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The Potentiation of Estrogen on Insulin-like Growth Factor I Action in MCF-7 Human Breast Cancer Cells Includes Cell Cycle Components*

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To gain insight into the mechanisms involved in the cross-talk between IGF-1 receptor (IGF-1R) and estrogen receptor signaling pathways, we used MCF-7-derived cells (SX13), which exhibit a 50% reduction in IGF-1R expression. Growth of NEO cells (control MCF-7 cells) was stimulated by both IGF-1 and estradiol (E2), and the addition of both mitogens resulted in a synergistic response. Estrogen enhanced IGF-1R signaling in NEO cells, but this effect was markedly diminished in SX13 cells. Estrogen was also able to potentiate the IGF-1 effect on the expression of cyclin D1 and cyclin E and on the phosphorylation of retinoblastoma protein in control but not in SX13 cells. IGF-1 increased the protein level of p21 and the luciferase activity of the p21 promoter, whereas it only reduced the protein level of p27 without affecting p27 promoter activity. Estrogen did not affect the p21 inhibitor, but it decreased the protein level of p27 and the p27 promoter luciferase activity. These effects of both mitogens were also observed at the level of association of both cyclin-dependent kinase inhibitors with CDK2 suggesting that IGF-1 and E2 affect the activity of both p21 and p27. Taken together, these data suggest that in MCF-7 cells, estrogen potentiates the IGF-1 effect on IGF-1R signaling as well as on the cell cycle components. Moreover, IGF-1 and E2 regulate the expression of p21 and p27 and their association with CDK2 differently.

Insulin-like growth factor-1 (IGF-1)[1] and estrogens are important mediators of cellular proliferation and are intimately linked to the progression of a number of human cancers, notably breast cancer (1). It has been shown that an inhibition of IGF-1 receptor (IGF-1R) signaling with anti-IGF-1R antibodies or antisense RNA to the IGF-1R restricts breast cancer cell growth both *in vitro* and *in vivo* (1). The IGF-1R is expressed in a high percentage of primary human breast tumors, and this expression is positively correlated with the level of estrogen receptor (ER) (2). In several tissues and cell lines, including normal breast (3), endometrial cancer cells (4), and estrogen-responsive breast cancer cells (MCF-7, ZR-75, and T47D) (5, 6), estrogen sensitizes the cells to the mitogenic effect of IGF-1. Consequently, the combined effects of estradiol (E2) and IGF-1 might stimulate the proliferation in mammary epithelium, thereby increasing the risk of breast cancer. The mechanisms involved in this sensitization at the level of IGF-1R signaling and cell cycle components have not yet been established.

The components of IGF-1R signaling pathways that transduce the mitogenic stimulus to the cell cycle machinery have been partially identified. IGF-1 initiates its growth-promoting effects through its cognate transmembrane tyrosine kinase receptor. Upon activation by ligand binding, the IGF-1R tyrosine kinase phosphorylates several intracellular substrates such as the insulin receptor substrate (IRS) proteins (IRS-1 through -4) and Shc (7). In breast cancer cell lines that express the ER (MCF-7, ZR-75 or T47-D), the mitogenic effects of IGF-1 are primarily mediated by IRS-1 (8, 9). Activated IRS-1 serves as a multisite docking protein for numerous Src homology 2 domain-containing proteins. These proteins include the p85 regulatory subunit of phosphatidylinositol 3′-kinase (PI3K) and the adapter protein, Grb2. Some of the downstream effectors of PI3K are the serine/threonine protein kinase Akt/PKB and p70/S6 kinase (10). The binding of IRS and Shc proteins to Grb2 and the associated guanine nucleotide exchange protein, mSos, results in activation of the Ras-Raf-MAP kinase pathway. The specific pathway involved in cell proliferation (*i.e.* PI3K *versus* MAP kinase) depends on the particular cell type (11). In myoblasts, adipocytes, and 3T3 fibroblasts, IGF-1-induced cellular proliferation is clearly mediated by the Ras-Raf-MAP kinase pathway (12–14). In contrast, in MCF-7 cells, the proliferative response to IGF-1 is mediated by PI3K (15). More specifically, it has been shown that in MCF-7 cells the PI3K pathway is involved in cyclin D1 synthesis and the hyperphosphorylation of the retinoblastoma protein (Rb) (15). Further studies have shown that Akt affects cyclin D translation in response to serum in MCF-7 cells (16).

The mechanisms by which E2 induces cellular proliferation have not been well established. E2 acts through nuclear hormone receptors (ERα and -β), which, upon activation, may induce the transcription of various genes, including growth factors, their receptors, and substrates (17). Recent studies have shown that these growth factors may, in turn, decrease ERα gene expression while increasing the activity of the receptor in MCF-7 cells (18). The effect of E2 on cellular proliferation may be mediated by the up-regulation of IGF-1R expression (5),

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[1] The abbreviations used are: IGF-1, insulin-like growth factor I; CDK, cyclin-dependent kinase; IRS-1, insulin receptor substrate 1; PI3K, phosphatidylinositol 3-kinase; CDKI, cyclin-dependent kinase inhibitors; PBS, phosphate-buffered saline; SFM, serum-free medium; Rb, retinoblastoma protein; ER, estrogen receptor; IGF-1R, insulin-like growth factor I receptor; IMEM, Iscove's minimal essential medium; FACS, fluorescence-activated cell sorter; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetraolium bromide; PMSF, phenylmethylsulfonyl fluoride; PAGE, polyacrylamide gel electrophoresis; MAP, mitogen-activated protein; E2, estradiol.

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IRS-1, and IRS-2 (19) or by down-regulating the expression of the inhibitory IGF-binding proteins (20). E2 also has direct effects on specific components of the cell cycle. For instance, some studies have shown that E2 stimulates cellular proliferation through early activation of CDK2 and CDK4, phosphorylation of Rb, and increased expression of certain cyclins (21–23).

In this study, we investigated the mechanisms involved in the potentiation of IGF-1s by E2 on cell proliferation of MCF-7 human breast cancer cells. We exposed MCF-7 cells to IGF-1 and E2 either separately or in combination, and then we analyzed various components of IGF-1R signaling (IGF-1R, IRS-1, PI3K, Akt, and Erk1/2) and certain cell cycle molecules (cyclin D1, Rb, cyclin E, and two cyclin-dependent kinase inhibitors (CDKIs), p21 and p27). In order to determine the role of the IGF-1R in estrogen signaling, we used MCF-7-derived cells that express a reduced level (50%) of IGF-1R (24). The results show a potentiation of action of IGF-1 by E2 not only on the immediate downstream targets of IGF-1R signaling but also on certain cell cycle components. Moreover, this potentiation is dependent on the level of IGF-1R expression. Our results also indicate that IGF-1 and E2 have differential actions on the CDKIs, p21 and p27.

EXPERIMENTAL PROCEDURES

Chemicals and Antibodies—The radionuclide [γ-^{32}P]ATP (6000 Ci/mmol) was purchased from PerkinElmer Life Sciences. Recombinant human IGF-1 was obtained from Genentech (South San Francisco, CA). 17β-Estradiol, phenylmethylsulfonyl fluoride (PMSF), leupeptin, aprotinin, protein A-agarose, and phosphatidylinositol were obtained from Sigma. ICI 182,780 was kindly supplied by Dr. Alan Wakeling at Zeneca Pharmaceuticals (Macclefields, UK). Silica TLC plates were obtained from Whatman. Rabbit polyclonal antibodies to cyclin D1 (HD11), cyclin E (C19), CDK2 (M2), p27KIP-1 (C19), Erk1 (C16), and the IGF-1 receptor β subunit (C20) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The rabbit polyclonal antibody to IRS-1 and mouse monoclonal antibodies to p21$^{WAF1/CIP1}$ and p85 were from Upstate Biotechnology, Inc. (Lake Placid, NY). The mouse polyclonal anti- phosphotyrosine antibody (PY20) was from Transduction Laboratories (Lexington, KY). Monoclonal anti-actin (clone AC) was obtained from Sigma. Rabbit polyclonal antibodies to phospho-Akt (Ser^{473}), Akt, phospho-Erk1/2 (Thr^{202}/Tyr^{204}), and phospho-Rb (Ser^{780}) were from New England Biolabs (Beverly, MA). All these antibodies were used with a 1/1000 dilution in Western blotting. Full-length promoter constructs for p21 and p27 were kindly donated by Dr. F. Kashanchi (NCI, National Institutes of Health, Bethesda) and Dr. T. Sakai (Prefectural University of Medicine, Japan), respectively.

Cell Culture—MCF-7 cells from ATCC (Manassas, VA) were cultured in IMEM supplemented with 10% fetal bovine serum, glutamine (2 mM), penicillin (100 IU/ml), and streptomycin (100 μg/ml). MCF-7 cells stably transfected with an antisense IGF-1R cDNA (SX13) (29) and corresponding control cell lines transfected with the empty vector (NEO) were maintained in the same medium supplemented with 800 μg/ml G418 (Geneticin, Life Technologies, Inc.).

Cell Proliferation Assays—For growth studies, cells were seeded in 96-well plates (8–10,000 cells per well) in IMEM phenol red-free medium containing 5% charcoal-stripped fetal bovine serum. One day later, the medium was switched to that containing the serum-free medium plus the anti-estrogen ICI 182,780, (10 nM) for 48 h to synchronize cells in the G_0 phase. The medium was then changed to that containing phenol red-free medium without serum and the various stimuli as described in the figure legends. As an indirect measure of growth, the 3-[4,5-dimethylthiazol 2-yl] 2,5-diphenyltetraolium bromide (MTT) assay was used as described previously (26).

Determination of Surface IGF-Rs by Flow Cytometry—Determination of the surface IGF-1Rs on NEO and SX13 cells was obtained by flow cytometry. Cells were trypsinized and washed once in PBS and once in FACS buffer (0.1% sodium azide, 2% bovine serum albumin in PBS). Next, cells (10^6 cells/sample) were incubated 30 min on ice with 5 μg/ml IGF-1R-PE-conjugated mouse IgG1 antibody (PharMingen, San Diego, CA) diluted in FACS buffer. Background staining was evaluated using a mouse IgG1 isotype control (5 μg/ml) (PharMingen, San Diego, CA). Cells were washed three times and resuspended in 0.5 ml with FACS buffer. Finally, cells were examined for fluorescence intensity on a FACSCalibur using CellQuest software (both from Becton Dickinson, Mountain View, CA).

Immunoprecipitation and Immunoblotting—Cell lysates were prepared in lysis buffer A (10 mM Tris (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 0.5% Nonidet P-40) containing various protease inhibitors (2 mM PMSF, 10 mg/ml leupeptin, 10 mg/ml aprotinin) and phosphatase inhibitors (100 mM sodium fluoride, 10 mM sodium pyrophosphate, 2 mM sodium orthovanadate). Lysates were centrifuged at 12,000 $\times$ *g* for 20 min at 4 °C, and then the protein concentration in the supernatants was determined using the BCA protein assay. After normalization for protein concentration (250 μg) various proteins were immunoprecipitated from the supernatants using 5 μg of appropriate antibodies at 4 °C overnight. The immunocomplexes were precipitated with 40 μl of protein A-agarose for 1 h at 4 °C. After two sequential washes using buffer A with a 1/2 dilution, the resulting pellets were boiled for 4 min in reducing Laemmli buffer containing 80 mM dithiothreitol. Proteins were resolved by SDS-PAGE and transferred to nitrocellulose membranes. Blots were blocked and probed with the various antibodies as indicated in the figure legends. After extensive washings, immunoreactivity was detected with the appropriate horseradish peroxidase-conjugated secondary antibodies followed by enhanced chemiluminescence. Densitometry was performed by scanning the radiographs and then analyzing the bands with the software MacBas version 2.52 (Fuji PhotoFilm). In some experiments, Western blotting was performed on whole cell lysates, using 50 μg of protein.

PI3K Activity—PI3′-kinase activity was determined as described previously (27). Cell lysates were prepared on ice in extraction buffer B composed of 20 mM Tris (pH 7.5), 137 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 150 mM Na_3VO_4, 1% Nonidet P-40, 10% glycerol (v/v), 2 mM PMSF, 10 μg/ml aprotinin in phosphate-buffered saline (PBS). Cell lysates were clarified by centrifugation for 35 min at 40,000 $\times$ *g* at 4 °C. IRS-1 was immunoprecipitated from aliquots of the resulting supernatants (each containing 250 μg of total protein) by incubating them overnight at 4 °C with the αIRS-1 antibody (1/1000). Immunoprecipitates were collected with protein A-agarose beads and washed successively as follows: once in PBS containing 1% Nonidet P-40 and 100 μM Na_3VO_4, twice in a buffer containing 100 mM Tris-HCl (pH 7.5), 500 mM $LiCl_2$, 100 μM Na_3VO_4, and finally, once in a buffer containing 10 mM Tris-HCl (pH 7.5), 100 mM NaCl, 1 mM EDTA, and 100 μM Na_3VO_4. The pellet was resuspended in 40 μl of a buffer containing 10 mM Tris-HCl (pH 7.5), 100 mM NaCl, and 1 mM EDTA. To each tube was added 10 μl of $MnCl_2$ (100 mM) and 20 μg of phosphatidylinositol. The reaction was initiated by the addition of 10 μl of ATP (440 μM) containing 30 μCi of [γ-^{32}P]ATP. Reactions were incubated for 10 min at room temperature and then were stopped by the addition of 20 μl of HCl (8 N) and 160 μl of $CHCl_3$:CH_3OH (1/1). After centrifugation (3,000 $\times$ *g* for 4 min at 4 °C), the organic phase was extracted and applied to a silica gel thin layer chromatography (TLC) plate. TLC plates were developed in $CHCl_3/CH_3OH/H_2O/NH_4OH$ (120/94/22.6/4) and dried, and the radioactivity was quantitated with a PhosphorImager apparatus (FujiFilm, Stamford, CT).

Analysis of p21 and p27 Promoter Activity—Three days prior to experiments, MCF-7-derived cells were stripped of endogenous steroids by passage in IMEM without phenol red containing 3% fetal bovine serum (charcoal-stripped). Cells were transiently transfected using the Effectene reagent according to the manufacturer's recommendations. Cells were plated in 6-well plates 24 h prior to transfection; each well received 1 μg of p21 or p27 promoter-luciferase construct and 0.5 μg of the pTK *Renilla* vector to normalize for transfection efficiency. All transfections included a reference sample with p0luc (for p21) or pGL2 basic (for p27). 16 h after transfection, cells were switched to serum-free medium for 24 h, followed by the addition of various stimuli for 24 h. Firefly and *Renilla* luciferase activities were measured using the Dual-luciferase System (Promega, Madison, WI), according to the manufacturer's instructions.

Statistical Analysis—All reported values are the means ± S.D. Statistical comparisons were made by a two-sided Student's *t* test. Statistical significant was assumed if a null hypothesis could be rejected at the $p < 0.05$.

RESULTS

The Synergistic Effect of IGF-1 and E2 on the Cellular Proliferation of MCF-7 Cells Is Abrogated by Reducing the Expression of the IGF-I Receptor—To characterize potential cross-talk and synergism between the IGF-1R and ER signaling on cellular proliferation, we used MCF-7 cells expressing an IGF-1R antisense cDNA (SX13) and control cells (NEO). SX13 cells

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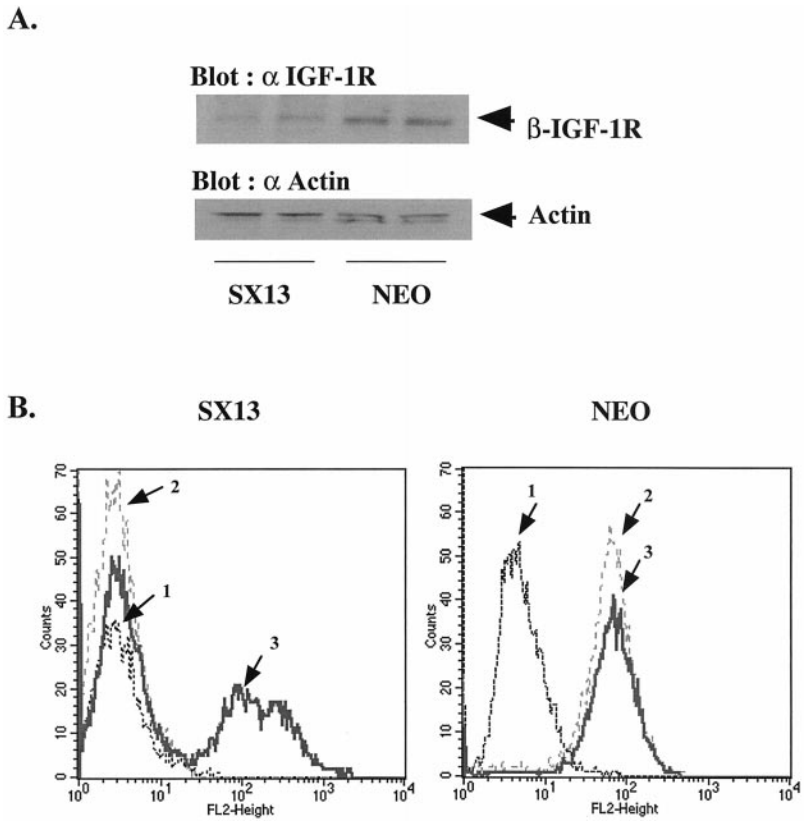

FIG. 1. **Expression of IGF-1R in SX13 cells.** *A,* IGF-1R protein expression in SX13 and NEO cells. Cells were lysed, and proteins were separated on SDS-PAGE and immunoblotted with an IGF-1R antibody. Samples contained equal levels of protein, as confirmed by reprobing each membrane with an anti-α-actin antibody. *B,* expression of surface IGF-1Rs in SX13 (*left panel*) and NEO (*right panel*) cells in response to E2 treatment. Both cell lines were synchronized in G_0 phase by serum deprivation and by using the anti-estrogen ICI 182,780 and then maintained in SFM in the absence or presence of E2 (10 nM) for 48 h. Cells were trypsinized and prepared to analyze their surface IGF-1R amount by flow cytometry as described under "Experimental Procedures." In both panels, *1* represents the isotype control (nonspecific); *2* represents SX13 or NEO unstimulated (starved for 48 h); and *3* represents SX13 or NEO cells treated with E2 for 48 h.

have a significantly reduced level of IGF-1R (50% reduction on Western blot analysis, Fig. 1*A* and Ref. 24). However, the surface receptors were undetectable by flow cytometry suggesting that most of IGF-1Rs in SX13 cells are internalized (Fig. 1*B, left panel, curve 1 versus curve 2*). NEO and SX13 cells were partially synchronized in G_0 phase by serum deprivation and using the anti-estrogen ICI 182,780 (10 nM) for 48 h. Under these conditions, more than 75% of cells were synchronized in G_0 phase (data not shown). Next, cells were incubated with E2 (10 nM), IGF-I (1 nM), or a combination of both stimuli for 3 days. MTT assays were performed on each day as an indirect measure of cell proliferation. In NEO cells, IGF-1 treatment resulted in a 1.7- ($p < 0.05$) and 1.8 ($p < 0.05$)-fold increase in cell number after 48 and 72 h, respectively, as compared with cells incubated with Serum-free Medium (SFM, Fig. 2). Treatment with E2 (10 nM) resulted in a 1.3- ($p < 0.05$) and 1.9 ($p < 0.05$)-fold increase in cell number after 48 and 72 h, respectively. No significant effect was observed after 24 h of stimulation with E2 (data not shown). Coexposure to both mitogens resulted in a 3.8- ($p < 0.05$) and 4.7 ($p < 0.05$)-fold increase in cell number after 48 and 72 h stimulation, respectively. These results indicate that IGF-1 and E2 exert a greater effect when cells are simultaneously exposed to these growth factors than when exposed to E2 or IGF-1 individually. Thus, E2 sensitizes the MCF-7 cell line to the mitogenic effect of IGF-1, at least after 48 h. At 72 h, the effect of both IGF-I and E2 may have been maximal, and thus no further synergism was observed. In SX13 cells, the effect of IGF-1 on cellular proliferation was abrogated; only E2 treatment resulted in a significant increase in cell number after 72 h (2.1 ($p < 0.05$)-fold compared with cells incubated in SFM). These results were confirmed in a second clone expressing a decreased IGF-1R level.

Effects of IGF-1 and E2 on the IGF-1 Receptor Signaling in NEO and SX13 Cells—We next identified the specific signaling components of the IGF-1R cascade that are sensitized by E2 treatment. SX13 and NEO cells were again treated with either

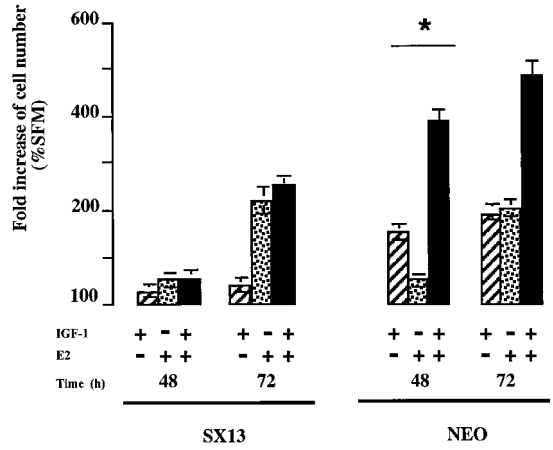

FIG. 2. **Abrogation of the synergistic effect of E2 and IGF-1 on cell growth in SX13 cells.** MCF-7 cells were maintained in SFM in the absence or presence of IGF-1 (1 nM), E2 (10 nM), or a combination of these both mitogens for 3 days, as described under "Experimental Procedures." Cell number was determined indirectly each day using the colorimetric MTT method. Results are expressed as the mean ± S.D. of percentage of cell number increase as compared with cells maintained in SFM. The results are obtained from three independent experiments with 5 measurements per experiment for each condition. *, $p < 0.05$ indicates a significant synergistic effect between IGF-1 and E2.

IGF-1 (5 min) or E2 (48 h) separately or sequentially (E2 for 48 h followed by IGF-1 for 5 min). The tyrosine phosphorylation state of the IGF-1R β subunit and IRS-1, PI3K activity, and the association of p85 with IRS-1, Akt, and Erk1/2 phosphorylation were then determined. Treatment with IGF-1 for 5 min resulted in tyrosine phosphorylation of the IGF-1 receptor β subunit and IRS-1 in NEO cells, whereas the effect of IGF-1 on phosphorylation of these proteins in SX13 cells was markedly reduced (Fig. 3, *panels 1* and *3*). In NEO cells, treatment with IGF-1 induced the phosphorylation of Erk1/2, and this induc-

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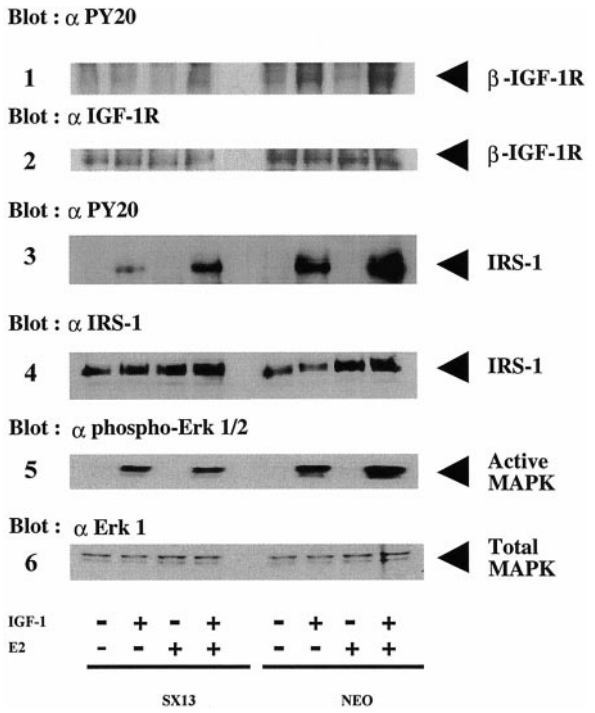

FIG. 3. **Effects of E2 and IGF-1 on the expression and phosphorylation of the IGF-1R β subunit, IRS-1 and Erk1/2, in NEO and SX13 cells.** NEO and SX13 cells were stimulated with IGF-1 (1 nM) for 5 min, E2 (10 nM) for 48 h, or with both agents. Cells were lysed and proteins separated on SDS-PAGE and immunoblotted with antibodies to phosphotyrosine (PY20, *panel 1* and *panel 3*), IGF-1R (*panel 2*), IRS-1 (*panel 4*), phospho-Erk1/2 (*panel 5*), and Erk1 (*panel 6*, this antibody cross-reacts to a lesser extent with Erk 2). The figure is representative of three independent experiments for IGF-1R and IRS-1 and two independent experiments for Erk1/2.

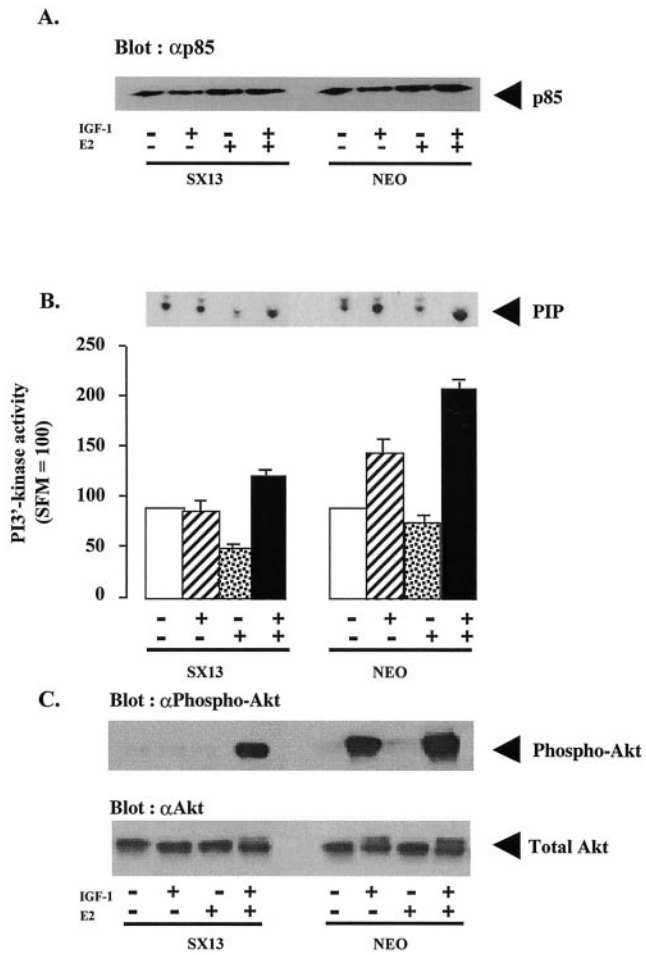

FIG. 4. **Effects of E2 and IGF-1 on the expression and activity of PI3K and on the expression and phosphorylation of Akt in NEO and SX13 cells.** NEO and SX13 cells were stimulated individually with IGF-1 (1 nM) for 5 min and E2 (10 nM) for 48 h or preincubated for 48 h with E2 (10 nM) and then stimulated with IGF-1 for the last 5 min. Cells were then lysed and directly submitted to SDS-PAGE, and the immunoblot was performed with the αp85 antibody (*A*). IRS-1 was also immunoprecipitated with the αIRS-1 antibody, as described under "Experimental Procedures." Immune complexes were used to determine PI3K activity associated with IRS-1 (*B*). The *lower panel* of *B* shows a quantitative analysis of ^{32}P-labeled inositol 1,3,4–5-trisphosphate levels, as determined by PhosphorImager. Results shown are representative of three independent experiments. *C, upper panel,* represents Ser^{473} phosphorylation of Akt in NEO and SX13 cells stimulated as described above. *C, lower panel,* represents the Western blot of the same membrane using an antibody that equally recognizes phospho- and dephospho-Akt. These results are representative of those obtained in three similar experiments.

tion was reduced by 50% in SX13 cells (Fig. 3, *panel 5*). The phosphorylation state of IGF-1R, IRS-1, and Erk1/2 was not regulated by E2 treatment alone in either SX13 or NEO cells. However, in both cell lines, after exposure to E2, IGF-1 stimulation resulted in enhanced tyrosine phosphorylation of the IGF-1R and IRS-1 compared with cells stimulated with IGF-1 alone (Fig. 3, *panels 1* and *3*). In NEO cells, treatment with E2 also increased the IGF-1-stimulated Erk1/2 phosphorylation, whereas in SX13 cells no potentiation of E2 was observed. In NEO cells, the increased phosphorylation of IRS-1 was paralleled with an increase in the total amount of IRS-1 immunoreactivity (Fig. 3, *panel 4*). This effect was markedly attenuated in SX13 cells. In contrast, treatment with E2 did not increase the total level of IGF-1R or the protein expression of Erk1/2 in either cell line (Fig. 3, *panels 2* and *6*). In NEO cells, we also show by flow cytometry analysis that the level of surface IGF-1Rs is unchanged by the treatment with E2 (Fig. 1, *right panel, curve 2 versus curve 3*). However, by using the same technique, in SX13 cells, 38% of cells are IGF-1R positives after E2 treatment. Thus, E2 induced a redistribution of IGF-1Rs to the cellular surface (Fig. 1, *left panel*, *curve 2 versus curve 3*). These results indicate that in SX13 cells, most of the IGF-1Rs are internalized, and treatment with E2 facilitates the translocation of IGF-1Rs to the plasma membrane at the cellular surface. Thus, we show that the potentiation of the effects of IGF-I by E2 can be explained, at least in part, by the increased phosphorylation of the IGF-IR, the increased IRS-1 expression, and the increased phosphorylation of IRS-1 and Erk1/2 in NEO cells. In SX13 cells, this potentiation of E2 on IGF-1 action, while seen with IGF-1R and IRS-1 phosphorylation, is not apparent with Erk1/2 phosphorylation or with cellular proliferation.

The regulatory subunit of PI3K (p85) is a signaling molecule that binds directly to IRS-1 and is important for the proliferative effects of IGF-1 on MCF-7 cells (8, 15). We therefore investigated the effects of E2 and IGF-I on the activation of PI3K and Akt, one of its downstream targets. Treating MCF-7 cells with IGF-1 for 5 min resulted in an increase in the association of p85 with IRS-1 in NEO cells (data not shown). This also increased PI3K enzyme activity associated with IRS-1 (Fig. 4*B*). This effect was not observed in SX13 cells. Phosphorylation of Akt at Ser^{473} was also induced by IGF-1 in NEO cells but not in SX13 cells (Fig. 4*C, upper panel*). The PI3K activity and Akt phosphorylation were unaffected by the presence of E2 in either cell line (Fig. 4, *B* and *C*). This is similar to the results observed on tyrosine phosphorylation of the IGF-1R and IRS-1. In NEO cells, estrogen enhancement of the IGF-1R and IRS-1 tyrosine phosphorylation (shown in Fig. 3, *panels 1* and *3*) was also associated with a significant increase in IGF-1-induced

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PI3K activity and an increase in the phosphorylation state of Akt (Fig. 4, *B* and *C*). Densitometric analysis revealed that IGF-1 induction of PI3K activity and phospho-Akt in the presence of E2 were 1.5-fold higher than that induced by IGF-1 alone. Interestingly, in SX13 cells, PI3K activity or phosphorylation of Akt induced by IGF-1 or E2 separately was undetectable, whereas these were both increased when E2 and IGF-I were given simultaneously (Fig. 4, *B* and *C*). This effect was associated with an increase in cell surface expression of IGF-1Rs following E2 treatment of SX13 (Fig. 1*B, left panel*). In NEO and SX13 cells, E2 treatment increased by 35% ($p < 0.05$) the total level of p85 immunoreactivity (Fig. 4*A*) but did not alter the total level of Akt immunoreactivity (Fig. 4*C, lower panel*). Thus, the synergistic effects of E2 and IGF-I are reflected at the level of downstream targets of the IGF-IR.

Effects of IGF-1 and E2 on Components of the Cell Cycle—We also investigated the mechanisms involved in the synergism of the mitogenic effects of IGF-1 and E2 at the level of various cell cycle components. The elements of the cell cycle we examined included cyclin D1, cyclin E, and phospho-Rb immunoreactivity levels in NEO and SX13 cells. In a time course experiment, when MCF-7 cells were treated separately with IGF-1 and E2, maximal cyclin D1 and cyclin E protein levels were induced after 3 and 24 h of stimulation, respectively (data not shown). Next, we tested the effect of simultaneous treatment with E2 and IGF-1 on these important cell cycle components. In NEO cells, treatment with IGF-1 and E2 separately for 3 h resulted in a 2.8- ($p < 0.05$) and 2.5 ($p < 0.05$)-fold increase in cyclin D1 immunoreactivity, respectively, as compared with cells maintained in SFM (Fig. 5*A*). Coexposure to both mitogens resulted in a 5.5-fold increase ($p < 0.05$) in cyclin D1 protein expression (Fig. 5*A*). In SX13 cells, treatment with IGF-1 and E2 increased cyclin D1 protein expression by 1.3- and 2-fold ($p < 0.05$), respectively. Moreover, E2 did not enhance the effects of IGF-1 in these cells (Fig. 5*A*). In NEO cells, after 24 h of exposure to IGF-1 and E2 separately, cyclin E protein expression was increased by 1.5- ($p < 0.05$) and 1.8 ($p < 0.05$)-fold, respectively, as compared with cells maintained in SFM (Fig. 5*B*). Simultaneously exposing cells to both stimuli resulted in a 2.7-fold increase ($p < 0.05$) in cyclin E protein expression. In SX13 cells, the effect of IGF-1 alone on cyclin E levels was undetectable, whereas E2 alone or combined with IGF-1 increased cyclin E protein expression by 1.5-fold ($p < 0.05$) (Fig. 5*B*). Finally, we determined the effects of IGF-1 and E2, alone or in combination, on the phosphorylation state of Rb using a phospho-specific Rb antibody. In NEO cells, IGF-1 or E2 treatment increased the phospho-Rb immunoreactivity by 3-fold ($p < 0.05$) as compared with cells grown in SFM (Fig. 5*C*). Coincubating NEO cells with IGF-1 and E2 induced a 4.5-fold increase ($p < 0.05$) in phospho-Rb immunoreactivity. Similar to the effects on cyclin D1 and cyclin E protein expression, phosphorylation of Rb was only increased by E2 treatment alone or in combination with IGF-1 in SX13 cells (Fig. 5*C*). Similar results were obtained with an antibody that recognizes phospho- and dephospho-Rb equally (PharMingen, San Diego, CA, data not shown). Thus, the potentiation of IGF-1-induced effects by E2 was also observed in several components of the cell cycle and was dependent on the level of expression of the IGF-I receptor.

Effects of IGF-1 and E2 on the Expression of p21 and p27 CDKIs and Their Association with CDK2—Certain growth factors have been shown to regulate the expression of CDKIs (28–29). Thus, we studied the effect of IGF-1 and E2 on levels of two CDKIs, p21 and p27. In NEO cells, treatment for 24 h with IGF-1 alone or in combination with E2 increased the level of p21 immunoreactivity by 2-fold ($p < 0.05$), as compared with unstimulated cells, whereas no effect of E2 treatment under the same conditions was detectable (Fig. 6*A*). Moreover, this effect of IGF-1 on p21 protein expression was totally abrogated in SX13 cells (Fig. 6*A*). We also determined the effect of IGF-1 and E2 on the association of p21 with CDK2 (Fig. 6*C*). In NEO cells, IGF-1 treatment induced a 2-fold increase ($p < 0.05$) in the association of p21 with CDK2, as compared with cells maintained in SFM. This effect was not observed in SX13 cells. Moreover, in NEO cells, the association of p21 with CDK2 was decreased by 30% after treatment with E2 but increased by 2-fold ($p < 0.05$) in response to the combination of IGF-1 and E2 suggesting no additive effect of both mitogens (Fig. 6*C*). These effects occurred in the absence of changes in the level of CDK2 protein. In a similar manner, we determined the effect of IGF-1 and E2 alone or in combination on p27 protein levels and on its association with CDK2. In NEO cells, both IGF-1 and E2 treatments alone or in combination decreased the level of p27 protein by 50% ($p < 0.05$, Fig. 6*B*). In SX13 cells, p27 protein levels were reduced by 50% ($p < 0.05$) in response to E2 treatment alone or when combined with IGF-1, whereas no effect of IGF-1 treatment alone was detectable on the p27 protein expression. In NEO cells, IGF-1 and E2 treatments each reduced the association of p27 with CDK2, by approximately 2-fold ($p < 0.05$) (Fig. 6*D*). In SX13 cells, this association was significantly reduced by E2 treatment alone or when combined with IGF-1. Again, these results occurred in the absence of changes in the level of CDK2 protein. Thus, the p21 protein expression and the formation of p21-CDK2 complex are increased by IGF-1 and unchanged or slightly reduced by E2, whereas the p27 protein expression and the formation of p27-CDK2 complex are reduced by both E2 and IGF-1. Moreover, no potentiation or additive effect of E2 and IGF-1 is observed on both inhibitors.

By having shown that p21 and p27 protein expressions were regulated by IGF-1 and E2, we next examined whether this effect occurred at the transcriptional level. The effects of IGF-1 and E2 on the p21 and p27 promoters were therefore investigated. MCF-7 cells were transiently transfected with p21 and p27 promoter reporter constructs. After transfection, serum-starved cells (NEO and SX13) were treated with IGF-1 (1 nM), E2 (10 nM), or both for 24 h. The results are presented in Fig. 7, *A* and *B,* for p21 and p27 promoters, respectively. In NEO cells, IGF-1 increased p21 luciferase activity by 2-fold ($p < 0.05$), whereas no effect of E2 was detectable. When given together IGF-1 and E2 induced an increase (1.8-fold, $p < 0.05$) in p21 luciferase activity (Fig. 7*A*). The activation of the p21 promoter-reporter gene by IGF-1 was dramatically reduced in SX13 cells, as compared with NEO (Fig. 7*A*). Treatment with E2 alone or combined with IGF-1 decreased activity of the p27 promoter by 50% ($p < 0.05$) in both NEO and SX13 cells (Fig. 7*B*). There was no detectable effect of IGF-1 on the p27 promoter in either cell line. Thus, the increase in p21 protein expression by IGF-1 and the reduction in p27 protein expression by E2 appear to be mediated, at least in part, at the transcriptional level. By contrast, the reduction in p27 protein expression by IGF-1 might involve post-transcriptional modifications.

DISCUSSION

Although the sensitization of MCF-7 human breast cancer cells to the mitogenic effects of IGF-1 by estradiol has been well established, the specific mechanisms involved remain unclear. Previous studies have reported that E2 up-regulates IGF-1 receptor expression (5) and IRS-1 levels (17, 19). In our study, E2 alone was found to have no effect on IGF-1R protein expression. This result can be explained by the use of the E2 antagonist, ICI 182,780 to synchronize the cells in G_0 phase. Indeed, treatment with ICI 182,780 at 10 nM for 48 h has been shown to decrease IGF-1R expression (30). We do, however, confirm

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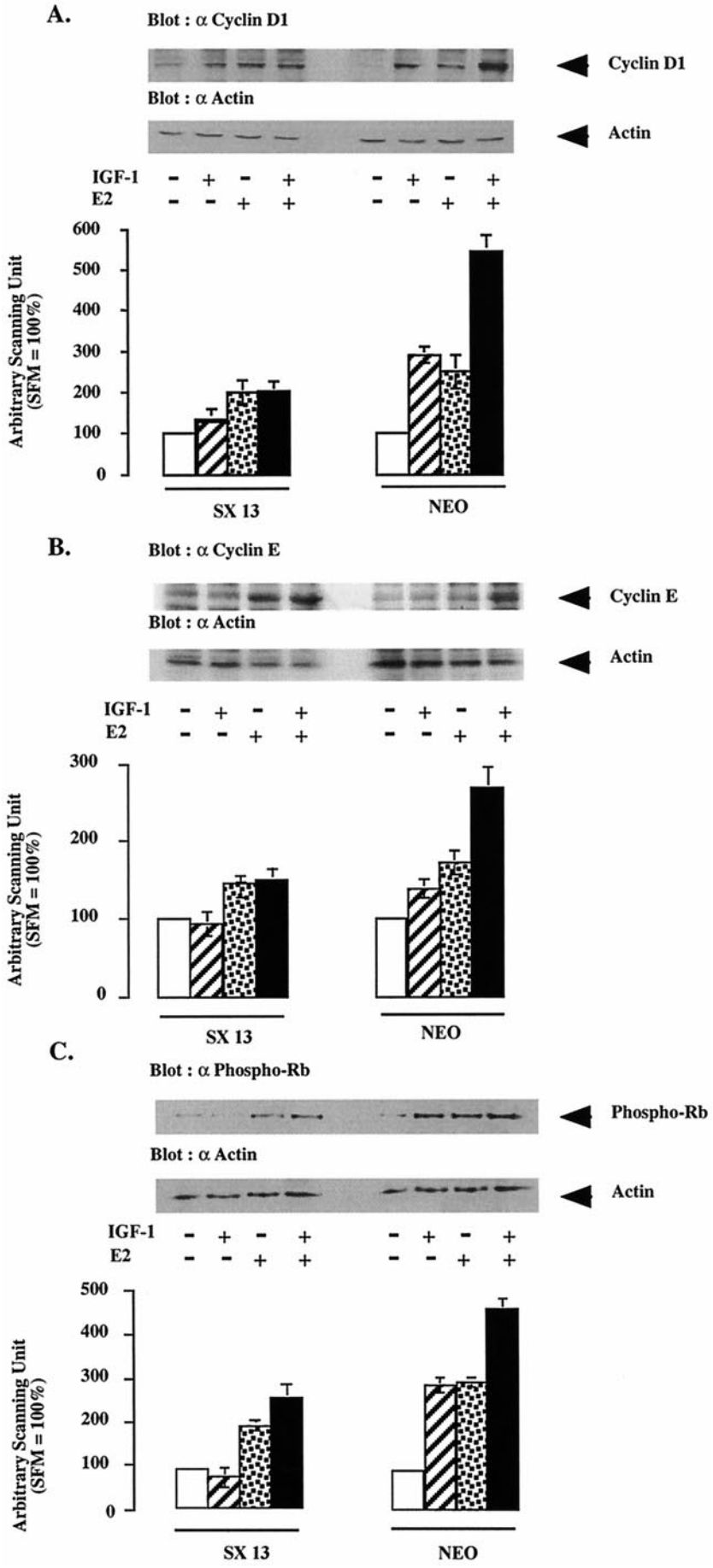

FIG. 5. **Effects of E2 and IGF-1 on cyclin D1, cyclin E, and on phospho-Rb levels in NEO and SX13 cells.** MCF-7-derived cells synchronized in the G_0 phase were stimulated with IGF-1 (1 nM), E2 (10 nM), or with the combination of both agents for 3 or 24 h as described below in each panel. *A,* cells were harvested after 3 h, and cyclin D1 levels were measured by Western blotting. *B* and *C,* cells were harvested after 24 h, and Western blot analysis was performed to determine the cyclin E and phospho-pRb immunoreactivity levels, respectively. Samples contained equal levels of protein, as confirmed by reprobing each membrane with an anti-α-actin antibody. In each panel, immunoreactivity was quantified by scanning densitometry and expressed as percent of that for cells maintained in SFM (*i.e.* unstimulated). These results are representative of three independent experiments.

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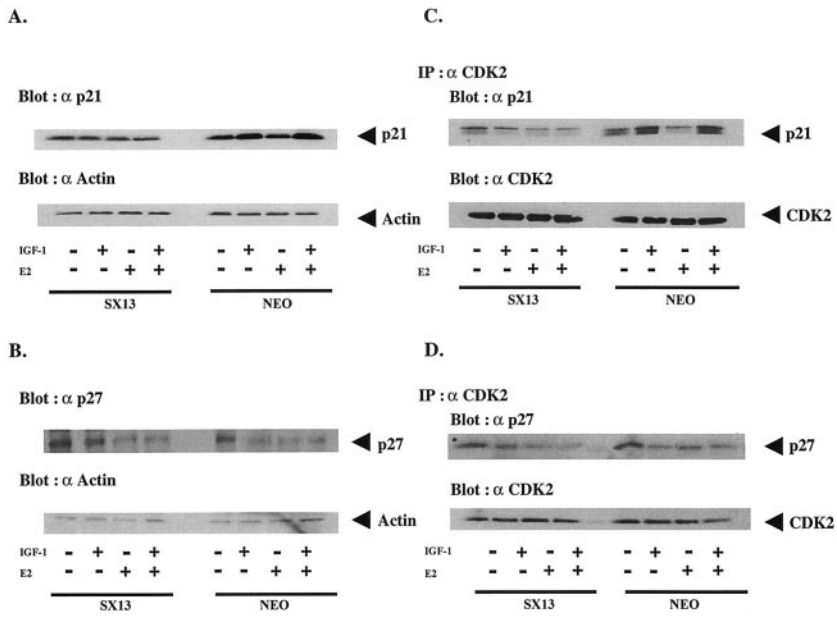

FIG. 6. **Effects of E2 and IGF-1 on the expression of p21 and p27 CDKIs and their association with CKD2 in NEO and SX13 cells.** MCF-7-derived cells, synchronized in the G_0 phase, were stimulated with either IGF-1 (1 nM), E2 (10 nM), or with the combination of both stimuli for 24 h. Western blot analyses for p21 (*A*) and for p27 (*B*) CDKIs protein were performed. Samples contained equal levels of protein, as confirmed by reprobing each membrane with an anti-αActin antibody. CDK2 association was determined with p21 (*C*) or with p27 (*D*) in both cell lines after the same treatment as shown in *A*. CDK2 was immunoprecipitated from whole cell lysates. Samples were then subjected to Western blotting with antibodies against either p21 or p27. Membranes were then reprobed with αCDK2 to evaluate CDK2 levels in each lane. These results are representative of three similar experiments for p21 and two for p27.

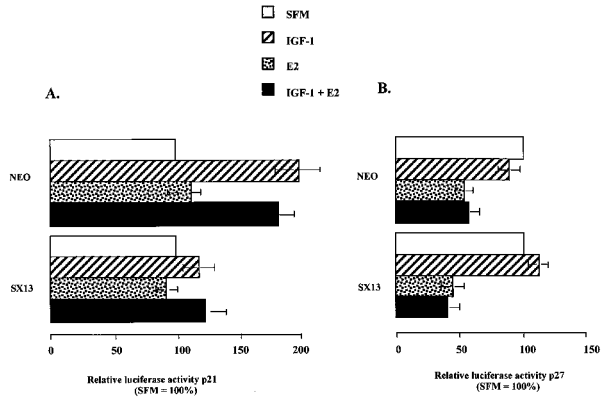

FIG. 7. **Effect of IGF-1 and E2 on p21 and p27 promoter activities in SX13 and NEO cells.** 1 μg of each plasmid (p21 or p27 promoter-reporter genes) was transiently transfected into NEO and SX13 cells with 0.5 μg of pTK-CMV. The resulting luciferase activities were analyzed in response to a 24-h treatment with IGF-1 (1 nM), E2 (10 nM), or with these factors in combination. Relative luciferase activities are shown as percentages of that of p21 (*A*) or p27 (*B*) obtained in unstimulated cells (maintained in SFM). Data are shown as mean ± S.D., from 3 independent experiments, using 5 measurements per experiment in each condition.

that E2 induces an increase in IRS-1 expression. We further show that E2 can induce the expression of other downstream IGF-1 signaling molecules, such as the p85 regulatory subunit of PI3K. The regulation of these molecules results in a potentiation of E2 on the stimulation of IGF-1 of IRS-1 tyrosine phosphorylation and the activation of PI3K, Akt, and Erk1/2. For Akt, these results are in good agreement with those of Ahmad *et al.* (31) showing that both IGF-1 and E2 act synergistically to increase Akt enzyme activity. Our results can also be explained by some nongenomic effects of estrogen. Indeed, in Cos7 and L6 cells, E2 induced the rapid association of ERα with the IGF-1R and in turn tyrosine phosphorylation of the IGF-1R (32). Activation of the IGF-1R signaling cascade, particularly the MAP kinase pathway, phosphorylates ERα and activates its transcriptional activity (32). Moreover, Richards *et al.* (33) show that *in vivo* in the mouse uterus, E2 stimulates the binding of IRS-1 and PI3K to the IGF-1R. In our model, by using MCF-7 cells that have reduced levels of IGF-1R expression (SX13) and few IGF-1Rs at the cell surface (undetectable by flow cytometry), the response to IGF-1 is significantly reduced for IGF-1R and IRS-1 tyrosine phosphorylation and undetectable for PI3K activity and Akt phosphorylation. However, when IGF-1 is combined with E2, PI3K activity and Akt phosphorylation are increased. This result can be explained by a redistribution of IGF-1Rs following E2 treatment of the cells and also by an increase in IRS-1 expression. Indeed, in MCF-7 cells, IRS-1 is the main substrate for IGF-1R signaling (8). Interestingly, this activation of Akt is not sufficient to potentiate the effect of E2 on the cellular proliferation. Although PI3K is thought to be the main pathway involved on the cellular proliferation in MCF-7, Lee *et al.* (19) show that MAP kinase may be also important for the synergistic effect of E2 and IGF-1 on the cellular proliferation. In SX13 cells, treatment with E2 did not enhance IGF-1-stimulated Erk1/2 phosphorylation. This result could explain the absence of potentiation of both mitogens on cell growth in SX13 cells.

In mammalian cells, it has been clearly shown that IGF-1 and E2 promote progression through the cell cycle by facilitating the transition from the G_1 to S phase of cell cycle (15, 23). The rate of transit through G_1 phase is regulated by coordinated action of CDKs in association with specific regulatory cyclin proteins. The primary known regulators of G_1 progression are the D-type cyclins (D1, D2, and D3), cyclin E, and their catalytic partners CDKs-2, -4, and -6 (34–36). The activity of these CDKs is regulated by changes in cyclin levels, interaction with CDKIs, and by regulatory phosphorylation (37). Two distinct classes of cellular CDKIs are known, the Cip/Kip and Ink4 families. The Cip/Kip family includes p21 and p27, which inhibit the formation of cyclin D-CDK4/6 and cyclin E-CDK2 complexes. The Ink4 family including $p16^{Ink4a}$, $p15^{Ink4b}$, $p18^{Ink4c}$, and $p19^{Ink4d}$ form specific binary complexes with CDK4 and CDK6, thereby inhibiting their functions (38). Active CDK-cyclin complexes associate with the E2F transcription factor and mediate hyperphosphorylation of pRb, leading to release of pRb-sequestered E2F. This is essential for the activation of genes required for progression through S phase (39). In our study, we also showed that the potentiation of the effects of IGF-1 and E2 on IGF-1R signaling was reflected at the level of the cell cycle components. MCF-7 cells were treated with both mitogens, either separately or in combination, and then the expression of cyclin D1 and cyclin E and the phosphorylation state of Rb were evaluated. After 3 h of stimulation, IGF-1 and E2 individually increased the expression of cyclin D1. Moreover, when IGF-1 and E2 were combined, we observed

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a further increase in cyclin D1 levels. The effects of IGF-1 and E2 on cyclin E and phospho-Rb immunoreactivity were also potentiated when IGF-1 and E2 were combined. Thus, we show for the first time that the potentiation of IGF-1 and E2 on the IGF-1R signaling system and on specific components of the cell cycle might be the basis for the synergistic effect of these mitogens on cell proliferation of MCF-7 cells.

In these studies, we also show for the first time that IGF-1 and E2 decrease the protein level of p27 as well as its association with CDK2 in MCF-7 cells. Since p27 inhibits the activities of CDKs-4, -6, and -2 (40) and thereby inhibits the formation the G_1 cyclin-CDK complexes, the reduction of p27 levels can explain, at least in part, the enhanced progression from the G_1 to S phase of MCF-7 cells in response to both IGF-1 and E2. Indeed, p27 plays a central role as a negative regulator of cell cycle progression in a variety of tissues and conditions (40). Many studies have shown that p27 protein levels are regulated by a variety of proliferative and anti-proliferative signals. It is thought that this regulation is primarily at the post-transcriptional level. However, some exceptions have been found in primary cultures of thymocytes (41), myeloid leukemic cell lines (42), and H82 cells (43) in which cAMP, vitamin D_3, and interferon (α2b) regulate p27 mRNA levels. In this report, we demonstrate that E2, but not IGF-1, inhibits a human p27 promoter-driven luciferase reporter gene when transiently transfected into MCF-7 cells. These results indicate that E2 and IGF-1 induce transcriptional and post-transcriptional regulation of p27 gene expression, respectively. Pagano *et al.* (44) showed that in mitogen-stimulated cells the p27 protein undergoes rapid degradation by the ubiquitin-proteasome pathway, whereas in resting cells, this proteolysis is dramatically reduced. The mechanisms involved in the ubiquitination of p27 have been well characterized (45). Our data suggest the possibility that IGF-1 may induce the ubiquitination of p27.

In the present study, we also show that treatment of MCF-7 cells with low concentrations of IGF-1 (1 nM, 24 h) stimulates activity of the p21 promoter. We also confirmed by Northern blot that IGF-1 increases the level of p21 mRNA (data not shown). In PC12 and HeLa cells, some studies have suggested that the p21 expression is regulated at the level of transcription, primarily through the MAP kinase pathway in response to growth factors (46). In a number of previous studies that have used various cell culture models, p21 has been described as a negative regulator of cell proliferation (47). For instance, in A431 cells, epidermal growth factor inhibited cell growth by increasing p21 protein levels and, more specifically, the half-life of p21 mRNA (28). Similarly, treatment with fibroblast growth factor induced an increase in p21 mRNA and protein levels and resulted in inhibition of cellular proliferation in MCF-7 cells (29). However, we, in the present study, and others (15) have observed that there is a positive correlation between mitogenic stimulation and p21 protein levels. However, this paradox can be resolved by the finding that, at low levels, p21 also promotes the assembly of active kinase complexes, whereas at higher concentrations it inhibits CDK activity (48). This increases kinase activity and consequently could promote cell cycle progression. In our study, we show that IGF-1 increases not only p21 protein levels but also the association of p21 with CDK2. It has been previously shown that p21 is a potent stoichiometric inhibitor of CDK2 activity (49). Some studies indicate that, at low concentrations, p21 may not inhibit CDK2, whereas high concentrations do inhibit CDK2 kinase activity (50). Our data raise questions about the ability of p21 to stoichiometrically inhibit CDK2.

In summary, we have elucidated some of the molecular mechanisms involved in the cross-talk between the estrogen receptors and the IGF-1 receptor in a cellular model for breast cancer. We provide evidence that in MCF-7 cells, estrogen potentiates early events in IGF-1R signaling as well as IGF-1R effects on certain components of the cell cycle machinery resulting in synergistic growth. Moreover, we show for the first time that IGF-1 and E2 reduce the protein level of p27 and its association with CDK2. We also demonstrate that IGF-1 increases p21 protein levels. Future studies will enable us to dissect further the cell cycle machinery involved in the pathways emanating from steroid and tyrosine kinase receptors that affect hormone-sensitive tissues.

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The Potentiation of Estrogen on Insulin-like Growth Factor I Action in MCF-7 Human Breast Cancer Cells Includes Cell Cycle Components

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