

Insulin-like Growth Factor 1 Differentially Regulates Estrogen Receptor-dependent Transcription at Estrogen Response Element and AP-1 Sites in Breast Cancer Cells*

Received for publication, June 29, 2006, and in revised form, December 12, 2006. Published, JBC Papers in Press, December 13, 2006, DOI 10.1074/jbc.M606244200

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Cross-talk between insulin-like growth factor 1 (IGF-1) and estrogen receptor α (ER) regulates gene expression in breast cancer cells, but the underlying mechanisms remain unclear. Here, we studied how 17- β -estradiol (E2) and IGF-1 affect ER transcriptional machinery in MCF-7 cells. E2 treatment stimulated ER loading on the estrogen response element (ERE) in the pS2 promoter and on the AP-1 motif in the cyclin D1 promoter. On ERE, similar amounts of liganded ER were found at 1–24-h time points, whereas on AP-1, ER binding fluctuated over time. At 1 h, liganded ER was recruited to ERE together with histone acetyltransferases SRC-1 and p300, ubiquitin ligase E6-AP, histone methyltransferase Carm1 (Carm), and polymerase (pol) II. This coincided with increased histone H3 acetylation and up-regulation of pS2 mRNA levels. At the same time, E2 moderately increased cyclin D1 expression, which was associated with the recruitment of liganded ER, SRC-1, p300, ubiquitin ligase E6-AP (E6L), Mdm2, and pol II, but not other regulatory proteins, to AP-1. In contrast, at 1 h, IGF-1 increased the recruitment of the ER-SRC-1-p300-E6L-Mdm2-Carm-pol II complex on AP-1, but not on ERE, and induced cyclin D1, but not pS2, mRNA expression. Notably, ER knockdown reduced the association of ER, E6L, Mdm2, Carm, and pol II with AP-1 and resulted in down-regulation of cyclin D1 expression. IGF-1 potentiated the effects of E2 on ERE but not to AP-1 and increased E2-dependent pS2, but not cyclin D1, mRNA expression. In conclusion, E2 and IGF-1 differentially regulate ER transcription at ERE and AP-1 sites.

Breast cancer development and progression depends on complex cross-talk between steroid hormones (e.g. 17- β -estradiol (E2)²) and growth factors, such as insulin-like growth fac-

tors (IGFs) (1–6). In estrogen receptor α (ER)-positive breast tumors and cancer cell lines, ER and IGF-1R are often coexpressed, and E2 acts in synergy with IGF-1 to stimulate maximal cell proliferation (5–7). The mechanism of ER/IGF-1 interaction includes regulation of the IGF-1 system by E2 and modulation of ER-dependent transcription by IGF-1. For instance, E2 stimulates the expression of the IGF-1 receptor, IGF-1 ligands, and a major IGF-1R signaling substrate, IRS-1 (6). Reciprocally, IGF-1 can activate ER in a ligand-independent manner and potentiate ER-dependent transcription through phosphorylation of ER coactivators and other ER regulatory proteins (8–12). These effects are balanced by down-regulation of ER expression (13) in response to IGF-1 stimulation (14, 15) and estrogen independence that occurs in cells overexpressing IGF-1R or IRS-1 (6).

On a genetic level, the interaction between ER and IGF-1 has been shown to affect the expression of E2-responsive genes, including genes whose transcription is directly regulated by ER (e.g. pS2) and those modulated by ER indirectly (e.g. a key cell cycle regulator, cyclin D1) (16–19).

In the case of pS2, activated ER induces transcription by directly binding to estrogen response elements (EREs) in the promoter. Recent studies suggested that liganded ER is recruited to pS2 ERE sites in an ordered and cyclical fashion together with several other cofactors, including coactivators of the p160 family (SRC1 and p/CIP); other histone acetyltransferases (e.g. p300, CBP, and p/CAF); histone methyltransferases (e.g. Carm); polymerase (pol) II; general transcription factors (e.g. pol II transcription factors and TATA-binding protein-associated factors); histones H3 and H4; nucleosome remodeling proteins SWI/SNF, p68 RNA helicase, and TRAP-mediator complexes that stimulate pol II; and many other chromatin-modifying enzymes (20, 21). The dynamics of ER transcriptional complex assembly has been well characterized for the period 1–3 h following E2 stimulation (21–24). Although it has been reported that IGF-1 can influence pS2 expression (25), it is not known whether IGF-1 can affect ER complex assembly on the pS2 ERE site.

Cyclin D1 is a key cell cycle regulator, often coexpressed with ER in breast cancer (26–29). E2 has been shown to stimulate cyclin D1 mRNA transcription through indirect interaction

tone acetyltransferase p300; pol II, polymerase II; SFM, phenol red-free serum-free medium; WB, Western blot; Ab, antibody; mAb, monoclonal antibody; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; siRNA, small interfering RNA.

* This work was supported by the Sbarro Health Research Organization. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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² The abbreviations used are: E2, 17- β -estradiol; Ach3, acetylated histone H3; Carm, histone methyltransferase Carm1; ChIP, chromatin immunoprecipitation assay; DAPA, DNA affinity precipitation assay; E6L, ubiquitin ligase E6-AP; ER, estrogen receptor α ; ERE, estrogen response element; IGF, insulin-like growth factor; IGF-1R, IGF-1 receptor; IRS-1, IGF-1R signaling substrate; IP, immunoprecipitation; Mdm2, ubiquitin ligase Mdm2; p300, his-

with several regulatory regions in the promoter, such as binding motifs for AP-1 (30) and cAMP-response element and SP-1 (31) motifs. The exact mechanism of ER binding and the composition of ER transcriptional complex at these indirect sites remain unclear. At AP-1 sites, ER might interact with DNA through the AP-1 complex (*i.e.* through Jun protein) or interact with AP-1 through SRC/histone acetyltransferase (32–35).

IGF-1 is known to modulate AP-1 activity in breast cancer cells (36, 37) and to stimulate cyclin D1 transcription and enhance cyclin D1 mRNA stability and protein levels (38–40). There is also evidence that cyclin D1 activity is regulated by cross-talk between E2 and IGF-1 pathways. For instance, IGF-1 is required for cyclin D1 nuclear accumulation in E2-stimulated MCF-7 cells (41). Furthermore, IGF-1-induced cyclin D1 expression in MCF-7 breast cancer cells depends on the presence of ER (38, 42, 43). However, the effects of IGF-1 on the recruitment of ER and other transcriptional modulators to transcription regulation sites in the cyclin D1 promoter are unknown.

In this study, we explored using *in vitro* and *in vivo* techniques how E2, IGF-1, and the combination of both factors regulate ER nuclear translocation, ER recruitment to the pS2 ERE and cyclin D1 AP-1 motifs, and ER complex assembly at these sites. We also assessed how ER knockdown affects IGF-1-dependent transcriptional regulation of cyclin D1 expression.

EXPERIMENTAL PROCEDURES

Cell Culture and Treatments—MCF-7 cells were routinely grown in Dulbecco's modified Eagle's medium/F-12 (both from Invitrogen) containing 5% calf serum. 70% confluent cultures were synchronized in phenol red-free serum-free medium (SFM) for 24 h (44, 45) and then stimulated with 10 nM E2 (Sigma), 50 ng/ml IGF (Bachem), or a combination of both mitogens.

Western Blotting (WB)—Synchronized cells were stimulated with E2 and/or IGF-1 for 1 and 4 h. Cytoplasmic and nuclear protein lysates were obtained as described previously (46). Protein expression was analyzed using 50 μ g of cytoplasmic cell lysates or 100 μ g of nuclear lysates. The following antibodies (Abs) were used: anti-ER F-10 monoclonal antibody (mAb) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), anti-GAPDH mAb (Research Diagnostics Inc.), and anti-nucleolin mAb (Santa Cruz Biotechnology).

DNA Affinity Precipitation Assay (DAPA)—Binding of nuclear ER to ERE and AP-1 DNA motifs was assessed *in vitro* using a modified DAPA protocol of Zhu *et al.* (47). Briefly, nuclear extracts were obtained from cells stimulated with E2 and/or IGF-1 for 1 h. 70 μ g of nuclear proteins were mixed with 2 μ g of specific biotinylated DNA probes (see below) in 400 μ l of Buffer D (20 mM HEPES, pH 7.9, 10% glycerol, 50 mM KCl, 0.2 mM EDTA, 1.5 mM MgCl₂, 10 mM ZnCl₂, 1 mM dithiothreitol, and 0.25% Triton X-100) and then incubated on ice for 45 min. After that, 50 μ l of streptavidin-agarose beads (Invitrogen) were added, and the samples were agitated for 2 h at 4 °C. Next, the agarose beads-protein complexes were collected by brief centrifugation and washed twice in Buffer D. Proteins were uncoupled from DNA probes by the addition of 40 μ l of SDS loading buffer and heating at 96 °C for 10 min. After removal of the beads, the supernatants were analyzed by WB for the presence of ER. The DNA motif probes were prepared by annealing

a biotinylated sense oligonucleotide (for ERE, 5'-Bio-CCC-CTGCAAGGTCACGGTGGCCACCCCGTGA-3'; for AP-1, 5'-Bio-TTAAAAAATGAGTCAGAATGGAGATCACT-3') with nonbiotinylated antisense oligonucleotide (for ERE, 5'-TCACGGGTGGCCACCGTGACCTTGCAGGGG-3'; for AP-1, 5'-AGTGATCTCCATTCTGACTCATTTTTTTTAA-3'). Unlabeled probes were used as negative controls. As an additional control, a 10-fold excess of unlabeled probes was added to the nuclear lysates 30 min prior to the addition of the labeled probes to block specific probe-protein interactions.

Chromatin Immunoprecipitation Assay (ChIP)—80% confluent cultures of MCF-7 cells were shifted to SFM for 24 h and then treated with 2.5 μ M α -amanitin for 2 h. Then the cells were stimulated with 10 nM E2 and/or 50 ng/ml IGF for 1–24 h or left untreated in SFM. Next, the cells were cross-linked with paraformaldehyde, and soluble chromatin was obtained as described by us before (48). The specific DNA-protein complexes were immunoprecipitated (IP) from soluble chromatin with the following Abs: anti-ER C terminus mAb F-10 (Santa Cruz Biotechnology) for ER; anti-pol II CTD4H8 mAb for pol II (UBI); and anti-p300 (UBI), anti-H3 (UBI), anti-E6L (UBI), anti-Mdm2 (Calbiochem), anti-Carm (UBI), and anti-SRC1 1135 mAb for SRC1 (UBI). The presence of specific DNA motifs in the precipitates was detected by PCR. The ERE-containing pS2 promoter fragment was amplified using the following primers: forward, 5'-GATTACAGCGTGAGCCA-CTG-3'; reverse, 5'-TGGTCAAGCTACATGGAAGG-3'. The AP-1 site in the cyclin D1 promoter was amplified using the following primers: forward, 5'-GAGGGGACTAATAT-TTCCAGCAA-3'; reverse, 5'-TAAAGGGATTTCAGCTT-AGCA-3'. The primers for GAPDH promoter were as follows: forward, 5'-GCTACTAGCGGTTTTACGGG-3'; reverse, 5'-AAGATGCGGCTGACTGTCAA-3'. The PCR conditions for pS2 and GAPDH promoters were 1 min at 94 °C, 1 min at 58 °C, and 50 s at 72 °C for 28 cycles, and conditions for cyclin D1 promoter were 45 s at 94 °C, 40 s at 58 °C, and 90 s at 72 °C for 35 cycles. In control samples, nonimmune IgG (rabbit for IRS-1 Abs and mouse for all other Abs; Santa Cruz Biotechnology) was used instead of the primary Abs.

Quantitative Real Time PCR—Synchronized MCF-7 cells were treated for 1 h and 4 h with 10 nM E2 and/or 50 ng/ml IGF-1. Total cellular RNA was isolated using TRIzol reagent (Invitrogen). 2 μ g of total RNA was reverse transcribed using the TaqMan reverse transcription kit (ABI) according to the vendor's instructions. 2 μ l of reverse transcription products were used to detect pS2 and cyclin D1 DNA. For pS2, we used the following primers: forward, 5'-GGTCGCCTTTGGAG-CAGAG-3'; reverse, 5'-AGGACCAGGGCGCAGATC-3'; probe, 5'-6-carboxyfluorescein-AGGCAATGGCCACCAT-GGAGAACA-tetramethyl-6-carboxyrhodamine-3'. For cyclin D1, we used the following primers: forward, 5'-ACCTG-AGGAGCCCCAACAA-3'; reverse, 5'-TCTGCTCCTGGCA-GGCC-3'; probe, 5'-6-carboxyfluorescein-TCCTACTACCG-CCTCACACGCTTCCTC-tetramethyl-6-carboxyrhodamine-3'. To normalize quantitative real time PCR, parallel TaqMan assays were run on each sample for the α -actin housekeeping gene. Changes in the pS2 and cyclin D1 mRNA content relative to α -actin mRNA levels were determined using the compara-

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tive CT method (64) to calculate changes in CT and ultimately -fold and percentage change. An average CT value for each RNA was obtained for replicate reactions.

ER Knockdown—ER expression in MCF-7 cells was reduced using the ER SMART pool siRNA (Dharmacon). ER siRNA (500 nM) was mixed with the transfection agent RNAiFect (Qiagen) (siRNA/RNAiFect ratio 1:3), incubated for 15 min at room temperature, and then transfected into 70% confluent cultures of MCF-7 cells. After 6 h, the cells were placed in SFM for 24 h and then treated with 50 ng/ml IGF for 1 h. The nontargeting siRNA number 2 (Dharmacon) was used as a control in all ER siRNA experiments.

RESULTS

IGF-1 Increases Nuclear Translocation of Liganded ER in MCF-7 Cells—We have previously observed efficient nuclear translocation of ER in response to E2 treatment (1–24 h) in MCF-7 breast cancer cells (48). Since IGF-1 is known to activate ER in a ligand-independent manner (6, 49–51), we assessed whether IGF-1 alone can stimulate ER nuclear transport and whether it can potentiate E2-induced ER translocation.

To test this, we analyzed ER protein abundance in cytoplasmic and nuclear protein lysates obtained from MCF-7 cells stimulated with E2, IGF-1, or E2 plus IGF-1 for 1 or 4 h (Fig. 1). In unstimulated cells (SFM), higher levels of ER were found in the cytoplasm than in the nucleus. As expected, E2 treatment significantly increased nuclear abundance of ER, decreasing ER cytoplasmic levels (Fig. 1). This effect was apparent at 1 and 4 h of stimulation.

IGF-1 alone stimulated modest nuclear translocation of unliganded ER, especially at 4 h of treatment. The addition of IGF-1 potentiated nuclear transport of liganded ER at 4 h but not at 1 h of treatment (Fig. 1).

E2 and IGF-1 Differentially Regulate Dynamics of pS2 and Cyclin D1 mRNA Expression—Since E2 and IGF-1 differentially affected nuclear accumulation of ER (Fig. 1), we hypothesized that the two factors might exert diverse effects on the transcription of ER-regulated genes, such as pS2 and cyclin D1. pS2 mRNA levels were significantly induced by E2 at 1 and 4 h and modestly up-regulated by IGF-1 at 4 h. The addition of IGF-1 significantly improved E2-induced pS2 mRNA expression at 1 h (Fig. 2). On the other hand, the expression of cyclin D1 mRNA was significantly augmented by IGF-1 at 1 and 4 h and moderately induced by E2 treatment at these time points. Interestingly, IGF-1-induced cyclin D1 RNA expression was reduced in the presence of E2.

E2 and IGF-1 Differentially Stimulate ER Binding to ERE and AP-1 Motifs—Next, we tested whether E2, IGF-1, and E2 plus IGF-1 differentially regulate ER loading on the ERE motif in the pS2 promoter and on the AP-1 site in the cyclin D1 promoter (Fig. 3). The experiments were performed using a DAPA assay, where short biotinylated DNA probes containing the pS2 ERE or cyclin D1 AP-1 sequences were hybridized with nuclear proteins obtained from MCF-7 cells stimulated with E2, IGF-1, or E2 plus IGF-1 for 1 or 4 h. The presence of ER in the resulting probe-protein complexes was assessed by WB.

We found that E2 induced efficient ER association with both ERE and AP-1 motifs, whereas IGF-1 moderately increased ER association with AP-1 but had only minor (albeit significant)

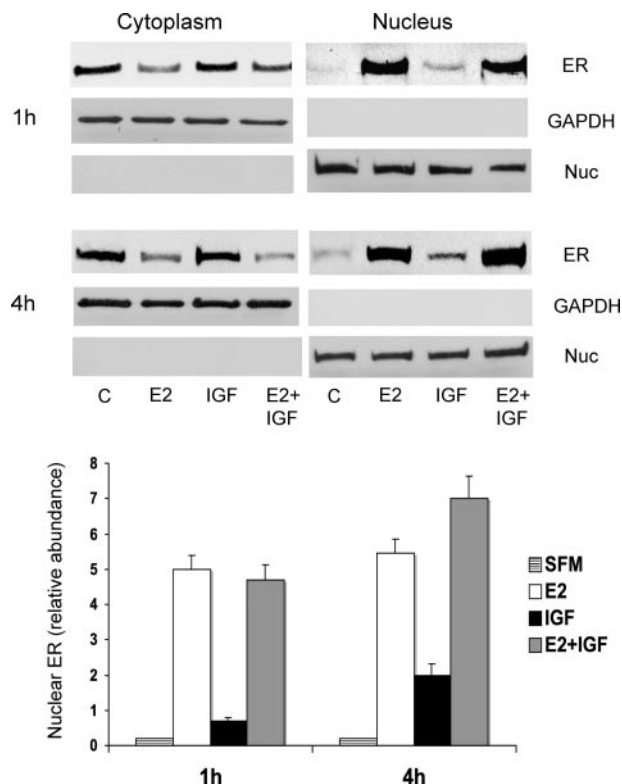


FIGURE 1. Subcellular localization of ER in E2- and/or IGF-1-treated MCF-7 cells. MCF-7 cells synchronized in SFM were treated with 10 nM E2 and/or 50 nM IGF-1 for 1 and 4 h or were left untreated (C). The expression of ER (~67 kDa), nucleolin (*Nuc*; ~39 kDa), and GAPDH (~36 kDa) was assessed by WB in 50 μ g of cytoplasmic protein lysates or 100 μ g of nuclear protein lysates, as described under "Experimental Procedures." The expression of GAPDH and nucleolin were used as controls of cytoplasmic and nuclear protein loading, respectively. The graphs represent relative abundance of nuclear ER under different conditions. Nuclear levels of ER were normalized to the expression of nucleolin (relative value = 1).

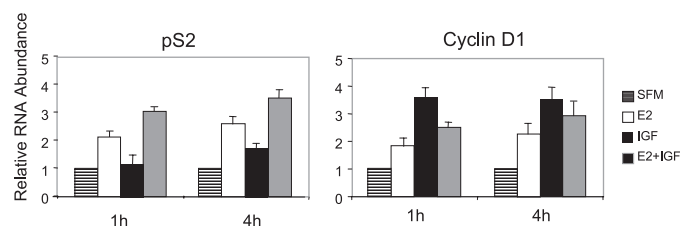


FIGURE 2. pS2 and cyclin D1 mRNA expression under different treatments. MCF-7 cells were treated with E2 and/or IGF-1 for 1 and 4 h, and the abundance of pS2 and cyclin D1 mRNA was assessed by quantitative real time PCR, as described under "Experimental Procedures." The mRNA levels were normalized to the expression of α -actin mRNA.

stimulatory effects on ER binding to ERE. Interestingly, IGF-1 increased E2-dependent association of ER to ERE but not to AP-1 (Fig. 3). The results were similar for 1 and 4 h of treatments (Fig. 3 and data not shown).

E2 and IGF-1 Differentially Induce ER Recruitment to pS2 ERE and Cyclin D1 AP-1 Sites—After ascertaining that liganded and unliganded ER binds pS2 ERE and cyclin D1 AP-1 sequences *in vitro*, we examined ER recruitment to these motifs *in vivo* using ChIP assays. Because the association of ER with specific chromatin regions might occur with different dynamics than binding of ER with isolated DNA oligonucleotides (as in DAPA), we analyzed ER association with ERE and AP-1 sites over the 0–24-h time course (Fig. 4).

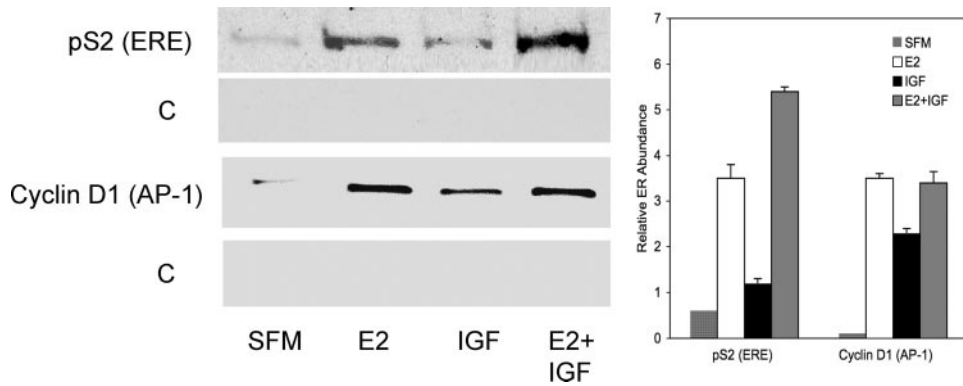


FIGURE 3. ER interacts with the pS2 ERE and cyclin D1 AP-1 motifs *in vitro*. MCF-7 cells were synchronized in SFM and treated with E2 and/or IGF-1 for 1 h, as described under "Experimental Procedures." Nuclear protein lysates were hybridized with biotinylated DNA probes corresponding to the pS2 ERE consensus sequence or the cyclin D1 AP-1 motif, as detailed under "Experimental Procedures." ER bound to the ERE or AP-1 probe was detected by WB. In control experiments (C), the lysates were hybridized with unlabeled probes.

E2 stimulated ER recruitment to pS2 ERE at 1, 4, 8, 16, and 24 h, with no significant differences seen over the time course. The addition of IGF-1 significantly improved the binding of liganded ER to ERE at 1 and 4 h but dramatically decreased ER abundance on ERE at 24 h. The maximum binding of ER to ERE in the presence of IGF-1 alone was noted at 8 h, with modest effects seen at 1, 4, and 16 h. At 24 h, ER was absent from ERE under IGF-1 treatment (Fig. 4).

In contrast, the recruitment of liganded ER to cyclin D1 AP-1 fluctuated

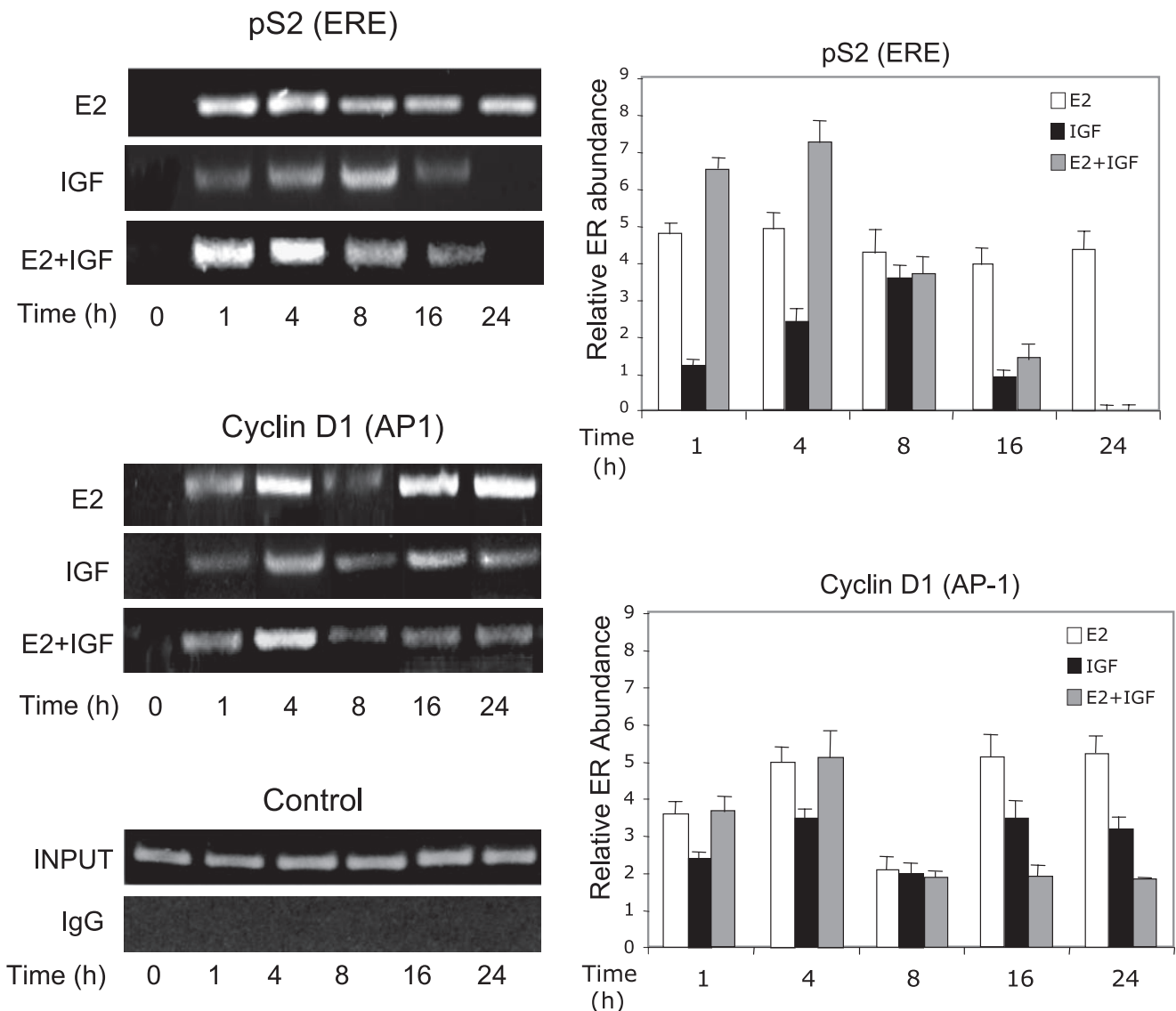


FIGURE 4. Time course of ER association with the pS2 ERE and cyclin D1 AP-1 motifs *in vivo*. Soluble chromatin was isolated from MCF-7 cells stimulated with E2, IGF-1, and E2 plus IGF-1 for 1, 4, 8, 16, and 24 h and from untreated cells (SFM). ER ChIP experiments were performed as described under "Experimental Procedures." DNA recovered from ER immune complexes was tested for the presence of the pS2 ERE promoter sequences (pS2 ERE) and the cyclin D1 AP-1 motif (cyclin D1 AP-1) by PCR with specific primers listed under "Experimental Procedures." In control experiments, the specific ER Ab was replaced by nonimmune IgG. The abundance of pS2 promoter sequences in all samples before immunoprecipitation is shown as control of input DNA.

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tuated over time, with the maximal association seen at 4, 16, and 24 h. The addition of IGF-1 significantly reduced the binding of liganded ER at 16 and 24 h. Under IGF-1 alone, modest binding of ER to AP-1 was found at all time points, with the maximum observed at 4 h (Fig. 4).

E2 and IGF-1 Synergize in the Recruitment of Several ER-associated Proteins to the pS2 ERE Site—Next, we studied how E2 and IGF-1 treatments affect association of various ER regulators/chromatin-modulating proteins to ERE and AP-1 sites (Fig. 5). We focused on p300, SRC-1, E6L, Mdm2, Carm, and pol II as well as acetylated histone H3 (AcH3), all of which are known to play a significant role in ER transcriptional activity (22, 52, 53). The experiments were performed at 1 and 4 h to address differential ER binding to ERE and AP-1 observed at these time points (Figs. 3 and 4).

In agreement with published observations regarding E2-regulated transcription, we observed variable association of several ER-associated proteins on ERE and AP-1 sites (21, 53, 54). On ERE, 1 h of E2 treatment stimulated the recruitment of SRC-1, p300, E6L, Carm, and pol II and increased H3 acetylation. At 4 h, the pattern of several associated proteins was similar, but the binding of p300 increased, whereas the recruitment of SRC-1 and the abundance of AcH3 decreased. The addition of IGF-1 augmented the abundance of liganded ER, p300, Carm, pol II, and AcH3. These IGF-1 effects were not seen at 4 h of treatment (Fig. 5A).

In response to 1 h IGF-1 alone, increased binding was seen for SRC-1, E6L, and AcH3; however, the abundance of these proteins was generally lower relative to that seen with E2. At 4 h of IGF-1 treatment, Carm II and pol II amounts increased, whereas E6L decreased (Fig. 5A). Interestingly, longer exposure to IGF-1 increased the presence of some regulatory proteins like SRC-1, Carm, pol II, and AcH3 relative to that at 1 h (Fig. 5A).

E2 and IGF-1 Differentially Recruit ER and Associated Proteins to the AP-1 Cyclin D1 Site—The effects of E2 and IGF-1 on the recruitment of ER and different ER transcriptional regulators

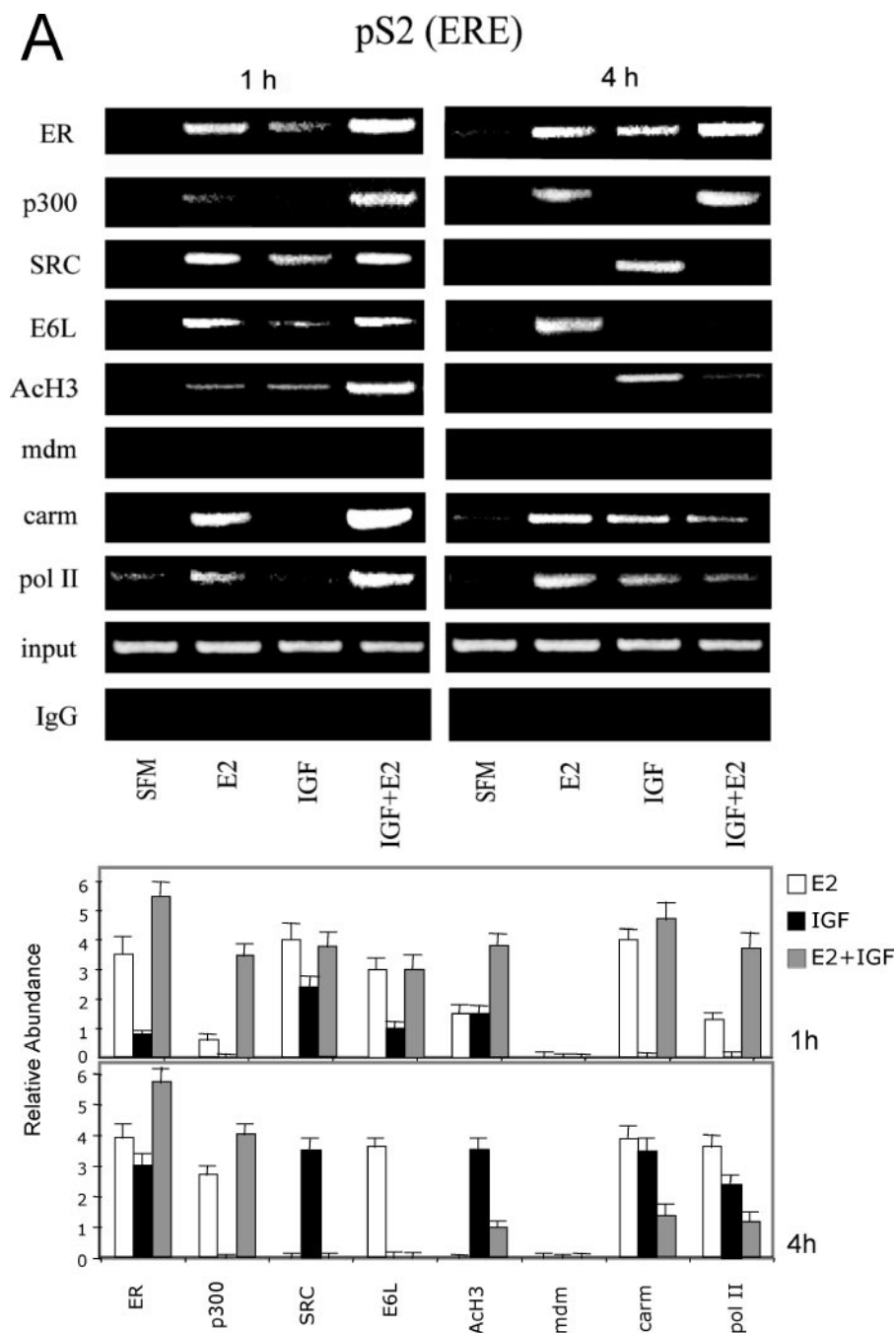


FIGURE 5. Identification of ER transcriptional cofactors and modulators recruited to the pS2 ERE and cyclin D1 AP-1 sites *in vivo*. ChIP assays determining the recruitment of ER, p300, SRC-1 (SRC), E6-AP ubiquitin ligase (E6L), acetylated histones H3 (Ac-H3), E3 ubiquitin ligase Mdm2 (*mdm*), histone methyltransferase Carm1 (*carm*), and pol II to the pS2 ERE sequence (A) and to the AP-1 cyclin D1 motif (B) at 1 and 4 h of treatment with E2 and/or IGF-1 were performed as described under "Experimental Procedures." In Fig. 4A, ChIP for the rapidly cycling SRC-1 coactivator was done at 45 min, as described in Ref. 21. In control experiments, the specific ER Ab was replaced by nonimmune IgG (IgG).

to cyclin D1 AP-1 differed from that observed for ERE. For example, at 1 h, E2 stimulated the binding of Mdm2, but not Carm, to AP-1. Similarly, at variance with ERE, IGF-1 alone induced the loading of p300, SRC-1, E6L, Mdm2, Carm, and pol II on this site (Figs. 5B and 7A).

Synergistic action of E2 and IGF-1 was observed only for E6L at 1 h and for Mdm2 and Carm at 4 h, whereas other regulators responded to either E2 or IGF-1 (Figs. 5B and 7A).

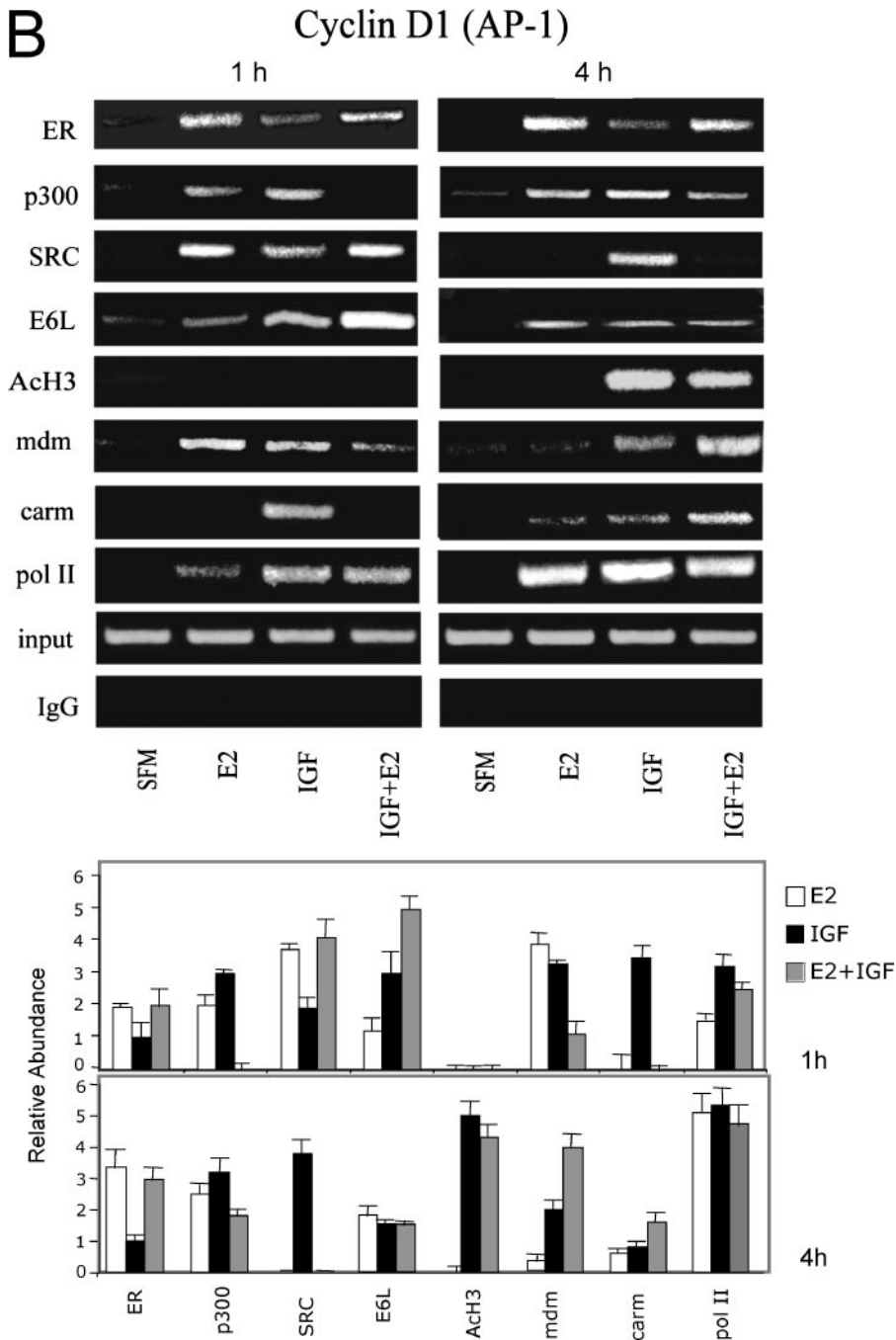


FIGURE 5—continued

Notably, IGF-1 alone was more efficient than E2 in the recruitment of several transcriptional regulatory proteins to AP-1, especially at 4 h of treatment (Fig. 5B).

ER Knockdown Inhibits Transcription of Cyclin D1 at the AP-1 Site—To examine the role of ER in IGF-1-dependent cyclin D1 transcription, we inhibited ER expression using RNA interference technology (Fig. 6). The ~80% reduction of ER protein levels resulted in the inhibition of basal pS2 transcription (data not shown) and significantly decreased IGF-1-dependent cyclin D1 mRNA expression (Fig. 6). The latter coincided with significantly reduced loading of ER, E6L, Mdm2, Carm, and pol II, but not p300 and SRC-1, on the cyclin D1

AP-1 site. In contrast, in cells treated with nontargeting siRNA, ER levels and IGF-1-dependent cyclin D1 transcriptional regulation resembled that in untransfected cells stimulated with IGF-1 (Figs. 5B and 6).

DISCUSSION

Cross-talk between ER and IGF-1 signaling systems has been shown to regulate several functions in breast cancer cells, including proliferation, survival, transformation, migration, cell-cell and cell-surface adhesion, and invasion (5, 6, 55). However, molecular mechanisms involved in ER/IGF-1 interaction are largely unknown. Here we studied how IGF-1 affects the association of liganded and unliganded ER with specific ER-responsive gene promoter motifs and corecruitment of ER regulatory proteins/DNA modulators to these sites. We also assessed the role of ER in IGF-1-induced transcription. We focused on gene regulatory domains that are ER- and/or IGF-1-responsive, specifically the ERE element in the pS2 gene promoter (region -405 to -394) that is a classic site of direct ER regulation (20, 21, 23, 48, 52) and the AP-1 binding motif (region -1071 to -931) in the cyclin D1 promoter that is known to be modulated by IGF-1 and indirectly by ER (32, 33, 56). In our cell model, only this cyclin D1 ER-responsive region was coregulated by E2 and IGF-1 *in vivo*, whereas others (e.g. SP-1) were activated only by E2 (data not shown).

Our studies yielded several novel findings that can be summarized as follows. 1) In MCF-7 breast cancer cells, IGF-1 improved ER nuclear translocation. 2) IGF-1 potentiated binding of liganded ER to the pS2 ERE site but not to the cyclin D1 AP-1 motif *in vitro*. 3) *In vivo*, binding of liganded ER to ERE was augmented by short stimulation with IGF-1 (1 and 4 h), whereas ER binding to AP-1 was not affected by IGF-1 cotreatment. 4) The addition of IGF-1 to E2 increased the abundance of p300, Carm, AchH3, and pol II at ERE at 1 h of treatment, which was paralleled by augmented pS2 mRNA expression. 5) Cotreatment with E2 and IGF-1 decreased the abundance of p300 on AP-1 but did not significantly affect cyclin D1 mRNA expression. 6) Stimulation with IGF-1 alone (1 h) increased

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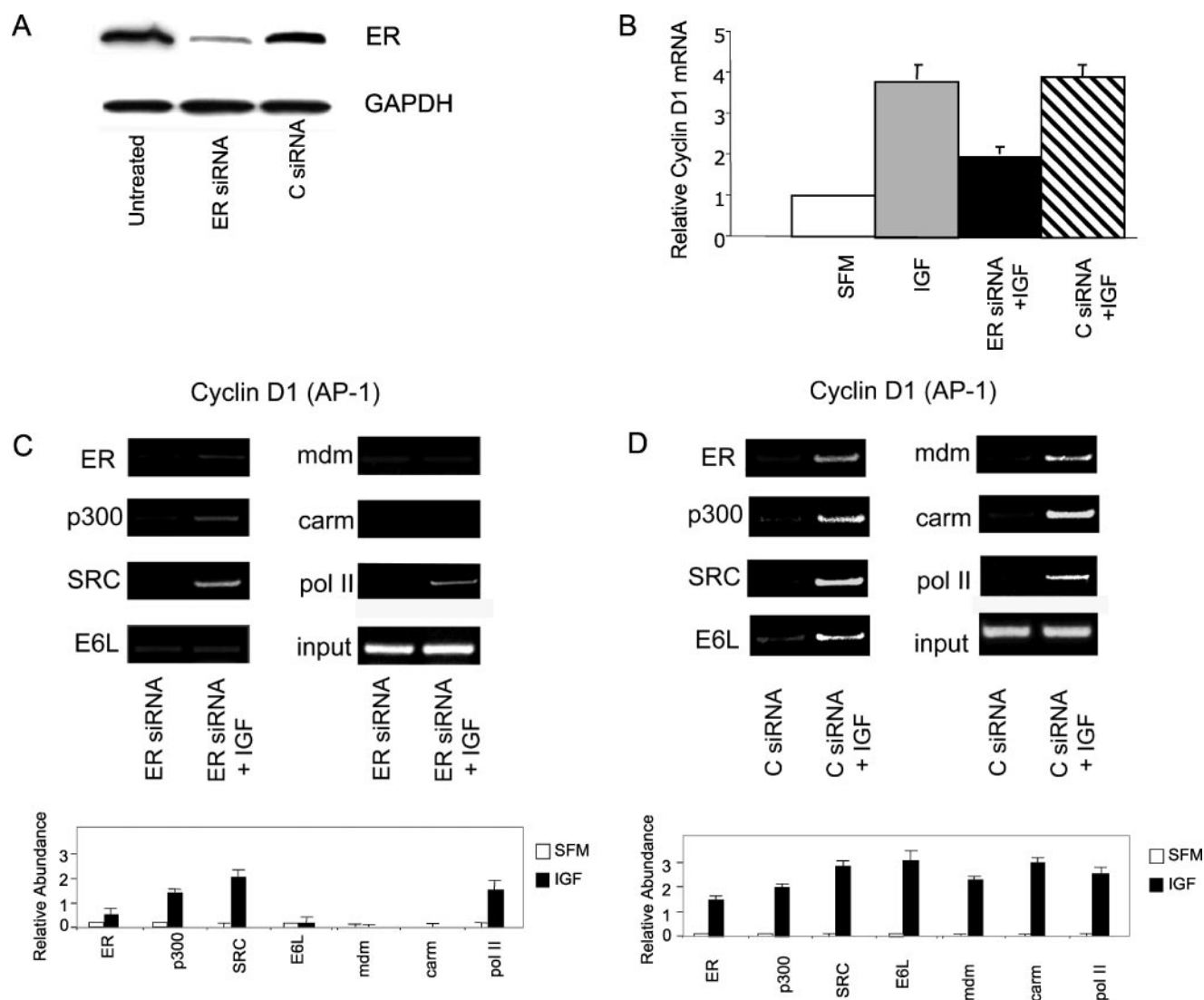


FIGURE 6. Effects of ER knockdown on IGF-1-induced cyclin D1 transcription. ER expression was down-regulated by RNA interference, as described under "Experimental Procedures." Nontargeting siRNA (C siRNA) was used as a control. The cells were transfected with ER siRNA or nontargeting siRNA or left untreated in SFM. Then all populations were stimulated with IGF-1 for 1 h. The levels of ER were assessed by WB (A), and the expression of cyclin D1 mRNA was analyzed by quantitative real time PCR (B). The recruitment of ER and other transcriptional regulators to AP-1 was studied by ChIP (C and D). Control ChIPs with nonimmune IgG were negative (data not shown).

the assembly of the ER·SRC-1·p300·E6L·Mdm2·Carm·pol II complex on AP-1 but not on ERE. This was associated with up-regulation of cyclin D1, but not pS2, mRNA levels. 7) ER knockdown reduced the recruitment of ER and several ER coregulators (E6L, Mdm2, Carm) to AP-1 and decreased IGF-1-dependent cyclin D1 mRNA transcription.

The stimulation of ER trafficking by IGF-1 could be related to enhanced ER phosphorylation (5, 15, 57) and/or direct interaction with IGF-1 signaling proteins like GSK-3, which has been shown to promote ER nuclear translocation in breast cancer cells (58). In our experiments, increased levels of liganded ER in the presence of IGF-1 were observed at 4 h of treatment but not evident at 1 h of stimulation, perhaps due to limited sensitivity of detection by WB. However, IGF-1 augmented binding of liganded ER to ERE at 1 h and 4 h, which suggests that IGF-1 not only can improve ER nuclear translocation but also might facilitate the associa-

tion of ER with DNA, for instance by initiation of chromatin modifications (Fig. 7). This suggestion is supported by our observations that IGF-1 increased the levels of AchH3 at ERE (Figs. 5A and 7). The addition of IGF-1 also augmented E2-dependent recruitment of p300, Carm, and pol II. The enhanced accumulation of these factors on ERE was probably related to a more efficient loading of liganded ER, since IGF-1 alone did not affect these proteins (Figs. 5A and 7). Thus, IGF-1 might potentiate E2-dependent transcription by improving the recruitment of liganded ER and associated proteins to ERE sites, at least at early time points.

A remarkably different picture can be seen at the cyclin D1 AP-1 site. This site has been shown to be indirectly activated by ER in different cell models (30, 32, 56). Indeed, in our experiments, liganded ER together with SRC-1, p300, E6L, Mdm2, and pol II can be found on AP-1 (Figs. 5B and 7). However, the presence of these proteins was correlated with

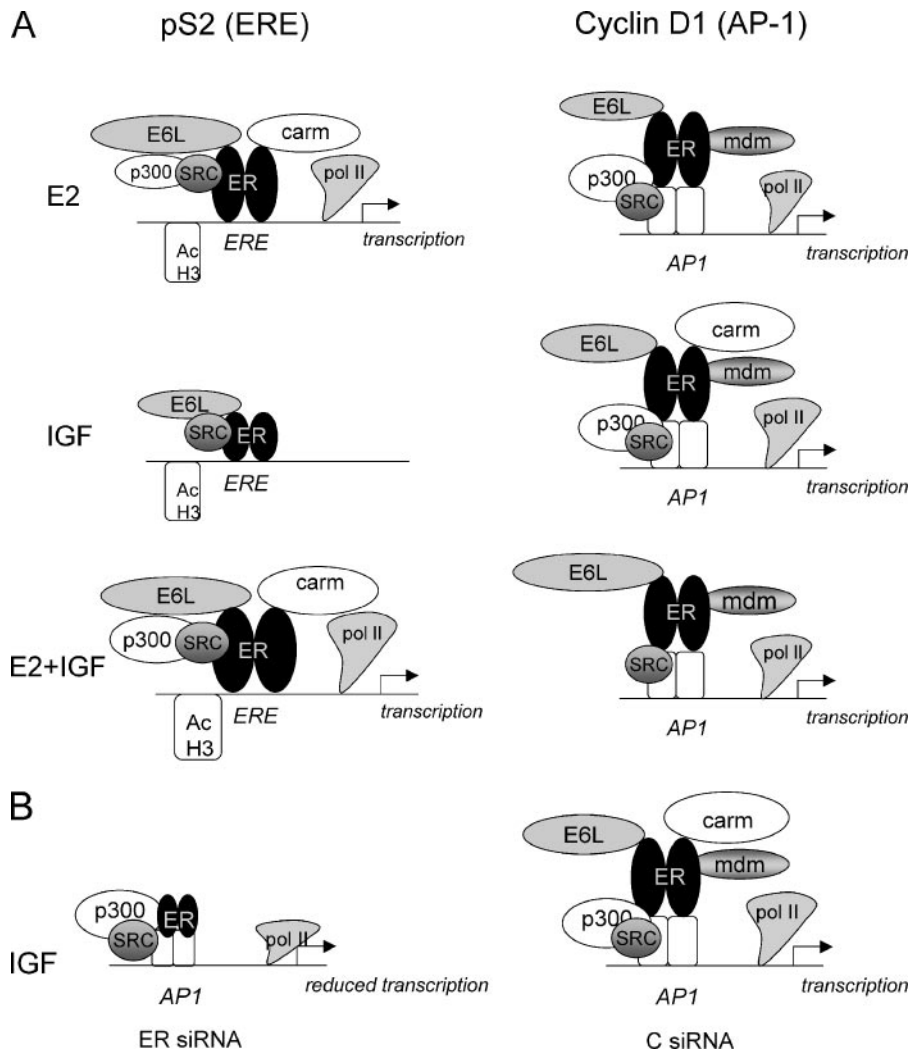


FIGURE 7. Model of transcriptional regulation at the pS2 ERE and cyclin D1 AP-1 sites under E2 and/or IGF-1 treatments. The model presented in A is based on the results obtained in this study and on previous reports (32, 52, 59–63). The IGF-1-dependent regulation of the cyclin D1 AP-1 site in cells with ER knockdown is shown in B.

only modest induction of cyclin D1 mRNA expression (Fig. 2). Furthermore, the addition of IGF-1 had no significant effect on E2-stimulated cyclin D1 mRNA levels over a 1–24-h time course (Fig. 2 and data not shown). On AP-1, IGF-1 reduced E2-dependent levels of p300 at 1 h, whereas at later time points, IGF-1 increased the abundance of p300 and AcH3 but decreased the binding of SRC-1. The exact mechanism of this phenomenon is unclear, but it could be speculated that under combined E2 and IGF-1 treatment, at least initially, the assembly of the fully active ER transcriptional complex occurs preferentially at sites of direct ER transcription (Fig. 7), which might be subsequently followed by redistribution of ER cofactors to the sites of indirect ER transcription. This would also implicate that some regulatory proteins, like p300 and SRC-1 that are common cofactors for steroid receptors and AP-1 (59–61), must be shared among several transcription sites. Notably, the recruitment of p300 and SRC-1 was not sufficient for optimal transcription (Fig. 7B), but their absence on ERE or AP-1 was usually related to reduced transcription (Fig. 7A).

As expected, treatment with IGF-1 alone stimulated the expression of cyclin D1 mRNA (38–40). Under IGF-1, the transcriptional complex on the cyclin D1 AP-1 site included p300 and SRC-1, both of which are required for cyclin D1 mRNA expression (32, 60). IGF-1 also induced the association of ER and its coregulators Carm, Mdm2, and E6L to the AP-1 site. ER knockdown resulted in reduced loading of ER, E6L, Mdm2, Carm, and pol II to AP-1 in IGF-1-treated cells. However, IGF-1 was still able to stimulate the recruitment of p300 and SRC-1 to AP-1 and support partial cyclin D1 expression (Figs. 6 and 7B).

IGF-1 was less capable in recruiting the ER complex at ERE, where only low amounts of ER, SRC-1, and E6L were found (Figs. 5A and 6). Interestingly, a long term (24-h) IGF-1 treatment reduced ER abundance at ERE possibly due to IGF-1-dependent down-regulation of ER (25).

In conclusion, our results demonstrate for the first time that IGF-1 and E2 differentially regulate the formation of transcriptional complexes at ERE and AP-1 sites. The synergism between E2 and IGF-1 in the regulation of gene transcription was noted for liganded ER at ERE but not at AP-1 sites. The requirement for ER for

IGF-1-dependent transcription was documented for the cyclin D1 AP-1 site.

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