to the pS2/TFF1 promoter, suggesting that this process is mediated by c-Jun. The delay in the recruitment of ERα to the pS2/TFF1 promoter (3 h) indicates that ERα does not play any role in the chromatin remodeling that precedes transcription initiation of this gene, but rather, in later stages of transcription. This hypothesis is reinforced by a recent report that investigated the distribution of a green fluorescent protein-ERα fusion transiently expressed in MCF-7 cells after treatment with estradiol or growth factors (47). Estradiol induced the formation of speckles, which are associated to a transcriptionally active form of ERα within 10 min. In contrast, after treatment with IGF-I, the maximum ERα redistribution was observed only after 90 min.

Fig. 9 presents our current working hypothesis. IGF-I induces an increase in c-Fos and c-Jun protein levels as well as their phosphorylation and the binding of AP1 to the TRE of transgenic mice (43). The difference could be due to an impairment of the IGF-I pathway in cell line MELN. Induction of GAPDH and pS2/TFF1 by IGF-I indicates that this is not the case. Thus, this discrepancy may come from the type of minimal promoter used. For our study we used a reporter gene under the control of the β-globin minimal promoter with an estrogen response element, whereas Klotz et al. (43) used the minimal thymidine kinase promoter. Another possibility could be that the promoter used to drive the transgene in mice contains a cryptic AP1 or SP1 response element. In addition, differences may be species-specific (human versus mouse) or tissue-specific (breast cancer cells versus normal uterus). The use of a cell line containing the luciferase gene under control of TPA response elements (that binds AP1) shows that IGF-I (and phorbol esters) induces luciferase activity and that this induction is not affected by anti-estrogens such as Fulvestrant. This finding confirms a previous report demonstrating that Fulvestrant does not inhibit IGF-I signaling (44) and that on this kind of promoter ERα does not play any role in IGF-I-induced activation of the AP1 complex. Studies on synthetic promoters report that the use of anti-estrogens stimulates AP1 transcriptional activity and that DNA binding activity of ERα is not required for this activation (45). Our studies on the endogenous promoter pS2/TFF1 demonstrate that the molecular mechanisms of cooperation between ERα and AP1 complex differ on endogenous estrogen-regulated promoters. First, a combined treatment of MCF7 cells by IGF-I, which activates the AP1 complex, and Fulvestrant, which blocks ERα action and causes its rapid degradation (35, 36), inhibits pS2/TFF1 transcription. Lowering protein levels of ERα by the use of siRNA in cells treated with IGF-I leads to the same result, demonstrating that ERα is crucial for IGF-I mediated activation of this gene. Moreover, invalidation of the AP1 complex by siRNA also causes loss of IGF-I mediated activation of the pS2/TFF1 gene. These results clearly indicate that on an endogenous promoter like pS2/TFF1 both ERα and AP1 are required for ligand-independent activation of this gene. Furthermore, for full cooperation between these transcription factors after IGF-I stimulation, both AP1 and ERα response elements are required. This suggests that DNA binding activity of both transcription factors is necessary.

The AP1 Complex and ERα Play Different Roles during Transcription Activation of the pS2/TFF1 Gene—We have previously described that both estradiol and IGF-I are able to induce chromatin remodeling of pS2/TFF1 promoter (28). Here we show that both estradiol and IGF-I are able to induce recruitment of Brg1 to the pS2/TFF1 promoter. In the presence of estradiol, ERα may recruit Brg1. Upon IGF-1 treatment, Brg1 could be recruited either by ERα or by AP1. However, the fact that Fulvestrant does not prevent IGF-I-induced recruitment of Brg1 and the fact that ERα is not recruited to pS2/TFF1 promoter after 1 h of IGF-1 treatment support a direct role of AP1 in this process. This suggests that transcriptional activation of the pS2/TFF1 gene via ERα and/or via the AP1 complex obeys different mechanisms.
IGF-I-induced Activation of the pS2/TFF1 Gene

FIGURE 9. Estrogenic activity of IGF-I: a model of ERα–AP1 cooperation. A, upon IGF-I stimulation, c-Fos and c-Jun are phosphorylated and can dimerize to form the AP1 complex. B, this complex binds to the pS2/TFF1 promoter at the TRE sequence and induces chromatin remodeling by recruiting Brg1. C, the increase in c-Jun protein levels and phosphorylation allows the recruiting and tethering of more ERα via its interaction with AP1 to the pS2/TFF1 promoter at the ERE sequence. D, thereby, DNA-bound ERα can recruit coactivators and maintain a high level of transcription of the pS2/TFF1 gene.

of pS2/TFF1 promoter. When bound to DNA, the AP1 complex can recruit factors involved in chromatin remodeling, such as Brg1. Next, the increase in c-Jun protein levels and phosphorylation allows the recruiting of more ERα and to tether it, via its interaction with AP1, to the pS2/TFF1 promoter at the ERE sequence. Thereby, DNA-bound ERα can recruit coactivators and maintain a high level of transcription of the pS2/TFF1 gene.

In this study we did not investigate ERα phosphorylation status under IGF-I stimulation. Therefore, we cannot exclude the possibility that some IGF-I-induced ERα phosphorylation may contribute to the formation of the complex with c-Jun in the absence of hormone, favoring indirect recruitment of ERα to the promoter.

Our work provides new insights in the field of ligand-independent regulation of ERα. It is well known that ERα and the AP1 complex cooperate during transcription activation. Here we demonstrate that after IGF-I stimulation both ERα and the AP1 complex are required for transcription activation of the pS2/TFF1 gene but play different roles in this molecular mechanism.

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