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17β-Estradiol (E2) Induces cdc25A Gene Expression in Breast Cancer Cells by Genomic and Non-Genomic Pathways

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Abstract Cdc25A is a potent tyrosine phosphatase that catalyzes specific dephosphorylation of cyclin/cyclin-dependent kinase (cdk) complexes to regulate G_1 to S-phase cell cycle progression. Cdc25A mRNA levels are induced by 17β-estradiol (E2) in ZR-75 breast cancer cells, and deletion analysis of the cdc25A promoter identified the −151 to −12 region as the minimal E2-responsive sequence. Subsequent mutation/deletion analysis showed that at least three different *cis*-elements were involved in activation of cdc25A by E2, namely, GC-rich Sp1 binding sites, CCAAT motifs that bind NF-Y, and E2F sites that bind DP/E2F1 proteins. Studies with inhibitors and dominant negative expression plasmids show that E2 activates cdc25A expression through activation of genomic ERα/Sp1 and E2F1 and cAMP-dependent activation of NF-YA. Thus, both genomic and non-genomic pathways of estrogen action are involved in induction of cdc25A in breast cancer cells. J. Cell. Biochem. 99: 209–220, 2006. © 2006 Wiley-Liss, Inc.

Key words: estrogen; activation; cdc25A; breast cancer

Cdc25A phosphatase is expressed in all eukaryotes and, in mammals, the cdc25A, cdc25B, and cdc25c forms are encoded by distinct genes [Sadhu et al., 1990; Galaktionov and Beach, 1991; Draetta and Eckstein, 1997]. Cdc25 phosphatases play a critical role in cell cycle progression by regulating phosphorylation of cyclin-dependent kinase (cdk)/cyclin complexes at specific phases of the cell cycle [Sadhu et al., 1990; Galaktionov and Beach, 1991; Nagata et al., 1991; Sebastian et al., 1993; Hoffmann et al., 1994; Jinno et al., 1994; Galaktionov et al., 1995a; Draetta and Eckstein, 1997; Gabrielli et al., 1997; Garner-Hamrick and Fisher, 1998; Lammer et al., 1998; Blomberg and Hoffmann, 1999]. Cdc25 phosphatases are dual-specific protein tyrosine phosphatases and catalyze rapid dephosphorylation of cyclin/cdk complexes on threonine[14] and tyrosine[15] [Galaktionov and Beach, 1991; Gautier et al., 1991; Gu et al., 1992; Sebastian et al., 1993]. Cdc25A regulation of G_1 to S-phase progression in the cell cycle is primarily associated with dephosphorylation and activation of the cdk2/cyclin A and cdk2/cyclin E complexes [Galaktionov and Beach, 1991; Hoffmann et al., 1994; Jinno et al., 1994; Blomberg and Hoffmann, 1999]. Cdc25 phosphatases are overexpressed in a wide variety of tumors, and overexpression of cdc25A and cdc25B in cooperation with ras immortalizes mouse embryo fibroblasts [Galaktionov et al., 1995b; Gasparotto et al., 1997; Kudo et al., 1997; Hernandez et al., 1998].

Mitogen activation of cells results in increased expression of cdc25A in G_1 for subsequent activation of cdk2 and progression of cells through S-phase. 17β-Estradiol (E2) induces cdc25A protein and mRNA levels in MCF-7 breast cancer cells [Wang et al., 1998; Foster

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et al., 2001]. Foster et al. [2001] studied interactions of cdc25A with multiple G_1 to S-phase cell cycle regulators in breast cancer cells and showed that cdk2 activity is required for activation of cdc25A which is a critical protein in hormone-dependent proliferation of MCF-7 breast cancer cells.

The mechanism of cdc25A transcriptional activation by E2 was investigated in this study in ZR-75 breast cancer cells. E2 induced cdc25A mRNA levels within 3–6 h after treatment and, in transient transfection studies, E2 also induced luciferase activity in cells transfected with pcdc25A-1, a construct containing the −460 to +129 region of the cdc25A gene promoter. Subsequent deletion and mutational analysis of the cdc25A gene promoter shows that the minimal E2-responsive −151 to −12 region of the promoter contains multiple E2F, Sp1, and NFY binding sites. Mutation and deletion analysis of the hormone-responsive region of the cdc25A gene promoter reveals that E2-dependent transactivation involves both genomic and non-genomic pathways of estrogen action. ERα/Sp1 is required for activation of the GC-rich sites and E2F-1 is activated by both induction of E2F-1 protein and release of phosphorylated retinoblastoma (Rb) protein. In contrast, motifs containing NFY binding sites mediate hormone-dependent induction through non-genomic activation of cAMP/protein kinase A (PKA) and phosphorylation of NF-YA. Hormone-dependent activation of genomic and non-genomic pathways of estrogen action are critical for breast cancer cell growth [Migliaccio et al., 1996; Castoria et al., 2001; Hall et al., 2001], and results of this study demonstrate that induction of cdc25A by E2 is dependent on both pathways.

MATERIALS AND METHODS

Cells, Chemicals, and Biochemicals

ZR-75 cells were obtained from American Type Culture Collection (ATCC, Manassas, VA). Cells were routinely maintained in RPMI 1640 medium with phenol red (Invitrogen, Carlsbad, CA) and supplemented with 10% fetal bovine serum (FBS) (Sigma, St. Louis, MO) plus antibiotic/antimycotic solution (Sigma) in an air-carbon dioxide (95:5) atmosphere at 37°C. For transient transfection studies, cells were grown for 1 day in DME/F12 medium without phenol red (Sigma) containing 2.5% FBS stripped with dextran-coated charcoal. ICI 182780 was kindly provided by Dr. Alan Wakeling (AstraZenaca Pharmaceuticals, Macclesfield, UK). The kinase inhibitors SQ22536 and H-8 were purchased from Calbiochem (La Jolla, CA). Luciferase and β-galactosidase enzyme assay systems were purchased from Promega Corp. (Madison, WI). [γ-^{32}P]ATP (3,000 Ci/mmol), were purchased from Perkin Elmer Life Sciences. Sp1 (sc-59), E2F1 (sc-251), NF-YA (sc-10779), and ERα (sc-543) antibodies were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). All other chemicals and biochemicals were the highest quality available from commercial sources.

Cloning and Plasmids

Cdc25A promoter variants, pcdc25A-2 (−209/+129), pcdc25A-3 (−184/+129), pcdc25A-4 (−31/+129), pcdc25A-5 (−151/−12), and pcdc25A-6 (−184/−65), were made by PCR amplification using pcdc25A-1 as template (Table I). The PCR products were purified and ligated into pGL2 basic vector (Promega Corp.) between *Xho*I and *Hind*III polylinker sites. Site-directed mutagenesis was performed using the two-step overlap extension PCR method (Table I). PCR primers were synthesized by Genosys/Sigma (The Woodlands, TX). All ligation products were transformed into competent *Escherichia coli* cells. Plasmids were isolated, and clones were confirmed by DNA sequencing (Gene Technologies Laboratory, Texas A&M University). The pcdc25A-1 was kindly provided by Joan Massagué (Cell Biology Program and Howard Hughes Medical Institute, Memorial Sloan-Kettering Cancer Center, New York, NY). Wild-type human estrogen receptor α (hERα) expression plasmid was provided by Dr. Ming-Jer Tsai (Baylor College of Medicine, Houston, TX). The ER deletion mutant ERα11C was provided by Professor Pierre Chambon (Institut de Genitique et de Biologie Moleculaire et Cellulaire, Illkirch, France). The DP1 mutants DP1Δ103-126 and DP1Δ127-411 bind E2F-1 but not DNA [Wu et al., 1996] and were kindly provided by Dr. Harlow (Harvard Medical School, Boston, MA). E132 is an E2F-1 mutant that also does not bind DNA [Cress et al., 1993] and was provided by Dr. Nevins (Duke University, Durham, NC). The dominant negative Sp1 construct (pEBG-Sp) expresses the DNA binding domain (amino acids 592–758) but not the activation domain of Sp1 [Petersohn and Thiel, 1996] and was provided by Dr. Thiel (University of Cologne,

TABLE I. Summary of Primers for Generating Variant Constructs of pcdc25A

	Primers[a]	Template
pcdc25A-2	Forward: 5′-GTA TCT CGA GCT CTT CTG CTC TGG GCT-3′ Reverse: 5′-CTT TAT GTT TTT GGC GTC TTC CA-3′	pcdc25A-1
pcdc25A-3	Forward: 5′-GTA GCT CGA GTT CTG AFGA GCC GAT GAC CTG-3′ Reverse: 5′-CTT TAT GTT TTT GGC GTC TTC CA-3′	pcdc25A-1
pcdc25A-4	Forward: 5′-GTA TCT CGA GAG CAG CTG GCC CCA CTG A-3′ Reverse: 5′-CTT TAT GTT TTT GGC GTC TTC CA-3′	pcdc25A-1
pcdc25A-5	Forward: 5′-GCT CGA GAG CCG CTT TCT TCT TCC CCT CT-3′ Reverse: 5′-GAT AAG CTT CTC AGT GGG GCC AGC TGC T-3′	pcdc25A-1
pcdc25A-6	Forward: 5′-GTA GCT CGA GTT CTG AFGA GCC GAT GAC CTGm3′ Reverse: 5′-CCG CAA GCT TGA ATC CAC CAA TCA GTA AGC-3′	pcdc25A-1
pcdc25A-1m1	5′-CGC CCG GCT GGG **TT**C GAG GTA-3′	pcdc25A-1
pcdc25A-1m2	5′-CTG CTC TGG GCT C**TT** CCC CCT TC-3′	pcdc25A-1
pcdc25A-1m3	5′-CTA GGA AAG GGG **TT**C GGG GCA G-3′	pcdc25A-1
pcdc25A-1m12	5′-CTG CTC TGG GCT C**TT** CCC CCT TC-3′	pcdc25A-1m1
pcdc25A-1m13	5′-CTA GGA AAG GGG **TT**C GGG GCA G-3′	pcdc25A-1m2
pcdc25A-1m23	5′-CTA GGA AAG GGG **TT**C GGG GCA G-3′	pcdc25A-1m2
pcdc25A-1m123	5′-CGC CCG GCT GGG **TT**C GAG GTA-3′	pcdc25A-1m23
pcdc25A-5m1	5′-CTA GGA AAG GGG **TT**C GGG GCA G-3′	pcdc25A-5
pcdc25A-5m2	5′-GAT TCC GT**A A**GG CGC CAA C-3′	pcdc25A-5
pcdc25A-5m3	5′-GAT TCC GT**A A**GG CGC CAA C-3′	pcdc25A-5m1
pcdc25A-5m4	5′-GTT GCT T**AC** TGA TAC GTG GAT TCC-3′ 5′-CCT CTC ATT G**TA** CCA GCC TAG CTG-3′	pcdc25A-5

[a]Mutations are underlined and substituted bases are indicated in bold.

Cologne, Germany). The dominant negative NF-YA construct (Δ4YA13m29) encodes for a dominant negative NF-YA protein [Mantovani et al., 1994] and was provided by Dr. Mantovani (University of Milan, Milan, Italy). The pM-NF-YA expression plasmid was made by PCR using primers (forward primer, 5′-GGA ATT CAT GGA GCA GTA TAC GAC A-3′; reverse primer, 5′-GCT CTA GAT TAG GAA ACT CGG ATG A-3′) to amplify full-length NF-YA using the NF-YA expression plasmid as a template. The amplified products were cloned into the pM vector (CLONTECH Laboratories, Palo Alto, CA) between *Eco*RI and *Xba*I cloning sites.

Reverse Transcriptase (RT)-PCR Analysis

Cdc25A PCR primers (forward primer, 5′-AGC CCA AAG AGT CAA CTA ATC CAG A-3′; reverse primer, 5′-CCG GTA GCT AGG GGG CTC ACA-3′) were used to amplify 500 bp of human cdc25A mRNA. RNA was extracted using RNAzol B (Tel-Test, Friendswood, TX), following manufacturer's protocol. RNA was reverse-transcribed at 42°C for 1 h using oligo-d (T) primer, followed by PCR amplification of RT product using 1.25 mM $MgCl_2$, 0.4 μM each gene-specific primer, 0.4 μM dNTPs, and 2.5 units Taq DNA polymerase (Promega Corp.). Primer sets for cdc25A were added to the mixture, and the gene product was amplified in a PTC-200 thermal cycler (MJ Research, Watertown, MA). The PCR product was run on 1% agarose gel and the signal was quantitated by densitometry and normalized by GSα (forward primer, 5′-GTG ATC AAG CAG GCT GAC TAT-3′; reverse primer, 5′-GCT GCT GGC CAAC CAC GAA GAT).

Transient Transfection Assays

For transfection experiments, ZR-75 (2.25×10^5) cells were initially seeded in 12-well plates. Twenty-four hours after seeding, ZR-75 cells were transfected by the calcium phosphate method with cdc25A promoter-luciferase reporter constructs, hERα expression vector, and pCDNA3/His/lacZ (Invitrogen) that was used as a standard reference control plasmid for determining transfection efficiencies. After 5 h, cells were shocked with 25% glycerol and washed with PBS. Fresh DME/F12 without phenol red and charcoal-stripped FBS containing DMSO or 10 nM E2 in DMSO were added to the cells and incubated for 48 h. Cells from each experiment were then harvested in 100 μl of 1× Reporter lysis buffer (Promega Corp.). Luciferase assays were performed on 20 μl of the cell extract using the Luciferase assay system. Light emission was detected on a Lumicount luminometer (Packard, Meriden, CT). β-Galactosidase assays were performed on 20 μl of cell extract using the luminescent

Galacton-Plus assay kit (Tropix, Bedford, MA). The luciferase activity observed in each treatment group was normalized to β-gal activity obtained from the same sample to correct for transfection efficiencies. Data are expressed as relative luc/β-gal activities or fold induction (by E2 or other chemicals) compared to the solvent (DMSO) control. The fold induction graphs (Figs. 2A, 3B, 4A,B) were used to emphasize the changes in this response which are not as apparent from the luc/β-gal ratios due to changes in basal luciferase activity resulting from the transfected dominant negative expression plasmids.

Gel Electrophoretic Mobility Shift Assay (EMSA)

ZR-75 cells were seeded in 100-mm tissue culture plates using DME/F12 without phenol red, supplemented with 2.5% charcoal-stripped FBS. After 24 h, cells were treated for 1 h with DMSO or 10 nM E2. Nuclear extracts were obtained using the NE-PER nuclear and cytoplasmic extraction kit (Pierce, Rockford, IL) according to the manufacturer's instructions. Nuclear extracts obtained from different treatment groups were incubated for 20 min in HEGD buffer with poly-(dI-dC), unlabeled oligonucleotides or antibodies for supershift assays. The mixture was then incubated for additional 20 min after addition of ^{32}P-labeled oligonucleotide. Oligonucleotides used for EMSA in this study were synthesized, purified, and annealed, and 5 pmol of specific oligonucleotides were ^{32}P-labeled at the 5′-end using T4 polynucleotide kinase and [γ-^{32}P]ATP. Reaction mixtures were separated on 5% polyacrylamide gels (acrylamide:bis-acrylamide 30:0.8) at 140 V in 1× TBE (0.09 M Tris-HCl, 0.09 M boric acid, and 2 mM EDTA, pH 8.3). Gels were dried and protein–DNA complexes were visualized using a Storm 860 instrument (Amersham Biosciences, Piscataway, NJ). Oligonucleotides used for EMSA in this study were listed as follow (mutations are underlined and substituted bases are indicated in bold).

Consensus Sp1: 5′-ATT CGA TCG GGG CGG GGC GAG C-3′; cdc25A: 5′-ACT AGG AAA GGG GGG CGG GGC AGC A-3′; cdc25A mut: 5′-CTA GGA AAG GGG <u>**TT**</u>C GGG GCA G-3′.

Chromatin Immunoprecipitation Assay (ChIP)

ZR-75 cells (2 × 10^{7} cells) were treated with DMSO (time 0) or 10 nM 17β-estradiol (E2) for varying times. Cells were then fixed with 1.5% formaldehyde, and the crosslinking reaction was stopped by addition of 0.125 M glycine. Nuclei were collected, and sonicated to desired length (500∼1,000 bp) of chromatin. The chromatin was pre-cleared by addition of protein A-conjugated beads (Upstate), and incubation at 4°C for 1 h with gentle agitation. The pre-cleared chromatin was immunoprecipitated with antibodies (Santa Cruz Biotechnology) to Sp1, ERα, NF-Y, and E2F1 at 4°C overnight, together with protein A-conjugated beads. The beads were then extensively washed, and protein-DNA crosslinks were reversed. PCR was performed with the purified DNA and the following primers: (1) cdc25A forward primers, 5′-CTT CTG AGA GCC GAT GAC CT-3′; reverse primer, 5′-CAC CTC TTA CCC AGG CTG TC-3′; amplifying a 225 bp region of the human cdc25A promoter from −186 to +39; (2) CNAP1 forward primers (Activemotif), 5′-ATG GTT GCC ACT GGG GAT CT-3′; reverse primer, 5′-TGC CAA AGC CTA GGG GAA GA-3′; amplifying a 174 bp region of the CNAP1 exon; (3) glyceraldehyde-3-phosphate dehydrogenase (GAPDH) forward primers (Activemotif), 5′-TAC TAG CGG TTT TAC GGG CG-3′; reverse primer, 5′-TCG AAC AGG AG GAG CAG AGA GCG A-3′; amplifying a 167 bp region of human GAPDH promoter. PCR products were resolved on a 2% agarose gel.

Statistical Analysis

Statistical significance was determined by ANOVA and Student's *t*-test, and the levels of probability are noted. The results are expressed as mean ± SE for at least three separate (replicate) experiments for each treatment.

RESULTS

Previous studies show that E2 induced cdc25A mRNA and protein levels in MCF-7 cells and the antiestrogen ICI182780 inhibited the hormone-induced response [Foster et al., 2001]. Results in Figure 1A show that E2 also induced cdc25A mRNA levels in ZR-75 cells, and a twofold increase was observed from 6 to 24 h after treatment. The −460 to +129 region of the cdc25A promoter contains multiple GC-rich motifs, two CCAAT motifs, and two E2F-1 binding sites (Fig. 1B) [Iavarone and Massague, 1999]. ZR-75 cells were transfected with pcdc25A-1 which contains the −460 to +129 promoter insert and E2 induced luciferase

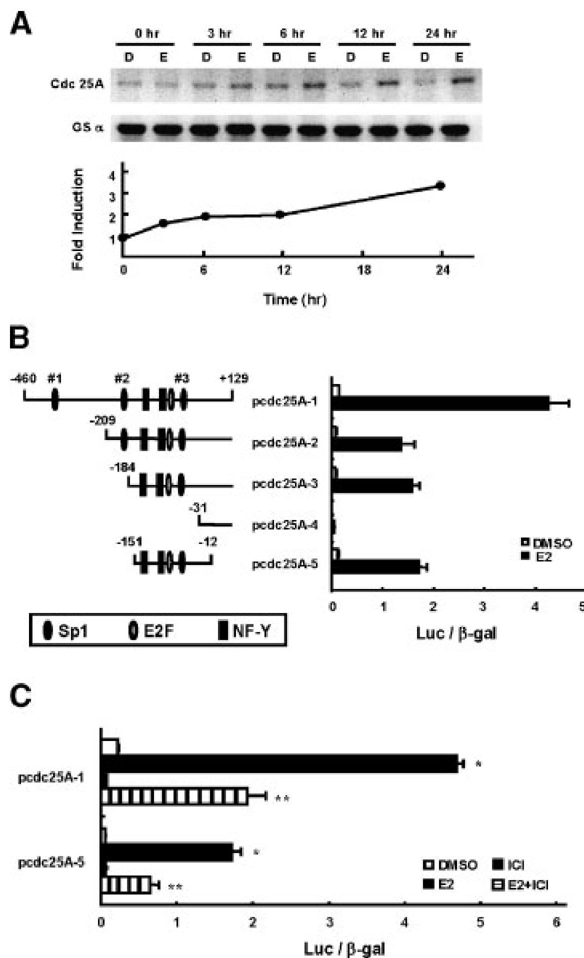

Fig. 1. Hormone inducibility of cdc25A in ZR-75 cells. **A**: Induction of mRNA levels. ZR-75 cells were treated with DMSO (solvent) or 10 nM E2 for different times, and mRNA levels were determined by RT-PCR analysis as described in the Materials and Methods. **B**: Deletion analysis of cdc25A promoter-reporter (luciferase) constructs. ZR-75 cells were transfected with the various constructs, cells were treated with DMSO or 10 nM E2, and luciferase activity determined as described in the Materials and Methods. **C**: Inhibition by ICI 182780. Cells were treated as described in (B); however, ICI 182780 and ICI 182780 plus E2 treatment groups were also added. Results in (B) and (C) are expressed as mean ± SE for at least three replicate determinations for each treatment group and significant ($P < 0.05$) induction by E2 (*) or inhibition by E2 plus ICI 182780 (**) are indicated.

activity. The deletion constructs pcdc25A-2, pcdc25A-3, and pcdc25A-4 containing the −209 to +129, −184 to +129, and −31 to +129 region, respectively, of the cdc25A promoter were also transfected into ZR-75 cells, and E2 induced activity in cells transfected with the former two constructs. The results show that basal activity was decreased approximately 40–50% after deletion of the upstream GC-rich site (#1), whereas deletion of GC-rich site #2 did not affect activity. Subsequent deletion of the −184 to −31 region of the promoter resulted in almost complete loss of basal and hormone-induced activity, suggesting that E2-responsiveness was associated with the GC-rich, CCAAT, and E2F-1 binding sites within this region of the cdc25A promoter. pcdc25A-5 was also highly E2-responsive in transient transfection assays and confirmed that the 3′ +129 to −11 region was not required for E2-induced transactivation. Thus, the −151 to −12 region of the cdc25A promoter was the minimal sequence required for E2-responsiveness, but this does not exclude hormone-responsiveness of other upstream (5′) *cis*-elements. Results in Figure 1C show that E2 induces transactivation in cells transfected with pcdc25A-1 and pcdc25A-5, and the hormone-induced response was significantly inhibited by the antiestrogen ICI 182780. These data confirm the role of E2/ER in mediating activation of cdc25A.

Previous studies in the laboratory have characterized activation of E2-responsive genes through interactions of ERα/Sp1 with GC-rich promoter sequences [Safe, 2001; Safe and Kim, 2004], and the cdc25A promoter contains three consensus Sp1 binding sites. Results in Figure 2A illustrate that mutation of one or more of the three GC-rich motifs at −384, −191, and −39 decreases hormone-responsiveness of several constructs compared to that observed in cells transfected with pcdc25A-1. These results suggest that hormone-dependent activation of ERα/Sp1 plays a role in mediating induction of cdc25A by E2, but other pathways also contribute to this response. Previous studies have demonstrated that ERα/Sp1-mediated transactivation, through interaction with GC-rich *cis*-elements, can also be observed for ERα11C/Sp1 in which the DNA binding domain of ERα has been deleted [Safe and Kim, 2004]. Figure 2B compares hormone-induced transactivation in ZR-75 cells cotransfected with pcdc25A-1 plus wild-type human ERα or ERα11C, and the induction of luciferase by E2 in cells cotransfected with the ERα deletion constructs confirms that the ERα/Sp1 pathway plays a role in hormonal regulation cdc25A. Gel mobility shift assays comparing the binding of nuclear extracts from ZR-75 cells to ^{32}P-labeled consensus Sp1 and cdc25A-Sp1 (−52 to −28) oligonucleotides show a similar pattern of protein–DNA interactions (Fig. 2C). Radiolabeled GC-rich (Sp1) and cdc25A oligonucleotides alone did

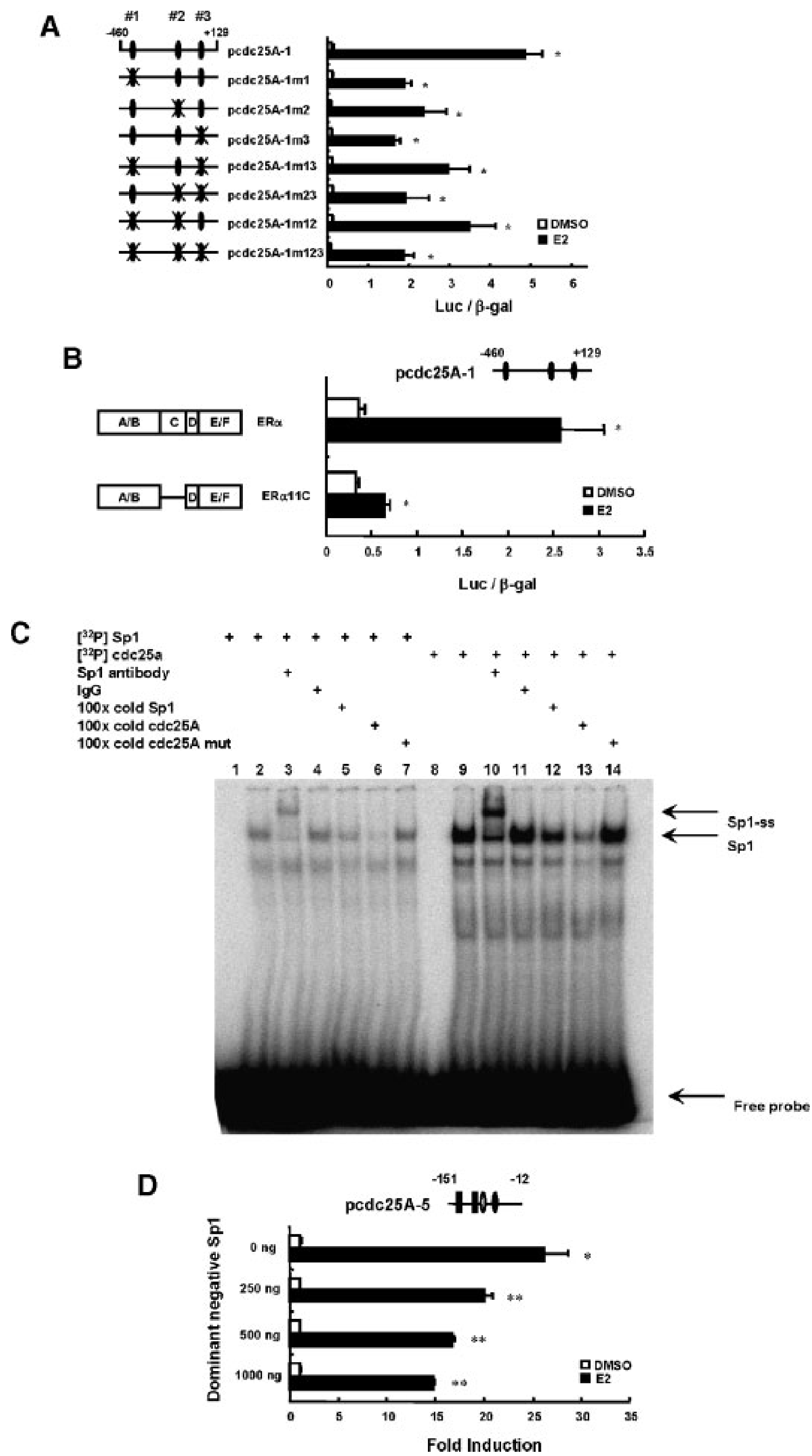

Fig. 2.

not give retarded bands (lanes 1 and 8); incubation with nuclear extracts gave one major retarded band (lanes 2 and 9) which was supershifted with Sp1 antibody (lanes 3 and 10) and unaffected by non-specific IgG (lanes 4 and 11). Both retarded bands were decreased after competition with 100-fold excess unlabeled Sp1 (lanes 5 and 12) and cdc25A (lanes 6 and 13) oligonucleotides but not by mutant cdc25A oligonucleotide (lanes 7 and 14). The role of Sp1 in mediating hormone-induced luciferase activity in cells transfected with pcdc25A-5 was also confirmed by decreased inducibility after cotransfection with dominant negative Sp1 expression plasmid (Fig. 2D). Approximately 40% of hormone-induced transactivation was observed in replicate studies, thus confirming a role for ERα/sp1 in mediating activation of cdc25A.

Mutation analysis of the −151 to −12 region of the promoter was also determined in ZR-75 cells transfected with pcdc25A-5 and four constructs containing mutations at the GC-rich (pcdc25A-5m1), E2F-1 (pcdc25A-5m2), GC-rich and E2F-1 (pcdc25A-5m3), and NFY (pcdc25A-5m4) motifs. E2 induced activity in cells transfected with wild-type and mutant constructs (Fig. 3A); however, the fold-induction was lower in cells transfected with the mutant plasmids. The role of NF-YA in mediating activation of cdc25A by E2 was further investigated (Fig. 3B) in cells transfected with constructs containing only the CCAAT sites (pcdc25A-6 and pcdc25A-5m3) and a dominant negative expression plasmid for NF-YA (Δ4YA13m29) [Mantovani et al., 1994]. The results showed that dominant negative NF-YA significantly inhibited hormonal activation of both pcdc25A-6 and pcdc25A-5m3. Previous studies in this laboratory confirmed expression of NF-YA in ZR-75 cells and showed that hormonal activation of CCAAT motifs in the E2F-1 gene promoter were due to cAMP/PKA-dependent activation of NF-YA through non-genomic pathways [Ngwenya and Safe, 2003]. Results in Figure 3C show that the PKA inhibitor SQ22536 inhibited induction of luciferase activity by E2 in cells transfected with pcdc25A-5m3, and both H8 (adenylcyclase inhibitor) and SQ22356 significantly inhibited hormonal activation of chimeric GAL4-NF-YA in ZR-75 cells transfected with expression plasmids for GAL4-NF-YA and a pGAL4-luc reporter plasmid (Fig. 3D). These results suggest that the CCAAT sites within the cdc25A promoter that bind NF-Y proteins are activated through non-genomic pathways of estrogen action. However, in cells transfected with pcdc25A-1 and pcdc25A-5, E2-induced activity was inhibited 12–15% in cells cotreated with 100 μM SQ22536 (data not shown).

The E2F1 binding site at −63 is another potential E2-responsive motif in the cdc25A promoter since E2F1 is induced by E2 in MCF-7 and ZR-75 cells [Wang et al., 1999; Ngwenya and Safe, 2003], and E2 also induces Rb protein phosphorylation which results in derepression of E2F1. E2 induces transactivation in ZR-75 cells transfected with pcdc25A-5, and cotransfection with dominant negative expression plasmids for the E2F1 binding partner DP1 (DPΔ103-126 and DRΔ127-411) or E2F1 (E132) [Johnson et al., 1993; Wu et al., 1996] significantly decreased transactivation (Fig. 4A). A second E2F1 motif at −3 in the cdc25A promoter is present in pcdc25A-4 which exhibit low activity (Fig. 1B) but is hormone inducible (approximately 2.5-fold). Results in Figure 4B show that the fold induction of luciferase activity by E2 in cells transfected with pcdc25A-4 was also significantly inhibited after cotransfection of the dominant negative DPΔ103-126, DPΔ127-411, and E132 expression plasmids. However, these plasmids also

Fig. 2. Role of ERα/Sp1 in mediating activation of cdc25A. **A**: Mutational analysis of GC-rich sites. ZR-75 cells were transfected with pcdc25A-1 or a series of mutant constructs, treated with DMSO or 10 nM E2, and luciferase activity determined as described in the Materials and Methods. **B**: Inducibility by ERα11C. Cells were treated as described in (A); however, both wild-type ERα and ERα11C were cotransfected. **C**: Gel mobility shift assay. Nuclear extracts from ZR-75 cells were incubated with ^{32}P-labeled oligonucleotides and other antibodies/oligonucleotides, and a gel mobility shift assay was carried out as described in the Materials and Methods. Sp1-DNA binding and antibody supershifted complexes are indicated by arrows. **Lanes 1** and **8** represent incubation of the free probe alone. **D**: Effects of dominant negative Sp1 expression. Cells were treated as described in (A); however, increasing amounts of dominant negative Sp1 expression plasmid (0–100 ng) were also cotransfected. Results in (A), (B), and (D) are expressed as mean ± SE for three replicate determinations for each treatment group. Significant ($P < 0.05$) induction by E2 (*) or inhibition of E2-induced activity by dominant negative Sp1 expression plasmid (**) are indicated.

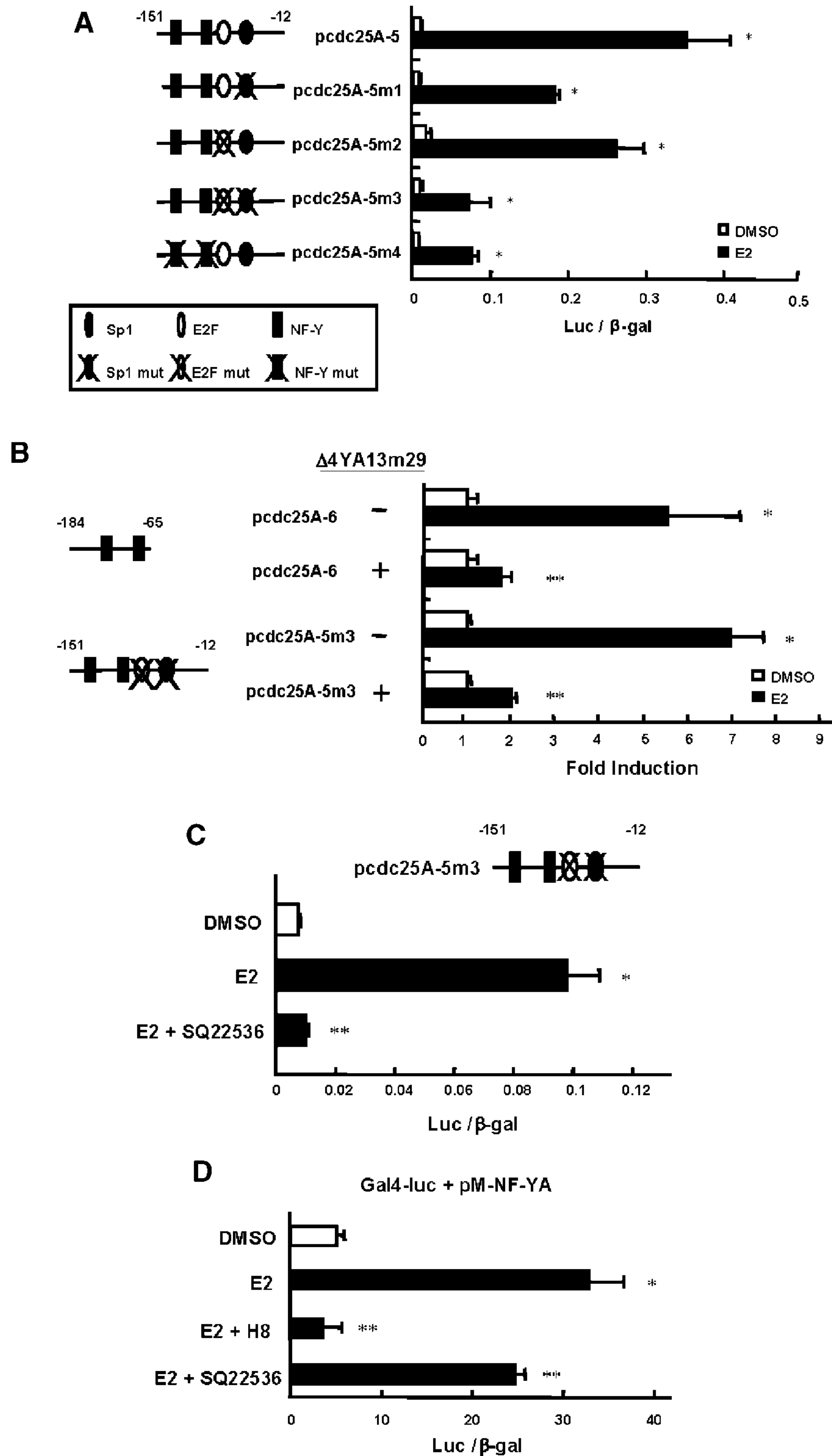

Fig. 3. Role of CCAAT sites in hormonal activation of cdc25A. **A**: Mutational analysis of −151 to −12 region of the cdc25A promoter. ZR-75 cells were transfected with the various constructs, treated with DMSO or E2, and luciferase activity determined as described in the Materials and Methods. **B**: Effects of dominant negative Δ4YA13m29 expression. Cells were transfected and treated as described in (A), and the effects of Δ4YA13m29 (dominant negative NF-YA) expression were also determined. **C**: Inhibition of transactivation by SQ22536. ZR-75 cells were transfected with pcdc25A-5m3 as described in (A) and treated with DMSO, E2, and E2 plus SQ22536. SQ22536 alone did not affect activity (data not shown). **D**: Inhibition by H8 and SQ22536. ZR-75 cells were transfected with GAL4-luc/pM-NF-YA as described in (A) and treated with DMSO, E2, E2 plus H8, and E2 plus SQ22536. H8 plus SQ22536 alone did not affect activity (data not shown). Results in (A–D) are mean ± SE for three replicate determinations for each treatment group, and significant induction by E2 (*) or inhibition of the induced response (**) are indicated.

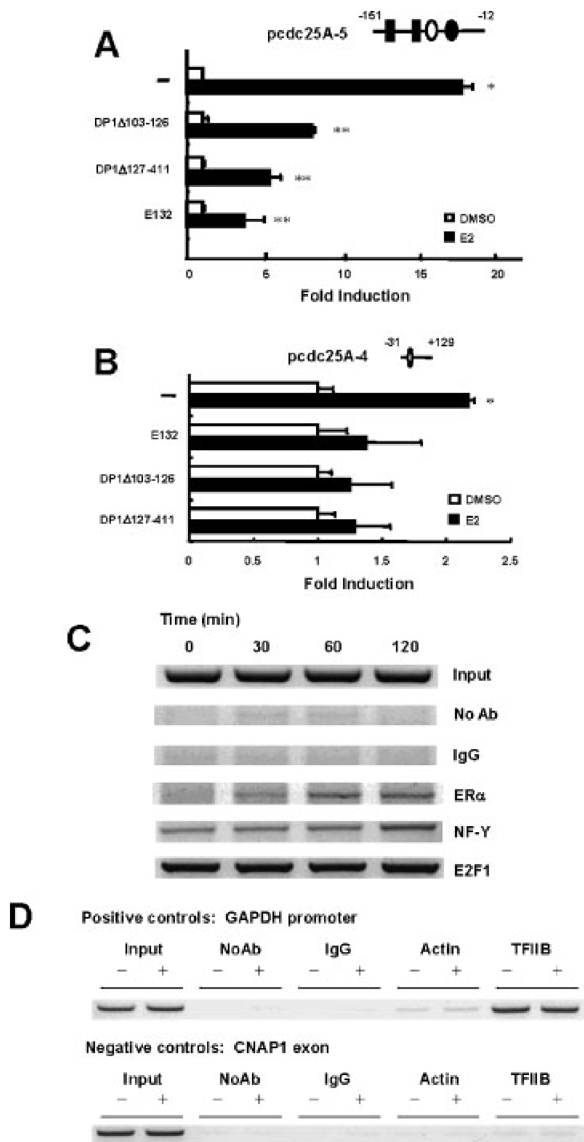

Fig. 4. Role of E2F1 in hormone activation of cdc25A and ChIP assay. pcdc25A-5 (**A**) and pcdc25A-4 (**B**) were transfected in ZR-75 cells, treated with DMSO or 10 nM E2, cotransfected with dominant negative expression plasmids for E2F1 (E132) or DP1 (DP1Δ103-126; DP1Δ127-411), and luciferase activity determined as described in the Materials and Methods. Results are expressed as mean ± SE for three replicate determinations for each treatment group, and significant ($P < 0.05$) induction by E2 (*) and inhibition of this response (**) are indicated. **C**: ChIP assay. ZR-75 cells were treated with DMSO (0 time) or 10 nM E2 for 30, 60, or 120 min, and the ChIP assay was carried out essentially as described in the Materials and Methods using antibodies to ERα, NF-YA, and E2F1, and non-specific IgG. Sp1 antibodies also showed consistent binding to the cdc25A promoter over the 0–120 min time period (data not shown). The primers amplified a 225 bp sequence from −186 to +39 in the cdc25A promoter. **D**: TFIIB promoter interactions. Control ChIP analysis showed that TFIIB specifically interacts with the GAPDH but not CNAP1 promoters as previously described [Hong et al., 2004].

significantly altered basal activity in solvent (DMSO)-treated cells. ChIP with primers targeted to the proximal region of the cdc25A promoter (Fig. 4C) confirmed that E2F1 and NF-YA were constitutively bound to the promoter and ERα binding is increased after treatment with E2. This is consistent with association of ERα which interacts with promoter bound Sp1 (data not shown). Results obtained using immunoprecipitation with TFIIB antibodies show that TFIIB binds to the GAPDH gene promoter but not exon 1 of CNAP1 as previously described [Hong et al., 2004], and this serves as a positive control for the ChIP assay. These results demonstrate that hormone-dependent induction of cdc25A gene expression in ZR-75 cells requires activation of both genomic and non-genomic pathways of estrogen action. The multiple E2-responsive *cis*-elements identified in this study demonstrate the complexity of hormonal regulation of cdc25A, and it is possible that other promoter regions and interactions between DNA-bound transcription factors may also be important.

DISCUSSION

E2 is a mitogen in ER-positive breast cancer cell lines and induced cell proliferation is accompanied by induction of multiple functional classes of genes and protooncogenes including those required for cell cycle progression and nucleotide biosynthesis [Migliaccio et al., 1996; Castoria et al., 2001; Hall et al., 2001; Safe, 2001; Safe and Kim, 2004]. For example, treatment of MCF-7 cells with E2 significantly enhances G_0/G_1 to S-phase progression and is accompanied by induction of cyclin D1, E2F1, cdk activities, Rb phosphorylation, downregulation of the cdk inhibitors p21 and p27 [Wang et al., 1998, 1999; Castro-Rivera et al., 2001; Foster et al., 2001; Ngwenya and Safe, 2003]. Previous studies also report that E2 also induces cdc25A [Wang et al., 1998; Foster et al., 2001], and this is consistent with the observed G_0/G_1 to S-phase progression. This article also shows that cdc25A gene expression is also induced by E2 in ZR-75 cells and pcdc25A-1 and related constructs are hormone-responsive and inhibited by the antiestrogen ICI 182780 (Fig. 1). This study has focused on investigating the molecular mechanisms of hormone-dependent activation of cdc25A by extensive promoter analysis.

The cdc25A promoter does not contain a consensus or non-consensus estrogen responsive element (ERE); however, several GC-rich sites that bind Sp1 protein are present in the E2-responsive proximal region of the promoter [Iavarone and Massague, 1999]. Previous studies in this laboratory have demonstrated that ERα/Sp1 interactions with GC-rich motifs in several gene promoters including cyclin D1 were required for hormone-induced transactivation [Safe, 2001; Safe and Kim, 2004]. Sp1 knockdown in MCF-7 cells [Abdelrahim et al., 2002] inhibited E2-induced G_0/G_1 to S-phase progression in MCF-7 cells, suggesting a possible role for ERα/Sp1 activation of cdc25A through interaction with one or more of the proximal GC-rich sites. The results (Fig. 2) demonstrate that this pathway contributes to hormonal activation of cdc25A; however, the induction response is still observed even with constructs in which all three GC-rich sites are mutated (Fig. 2A).

The minimal E2-responsive region of the cdc25A promoter (−151 to −12) contains GC-rich, CCAAT and E2F1 motifs, and a second E2F1 site at −3 is also present outside this minimal promoter. Previous studies showed that E2F1–Rb complexes at the −3 site were important for inhibition of cdc25A by the bovine papillomavirus E2 protein in cervical adenocarcinoma cells [Wu et al., 2000]. In ZR-75 cells, only minimal basal activity was observed in cells transfected with a construct (pcdc25A-4) containing the −3 but not −62 E2F1 site (Fig. 1B); however, pcdc25A-4 was E2-responsive (Fig. 4B). Both dominant negative DP-1/E2F1 expression plasmids decreased hormone-induced transactivation in ZR-75 cells transfected with pcdc25A-4 (Fig. 4B) and pcdc25A-5 (Fig. 4A) confirming a role for E2F1 in mediating E2-dependent induction of cdc25A. ChIP assays confirm interaction of E2F1 with the cdc25A promoter (Fig. 4C), suggesting that induction of cdc25A is due, in part, to E2-dependent Rb phosphorylation and subsequent derepression of E2F1. Both NF-YA and E2F1 are constitutively bound to the cdc25A promoter and their band intensities are not significantly increased after treatment with hormone. This observation is consistent with hormonal activation of both transcription factors through phosphorylation of Rb and NF-YA. Moreover, since E2 also induces E2F1 mRNA/protein expression in ZR-75 cells [Wang et al., 1999; Ngwenya and Safe, 2003], this pathway may also contribute to the induction response at later time points, but would not be apparent in this study due to the shorter duration (2 h) of the ChIP experiment.

Constructs containing the two CCAAT sites were also E2-responsive, and expression of dominant negative NF-YA significantly blocked hormone-dependent activation (Fig. 3). Previous studies show that NF-YA bound to CCAAT sequences in the E2F1 gene promoter [Wang et al., 1999; Ngwenya and Safe, 2003] was also activated through non-genomic pathways of estrogen action that involved activation of cAMP/PKA [Ngwenya and Safe, 2003]. Hormonal activation of cAMP/PKA has previously been observed [Aronica and Katzenellenbogen, 1991, 1993; Aronica et al., 1994; El-Tanani and Green, 1997] and involves phosphorylation of downstream transcription factors including NF-YA. Results in Figure 3A show that E2 induced transactivation in cells transfected with pcdc25A-6 and pcdc25A-5m3 or GAL4-NF-YA/pGAL4-luc. The results, coupled with the effects of cAMP/PKA inhibitors and dominant negative NF-YA expression, confirm that induction of cdc25A by E2 also involves activation of cAMP/PKA and subsequent kinase-dependent activation of NF-YA. Previous studies have shown that activation of human TIMP [Zhong et al., 2000] and hormone-dependent activation of E2F1 [Ngwenya and Safe, 2003] were dependent on cAMP/PKA phosphorylation of NF-YA bound to CCAAT motifs. However, based on PKA inhibitor studies in cells transfected with pcdc25A1 and pcdc25A-5 and treated with E2, we conclude that the contribution of the non-genomic (cAMP/PKA) pathway of estrogen action was relatively small (<15%). The activation of cdc25A by genomic and non-genomic pathways of estrogen action is not unprecedented since c-fos, cyclin D1, bcl-2, and E2F1 are also induced by E2 through both nuclear and extranuclear ER pathways in breast cancer cells [Dong et al., 1999; Wang et al., 1999; Castro-Rivera et al., 2001; Duan et al., 2001, 2002; Ngwenya and Safe, 2003]. These genes are important for cell growth/survival and clearly highlight the importance of both pathways for ER-positive breast cancer cell proliferation.

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