

Cyclin D1 Stimulation of Estrogen Receptor Transcriptional Activity Independent of cdk4†

ELIZABETH NEUMAN,¹ MOHAMED H. LADHA,¹ NANCY LIN,¹ TODD M. UPTON,¹
SUSAN J. MILLER,¹ JAMES DiRENZO,¹ RICHARD G. PESTELL,² PHILIP W. HINDS,³
STEVEN F. DOWDY,⁴ MYLES BROWN,¹ AND MARK E. EWEN^{1*}

The Dana-Farber Cancer Institute and Harvard Medical School¹ and Department of Pathology, Harvard Medical School,³ Boston, Massachusetts 02115; The Albert Einstein Cancer Center, Departments of Medicine and Developmental and Molecular Biology, Albert Einstein College of Medicine, Bronx, New York 10461²; and Howard Hughes Medical Institute and Division of Molecular Oncology, Departments of Pathology and Medicine, Washington University School of Medicine, St. Louis, Missouri 63110⁴

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Cyclin D1 plays an important role in the development of breast cancer and is required for normal breast cell proliferation and differentiation associated with pregnancy. We show that ectopic expression of cyclin D1 can stimulate the transcriptional activity of the estrogen receptor in the absence of estradiol and that this activity can be inhibited by 4-hydroxytamoxifen and ICI 182,780. Cyclin D1 can form a specific complex with the estrogen receptor. Stimulation of the estrogen receptor by cyclin D1 is independent of cyclin-dependent kinase 4 activation. Cyclin D1 may manifest its oncogenic potential in breast cancer in part through binding to the estrogen receptor and activation of the transcriptional activity of the receptor.

The three D cyclins are differentially expressed in a cell lineage-specific manner as part of a delayed early response to mitogens. D-type cyclins are rate limiting and essential for progression through the G₁ phase of the cell cycle (48, 49). One of the known biochemical functions of D cyclins is to bind to and activate cyclin-dependent kinase 4 (cdk4) and cdk6. In addition, cyclins D1, D2, and D3 can bind to the retinoblastoma protein Rb, and related proteins, in the absence of kinase *in vitro*. This binding is thought to direct cdk4 and cdk6 to Rb, allowing for efficient phosphorylation of the substrate. In support of the notion that Rb is a critical downstream target of D cyclins, cells lacking functional Rb do not require cyclin D-dependent kinases for passage from G₁ into S phase (50). Emerging evidence suggests that D-type cyclins are not redundant. The three D cyclins have different affinities for Rb (15, 17, 31). Ectopic expression of cyclins D2 and D3, but not cyclin D1, can inhibit granulocyte differentiation (32). Cyclin D1- and D2-deficient mice show different, specific developmental defects (19, 51, 52). Cyclin D1, and not cyclins D2 and D3, is overexpressed in a high percentage of certain tumors (24).

Cyclin D1 is amplified or overexpressed in a high percentage (>50%) of human breast adenocarcinomas (3, 8, 12, 21, 41, 57) and is oncogenic *in vivo*, in breast epithelial cells, and *in vitro* (26, 38, 56). While cyclin D1 is not essential for the development of most murine tissues and organs, female *cyclin D1*^{-/-} mice are markedly deficient in breast epithelial cell proliferation associated with pregnancy (19, 52). Specifically, ductal side branching and lobuloalveolar development are severely impaired in these mice despite normal levels of circulating ovarian hormones. It has been suggested that steroid hormone-induced breast epithelial cell proliferation and/or differentiation during pregnancy requires the action of cyclin D1.

Here, we describe the functional interaction of cyclin D1 with the estrogen receptor.

MATERIALS AND METHODS

Plasmids. The following plasmids have been described previously: -1745CD1Luc (human cyclin D1 promoter-luciferase reporter) (2); p(ERE)₂-tk-luc (estrogen response element [ERE]-luciferase reporter) (34), a gift from P. Chambon; pCMV-hER (60), a gift from D. J. Shapiro; BBCAT (β-casein promoter-chloramphenicol acetyltransferase [CAT] construct) (47), a gift from M. J. Bissell and C. Myers, Monsanto; pRc/CMV-cyclin D1, D2, and D3 (15); pRc/CMV-cyclin D1-KE (26); pRc/CMV-cyclin A (27); glutathione S-transferase (GST)-Rb (17, 29); and pcDNA3-p16 (46). To construct pcDNA3.1-hER-ΔAF-1, a 1,816-bp *EcoRI* fragment containing the full-length cDNA encoding human estrogen receptor was subcloned from pHEG0 (34) into pALTER-1 (Promega). Oligonucleotide-directed mutagenesis was used to introduce an *SpeI* restriction site flanking codons 173 and 174, so that subsequent subcloning of this mutant at the introduced *SpeI* site resulted in hER-ΔAF-1 (amino acids 174 to 596). The sequence of the mutagenic oligonucleotide was 5'-CAGTACCAATGACAAGACTAGTATGGCTATGGAATC-3'. The hER-ΔAF-1 cDNA was excised as a *SpeI*-to-*EcoRI* fragment and ligated into pcDNA3.1 (Invitrogen) cut with *XbaI* and *EcoRI*. Constructs encoding hemagglutinin (HA)-tagged human D-type cyclin fusion proteins (to allow detection with the mouse monoclonal antibody 12CA5) were made by adding the amino acid sequence GYPYDVP-DYA to the C termini of the proteins. For cyclin D1, PCR was performed using the 5' primer CGCAAACACGCGCAGACC located upstream of the region to be modified and the 3' primer GAAGCTCCGGATCAGATATCGGCGTAGTCCGGGACGTCGTAGGGGTAGCCGATGTCACGTC, encoding the tag and a *BspEI* restriction site used for subcloning. The PCR product (Vent polymerase; New England Biolabs) was cleaved with restriction enzymes *StuI* and *BspEI*, purified, and substituted for the wild-type fragment in the construct pRc/CMV-cyclin D1. As a result, 80 bp of the 3' untranslated sequence of cyclin D1 located between the stop codon and the *BspEI* site was lost. Dideoxynucleotide sequencing by standard techniques confirmed that this construct contained the HA sequence. The HA tag sequence was added to pRc/CMV-cyclin D2 by subcloning a double-stranded oligonucleotide that encoded the tag and was flanked by *EcoRV* and *MluI* restriction sites. Upon substitution of this oligonucleotide for the wild-type sequence, the tag was present at the C terminus of the protein and 30 bp of 3' untranslated sequence was deleted immediately after the stop codon and preceding the *MluI* site. The single-stranded oligonucleotides used were ATCGACCTGGGCTACCCCTACGACGTCGCCGGACTACGCCGACCTGTGAA and CGCGTTCACAGGTCGGCGTAGTCCGGGACGTCGTAGGGGTAGCCGATGTCGAT. An oligonucleotide flanked by restriction sites for *ApaI* and *XbaI* was used to create an HA-tagged cyclin D3 by the method described for cyclin D2. Substitution of this oligonucleotide into pRc/CMV-cyclin D3 resulted in a cDNA encoding a C-terminally tagged protein lacking 855 bp of 3' untranslated sequences between the stop codon and the *XbaI* site.

* Corresponding author.

† M. E. Ewen dedicates this work to a friend and teacher, Charles Brenton Huggins, Nobel Laureate.

Deletion of 735 nucleotides internal to this region had previously been found to increase expression from the parental cyclin D3 cDNA. The oligonucleotides used to construct HA-tagged cyclin D3 were CAGCCAGACCAGCACTCTAC AGATGTACAGCCATACACCTGGGCTACCCCTACGACGTCGCCGGAC TACGCCACCTGTAGT and CTGACTACAGGTGGGCGTAGTCCG GGAGCTCGTAGGGGTAGCCAGGTGTATGGCTGTGACATCTGTAG GAGTGCTGGTCTGGCTGGGCC.

SCp2 cell differentiation. To generate stable pools of SCp2 cells expressing BBCAT, cells were transfected with calcium phosphate precipitates (22) by using 3 μ g of pBabe-neo (for neomycin selection) or pBabe-puro (for puromycin selection) and 27 μ g of BBCAT. The precipitate was removed after 4 h, and the cells were glycerol shocked (25% glycerol in 25 mM HEPES [pH 7.6]–140 mM NaCl) for 90 s, washed three times, and fed in Dulbecco modified Eagle medium (DMEM)-F12 supplemented with 5% fetal bovine serum and insulin (5 μ g/ml). The following day, selection was started with either G418 (400 μ g/ml) or puromycin (1.5 μ g/ml). After 2 to 3 weeks of selection, colonies were pooled and used directly in differentiation assays. Alternatively, these cells were used to generate stable pools of SCp2 cells expressing BBCAT plus either –1745CD1Luc or p(ERE)₂-tk-luc, as described above. For differentiation studies, pooled populations were plated either on plastic (1.3 $\times 10^6$ cells per 60-mm-diameter dish; 5.2 $\times 10^6$ cells per 100-mm-diameter dish) or on plates precoated with an extracellular matrix (ECM) (Matrigel; Collaborative Research) from the Englebreth-Holm-Swarm tumor (1.3 $\times 10^6$ cells per 60-mm-diameter dish; 4.3 $\times 10^6$ cells per 100-mm-diameter dish) in DMEM-F12 containing 2% serum. The following day, the cells were induced to differentiate by the addition of DMEM-F12 without serum plus insulin (5 μ g/ml), hydrocortisone (1 μ g/ml), and prolactin (3 μ g/ml) (differentiation medium) (for details, see reference 13). At the times indicated below, cells were removed from the ECM by incubation for 2 h at 37°C with the enzyme dispase (Collaborative Research) or removed from plastic by cell scraping with a rubber policeman prior to the various analyses being performed.

Estrogen receptor transcriptional activation assays. CV1P and SAOS-2 cells were maintained in DMEM supplemented with 10% Fetal Clone I (Hyclone), penicillin, and streptomycin. Twenty-four hours prior to transfection, cells were split and fed with phenol red-minus DMEM supplemented with charcoal-dextran-stripped fetal bovine serum (HyClone) plus antibiotics. Cells were transfected with the indicated plasmids by the calcium phosphate protocol (22). Sixteen to eighteen hours posttransfection, the precipitate was washed from the cells and estradiol or antiestrogens were added as indicated. Forty-eight hours later, luciferase activity was measured (16). β -Galactosidase activity was also determined (16) and used as an internal control to which luciferase activity was normalized.

cdk4 assays. Kinase assays were essentially as described previously (40). At the indicated times after the induction of differentiation, SCp2 cells were harvested as described above. The cells were pelleted by centrifugation at 1,500 $\times g$ for 5 min and then washed three times with cold phosphate-buffered saline. The pellet was resuspended in 800 μ l of DIP buffer (50 mM HEPES [pH 7.2], 150 mM NaCl, 1 mM EDTA, 2.5 mM EGTA, 10% glycerol, and 0.1% Tween 20) containing freshly added 1 mM dithiothreitol (DTT), 1 mM NaF, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 0.5 mM sodium orthovanadate, 10 mM β -glycerophosphate, 1 μ g of aprotinin per ml, and 1 μ g of leupeptin per ml. The sample was carried out for 30 min at 4°C with rocking. Samples were passed through a 23-gauge needle five times and then pelleted by centrifugation at 16,000 $\times g$ for 10 min. The resulting supernatant was precipitated with an antibody specific for cdk4 (C-22; Santa Cruz Biotechnology, Inc.) for 90 min at 4°C with rocking. Immune complexes were collected with protein A-Sepharose beads (50 μ l of a 10% slurry containing beads pre-equilibrated in DIP with 4% bovine serum albumin [BSA]) while the mixture was rocked at 4°C for 90 min. The beads were then washed four times with DIP buffer containing fresh inhibitors (as described above) and twice with kinase buffer (50 mM HEPES [pH 7.2], 10 mM MgCl₂, 5 mM Mn Cl₂, and freshly added 1 mM DTT) and suspended in 25 μ l of kinase buffer containing 20 μ M ATP, 10 μ Ci of [γ -³²P]ATP, and 1 μ g of substrate, GST-Rb (C terminus) fusion protein [GST-Rb(C)] (see below). The reaction proceeded for 30 min at 37°C and was stopped with the addition of 2 \times sodium dodecyl sulfate (SDS) gel sample buffer. After being boiled for 5 to 10 min, the samples were electrophoresed by using an SDS–10% polyacrylamide gel. The phosphorylated GST-Rb was visualized by autoradiography. The substrate, GST-Rb(C), was prepared by growing *Escherichia coli* DH5 α transformed with pGEX-Rb (792-928) overnight at 37°C in Luria broth containing ampicillin. The culture was then diluted 1:10 in Luria broth with ampicillin and grown for 1 to 2 h at 37°C. GST fusion protein was induced by growth in 100 μ M isopropylthioglycoside (IPTG) for 3 to 4 h at 37°C. The bacteria were pelleted by centrifugation at 5,000 $\times g$ for 5 min at 4°C and resuspended in cold HEMG buffer (25 mM HEPES [pH 7.6], 0.1 mM EDTA, 12.5 mM MgCl₂, 100 mM KCl, and 10% glycerol) with freshly added 1 mM DTT, 1 μ g of leupeptin per ml, 1 μ g of aprotinin per ml, 400 μ M sodium orthovanadate, 500 μ M PMSF, and 50 mM NaF and lysed by sonication on ice. The lysate was cleared by centrifugation at 10,000 $\times g$ for 5 min at 4°C and incubated with 500 μ l of a 50% slurry containing glutathione-Sepharose 4B (Pharmacia) pre-equilibrated with kinase buffer. After the mixture was rocked at 4°C for 15 min and washed three times with cold kinase buffer, the GST-Rb(C) was eluted by resuspending the beads in 300 μ l of kinase buffer containing 200 mM reduced glutathione and 1% Tween 20 and rocking the

mixture at 4°C for 10 min. The eluate was aliquotted and stored at –70°C. The amount of purified GST-Rb(C) in samples was quantitated by Bradford assay, as well as SDS-polyacrylamide gel electrophoresis, in comparison with bovine serum albumin of known concentration. Proteins were visualized by Coomassie blue staining.

Western blot analysis. Cells were washed twice in ice-cold phosphate-buffered saline and lysed in 300 μ l of RIPA-B buffer (20 mM sodium phosphate [pH 7.4], 150 mM NaCl, and 1% Triton X-100). Equal amounts of protein (as determined by Bradford assay) were separated by SDS-polyacrylamide gel electrophoresis. The proteins were transferred to a polyvinylidene difluoride membrane (NEN) and detected with antibodies specific for either cyclin D1 (DCS-6; NeoMarkers), CDK4 (C-22; Santa Cruz Biotechnology, Inc.), or estrogen receptor (TE111.5D11; NeoMarkers) by using a chemiluminescence detection system (DuPont, NEN).

Immunoprecipitations and reimmunoprecipitations. SAOS-2 cells were transfected (22) with 2 μ g of pCMV-hER and 5 μ g of pRc/CMV-cyclin D1, 10 μ g of pRc/CMV-cyclin D2, 10 μ g of pRc/CMV-cyclin D3, or 10 μ g of pRc/CMV-cyclin A (six plates of each). Forty-eight hours posttransfection, the cells were metabolically labeled with [³⁵S]methionine for 3 h and then lysed in 50 mM Tris-HCl (pH 8.0)–200 mM NaCl–5 mM EDTA–0.5% Nonidet P-40 containing 50 μ g of PMSF per ml, 10 μ g of aprotinin per ml, 20 mM NaF, 20 mM β -glycerophosphate, and 0.1 mM sodium orthovanadate. The lysates were subjected to immunoprecipitation with a monoclonal antibody to the estrogen receptor (TE111.5D11; NeoMarkers) at 4°C for 45 min with rocking. Following a 30-min incubation (4°C) with protein A-Sepharose, samples were washed five times in NETN (10 mM Tris-HCl [pH 8.0], 100 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40), resuspended in 100 μ l of 50 mM Tris-HCl (pH 7.5)–5 mM DTT–0.5% SDS, and boiled for 10 min. Ten percent of this sample was removed for direct analysis in an SDS-polyacrylamide gel, and to the remainder was added 1 ml of lysis buffer (above). The protein A-Sepharose was removed from the lysate by brief centrifugation at 16,000 $\times g$, and replicate samples of the cleared lysate were subjected to a second immunoprecipitation (4°C for 60 min with rocking) with appropriate monoclonal antibodies to either the estrogen receptor (TE111.5D11 and AER314; NeoMarkers) or HA (12CA5), cyclin A (C160; gift from E. Harlow), or adenovirus E1A (M73). After immune complexes were collected with protein A-Sepharose for 45 min, samples were washed five times in NETN, resuspended in SDS gel sample buffer, boiled, and analyzed by electrophoresis using an SDS–10% polyacrylamide gel. Replicate samples were also analyzed following a first immunoprecipitation with 12CA5 or C160 and a second immunoprecipitation with antibodies to either the estrogen receptor, HA, or adenovirus E1A (see above). Similar experiments were also performed with polyclonal sera to each of the D-type cyclins (gift from C. Sherr), with the same results.

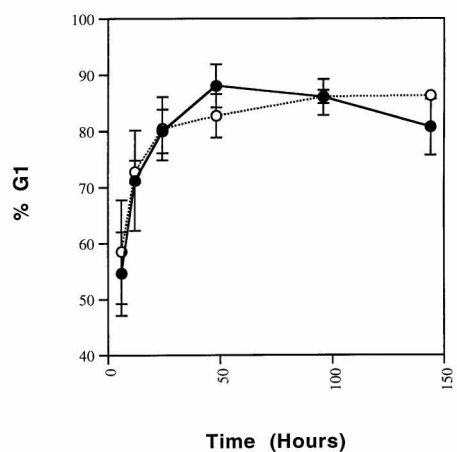
In similar experiments, COS-1 cells were transfected with plasmids encoding cyclin D1 and either wild-type estrogen receptor or pcDNA3.1-hER- Δ AF-1, a mutant estrogen receptor lacking AF-1. The cells were metabolically labeled and lysed, and cleared lysates were subjected to immunoprecipitation with cyclin D1 antibody or antibody to the estrogen receptor as described above. Immune complexes were resolved in a denaturing gel.

RESULTS

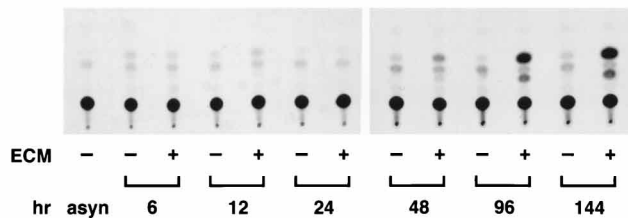
In order to investigate the possible relationship between cyclin D1 and the action of steroid hormone receptors, we used the murine cell line SCp2. This cell line is derived from the breasts of mice in midpregnancy and is thought to be of ductal epithelial origin (13). SCp2 cells express transcriptionally active estrogen receptor (44). In the presence of lactogenic hormones and an ECM, SCp2 cells show the essential features of mammary gland differentiation: the formation of alveoli made of polarized cells capable of vectorial secretion and sequestration of milk proteins.

Approximately 57% of an asynchronous population of SCp2 cells, cultured on plastic, was in the G₁ phase of the cell cycle. When plated on an ECM in the presence of lactogenic hormones and in the absence of serum, the cells withdrew from the cell cycle within 50 h, with >85% of the cells in G₁ as determined by flow cytometry (Fig. 1A). As a marker of breast cell differentiation, β -casein promoter activity in cells previously transfected with a β -casein reporter construct was measured. By 4 days after the induction of differentiation, SCp2 cells showed significant β -casein promoter activity that continued to increase to day 6 (Fig. 1B), consistent with previous results (13). In contrast, SCp2 cells cultured in differentiation medium on plastic had no detectable β -casein promoter activity.

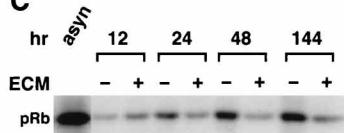
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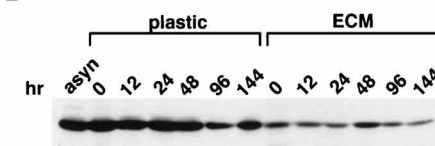
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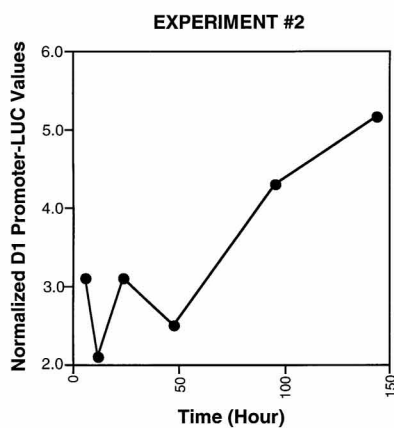
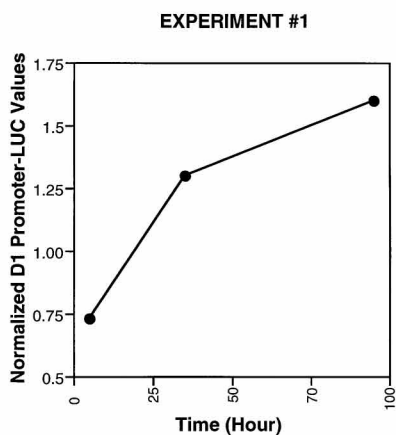
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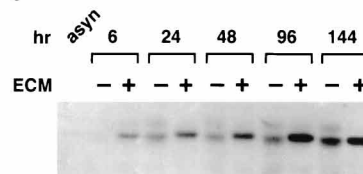
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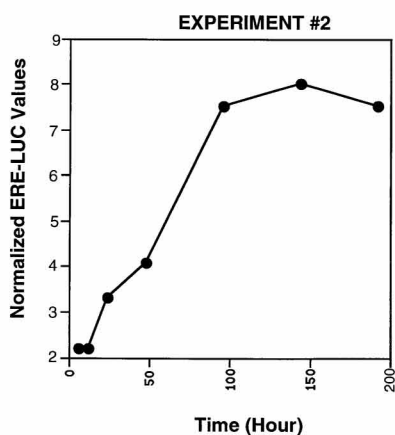
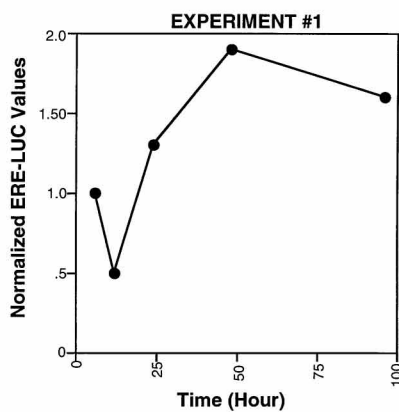
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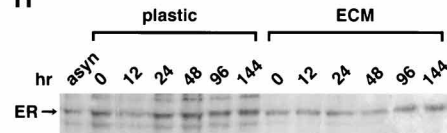
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H



cdk4 activity was measured during a time course of SCp2 cell differentiation. cdk4 was immunoprecipitated from cleared lysates prepared from SCp2 cells at various times after being plated on an ECM in the presence of lactogenic hormones. Immunoprecipitates, normalized for protein content, were incubated with recombinant GST-Rb used as a substrate in the kinase reaction (40). As shown in Fig. 1C, cdk4 activity decreased significantly in cells cultured for 12 h in differentiation medium on ECM compared to that in asynchronously growing cells in maintenance media (all of the experimental samples in this figure were separated on the same gel and exposed to film for the same length of time). cdk4 activity continued to decrease during the first 48 h of differentiation on ECM and remained low relative to that of either cells cultured on plastic in differentiation medium or asynchronous populations of cells, through 6 days of differentiation. As a control, cdk4 activity was measured in cells that were cultured in differentiation medium on plastic and did not show an induction of β -casein promoter activity (Fig. 1B). Although the amount of cdk4 activity in cells cultured for 12 h in differentiation medium on plastic was also decreased relative to that of asynchronously growing cells, cdk4 activity in these cells steadily increased during the time course of differentiation. These results were in part attributable to cdk4 protein levels. Although the amount of cdk4 protein was smaller in cells plated on ECM and cultured overnight in 2% serum than in asynchronously growing cells, during the time course of differentiation there was no change in cdk4 protein levels (Fig. 1D). As expected, the decrease in cdk4 activity in cells induced to differentiate on ECM in the presence of lactogenic hormones was seen within the same window of time during which these cells arrest in G₁.

To further characterize cell cycle components during SCp2 cell differentiation, we measured cyclin D1 promoter activity during differentiation. This was in part motivated by the observation that steroid hormone agonists and antagonists affect the expression of cyclin D1 in breast cancer cell lines (43, 58). A human cyclin D1 reporter construct (2) was introduced into SCp2 cells, and resultant pooled populations were induced to differentiate in the presence of an ECM and lactogenic hormones. Figure 1E shows the results obtained with two independently derived pools of cells expressing the cyclin D1 promoter. The activity of the cyclin D1 promoter was induced within 24 h after the induction of differentiation (Fig. 1E), in contrast to that of an asynchronous population, which showed a low, constant level (not shown). Some experimental variation in the activity of the cyclin D1 promoter was noted during the first 24 h of differentiation (Fig. 1E and data not shown). Cyclin D1 promoter activity continued to increase through 48 (experiment 1) and 144 (experiment 2) h of differentiation (Fig. 1E). This elevation in cyclin D1 promoter activity occurred before β -casein promoter activity was first detected (96 h of differentiation; Fig. 1B). These results paralleled the

changes in cyclin D1 protein levels during differentiation. Cyclin D1 protein was induced when cells were cultured overnight on ECM in 2% serum, compared to the protein level in asynchronously growing cells (Fig. 1F). During differentiation on ECM, the level of cyclin D1 protein continued to increase. In contrast to the levels in cells induced to differentiate on ECM, there was no change in cyclin D1 protein levels in cells cultured in differentiation medium on plastic (Fig. 1F). These results are consistent with the observation that in mice, cyclin D1 expression is high during pregnancy but not during lactation (53).

In a parallel study, we also measured estrogen receptor activity during a time course of differentiation both in the presence and in the absence of estradiol (Fig. 1G and data not shown). A reporter containing two EREs upstream of a luciferase gene (34) was transfected into SCp2 cells, and pooled drug-resistant clones were analyzed. Figure 1G shows the results obtained with two independently derived pools of cells. Interestingly, there was a peak of estrogen receptor transcriptional activity in the absence of exogenous estradiol within the first 3 days of differentiation (Fig. 1G). The activity continued to increase through 48 h of differentiation and then either decreased slightly (experiment 1) or reached a plateau (experiment 2). The change in the level of estrogen receptor activity roughly paralleled that seen for cyclin D1 promoter activity and protein levels. During the first 24 h of differentiation, there was significant variation in the activity of the integrated ERE-luciferase reporter, thus making it difficult to draw conclusions from these earlier time points. Importantly, neither cyclin D1 promoter activity nor estrogen receptor transcriptional activity increased in cells cultured in differentiation medium on plastic compared to that of asynchronously growing cells (not shown). The results were not attributable to changes in estrogen receptor protein levels, since they did not change when SCp2 cells were cultured in differentiation medium on plastic or ECM for the indicated times, in comparison to asynchronously growing cells (Fig. 1H).

Our observation of simultaneous induction of cyclin D1 and repression of cdk4 activity and expression as SCp2 cells withdraw from the cell cycle during differentiation suggested that cyclin D1 may have a role in breast epithelial cell differentiation independent of its ability to activate cdk4. The correlation between the increase in cyclin D1 levels and an increase in ligand-independent estrogen receptor activity during SCp2 cell differentiation prompted us to explore a possible functional relationship between cyclin D1 and the estrogen receptor.

As one possible explanation of the observations noted above, we first sought to determine whether cyclin D1 functions upstream of the estrogen receptor to stimulate its transcriptional activity. To this end, CV1P cells were transfected with plasmids encoding the human estrogen receptor (60) and an ERE-luciferase reporter (34) together with an expression

FIG. 1. Characterization of murine SCp2 breast epithelial cells during differentiation. (A to C) SCp2 cells containing an integrated β -casein reporter were plated on either an ECM or tissue culture plastic in the presence of lactogenic hormones (differentiation medium). At the indicated time points, replicate plates of cells were processed for fluorescence-activated cell sorter (FACS) analysis (ECM, closed circle; plastic, open circles) (A), CAT assays measuring β -casein promoter activity as a marker of differentiation (B), or cdk4 kinase assays using recombinant GST-Rb as a substrate (C). All lanes of the Rb kinase assay are from the same gel and have been exposed to film for the same length of time. (D, F, and H) Whole-cell lysates isolated from asynchronously growing SCp2 cells or SCp2 cells cultured in differentiation medium on either plastic or ECM for the times indicated were resolved on an SDS-10% acrylamide denaturing gel. Proteins were detected by Western blot analysis with antibodies specific for cdk4 (D), cyclin D1 (F), or estrogen receptor (H), using a chemiluminescence detection system. (E and G) Independently derived pools of cells stably expressing the BBCAT construct plus either -1745CD1Luc or p(ERE)₂-tk-luc were used in differentiation assays as described in Materials and Methods. At the indicated times, cells were harvested and assayed for luciferase activity to measure cyclin D1 promoter activity (E) or estrogen receptor transcriptional activity (G). Activity was normalized for protein concentration as determined by Bradford analysis and to the level of activity in an asynchronous population, which was set to 1. Graphs are representative of the results obtained with two independently derived pooled populations (designated experiments 1 and 2) for each reporter construct being tested. Cells were also assayed for β -casein promoter activity as a measure of appropriate differentiation (not shown). ER, estrogen receptor; asyn, asynchronously growing cells; LUC, luciferase; hr, hours.

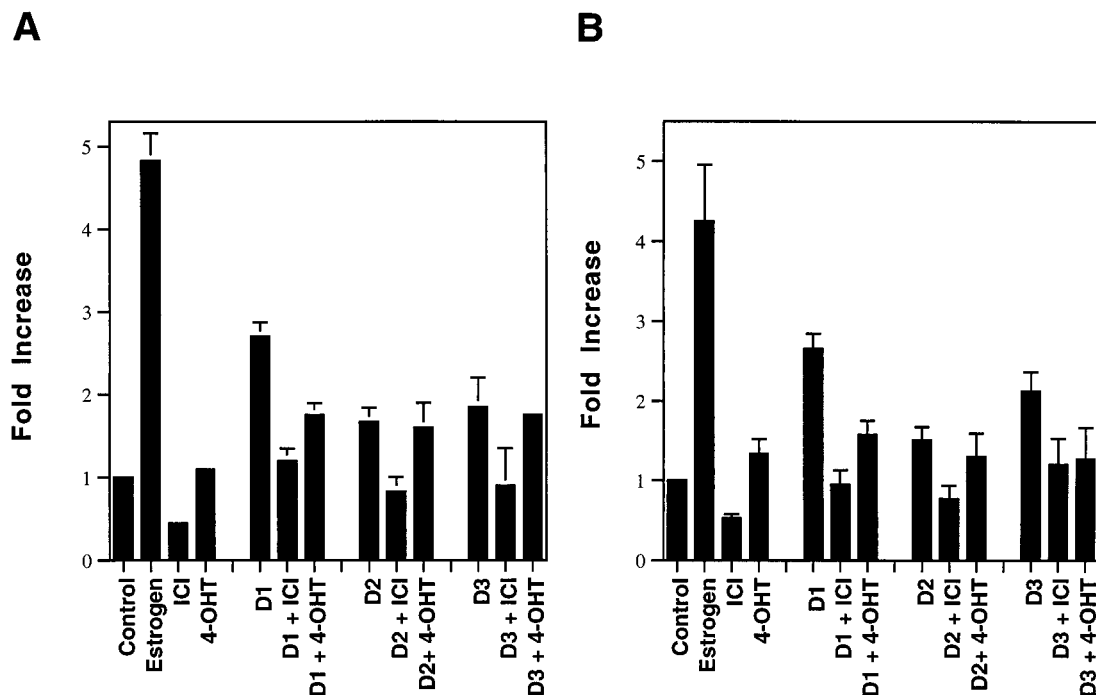


FIG. 2. Cyclin D1 stimulation of estrogen receptor transcriptional activity independent of its influence on the cell cycle. (A) Estrogen receptor activation by cyclin D1 and, to a lesser extent, cyclins D2 and D3. CV1P cells were transfected with pCMV-hER (1 μ g), encoding the human estrogen receptor; p(ERE)₂-tk-luc (3 μ g), an ERE luciferase reporter; pCMV- β Gal (3 μ g) together with a plasmid encoding human cyclin D1, D2, or D3; or the empty vector pRC/CMV (5 μ g). After the precipitate was washed off, the cell cultures were either left untreated or had estradiol (10 nM), 4-hydroxytamoxifen (4-OHT; 100 nM), or ICI 182,780 (ICI; 100 nM) added to them. Forty-eight hours later, luciferase activity was measured, and the values were normalized to β -galactosidase activity. The fold activation was calculated with respect to the luciferase activity in the absence of D-type cyclins, estradiol, 4-OHT, or ICI, which was set to 1. The graph shows the averages plus standard errors of at least three independent experiments. (B) Same as for panel A except that SAOS-2 cells were used. The graph shows the averages plus standard errors of at least three independent experiments.

vector encoding human cyclin D1, D2, or D3 (15). Coexpression of cyclin D1 consistently led to a two- to threefold increase in estrogen receptor transcriptional activity in the absence of added estradiol compared to the activity in vector control transfections (Fig. 2A). This increase in transcriptional activity was approximately half the activation seen in the presence of estradiol and in the absence of ectopically expressed cyclin D1. Coexpression of cyclin D1 and estradiol did not result in an additive effect, since maximal activation in the presence of estradiol appeared not to be influenced by expression of exogenous cyclin D1 (Fig. 2A and data not shown). Similar results were found with SCp2 cells and MCF-7 cells (44). Both cyclins D2 and D3, expressed at levels similar to that of cyclin D1, were found to induce estrogen receptor transcriptional activity in the absence of ligand; however, the effect was less pronounced than that of cyclin D1. In similar experiments performed using the progesterone receptor, ectopic expression of cyclin D1 had no effect on the transcriptional activity of this steroid hormone receptor (44). These results suggested that cyclin D1 can activate the estrogen receptor in a ligand-independent fashion.

The estrogen receptor has two transactivation domains, AF-1 and AF-2, the latter of which is induced by estrogen binding. The antiestrogen 4-hydroxytamoxifen is thought to block primarily the activity of AF-2, and pure antiestrogens such as ICI 182,780 and ICI 164,384 inhibit the activity of both AF-1 and AF-2 (5, 7, 20). We investigated the effect of antiestrogens on the cyclin D1-induced activation of the estrogen receptor. Both ICI 182,780 and, to a lesser extent, 4-hydroxytamoxifen inhibited the effect of cyclin D1 on estrogen receptor

activity in the presence or absence of estradiol (Fig. 2A and data not shown).

On the basis of the known biochemical and biological functions of cyclin D1, several scenarios for how cyclin D1 might activate estrogen receptor transcriptional activity can be envisioned. To determine if cyclin D1 influences the estrogen receptor by its effect on the cell cycle, we made use of SAOS-2 cells. These cells do not express wild-type Rb and have undetectable levels of cyclins D1 and D2 and moderate levels of cyclin D3 (17). Importantly, expression of D-type cyclins in SAOS-2 cells does not alter their cell cycle profile (17). This is consistent with the observations that Rb-minus cells do not have a requirement for cyclin D-cdk4 activity (39) and that SAOS-2 cells express high levels of the cdk4- and cdk6-specific inhibitor p16 (54). Transient transfection of a cyclin D1 expression plasmid together with plasmids encoding the estrogen receptor and an ERE reporter led to an approximately threefold increase in estrogen receptor transcriptional activity relative to that of control transfections (Fig. 2B). Similar results were observed with COS-1, VA13, and HeLa cells, in which the function of Rb is compromised by the expression of viral oncoproteins (44). In SAOS-2 cells, expression of cyclins D2 and D3 showed a less pronounced effect than cyclin D1 on estrogen receptor activity. These results suggest that cyclin D1 can stimulate estrogen receptor transcriptional activity in a cell cycle- and Rb-independent manner.

Given that SAOS-2 cells express high levels of the cdk4- and cdk6-specific inhibitor p16, ectopic expression of cyclin D1 is likely not to activate these kinases in these cells. In addition, cyclin D1, unlike cyclins D2 and D3, cannot activate cdk2 (17).

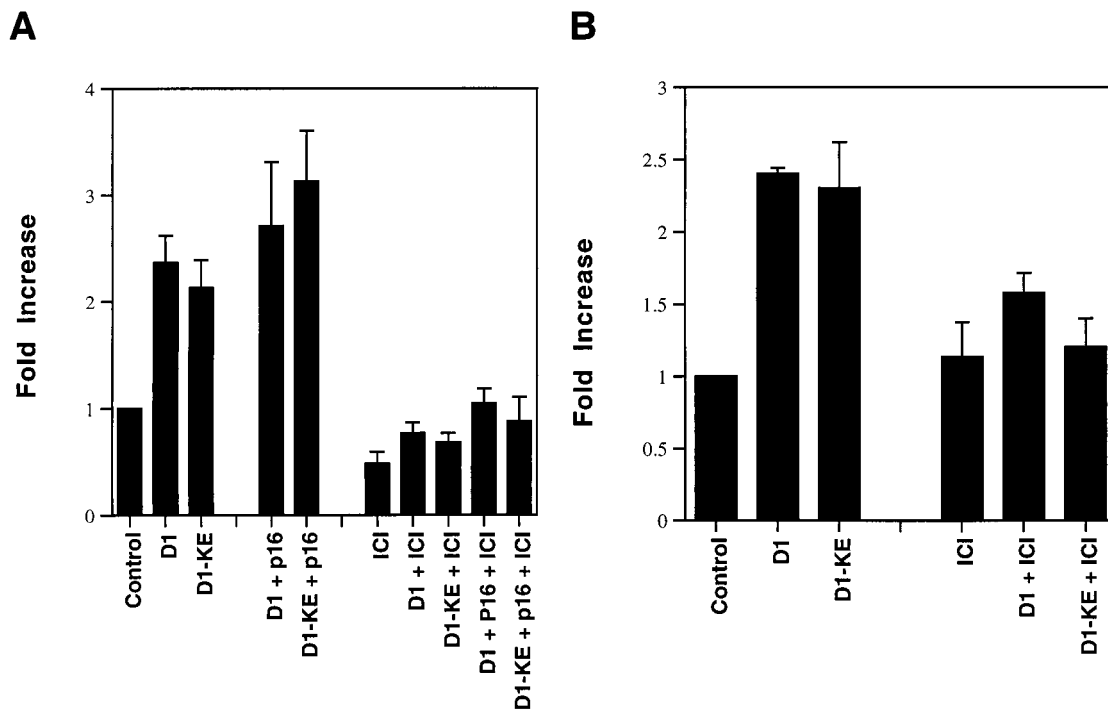


FIG. 3. Cyclin D1 stimulation of estrogen receptor transcriptional activity independent of cdk4 activation. (A) Cyclin D1 stimulation of estrogen receptor transcriptional activity independent of cell cycle influence and cdk4 activation. SAOS-2 cells were transfected with pCMV-hER, p(ERE)₂-tk-luc, or pCMV-βGal, as described for Fig. 2A, together with pRc/CMV-cyclin D1 or pRc/CMV-cyclin D1-KE (D1-KE) in the presence or absence of an equivalent amount (5 μg) of p16-encoding plasmid pcDNA3-p16 or pRc/CMV (Invitrogen). Replicate plates of the transfected cultures were treated with estradiol (10 nM; not shown) or ICI 182,780 (ICI; 100 nM). Fold activation in luciferase activity was determined as described for Fig. 2A. The graph shows the averages plus standard errors of at least three independent experiments. (B) Activation of the estrogen receptor by a cyclin D1 mutant incapable of activating cdk4 in CV1P cells. CV1P cells were transfected as indicated below the graph, and fold activation in luciferase activity was determined. The graph shows the averages plus standard errors of at least three independent experiments.

Thus, cdk4 and cdk6 are the only known kinases that cyclin D1 can activate. These observations, together with the results shown in Fig. 2B, suggest that cyclin D1 might activate the estrogen receptor in a cdk-independent manner. To explore this possibility further, we determined whether overexpression of p16 influenced the ability of cyclin D1 to stimulate estrogen receptor transcriptional activity. As shown in Fig. 3A, coexpression of p16, driven from a cytomegalovirus (CMV) promoter (46), with cyclin D1 did not affect the ability of this cyclin to stimulate estrogen receptor activity. Under these conditions, the levels of p16 were 5- to 10-fold greater than the endogenous cdk4 levels (44). Furthermore, we have not detected measurable cdk4 activity in SAOS-2 cells in either the absence or the presence of exogenous p16 (44).

As an alternative approach to demonstrating that cyclin D1 can stimulate the estrogen receptor in the absence of cdk4 activation, we made use of a cyclin D1 mutant in which the lysine at amino acid position 112 was changed to glutamic acid. This genetic alteration in the evolutionarily conserved lysine residue resulted in a mutant, termed cyclin D1-KE, that retains the ability to bind cdk4 but not the ability to activate cdk4 (14, 26). Expression of cyclin D1-KE in either CV1P or SAOS-2 cells was as effective as wild-type cyclin D1 in stimulating the transcriptional activity of the estrogen receptor in a ligand-independent manner (Fig. 3). Similarly to the situation for the wild-type protein, the stimulation in transcriptional activity by cyclin D1-KE could be inhibited by ICI 182,780. Together, these results suggest that cyclin D1-mediated stimulation of the transcriptional activity of the estrogen receptor is independent of the ability of cyclin D1 to activate cdk4 and cdk6.

Our observation that the ability of cyclin D1 to affect estrogen receptor activity did not involve its ability to activate cdk4 suggested that the communication between cyclin D1 and the estrogen receptor might be direct. To test this possibility, we examined whether cyclin D1 and the estrogen receptor could be found in the same complex by coimmunoprecipitation. Cell were transfected with plasmids encoding the estrogen receptor together with cyclin D1, D2, or D3. HA-tagged versions of the three D-type cyclins were used to ensure that the abilities to immunoprecipitate each of these cyclins were equivalent. The cells were metabolically labeled with [³⁵S]methionine, and then lysates were prepared. Cleared lysates were subjected first to immunoprecipitation with either anti-estrogen receptor or HA monoclonal antibody. The anti-HA precipitates were then analyzed to determine if the estrogen receptor coprecipitated with cyclin D1, D2, or D3. The precipitates were boiled in the presence of SDS, diluted in lysis buffer, and then subjected to a second round of immunoprecipitation with anti-estrogen receptor monoclonal antibody (Fig. 4A, lanes 8, 11, and 14) or control antibody (lanes 9, 12, 15). Alternatively, the first immunoprecipitation was performed with anti-estrogen receptor antibody, and after boiling, the second immunoprecipitation was performed with anti-HA antibody (Fig. 4A, lanes 17, 20, and 23) or control antibody (lanes 18, 21, and 24). A fraction of the first immunoprecipitation was saved for analysis (Fig. 4A, lanes 1 to 6). The levels of input estrogen receptor were determined by anti-estrogen receptor immunoprecipitation followed by anti-estrogen antibody precipitation (Fig. 4A, lanes 7, 10, and 13), and the same procedure was used for the input cyclins (lanes 16, 19, and 22). Cyclin D1 and, to a lesser

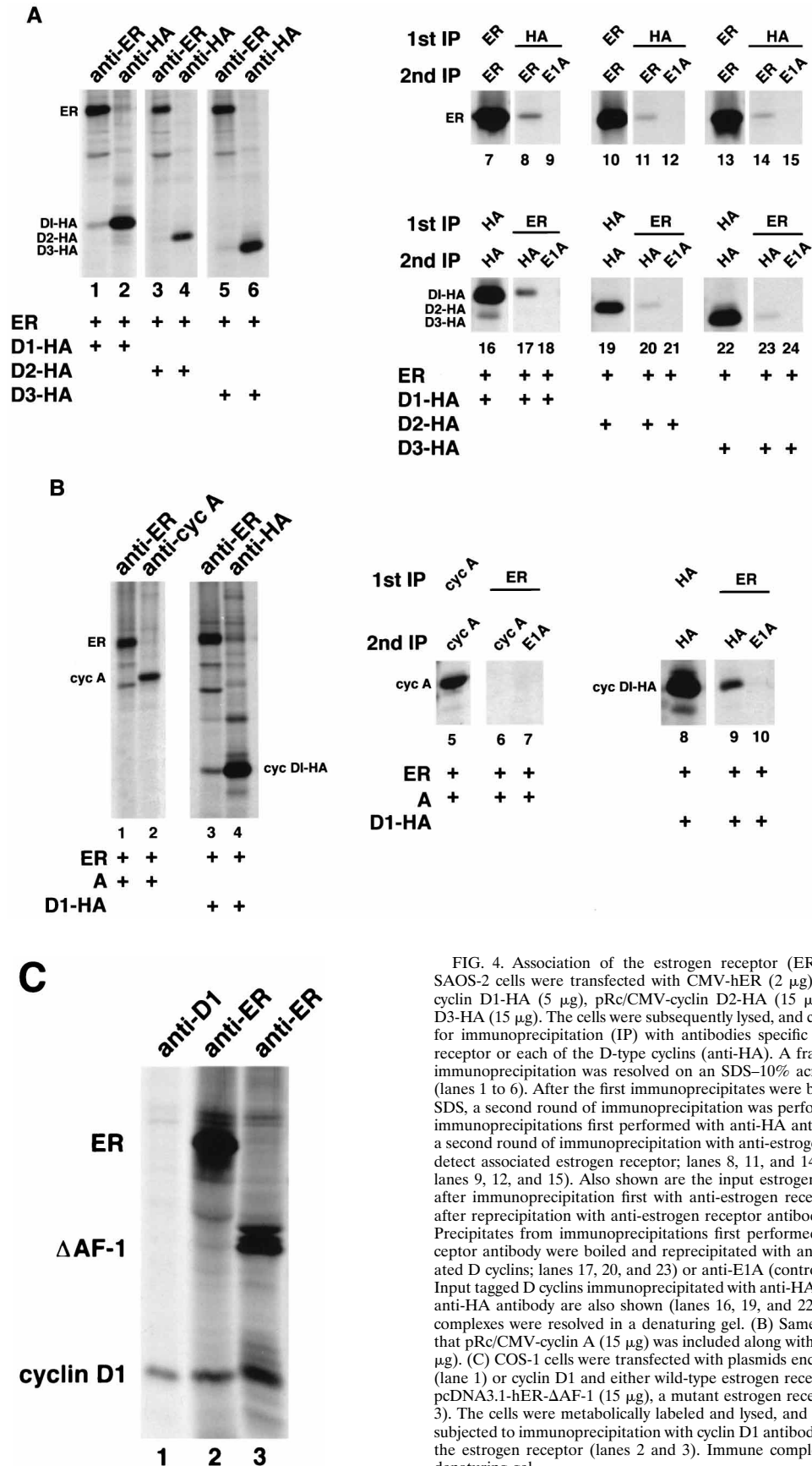


FIG. 4. Association of the estrogen receptor (ER) with cyclin D1. (A) SAOS-2 cells were transfected with CMV-hER (2 μ g) plus either pRc/CMV-cyclin D1-HA (5 μ g), pRc/CMV-cyclin D2-HA (15 μ g), or pRc/CMV-cyclin D3-HA (15 μ g). The cells were subsequently lysed, and cleared lysates were used for immunoprecipitation (IP) with antibodies specific for either the estrogen receptor or each of the D-type cyclins (anti-HA). A fraction (10%) of the first immunoprecipitation was resolved on an SDS-10% acrylamide denaturing gel (lanes 1 to 6). After the first immunoprecipitates were boiled in the presence of SDS, a second round of immunoprecipitation was performed. Precipitates from immunoprecipitations first performed with anti-HA antibody were subjected to a second round of immunoprecipitation with anti-estrogen receptor antibody (to detect associated estrogen receptor; lanes 8, 11, and 14) or anti-E1A (control; lanes 9, 12, and 15). Also shown are the input estrogen receptor levels, shown after immunoprecipitation first with anti-estrogen receptor antibody and then after reprecipitation with anti-estrogen receptor antibody (lanes 7, 10, and 13). Precipitates from immunoprecipitations first performed with anti-estrogen receptor antibody were boiled and reprecipitated with anti-HA (to detect associated D cyclins; lanes 17, 20, and 23) or anti-E1A (control; lanes 18, 21, and 24). Input tagged D cyclins immunoprecipitated with anti-HA and reprecipitated with anti-HA antibody are also shown (lanes 16, 19, and 22, respectively). Immune complexes were resolved in a denaturing gel. (B) Same as for panel A, except that pRc/CMV-cyclin A (15 μ g) was included along with pRc/CMV-cyclin D1 (5 μ g). (C) COS-1 cells were transfected with plasmids encoding cyclin D1 (15 μ g) (lane 1) or cyclin D1 and either wild-type estrogen receptor (15 μ g) (lane 2) or pCDNA3.1-hER- Δ AF-1 (15 μ g), a mutant estrogen receptor lacking AF-1 (lane 3). The cells were metabolically labeled and lysed, and the cleared lysates were subjected to immunoprecipitation with cyclin D1 antibody (lane 1) or antibody to the estrogen receptor (lanes 2 and 3). Immune complexes were resolved in a denaturing gel.

extent, cyclins D2 and D3 were found to associate with the estrogen receptor in the first immunoprecipitation with anti-estrogen receptor antibody (Fig. 4A, compare lanes 1, 3, and 5, lanes 8, 11, and 14 and lanes 17, 20, and 23). With the use of specific antibodies to each of the D-type cyclins and non-HA-tagged versions, cyclin D1 and, to a lesser extent, cyclins D2 and D3 were found to associate with the estrogen receptor (not shown). Together, these results parallel those found in the transactivation studies in which cyclins D2 and D3 were found to activate estrogen receptor transcriptional activity to a lesser extent than was found for cyclin D1.

Given the above observations and the fact that D-type cyclins show a significant degree of sequence homology, we determined if cyclin A might form a complex with the estrogen receptor. In an experiment performed in parallel with an experiment for cyclin D1, cyclin A was found not to associate to any detectable degree with the estrogen receptor (Fig. 4B). This is not attributable to the antibody to cyclin A which was used, for two reasons. First, the monoclonal antibody is very efficient in precipitating denatured cyclin A (Fig. 4B). Second, unlike for cyclin D1, anti-estrogen receptor immunoprecipitates were found not to contain cyclin A.

To begin to explore the genetics of the estrogen receptor-cyclin D1 interaction, COS-1 cells were cotransfected with plasmids encoding cyclin D1 and either the wild-type estrogen receptor or a mutant lacking the AF-1 transactivation domain. The cells were metabolically labeled, and then cleared lysates were subjected to immunoprecipitation with a monoclonal antibody to the estrogen receptor. Immune complexes, resolved in a denaturing gel, were found to contain a protein which comigrated with cyclin D1 (Fig. 4C, lanes 2 and 3) when compared to a cyclin D1 immunoprecipitation (lane 1).

DISCUSSION

Our results suggest that cyclin D1 can activate estrogen receptor transcriptional activity. Transcriptional activation appears to be mediated by the binding of cyclin D1 to the estrogen receptor. Consistent with the idea that activation of the estrogen receptor can be mediated by binding to cyclin D1, the relative abilities of the three D-type cyclins to activate the estrogen receptor correlated with their relative abilities to associate with the estrogen receptor. In addition, the data suggesting that cyclin D1 activates estrogen receptor transcriptional activity in the absence of activation of cdk4 also suggest a mechanism involving direct protein-protein interaction. That cyclin D1 and the cyclin D1-KE mutant might activate an as yet unidentified kinase which is not inhibited by p16 and is involved in estrogen receptor activation remains a formal possibility.

The experiments performed linking cyclin D1 to activation of the estrogen receptor were motivated by the characterization of SCp2 murine breast cells during differentiation (Fig. 1). At present, the increase in cyclin D1 levels seems to correlate with increased estrogen receptor transcriptional activity during differentiation. We are trying to determine if there is a direct causal relationship between these two events in an effort to demonstrate a physiological role for cyclin D1 in the activation of the estrogen receptor. The results, however, like those in previous reports (28, 62), raise the possibility that D-type cyclins regulate gene expression via interaction with transcription factors.

During completion of this work, Zwijsen et al. (62) published data similar, but not identical, to those reported here. Although we did occasionally see cooperativity between cyclin D1 and estradiol in the activation of the estrogen receptor, this

was not seen consistently. In the data reported by Zwijsen et al. (62), a synergistic effect of cyclin D1 and estradiol on the transcriptional activation of the estrogen receptor was found. We have consistently found that cyclins D2 and D3 activate the estrogen receptor to some extent, while Zwijsen et al. found that cyclins D2 and D3 were completely defective with respect to activation of the estrogen receptor. Lastly, we have consistently found that 4-hydroxytamoxifen and, to a greater extent, ICI 182,780 significantly inhibited cyclin D1-mediated activation of the estrogen receptor, which is in contrast to the findings of Zwijsen et al. Of interest, ICI 182,780 does not appear to inhibit the association of cyclin D1 and the estrogen receptor (negative data not shown). It will be particularly important to further analyze the abilities of various antiestrogens to inhibit cyclin D1 activation of the estrogen receptor. In the setting of primary breast adenocarcinomas which specifically recur with resistance to antiestrogen treatment, following an initial treatment with antiestrogens, it will be particularly interesting to determine if overexpression of cyclin D1 might be responsible for the development of resistance to hormonal treatment.

Cyclin D1 can transcriptionally activate the estrogen receptor in the absence of an agonist, suggesting the possibility that cyclin D1 operates through the ligand-independent AF-1 transactivation domain of the estrogen receptor. However, activation of the estrogen receptor by cyclin D1 was at least partially inhibited by the antagonist 4-hydroxytamoxifen (Fig. 2), and cyclin D1 was found to associate with an estrogen receptor mutant lacking AF-1 (Fig. 4B), suggesting the possibility that cyclin D1 stimulates AF-2-dependent transactivation. Efficient steroid hormone-dependent activation (AF-2) of the estrogen receptor has been shown to involve the direct or indirect association with a number of nuclear receptor coactivators: RIP140/160, SRC1, TRIP1, and TIF1 (10, 23, 36, 37, 45, 55) and CBP/p300 (11, 25, 30, 59). Importantly, the association of these coactivators with nuclear receptors is ligand dependent, which is in contrast to the situation with cyclin D1 and the estrogen receptor. Whether the binding of the estrogen receptor to cyclin D1 is direct or involves a functional interaction with coactivators or CBP/p300 remains to be determined. The possible involvement of coactivators in cyclin D1-estrogen receptor association might explain why a high percentage of input cyclin D1 and estrogen receptor in transfected cells was not found associated.

There exist data suggesting that estrogen-dependent activation of the estrogen receptor leads to stimulation of the Ras/mitogen-activated protein (MAP) kinase pathway (42). The Ras/MAP kinase pathway has been implicated in the regulation of cyclin D1 expression (2, 35). There are also data suggesting that growth factors such as epidermal growth factor and insulin-like growth factor can stimulate AF-1-dependent transcription of the estrogen receptor via activation of and direct phosphorylation by MAP kinase (9, 33). Together, these data suggest that the estrogen receptor functions upstream of cyclin D1. However, they also suggest that MAP kinase operates both upstream and downstream of the estrogen receptor. Our data indicate that cyclin D1 operates upstream of the estrogen receptor. Consistent with this observation, some groups have shown that overexpression of cyclin D1 in breast cancer correlates with an estrogen receptor-positive status (1, 4, 6, 8, 12, 18, 41, 61). Importantly, most estrogen receptor-positive breast cancers occur in postmenopausal women who have low estrogen levels. Thus, overexpression of cyclin D1 in postmenopausal breast cancer may allow for abnormal estrogen receptor activity in the presence of low estrogen levels. It remains to be determined whether the presence of the estro-

gen receptor is required for the oncogenic action of cyclin D1 in breast cancer.

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