Insulin/Insulin-like Growth Factor-I and Estrogen Cooperate to Stimulate Cyclin E-Cdk2 Activation and Cell Cycle Progression in MCF-7 Breast Cancer Cells through Differential Regulation of Cyclin E and p21WAF1/Cip1.

Received for publication, January 31, 2001, and in revised form, May 3, 2001
Published, JBC Papers in Press, May 3, 2001, DOI 10.1074/jbc.M100925200

Angela Lai§, Boris Sarcevic, Owen W. J. Prall§, and Robert L. Sutherland¶
From the Cancer Research Program, Garvan Institute of Medical Research, St. Vincent's Hospital, Sydney, New South Wales 2010, Australia

Estrogens and insulin/insulin-like growth factor-I (IGF-I) are potent mitogens for breast epithelial cells and, when co-administered, induce synergistic stimulation of cell proliferation. To investigate the molecular basis of this effect, a MCF-7 breast cancer cell model was established where serum deprivation and concurrent treatment with the pure estrogen antagonist, ICI 182780, inhibited growth factor and estrogen action and arrested cells in G0/G1 phase. Subsequent stimulation with insulin or IGF-I alone failed to induce significant S-phase entry. However, these treatments increased cyclin D1, cyclin E, and p21 gene expression and induced the formation of active Cdk4 complexes but resulted in only minor increases in cyclin E-Cdk2 activity, likely due to recruitment of the cyclin-dependent kinase (CDK) inhibitor p21WAF1/Cip1 into these complexes. Treatment with estradiol alone resulted in a greater increase in cyclin D1 gene expression but markedly decreased p21 expression, with a concurrent increase in cyclin E and Cdk2 activity and subsequent synchronous entry of cells into S phase. Co-administration of insulin/IGF-I and estrogen induced synergistic stimulation of S-phase entry coincident with synergistic activation of high molecular mass (350 kDa) cyclin E-Cdk2 complexes lacking p21. To determine if the ability of estrogen to deplete p21 was central to these effects, cells stimulated with insulin and estradiol were infected with an adenovirus expressing p21. Induction of p21 to levels equivalent to those following treatment with insulin alone markedly inhibited the synergism between estradiol and insulin on S-phase entry. Thus the ability of estradiol to antagonize the insulin-induced increase in p21 gene expression, with consequent activation of cyclin E-Cdk2, is a central component of the synergistic stimulation of breast epithelial cell proliferation induced by simultaneous activation of the estrogen and insulin/IGF-I signaling pathways.

* This work was supported by grants from the National Health and Medical Research Council of Australia, the New South Wales Cancer Council, and the Leo and Jenny Leukemia Foundation of Australia. 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.

† Recipient of an Australian Postgraduate Award.
‡ Recipient of a Dora Lush Postgraduate Scholarship from the National Health and Medical Research Council of Australia.
¶ To whom correspondence should be addressed: Cancer Research Program, Garvan Institute of Medical Research, 384 Victoria St., Darlinghurst, Sydney, New South Wales 2010, Australia. Tel.: 61-2-92958322; Fax: 61-2-92958321; E-mail: r.sutherland@garvan.org.au.

Estrogens are critical for the development and normal physiological function of female reproductive tissues including the mammary gland and uterus (1). Estrogens also play a pivotal role in the initiation and progression of breast cancer, where their mitogenic properties are thought to play a causative role in the disease process (2). Although the mechanisms through which estrogens stimulate cell proliferation are becoming increasingly well understood (3–7), there is compelling evidence that several mitogenic growth factors, particularly those of the epidermal growth factor and IGF-1 families, interact with ER-mediated signaling to regulate cell proliferation in target tissues (8, 9). The molecular basis of these interactions is currently of major research interest (10–12), but the mechanisms remain to be fully defined.

In breast epithelial cells, insulin (13) and IGF-I (14) are potent mitogens and synergize with estrogen to stimulate cell proliferation (15, 16). Several potential mechanisms have been proposed to account for these effects. Estrogens alter the expression of IGF ligands, receptors, and binding proteins, suggesting potential autocrine and paracrine mechanisms for estrogen-stimulated mitogenesis (17). By up-regulating IGF-IR, estrogen can sensitize MCF-7 cells to the mitogenic effects of high concentrations of insulin (acting through the IGF-IR) and IGF-I (16, 18). Other studies have shown that estrogen may impinge directly on IGF-I signal transduction downstream of the IGF-IR.

Ligand stimulation of the IGF-IR activates its tyrosine kinase activity, leading to phosphorylation of key intracellular substrates including insulin receptor substrate proteins 1–4 (19). Phosphorylated insulin receptor substrate-1 acts as a docking site for several SH2-containing proteins including the p85 subunit of phosphatidylinositol 3-kinase and Grb2 (20). These molecules in turn link IGF-IR to specific pathways controlling cellular proliferation, i.e. the phosphatidylinositol 3-kinase-Akt/protein kinase B and the Ras-Raf-mitogen-activated protein kinase pathways. In MCF-7 cells, proliferation in response to IGF-I appears to be mediated predominantly via the phosphatidylinositol 3-kinase pathway (21). Estrogen can activate insulin receptor substrate-1 by increasing gene expression and protein phosphorylation (12, 22) and stimulates the formation of IGF-IR-insulin receptor substrate-1-phosphatidylinositol 3-kinase complexes (23). Ligand-bound ERα, but not ERβ, can activate IGF-I signal transduction via a rapid nongenomic effect mediated by direct interaction of ERα with the IGF-IR.

* The abbreviations used are: IGF, insulin-like growth factor; IGF-IR, IGF-I receptor; ER, estrogen receptor; CDK, cyclin-dependent kinase; p21WAF1/Cip1; p27Kip1; pRb, retinoblastoma protein; PAGE, polyacrylamide gel electrophoresis; DTT, dithiothreitol.
This interaction appeared dependent on ERα phosphorylation by ERK1/2 and resulted in IGF-IR phosphorylation and activation of the Ras-Raf mitogen-activated protein kinase cascade (11). IGF-I and estradiol can also act synergistically to increase Akt protein expression and enzymatic activity (24). Finally, direct activation of p21*mitogen-activated protein kinase-signalizing pathways by estrogen has also been proposed (25).

In addition to estrogen regulation of components of insulin/IGF-I-signalizing pathways there is also evidence that polyepitope growth factors acting via type I receptor tyrosine kinases modulate estrogen action. Ligands for both the epidermal growth factor receptor and IGF-I can regulate ER gene expression (26, 27) and ER-mediated transcription (28) in an estrogen-independent manner. Studies in which functional domains of ER were deleted revealed a requirement for the activation function-1 domain in ligand-independent activation of ER by epidermal growth factor (29). Furthermore, ER function can be modulated by mitogen-activated protein kinase phosphorylation of Ser-118 within the activation function-1 domain after epidermal growth factor (30) or insulin/IGF-I (31) stimulation. This specific phosphorylation event provides one potential mechanism for growth factor activation of ER signaling.

The data outlined above provide mechanisms by which estrogen and insulin/IGF-I-signalizing pathways are cross-modulated. However, their mitogenic effects are ultimately mediated at the level of regulation of kinases that govern transition from G0/G1 to S phase of the cell cycle, i.e., the G1 cyclin-dependent kinases (CDKs) cyclin D-Cdk4/6 and cyclin E-Cdk2 (32, 33). Several of these cell cycle regulatory molecules have been identified previously as targets of insulin/IGF-I (34) or estrogen-induced mitogenesis in breast cancer cells (3–5, 7). These mitogenic signals are thought to converge on cyclin D-Cdk4/6 since activity of this kinase is required for either estrogen- or estrogen-induced mitogenesis (35). Insulin or IGF-I stimulate cyclin D1, D3, and E gene expression (34), in common with other mitogens in a spectrum of cell types. In MCF-7 cells, increased cyclin D1 gene expression and stabilized cyclin D1 mRNA in response to IGF-I is mediated via the phosphatidylinositol 3-kinase pathway (36). Estrogen also stimulates cyclin D1 gene expression and subsequent activation of cyclin D-Cdk4 (3, 5) as well as inducing early activation of cyclin E-Cdk2. However, unlike the situation observed after growth factor factor stimulation, this is not preceded by a major increase in cyclin E gene expression (5). Rather, estrogen-induced Cdk2 activation involves a novel mechanism wherein a small fraction of the total cyclin E-Cdk2 pool is present in a high molecular mass complex depleted of the CDK inhibitor, p21*WAF1/Cip1 (5, 7). The known synergistic interactions between insulin/IGF-I and estrogen in stimulating breast cancer cell proliferation may, thus, converge at both cyclin D-Cdk4/6 and cyclin E-Cdk2, although this has not been investigated to date.

In this study we investigated the cell cycle regulatory events accompanying synergistic interactions between estrogen and insulin/IGF-I in mediating cell cycle progression in MCF-7 cells. Cells were initially arrested in a quiescent state by serum withdrawal and treatment with the pure estrogen antagonist, ICI 182780 (37). Subsequent treatment with insulin/IGF-I and estradiol resulted in synergistic stimulation of S-phase entry. Analysis of the expression and function of cyclin D1-Cdk4 and cyclin E-Cdk2 during the G1/S-phase transition revealed cooperative regulation by insulin/IGF-I and estrogen of several molecules regulating these kinases. Of primary significance was the observation of synergy at the level of cyclin E-Cdk2 activation as a likely key mediator of the synergistic cell cycle progression.

EXPERIMENTAL PROCEDURES

Cell Culture—Stock solutions of TGF-β (4, 5, 5, 5-pentafluoropropyl-sulfanyl)nonilnoylnonene-1,3,5,7-tetra-5,7-diol (ICI 182780) and 17β-estradiol (estradiol) were prepared as follows. ICI 182780 (a gift of Dr. Alan Wakeling, Astra-Zeneca Pharmaceuticals, Alderley Park, Cheshire, UK) was dissolved in ethanol at 10 mg/mL and a working dilution of 10 μM in RPMI 1640 medium was prepared before each experiment. Estradiol (10 μM) was dissolved in ethanol at 2 × 10−6 M. All stocks of a stock solution of insulin (Novo Nordisce Pharmaceuticals Pty Ltd. North Rocks, New South Wales, Australia) at 4 mg/mL were added directly to the culture medium. Stock cultures of MCF-7 cells (Michigan Cancer Foundation, Detroit, Michigan) were cryo-preserved and maintained as described previously (38). Cells were cultured in RPMI 1640 medium supplemented with 5% fetal bovine serum, insulin (10 μg/mL), and gentamicin (10 μg/mL). For experiments investigating the effects of ICI 182780, estradiol, and insulin on cell cycle-protein levels, cyclin-Cdk complex formation, and kinase activity employed 150 cm2 tissue culture flasks seeded with 2 × 106 cells. Experiments investigating the effects of estradiol and insulin on cell cycle protein levels, cyclin-Cdk complex formation, and kinase activity employed 150 cm2 tissue culture flasks seeded with 2 × 106 cells. The growth kinetics, including changes in cell cycle-phase distribution, were identical under both experimental conditions.

To investigate the effects of insulin and estradiol on cell cycle progression, cells were allowed to proliferate for 2 days, after which the medium was removed, and cells were washed twice with phosphate-buffered saline. Medium was replaced with a defined serum-free phenol-red-free RPMI 1640 medium supplemented with transferrin (24 μg/mL) and gentamicin (10 μg/mL). This medium was changed once daily for 2 days and ~20 h of serum deprivation in this defined medium. ICI 182780 was added directly to the medium to a final concentration of 10 nm. After 24 h of ICI 182780 pretreatment, vehicle, estradiol (100 nm), insulin (10 μg/mL), or IGF-I (10 nm) was added directly to the medium, and after incubation for the desired time, cells were harvested by brief incubation with trypsin (0.05% w/v), EDTA (0.02% w/v). Cells were prepared for analytical DNA flow cytometry as previously described (37). Flow cytometry was performed on a FACSCalibur laser-based flow cytometer (Becton Dickinson, Immunocytometry Systems, Mountainview, CA), and cell cycle-phase distribution was analyzed with a software program Modfit (Becton Dickinson, Immunocytometry Systems, Mountainview, CA).

Western Blot Analysis—Cells were lysed as follows. Cell monolayers were washed twice in ice-cold phosphate-buffered saline then scraped into ice-cold lysis buffer A (50 mM HEPES, pH 7.5, 150 mM NaCl, 10% (v/v) glycerol, 1% Triton X-100, 1.5 mM MgCl2, 1 mM EGTA, 10 μg/mL aprotinin, 10 μg/mL leupeptin, 1 mM phenylmethylsulfonylfluoride, 200 μM sodium orthovanadate, 10 mM pyrophosphate, 100 mM NaF, and 1 mM DTT). The lysates were incubated for 5 min on ice, and cellular debris was cleared by centrifugation (15,000 × g, 5 min, 4 °C). Equal amounts of total protein (20–40 μg) were separated by SDS-PAGE then transferred to nitrocellulose membranes. Western blots were probed with antibodies: cyclin D1 (DCS-6) (Novacstra Laboratories Ltd, Newcastle-upon-Tyne, UK); cyclin D1 (C-22) (Santa Cruz Biotechnology Inc., Santa Cruz, CA); cyclin E (HE12), Cdk2 (M2), Cdk4 (C-22), p107 (C-18), and p130 (C-20) (Santa Cruz Biotechnology Inc., Santa Cruz, CA); cyclin D1 (DCS-6) (Novacstra Laboratories Ltd, Newcastle-upon-Tyne, UK); p21*WAF1/Cip1 (catalog number C24420) and p27*Kip1 (catalog number K25020) (Transduction Laboratories, Lexington, KY); and secondary antibodies anti-rabbit horseradish peroxidase and anti-mouse horse-radish peroxidase (Santa Cruz Biotechnology Inc., Santa Cruz, CA).

Protein abundance was quantitated by analysis of autoradiographs by densitometry (Molecular Dynamics, Sunnyvale, CA). Quantitation of protein levels by this method was linear over the analyzed range of protein concentrations and exposure times.

Cyclin-dependent Kinase Assays—MCF-7 cell monolayers were washed twice with phosphate-buffered saline and then scraped into ice-cold phosphate-buffered saline and pelleted by centrifugation (15,000 × g, 5 min). For Cdk4 activity assays, the pellets were frozen in liquid nitrogen for storage at −70 °C. Immediately before assaying, the pellets were thawed and resuspended in 1 mL of ice-cold lysis buffer B (50 mM HEPES, pH 7.5, 1 mM DTT, 150 mM NaCl, 1 mM EDTA, 2.5 mM EGTA, 0.1% Tween 20, 10% glycerol, 10 mM β-glycerophosphate, 1 mM NaF, 0.1 mM sodium orthovanadate, 10 μg/mL leupeptin, 10 μg/mL aprotinin, 1 mM DTT, and 0.1 mM phenylmethylsulfonylfluoride). The lysate was placed on ice and vortexed vigorously at intervals for 60 min then centrifuged at 15,000 × g for 5 min at 4 °C. For cyclin E-Cdk2 activity assays, the pellet was resuspended in 1 mL of ice cold lysis buffer A and
frozen in liquid nitrogen for storage at −70 °C. The lysate was placed on ice, vortexed vigorously at intervals for 10 min, and centrifuged at 15,000 g for 5 min at 4 °C, and the supernatant was collected.

Equivalent amounts of protein were precleared by incubation with protein A-Sepharose (Zymed Laboratories Inc., San Francisco, CA) for 45 min at 4 °C. Cdk4 or cyclin E complexes were immunoprecipitated with either rabbit polyclonal human Cdk4 (H-22) or anti-cyclin E polyclonal antibody (C-19), respectively (Santa Cruz Biotechnology Inc.), for 2 h at 4 °C. Similarly, control samples were immunoprecipitated with antibodies preincubated with the respective Cdk4 or cyclin E peptides (Santa Cruz Biotechnology Inc.), also for 2 h at 4 °C. This was followed by a 30-min incubation at 4 °C with protein A-Sepharose for conjugation to the antibodies. Cdk4 immunoprecipitates were washed twice with ice-cold lysis buffer B, twice with ice-cold 50 mM HEPES pH 7.5, and 1 mM DTT, and the supernatant was aspirated. The immunoprecipitates were used for kinase assays with glutathione S-transferase-pRbT73-phosphate (generated from a plasmid kindly supplied by Dr. Ed Harlow, Massachusetts General Hospital Cancer Center, Charlestown, MA) as substrate. Cyclin E immunoprecipitates were washed twice with ice-cold lysis buffer A containing 1 mM NaCl, once with ice-cold lysis buffer A, and then twice with ice-cold 50 mM HEPES, pH 7.5, 1 mM DTT. Histone H1 (Sigma) was employed as the substrate for the kinase activity assays on cyclin E immunoprecipitates.

Each kinase reaction was initiated by resuspending the beads in 30 μl of kinase buffer (50 mM HEPES, pH 7.5, 1 mM MgCl₂, 20 μM ATP, 10 μCi of [γ-³²P]ATP, 0.1 mM sodium orthovanadate, 1 mM NaF, 10 mM β-glycerophosphate) and 10 μg of histone H1 (cyclin E immunoprecipitates) or 10 μg of pRb (Cdk4 immunoprecipitates) as substrate. After incubation for 15 min at 30 °C, the reactions were terminated by the addition of 15 μl of 3× SDS sample buffer (187 mM Tris-HCl, pH 6.8, 30% (v/v) glycerol, 6% SDS, 15% (v/v) β-mercaptoethanol). The samples were then heated at 95 °C for 2 min and separated using 12% SDS-PAGE, and the dried gel was exposed to x-ray film for autoradiography. Relative band intensities were quantitated by densitometric analysis as described above.

Detection of Cdk4-, cyclin E-, and p130-associated Proteins—Immunoprecipitation of Cdk4, cyclin E, and p130 was performed using the method described above (for immunoprecipitating cyclin E and Cdk4 for kinase activity assays), except that the cyclin E and p130 antibodies were chemically cross-linked to protein A-Sepharose to reduce background (40). The antibodies used were rabbit polyclonal antisera to human Cdk4 (H-22), human cyclin E (C-19), and human p130 (C-20) from Santa Cruz Biotechnology Inc. The supernatants obtained after immunoprecipitation were Western-blotted to determine immunoprecipitation efficiency of each antibody, with a 90–95% efficiency consistently achieved. The immunoprecipitated proteins were resuspended in 1× SDS sample buffer, separated by SDS-PAGE, and transferred to nitrocellulose membrane, and the proteins were detected using the antibodies described for Western-blotting above.

Determination of quiescent cells (37). As is evident in Fig. 1A, stimulation of insulin/IGF-I and/or estradiol/IGF-I stimulation. MCF-7 cells were cultured in serum-free medium for 72 h with 10 mM ICI 182780 added for the final 24 h. After this pretreatment, cells were stimulated with vehicle control (C), estradiol alone (E2), insulin alone (I), insulin and estradiol (I + E2), IGF-I alone (IGF-I), or IGF-I and E2 (IGF-I + E2). A, cells were harvested after 26 h stimulation for DNA analysis by flow cytometry. B, percentage of cells in S phase after mitogenic stimulation for various periods with control (Δ), insulin (▲), estradiol (□), and insulin plus estradiol (■). This experiment is representative of at least three independent experiments.

RESULTS

Synergistic Effects of Estradiol and Insulin/IGF-I on G₁-S Phase Progression—Previous studies have shown that treatment of exponentially growing MCF-7 cells with ICI 182780, a pure estrogen antagonist that inhibits estrogen-mediated gene transcription (42), arrests cells in the G₀/G₁ phase of the cell cycle (37, 43). Cell cycle progression is re-initiated after the addition of estradiol to ICI 182780-treated cells in serum-supplemented medium (5). In the present study this model was modified to examine the interaction of insulin/IGF-I and estrogen in mediating cell cycle progression in a serum-free environment. Exponentially growing MCF-7 cells were growth-arrested by serum deprivation for 72 h to inhibit growth factor-signaling pathways with concomitant treatment for the final 24 h with ICI 182780 to inhibit estrogen action. This treatment reduced S phase from 40 to 3–5% and led to accumulation of quiescent cells (37). As is evident in Fig. 1A, stimulation of arrested cells with insulin (10 μg/ml) or IGF-I (1 nM) alone

![Fig. 1. Reinitiation of cell cycle progression of growth-arrested MCF-7 cells with estradiol and/or insulin/IGF-I stimulation.](http://www.jbc.org/)
resulted in a minor increase in S-phase cells to ~10–12% at 26 h. Insulin dose-response experiments in serum-free medium without ICI 182780 indicated a maximal response between 100 ng/ml and 10 μg/ml insulin (data not shown), and a similar maximal response was achieved with 1 nM IGF-I. A supraphysiological concentration (10 μg/ml) of insulin, known to activate both insulin and IGF-I receptor signaling in breast epithelial cells (44, 45), was used in all subsequent experiments, in agreement with the design of earlier studies demonstrating synergistic effects on breast epithelial cell proliferation (15, 16, 46).

In marked contrast, estradiol (100 nM) alone stimulated a significantly greater S-phase entry of 43%. However, when administered together, insulin/IGF-I and estradiol stimulated the greatest G1-S progression, with a maximum 70% S phase after 26 h. Also evident in Fig. 1A was the more highly synchronous entry of cells into S phase after co-stimulation with insulin/IGF-I and estradiol compared with estradiol alone.

A time course of S-phase entry is presented in Fig. 1B. The first significant increase in the proportion of cells in S phase was apparent 14 h after estradiol treatment alone or in combination with insulin. The S-phase fraction reached a maximum between 24 and 26 h and declined thereafter, coincident with a rise in G2/M phase (data not shown). Interestingly, S-phase entry stimulated by insulin and estradiol was significantly greater than the sum of the two individual S-phase responses of either insulin or estradiol alone at all time points between 14 and 28 h. Together these data demonstrate a synergistic effect of insulin/IGF-I and estradiol on MCF-7 cell cycle progression and characterize an experimental paradigm for further elucidating potential molecular mechanisms responsible for these effects.

**Fig. 2.** Expression and phosphorylation of pocket proteins after estradiol and/or insulin/IGF-I stimulation. A. MCF-7 cells treated according to the protocol outlined in Fig. 1 were harvested at the time points indicated, and whole cell lysates were prepared, subjected to electrophoresis on 7% SDS-PAGE, and Western-blotted for pRb, p107 and p130 as described under “Experimental Procedures.” B, graphical presentation of total protein levels after mitogenic stimulation. C, the ratio of hyperphosphorylated pRb and p130 to total protein is represented graphically. ▲, vehicle control; △, insulin; □, estradiol; and ■, insulin plus estradiol. This experiment is representative of data derived from three independent experiments.
phorylated in arrested cells and hyperphosphorylated during G₁-S transition (32). In agreement with these findings, pRb and p130 (Fig. 2A) existed predominantly in their hypophosphorylated states in growth-arrested MCF-7 cells (Fig. 2C). Insulin stimulation resulted in only a minor increase in the phosphorylation of pRb and p130, whereas estradiol induced a significant increase in the phosphorylation of both proteins, as evidenced by the predominance of hyperphosphorylated forms after 15–20 h. Stimulation of cells with both insulin and estradiol had the most profound effect on the degree of pRb and p130 phosphorylation (Figs. 2, A and C). The hyperphosphorylated form was evident as early as 10–15 h after combined treatment, and subsequently, the vast majority of total pRb and p130 was in the hyperphosphorylated state. Although differentially phosphorylated species of p107 were not discernable in these experiments, the abundance of this protein was low in arrested cells, increased slightly by insulin treatment, and induced 4-fold by estrogen treatment (Fig. 2B). There appeared to be no further increase after insulin and estrogen treatment. A similar pRb protein expression profile was also observed. In contrast, p130 protein levels were increased at 5–10 h by all treatments and then decreased to control levels (Fig. 2B). Together these data demonstrate that insulin and estradiol differentially regulate pocket protein abundance and phosphorylation (pRb, p130), in accordance with their differential effects on S-phase entry.

Effects of Estradiol and Insulin on Activation of Cdk4 and Cdk2—Since changes in pocket protein phosphorylation are mediated predominantly by the G₁ CDKs (33), we examined effects on cyclin D1-Cdk4 and cyclin E-Cdk2 activities. After serum deprivation and anti-estrogen pretreatment, Cdk4 activity in vehicle-treated control cells was low and continued to decline over 25 h to levels indistinguishable from those seen in the peptide-blocked controls (Fig. 3A). After insulin stimulation, Cdk4 activity was increased significantly between 5 and 15 h and then declined to control levels by 25 h (Figs. 3, A and C). Estradiol stimulated Cdk4 activity between 5 and 25 h. Co-stimulation with insulin and estradiol resulted in a gradual increase in Cdk4 activity to reach a maximum 2-fold increase by 10 h, thereafter remaining elevated. These data suggest that both insulin and estrogen stimulate a modest increase in Cdk4 activity, although there was no evidence of synergy.

In arrested cells cyclin E-Cdk2 activity was essentially undetectable (Fig. 3, B and C). After insulin stimulation, cyclin E-Cdk2 activity increased at 10 h, reaching a maximum 2-fold at 15–20 h, after which it returned to control levels by 25 h. An initial 2-fold increase was evident at 5 h after estradiol stimulation, with a further increase to a 5-fold maximum at 25 h. Stimulation of cells with estradiol and insulin together resulted in the most dramatic effect on cyclin E-Cdk2 activation; a 3-fold activation was observed as early as 5 h, and cyclin E-Cdk2 activity continued to increase to an 8-fold maximum at 20 h, thereafter declining to 4-fold at 25 h as cells began to exit S phase. Thus, insulin and estradiol together result in synergistic activation of cyclin E-Cdk2, and this precedes S-phase entry by at least 5 h (compare Figs. 3C and 1B).

Effects of Estradiol and Insulin on Cyclin D1-Cdk4 Complexes—CDK activity is determined predominantly by the com-
Regulation of G1 cyclins, CDK1 inhibitors, and cyclin D1-Cdk4 complexes with estradiol and/or insulin stimulation. MCF-7 cells treated according to the protocol outlined in Fig. 1 were harvested at the time points indicated, and whole cell lysates were prepared for Western blot (A) and immunoprecipitation with an anti-Cdk4 antibody as described under "Experimental Procedures" (B). The samples were subjected to electrophoresis on 12% SDS-PAGE and Western-blotted for cyclins D1 and E and CDK inhibitors p27 and p21 (A) and cyclin D1, p27, and p21, as indicated (B). Graphical presentations of total protein levels (A) and Cdk4-associated proteins (B) are shown below the Western blots. △, vehicle control; ▲, insulin; ■, estradiol; and ●, insulin plus estradiol. This experiment is representative of three independent experiments.

corresponds with the insulin-induced peak of Cdk4 activity noted between 5 and 15 h (Figs. 3, A and C). Estradiol stimulated a 5-fold increase in total cyclin D1 but decreased total p21 levels dramatically such that they were essentially undetectable after 15 h (Fig. 4A). Despite an increase in total cyclin D1, estradiol induced only a minor increase in cyclin D1 association with Cdk4, presumably due to the lack of availability of p21 as an assembly factor (47). A pronounced 8-fold induction of cyclin D1 by co-treatment with insulin and estradiol was accompanied by a biphasic effect upon p21. Levels of p21 were initially elevated 4-fold at 5 h and thereafter gradually declined (Fig. 4A). Cyclin D1 and p21 association with Cdk4 was also biphasic, an initial increase coincident with an increase in Cdk4 activity, declining to control levels by 25 h despite elevated Cdk4 activity (Fig. 3C and 4B). The initial event is likely due to insulin induction of p21, and the latter, to estradiol inhibition of p21 gene expression. These results demonstrate that insulin and estradiol have differential effects on cyclin D1-Cdk4 assembly, and this is likely mediated in large part through differential effects on total cellular p21 protein levels.

Effects of Estradiol and Insulin on Cyclin E-Cdk2 Complexes—In addition to cyclin D1-Cdk4, cyclin E-Cdk2 also plays a pivotal role in mediating G1 to S-phase progression (33). Total protein levels of both p27 (Fig. 4A) and Cdk2 (data not shown) were not significantly regulated by insulin or estradiol stimulation. However, cyclin E and p21 were differentially regulated. In control cells, low cyclin E, Cdk2 (data not shown), and p21 protein levels were evident in both whole cell lysates (Fig. 4A) and cyclin E complexes (Fig. 5A). The amount of p27 and p21 associated with cyclin E remained constant over 25 h. Cyclin E and p21 were consistently up-regulated by insulin to ~3- and 4-fold above control, respectively (Fig. 4A). Insulin also caused a profound increase in p21 association with cyclin E-Cdk2, first apparent at 10 h and increasing through to 25 h (Fig. 5A). Estradiol had only minor effects on cyclin E protein expression but significantly decreased p21 expression. Down-regulation of p21 resulted in decreased association with cyclin E to almost undetectable levels by 15 h, consistent with increasing cyclin E-Cdk2 activity (Figs. 3, B and C). Co-stimulation with insulin and estradiol revealed a 2-fold up-regulation of cyclin E, which declined after 20 h (Fig. 4A). This was associated with a 2-fold increase in the amount of p21 associated with cyclin E-Cdk2 at 5 and 10 h (Fig. 5A). Thereafter, both total p21 and its association with cyclin E-Cdk2 gradually declined to undetectable levels by 25 h. These changes are consistent with increasing cyclin E-Cdk2 activity (Fig. 3, B and C). Furthermore, examination of the ratio of p21 to cyclin E revealed a time-dependent decline in the amount of p21 bound to cyclin E-Cdk2 complexes after estradiol stimulation alone or in combination with insulin, but there was little difference between these two treatments (Fig. 5B).

In marked contrast to the differential effects on p21 gene expression and Cdk complex formation, neither insulin nor estradiol had major effects on the total cellular levels of p27 or its accumulation into cyclin D1-Cdk4 or cyclin E-Cdk2 complexes (Figs. 4 and 5). The only exception was at late time points, significantly after S-phase entry, where estradiol alone or in combination with insulin decreased p27 recruitment into cyclin E-Cdk2 complexes. These data imply that p27 plays a relatively minor role in regulating the early cell cycle events responsible for synergism between insulin/IGF-I and estradiol.

Insulin and Estradiol Act Synergistically to Induce and Activate High Molecular Mass Cyclin E-Cdk2 Complexes—Since our previous work has shown that estradiol treatment results in the formation of high specific activity, high molecular mass cyclin E-Cdk2 complexes that represent only a minority of the
Insulin/IGF-I and Estrogen Synergy on G₀/G₁-S-phase Progression

Unusually, these complexes showed little or no cyclin E-Cdk2 activity. Additionally, in control- and insulin-treated cells, some p21 and p27 molecules (in the case of p27, constituting a significant proportion of the total protein) eluted at low molecular mass, i.e. ~70 kDa (Fig. 6C, fractions 28–32 and Fig. 6D, fractions 26–30). The presence of these species presumably indicates cyclin E-Cdk2 complex saturation by p21 and/or p27. These low molecular mass forms of p21 and p27 were not detected after estradiol stimulation or with estradiol and insulin, indicating that after these treatments, all the p21 and p27 was bound in higher molecular mass complexes.

The higher molecular mass of the active cyclin E complexes is due in part to p130 association (6). To investigate whether the increased complex formation induced by estradiol and insulin treatment was due to increased association with p130, we immunoprecipitated p130 from the eluted fractions and Western-blotted for cyclin E. Cyclin E was associated with p130 in the active high molecular mass 350-kDa complexes (Fig. 7A) but not in the inactive 160-kDa complexes (Fig. 7B). In addition, a greater degree of cyclin E association with p130 was induced by insulin and estradiol stimulation compared with estradiol alone. This is in marked contrast to control and insulin-stimulated cells, where there was little or no detectable cyclin E association with p130. These data demonstrate that an increased association of cyclin E with p130 is correlated with the synergistic activation of cyclin E-Cdk2 complexes.

Adenoviral Expression of p21 Inhibits Synergism between Insulin and Estrogen on Cell Cycle Progression—Differential induction of p21 by insulin and estrogen (Figs. 4A and 6C) resulted in differential regulation of the cyclin D1-Cdk4 and cyclin E-Cdk2 complexes (Figs. 4B and 5A). The synergistic activation of cyclin E-Cdk2 (Figs. 3B and 6B) by insulin and estradiol appears to depend on the estrogen-induced down-regulation of p21 (Fig. 5A) with consequent loss of p21 and accumulation of p130 in high molecular mass active cyclin E-Cdk2 complexes (Figs. 6 and 7). To investigate more directly the possible role of p21 in the synergism between insulin and estradiol, we first attempted to mimic the effects of estradiol by decreasing p21 levels using antisense oligonucleotides to the p21 mRNA. Unfortunately these experiments were unsuccessful, as MCF-7 cells that had undergone serum deprivation and ICI 182780 treatment could not tolerate the further manipulations required to deliver the p21 antisense oligonucleotides to the cell.

In an alternative approach, cells treated with insulin and estradiol were infected with an adenovirus expressing p21 to restore p21 levels to those observed after treatment with insulin alone. In agreement with earlier experiments, insulin induced p21 levels ~4-fold at 24 h (compare Figs. 4A and 8A) and a hypophosphorylated form of pRb (compare Figs. 2A and 8B). This was consistent with a low (8%) S-phase fraction (compare Figs. 1A and 8C) in uninfected cells or those infected with a control adenovirus lacking p21. Similarly, low levels of p21 were seen in estradiol plus insulin-stimulated cells regardless of infection with control adenovirus (Fig. 8A), and this was accompanied by a synergistic 51% S-phase entry after 24 h (Fig. 8C). This synergistic S-phase fraction was coincident with the appearance of pRb in a predominantly hyperphosphorylated state (Fig. 8B). Infection of insulin plus estradiol-stimulated cells with the p21 adenovirus resulted in the expression of a faster migrating p21 species (~19 kDa), distinct from the endogenous p21. This p21 adenovirus contains a truncated human p21 cDNA that lacks the C-terminal 21 amino acids but preserves the CDK and cyclin binding domains sufficient for full CDK inhibitory activity (48, 49). Infection, which led to increases in p21 protein to a maximum level almost equivalent...
to that induced by insulin alone, resulted in inhibition of insulin plus estradiol-stimulated cell cycle progression as reflected in the decreased S-phase fraction (Fig. 8C). Infection with 12 × 10⁶ plaque-forming units/ml p21 adenovirus induced p21 levels 4-fold and reduced the S-phase fraction from 51 to 27%. This reduction in the S-phase fraction was coincident with a decrease in the hyperphosphorylated form of pRb (Fig. 8B). These data demonstrate that modulation of p21 levels inhibits the synergistic effects of insulin and estradiol on cell cycle progression, identifying a pivotal role for p21 in the cooperative interaction underlying the synergy between insulin/IGF-I and estradiol.

**DISCUSSION**

The importance of estrogen action in the regulation of breast cancer cell proliferation has been demonstrated both in vitro and in vivo (50). Epidemiological studies demonstrate a causative role for estrogen exposure in the etiology of breast cancer (2), whereas the clinical efficacy of oophorectomy and pharmacological agents that inhibit the synthesis or action of estrogen further emphasize the fundamental importance of estrogen-induced mitogenesis in breast cancer (51). It is now evident, however, that estrogens do not exert their mitogenic actions alone but act in concert with other hormones, growth factors, and cytokines to regulate breast epithelial cell proliferation.
treatment with insulin/IGF-I alone increased cyclin D1, cyclin E, and p21 expression (Fig. 4), a pattern of expression typical of the proliferative response to diverse mitogenic growth factors. Furthermore, active p21-associated cyclin D1-Cdk4 complexes were formed (Figs. 3 and 4). These data provide convincing evidence of an intact IGF-IR mitogenic-signaling pathway following anti-estrogen pretreatment and imply that the ability of anti-estrogens to block insulin/IGF-I-induced mitogenesis in breast cancer cells occurs predominantly downstream of Cdk4 activation.

The most likely explanation for the inability of insulin/IGF-I to induce cell cycle progression is the inability to activate cyclin E-Cdk2 complexes, an essential requirement for cell cycle progression (33). Some insight into reasons for the failure of insulin to activate cyclin E-Cdk2 in anti-estrogen-pretreated cells can be gained from examination of anti-estrogen effects on cyclin-CDK complex composition and activity. An integral component of the growth inhibitory response of ICI 182780 is decreased cyclin D1 gene expression (43) and the consequent rapid redistribution of p21 from cyclin D1-Cdk4 to cyclin E-Cdk2 complexes, inhibiting the activity of the latter kinase (5, 37). This is followed at later time points by a 4–5-fold increase in total cellular p21, a modest increase in p27 (37, 43), and the accumulation of inactive cyclin E-Cdk2 complexes loaded with p21 and p27 inhibitors (5, 37). Thus, although the induction of cyclin D1 and cyclin E by insulin/IGF-I might be expected to change the dynamics of CDK complex formation through the sequestration of p21 and p27 into newly formed cyclin D1-Cdk4 complexes and hence facilitate the formation of active cyclin E-Cdk2 complexes, the concurrent increased p21 gene expres-

![Fig. 8](http://www.jbc.org/)

**Fig. 8.** Adenovirus expressing p21 inhibits synergism between insulin/IGF-I and estradiol. MCF-7 cells treated according to the protocol outlined in Fig. 1 were subsequently infected with the indicated amount of either p21 expressing or control adenovirus 4 h after insulin (I) or insulin and estradiol (I + E) treatment. These cells were harvested at 24 h, and whole cell lysates were prepared, subjected to electrophoresis on 12% SDS-PAGE, and Western-blotted for p21 (A) and pRb (B) as described under "Experimental Procedures." C, cells treated according to the above protocol were harvested after a 24-h stimulation for DNA analysis by flow cytometry. This experiment is representative of at least three independent experiments. pfu, plaque-forming units.

![Fig. 9](http://www.jbc.org/)

**Fig. 9.** Proposed model of synergy between insulin and estrogen in stimulating G1-S-phase progression. Graphical representation of the proposed molecular mechanisms described in the text underlying the regulation of cyclin D1-Cdk4, cyclin E-Cdk2 activity by insulin and estrogen. See "Discussion" for details. LMW, low molecular mass.
sion favors maintenance of inactive cyclin E-Cdk2. Indeed it is evident from the gel filtration data presented in Fig. 6 that after stimulation with insulin alone, a significant proportion of both p21 and p27 remains in a state unbound to cyclin E-Cdk2, apparently in excess of that required to inhibit the majority of the cyclin E-Cdk2 complexes. We and others have previously shown that anti-estrogen-arrested cells can be stimulated by estrogens to reenter the cell cycle in a semi-synchronous manner (5). Numerous lines of evidence indicate that cyclin E-Cdk2 activation is critical to G\textsubscript{1}-S-phase progression (33). Cyclin E-Cdk2 is activated by estradiol in MCF-7 cells several hours before S-phase entry (4, 5, 7), providing a potential mechanism for estrogen-mediated early cell cycle progression. In the current model, estrogen stimulated cyclin E-Cdk2 activity in the absence of significant increases in cyclin E or Cdk2 protein levels, a result in agreement with previous observations in a serum-containing medium (5). This is in marked contrast to previous studies in fibroblasts where mitogenic activation of cyclin E-Cdk2 resulted predominantly from increases in cyclin E gene expression (54, 55).

The mechanism of activation of cyclin E-Cdk2 by estrogen has been investigated in some detail (5, 7) and involves at least two distinct mechanisms. In the acute phase of the response no detectable changes in the total cellular levels of the major components of the cyclin E-Cdk2 complex, i.e. cyclin E, Cdk2, p21, and p27, were apparent, but significant changes occurred in association of the CDK inhibitors with CDK complexes. Thus, as a result of estrogen-induced cyclin D1 gene expression, p21 and to a lesser extent p27 are sequestered into cyclin D1-Cdk4 complexes (5, 7). In addition, estrogen inhibits p21 gene transcription, rapidly depleting the pool of newly synthesized p21 and facilitating the formation of active cyclin E-Cdk2 complexes lacking p21.2 When both potential pathways were inhibited by the simultaneous inhibition of estrogen-induced cyclin D1 gene expression with antisense oligonucleotides and inhibition of p21 and to a lesser extent p27 remains in a state unbound to cyclin E-Cdk2, resulting from increases in cyclin E gene expression (54, 55).

A consequence of these events is that a minority of the total cyclin E-Cdk2 complexes are active and, despite being depleted of p21 and p27, elute at a higher molecular mass, i.e. ~350 kDa on gel filtration chromatography. Although all the components of this active oligomeric complex have yet to be defined, the pRb-related protein, p130, is associated with active cyclin E-Cdk2 after estrogen activation. This interaction is likely facilitated by the high abundance of p130 after anti-estrogen arrest (37) and through competition between p21 and p130 for a common binding site on cyclin E-Cdk2, recognizing the RXL motif common to both proteins (48, 56).

Delineation of the mechanisms through which insulin/IGF-I and estrogen regulate cyclin D1-Cdk4 and cyclin E-Cdk2 activity provides insight into the synergistic activation of the latter complex after combined treatment, illustrated in the model presented in Fig. 9. As noted above, insulin/IGF-I failed to initiate substantial S-phase entry despite the induction of cyclin D1 and formation of active p21-associated cyclin D1-Cdk4 complexes. Although cyclin E expression increased, little activation of cyclin E-Cdk2 occurred, presumably due to the presence of high levels of p21 and consequent formation of inactive p21-associated cyclin E-Cdk2 complexes. Treatment with estrogen alone resulted in synchronous entry of the majority of cells into S phase. Like insulin/IGF-I, estrogen induced cyclin D1 expression and led to the formation of active p21-associated cyclin D1-Cdk4 complexes. However, in contrast with insulin/IGF-I, estrogen activated cyclin E-Cdk2 via a combination of p21 sequestration into newly formed cyclin D1-Cdk4 complexes and inhibition of p21 gene expression, favoring the formation of high molecular mass, active cyclin E-Cdk2-p130 complexes (Fig. 9).

Data presented in this manuscript indicate that the synergy between insulin/IGF-I and estrogen in initiating cell cycle progression is not due to cooperative activation of cyclin D1-Cdk4 despite evidence that cyclin D1-Cdk4 activity is essential to the effects of both mitogens (35). Rather, the synergy is associated with enhanced activation of cyclin E-Cdk2 via a significant increase in high molecular mass cyclin E complexes containing p130. Two distinct mechanisms contribute to this effect; one mechanism is the induction of cyclin E after estrogen/IGF-I stimulation, and the other mechanism is the ability of estrogen to attenuate the insulin-induced increase in total cellular p21 (Fig. 4) and the concurrent recruitment of p21 into cyclin E-Cdk2 complexes (Fig. 5). Since cyclin E and its associated Cdk2 activity are rate-limiting for G\textsubscript{1} to S-phase progression (33), the markedly increased formation of active cyclin E-Cdk2 complexes after combined treatment (Figs. 3 and 6) would be expected to increase pRb phosphorylation and S-phase entry, as demonstrated in Figs. 1 and 2. Our ability to inhibit the synergistic interaction between insulin/IGF-I and estrogen after infection with a p21-expressing adenovirus adds further support for a pivotal role for estrogen depletion of p21 in this synergistic mitogenic response.

Acknowledgments—We thank Elizabeth Musgrove for critical reading of the manuscript and Jason Carroll for assistance in the preparation of this manuscript.

REFERENCES

1. Coze, J. F., and Korach, K. S. (1990) Endocrin. Rev. 20, 358–417
2. Colditz, G. A. (1990) J. Natl. Cancer Inst. 90, 814–825
3. Altucci, L., Addeo, R., Cicatiello, L., Davouso, S., Parker, M. G., Truss, M., Beato, M., Sica, V., Bracchini, F., and Weisz, A. (1996) Oncogene 12, 2315–2324
4. Foster, J. S., and Wimalasena, J. (1996) Mol. Endocrinol. 10, 488–498
5. Prall, O. W. J., Sarcevic, B., Musgrove, E. A., Watts, C. K. W., and Sutherland, R. L. (1997) J. Biol. Chem. 272, 10882–10894
6. Prall, O. W. J., Rogan, E. M., Musgrove, E. A., Watts, C. K. W., and Sutherland, R. L. (1998) Mol. Cell. Biol. 18, 4499–4508
7. Planas-Silva, M. D., and Weinberg, R. A. (1997) Mol. Cell. Biol. 17, 4659–4669
8. Stewart, A. J., Westley, B. R., and May, F. E. (1997) Br. J. Cancer 66, 640–648
9. Ignar-Trowbridge, D. M., Nelson, K. G., Bidwell, M. C., Curtis, S. W., Washburn, T. F., McLauchlan, J. A., and Korach, K. S. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 4655–4662
10. Dupont, J., Karas, M., and LeRoith, D. (2000) J. Biol. Chem. 275, 35893–35901
11. Kahlert, S., Nuedling, S., van Eickels, M., Vetter, H., Meyer, R., and Grohe, C. (2000) J. Biol. Chem. 275, 18447–18453
12. Melloy, C. A., May, F. E., and Westley, B. R. (2000) J. Biol. Chem. 275, 12565–12571
13. Osborne, C. K., Bolan, G., Monaco, M. E., and Lippman, M. E. (1976) Proc. Natl. Acad. Sci. U. S. A. 73, 4536–4540
14. van der Burg, B., Ierbrucker, L., van Seem-Miltung, A. J. P., de Laat, S. W., and van Zoolen, E. J. J. (1990) Cancer Res. 50, 7770–7774
15. van der Burg, B., Rutteman, G. R., Blankenstein, M. A., de Laat, S. W., and van Zoolen, E. J. (1998) J. Cell. Physiol. 134, 101–108
16. Stewart, A. J., Johnson, M. D., May, F. E., and Westley, B. R. (1990) J. Biol. Chem. 265, 21172–21177
17. Yee, D. C., and Lee, M. (2000) J. Mamm. Biol. Neoplasia 5, 107–115
18. Dawes, M. R., Westley, B. R., and May, F. E. (1996) Endocrinology 137, 1177–1186
19. White, M., and Yenush, L. (1998) Curr. Top. Microbiol. Immunol. 228, 179–208
20. Jackson, J. G., White, M. F., and Yee, D. (1998) J. Biol. Chem. 273, 11994–11993
21. Dufourny, B., Alblas, J., van Treefelen, H. A., van Schaik, F. M., van der Burg, B., Steenbergh, P. H., and Sussenbach, J. S. (1997) J. Biol. Chem. 272, 31163–31171
22. Lee, A. V., Jackson, J. G., Gooch, J. L., Hilsenbeck, S. G., Coronado-Heinsohn, E., Osborne, C. K., and Yee, D. (1999) Mol. Endocrinol. 13, 787–796
23. Richards, R. G., Walker, M. P., Sebastian, J., and DiAugustine, R. P. (1998) J. Biol. Chem. 273, 11962–11969
24. Ahmad, S., Singh, N., and Glazer, R. I. (1999) Biochem. Pharmacol. 58, 425–430
25. Migliaccio, A., Di Domenico, M., Casteria, G., de Falco, A., Bontempio, P., Noda, E., and Auricchio, F. (1996) EMBO J. 15, 1292–1300
26. Stoica, A., Saceda, M., Falckro, A., Jeynor, M., and Martin, M. B. (2000) J. Cell. Biochem. 76, 605–614
27. Stoica, A., Saceda, M., Doraisivwanyi, V. L., Coleman, C., and Martin, M. B.
28. Lee, A. V., Weng, C. N., Jackson, J. G., and Yee, D. (1997) J. Endocrinol. 152, 39–47
29. Ignar-Trowbridge, D. M., Teng, C. T., Ross, K. A., Parker, M. G., Korach, K. S., and McLachlan, J. A. (1993) Mol. Endocrinol. 7, 992–998
30. Kato, S., Endoh, H., Masuhiro, Y., Kitamoto, T., Uchiyama, S., Sasaki, H., Masushige, S., Gotoh, Y., Nishida, E., and Kawashima, H. (1995) Science 270, 1491–1494
31. Ma, Z. Q., Santagati, S., Patrone, C., Pollio, G., Vegeto, E., and Maggi, A. (1994) Mol. Endocrinol. 8, 910–918
32. Nevins, J. R. (1998) Cell Growth Differ. 9, 585–593
33. Sherr, C., and Roberts, J. (1999) Genes Dev. 13, 1501–1512
34. Musgrove, E. A., Hamilton, J. A., Lee, C. S., Sweeney, K. J., Watts, C. K., and Sutherland, R. L. (1993) Mol. Cell. Biol. 13, 3577–3587
35. Lukas, J., Bartkova, J., and Bartek, J. (1996) Mol. Cell. Biol. 16, 6917–6925
36. Dufourny, B., van Teeffelen, H. A., Hamelers, I. H., Sussenbach, J. S., and Steenbergh, P. H. (2000) J. Endocrinol. 166, 329–338
37. Carroll, J. S., Prall, O. W., Musgrove, E. A., and Sutherland, R. L. (2000) J. Biol. Chem. 275, 38221–38229
38. Sutherland, R. L., Hall, R. E., and Taylor, I. W. (1983) Cancer Res. 43, 3998–4006
39. Musgrove, E. A., Wakeling, A. E., and Sutherland, R. L. (1989) Cancer Res. 49, 2398–2404
40. Harlow, E., and Lane, D. (1988) Antibodies: A Laboratory Manual, pp. 526–527, Cold Spring Harbor Laboratory, NY
41. Falck-Pedersen, E. (1998) in Cells: A Laboratory Manual (Spector, D. L., Goldman, R. D., and Leinwand, L. A., eds) pp. 90.1–90.28, Cold Spring Harbor Laboratory, NY
42. McDonnell, D. P., Clemm, D. L., Hermann, T., Goldman, M. E., and Pike, J. W. (1995) Mol. Endocrinol. 9, 659–669
43. Watts, C. K., Brady, A., Sarcevic, B., deFazio, A., Musgrove, E. A., and Sutherland, R. L. (1995) Mol. Endocrinol. 9, 1804–1813
44. Milazzo, G., Yip, C. C., Maddux, B. A., Vigneri, R., and Goldfine, I. D. (1992) J. Clin. Invest. 89, 899–908
45. Adamo, M., Roberts, C. T. J., and LeRoith, D. (1992) Biofactors 3, 151–157
46. Thorsen, T., Lahooti, H., Rasmussen, M., and Aakvaag, A. (1992) J. Steroid Biochem. Mol. Biol. 41, 537–540
47. LaBaer, J., Garrett, M. D., Stevenson, L. F., Slingerland, J. M., Sandho, C., Chou, H. S., Pataa, A., and Harlow, E. (1997) Genes Dev. 11, 847–862
48. Adams, P. D., Sellers, W. R., Sharma, S. K., Wu, A. D., Nalin, C. M., and Kaelin, W., Jr. (1996) Mol. Cell. Biol. 16, 6623–6633
49. Luo, Y., Hurwitz, J., and Massague, J. (1996) Nature 375, 159–161
50. Prall, O. W. J., Rogan, R. M., and Sutherland, R. L. (1998) J. Steroid Biochem. Mol. Biol. 65, 169–174
51. Nicholson, R. L., Gee, J. M., Bryant, S., Francis, A. B., McClelland, R. A., Knowlden, J., Wakeling, A. E., and Osborne, C. K. (1996) Ann. N. Y. Acad. Sci. 784, 325–335
52. Yu, H., and Rohan, T. (2000) J. Natl. Cancer Inst. 92, 1472–1489
53. Guvakova, M. A., and Sormaz, E. (1997) Cancer Res. 57, 2606–2610
54. Dulic, V., Lees, E., and Reed, S. I. (1992) Science 257, 1958–1961
55. Koff, A., Giordano, A., Desai, D., Yamashita, K., Harper, J. W., Elledge, S. N., Nishimoto, T., Morgan, D. O., Franza, B. R., and Roberts, J. M. (1992) Science 257, 1689–1694
56. Shiyano, P., Bagchi, S., Adami, G., Kokontis, J., Hay, N., Arroyo, M., Morozov, A., and Raychaudhuri, P. (1996) Mol. Cell. Biol. 16, 737–744
Insulin/Insulin-like Growth Factor-I and Estrogen Cooperate to Stimulate Cyclin E-Cdk2 Activation and Cell Cycle Progression in MCF-7 Breast Cancer Cells through Differential Regulation of Cyclin E and p21WAF1/Cip1

Angela Lai, Boris Sarcevic, Owen W. J. Prall and Robert L. Sutherland

J. Biol. Chem. 2001, 276:25823-25833. doi: 10.1074/jbc.M100925200 originally published online May 3, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M100925200

Alerts:
  • When this article is cited
  • When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 54 references, 29 of which can be accessed free at http://www.jbc.org/content/276/28/25823.full.html#ref-list-1