Potentiation of Human Estrogen Receptor α Transcriptional Activation through Phosphorylation of Serines 104 and 106 by the Cyclin A-CDK2 Complex*

Inez Rogatsky‡‡, Janet M. Trowbridge‡‡§, and Michael J. Garabedian¶
Department of Microbiology and the Kaplan Comprehensive Cancer Center, New York University School of Medicine, New York, New York 10016

Both estradiol binding and phosphorylation regulate transcriptional activation by the human estrogen receptor α (ER). We have previously shown that activation of the cyclin A-CDK2 complex by overexpression of cyclin A leads to enhanced ER-dependent transcriptional activation and that the cyclin A-CDK2 complex phosphorylates the ER N-terminal activation function-1 (AF-1) between residues 82 and 121. Within ER AF-1, serines 104, 106, and 118 represent potential CDK phosphorylation sites, and in this current study, we ascertain their importance in mediating cyclin A-CDK2-dependent enhancement of ER transcriptional activity. Cyclin A overexpression does not enhance transcriptional activation by an ER derivative bearing serine-to-alanine changes at residues 104, 106, and 118. Likewise, the cyclin A-CDK2 complex does not phosphorylate this triple-mutated derivative in vitro. Individual serine-to-alanine mutations at residues 104 and 106, but not 118, decrease ER-dependent transcriptional enhancement in response to cyclin A. The same relationship holds for ER phosphorylation by cyclin A-CDK2 in vitro. Finally, enhancement of ER transcriptional activation by cyclin A is evident in the absence and presence of estradiol, as well as in the presence of tamoxifen, suggesting that the effect of the cyclin A-CDK2 on ER transcriptional activation is AF-2-independent. These results indicate that the enhancement of ER transcriptional activation by the cyclin A-CDK2 complex is mediated via the AF-1 domain by phosphorylation of serines 104 and 106. We propose that these residues control ER AF-1 activity in response to signals that affect cyclin A-CDK2 function.

The estrogen receptor α (ER),1 a transcription factor that controls the expression of a number of genes involved in cellular differentiation and proliferation in a wide variety of tissues (1–4), is regulated by ligand binding and phosphorylation. The receptor is structurally similar to other members of the nuclear receptor superfamily in that separate receptor activities such as DNA and ligand binding are localized to distinct regions of the protein (5). ER contains at least two transcriptionally active domains: constitutively active AF-1 in the N terminus of the protein and ligand-dependent AF-2 at the ER C terminus. AF-1 and AF-2 can act independently or synergize to effect transcriptional activation (6, 7). Interestingly, they are differentially affected by certain ligands such as tamoxifen, which blocks AF-2 action but activates AF-1, accounting for the mixed agonist-antagonist properties of this agent (8, 9).

Although ligand binding is considered essential for the full activation of ER, it has long been recognized that the receptor is subject to post-translational alterations, such as phosphorylation, which also regulate its activity (10). The phosphorylation of three N-terminus-located residues, serines 104, 106, and 118, which are the focus of our current studies, appears to regulate receptor-dependent transcriptional activation (11, 12). This additional level of regulation most likely serves to modulate receptor activity in a cell- and physiologically-specific manner. Indeed, it has been suggested that phosphorylation of steroid receptors may determine promoter specificity, cofactor interaction, strength and duration of receptor signaling, and ligand-independent receptor transactivation. Since ER can serve as a transcriptional repressor as well as an activator, effecting cellular proliferation in some settings and arrest or differentiation in others (13–17), this level of complexity and flexibility is not surprising.

Much work has been directed toward elucidating which circumstances induce ER phosphorylation and which receptor sites are the targets for this modification. Although a number of potential phosphorylation sites have been identified, the kinases that modify these residues are not fully established. In addition, ER phosphorylation patterns appear to be cell-type specific. Serine residues are the predominantly modified amino acids present in ER, and four of these (Ser-104, Ser-106, Ser-118, and Ser-167) are clustered in the N terminus within AF-1 of the receptor (12). The sequence context surrounding serines 104, 106, and 118 suggests that they may be targeted by the serine/proline-directed protein kinases, which include mitogen-activated protein kinase family members, glycogen synthase kinase-3, and the cyclin-dependent kinases (CDKs). Indeed, Ser-118 has been shown to be phosphorylated by the mitogen-activated protein kinase family member, extracellular signal-regulated kinase 1 (ERK-1), in vitro and to facilitate ER ligand-independent activation in vivo (18, 19). Recent findings also suggest that Ser-118 is phosphorylated by a kinase distinct from mitogen-activated protein kinase upon estradiol treatment, suggesting that Ser-118 is the target for multiple kinases in vivo (20). Serine 167 has been shown to be phosphorylated by p90s6k in vitro and to regulate ER AF-1-dependent transcrip-
Phosphorylation of ER by Cyclin A-CDK2

Phosphorylation in vitro (21); interestingly, this site also lies within the consensus sequence targeted by both calmodulin-dependent protein kinase II and casein kinase II and has been reported to be phosphorylated by the latter in vitro, although the physiological significance of this finding remains uncharacterized (22). Three of the putative phosphorylation sites, serines 104, 106, and 118, are critical for ER-dependent transcriptional enhancement and are phosphorylated in COS-1 cells (11). In an attempt to identify the kinase(s) responsible for this alteration, we have previously shown that the cyclin A-CDK2 complex phosphorylates ER between residues 82 and 121 in vitro and that overexpression of cyclin A in vivo results in ligand-independent hyperphosphorylation of the receptor (23). Regulatory effects of cyclin-CDK complexes upon steroid/nuclear receptors have been described for three other family members. The glucocorticoid receptor is phosphorylated by two cyclin-CDK complexes, A-CDK2 and E-CDK2 (24). The progestrone receptor is phosphorylated by the cyclin A-CDK2 complex, and the retinoic acid receptor is phosphorylated by cyclin H-CDK7, leading to ligand-dependent enhancement of receptor transcriptional activation (25, 26).

To identify ER residues phosphorylated by the cyclin A-CDK2 complex, we have generated a series of phosphorylation site-specific mutant ER derivatives at serines 104, 106, and 118, the three potential CDK phosphorylation sites. We examined the effect of cyclin A overexpression on ER transcriptional activation of these serine-to-alanine mutants, individually and collectively, in cultured mammalian cells and also determined whether these sites are phosphorylated by the cyclin A-CDK2 complex in vitro. Our results suggest that the effect of cyclin A-CDK2 on ER transcriptional activation is mediated by phosphorylation of serines 104 and 106.

EXPERIMENTAL PROCEDURES

Plasmids and Generation of ER Phosphorylation Site Mutants—Phosphorylation site mutants were generated via a two-step polymerase chain reaction process wherein overlapping primers (a “top” strand and a “bottom” strand; Genelink, Thornwood, NY) bearing the mutation of interest were mixed and amplified. The reaction was carried out on a Perkin-Elmer GeneAmp 2400 System using Perkin-Elmer reagents and Taq DNA polymerase. Intermediate polymerase chain reaction products were separated from excess primer and template using the Qiagen polymerase chain reaction purification kit. B. Katzenellenbogen (University of Illinois, Urbana) kindly provided a double mutant, pCMV5-ER S104A/S106A, Triple phosphorylation site mutants in the context of pGex4T-1 (Amersham Pharmacia Biotech) and pcDNA3 (Invitrogen) were constructed by subcloning. All phosphorylation site mutants were sequenced to verify the existence of the desired base alterations and to guard against the inclusion of untoward mutations (Sequenase Version 2.0 DNA sequencing kit, U. S. Biochemical Corp.). pcDNA3-wt ER, pcDNA3-ER S104A, pcDNA3-ER S106A, pcDNA3-ER S118A, and pcDNA3-ER S104A/S106A/S118A expression plasmids were used to produce full-length human ER derivatives, and an XETL reporter plasmid containing one consensus ERE upstream of firefly luciferase gene was used to assay for ER transcriptional activity. The pCMV-Myc-cycA plasmid expressed Myc-tagged cycA A. A pCMV empty vector was used to equalize the total amount of DNA transfected in each experiment. pCMV-LucZ plasmid produced β-galactosidase and was used as an internal control for transfection efficiency.

Cell Culture, Transient Transfections, and ER Activity Assays—U-2 OS human osteosarcoma cells (ATCC HTB 96) were maintained in Dulbecco’s modified Eagle’s medium (Life Technologies, Inc.) supplemented with 10% fetal bovine serum (HyClone), 50 units/ml each penicillin and streptomycin, and 2 mM L-glutamine (Life Technologies, Inc.).

For transient transfections, U-2 OS cells were seeded into 60-mm dishes (120,000 cells/dish) in Dulbecco’s modified Eagle’s medium, 10% fetal bovine serum. One hr before transfection, cells were re-fed with phenol red-free Dulbecco’s modified Eagle’s medium supplemented with 10% charcoal-stripped fetal bovine serum and transfected with indicated plasmids via the calcium phosphate precipitation method as described elsewhere (27). Five hr post-transfection, cells were washed three times with phosphate-buffered saline to remove calcium phosphate precipitates, allowed to recover overnight in phenol red-free Dulbecco’s modified Eagle’s medium, 10% stripped fetal bovine serum, and incubated with fresh medium containing 100 nM 17β-estradiol (E2, resuspended in 100% ethanol) or 1 μM 4-hydroxy-tamoxifen (Calbiochem-Novabiochem; resuspended in 100% ethanol) where indicated for an additional 12 h.

Transfected cells were washed twice with phosphate-buffered saline and lysed directly on the plates in 250 μl of 1× reporter lysis buffer (Promega). Luciferase activity was quantified in a reaction mixture containing 25 mM glycylglycine, pH 7.5, 15 mM MgSO4, 1 mM ATP, 0.1 mM dithiothreitol, 1 mM sodium orthovanadate, 0.5 mM ZnCl2, 4-hydroxy-tamoxifen (Calbiochem-Novabiochem), and 10% charcoal-stripped bovine serum albumin. A Lumar 9507 luminometer (EG&G Berthold) was used with 1 mM t-luciferin (Analytical Luminescence Laboratory) as substrate. Luciferase assays were performed, normalized to β-galactosidase (28) activity, and expressed as relative luminescence units.

Immunoblotting—To prepare protein extracts from transfected cells, U-2 OS cells were washed twice with phosphate-buffered saline and lysed directly on the plates in 200 μl of ice-cold lysis buffer (150 mM NaCl, 50 mM Hepes, pH 7.5, 1 mM EDTA, 1 mM EGTA, 1% glycerol, 1% Triton X-100, 1 mM NaF, 25 mM ZnCl2 supplemented with protease inhibitors (1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin A, 1 mM phenylmethylsulfonyl fluoride) and a phosphatase inhibitor, 1 mM sodium orthovanadate. The lysates were collected, incubated on ice for 15 min, and precleared by centrifugation (10,000×g for 15 min at 4 °C), with 5% sucrose added to the lysis buffer, and 200 μl of the whole cell extract was boiled for 3 min with 50 μl of 5× SDS sample buffer. For immunoblotting, protein extracts were fractionated by 10% SDS-polyacrylamide gel electrophoresis, transferred to Immobilon membrane (Millipore), and probed with the Myc-specific mouse monoclonal antibody to detect transfected Myc-tagged cyclin A or with anti-ER mouse monoclonal or rabbit polyclonal antibodies (Santa Cruz Biotechnology, Inc. catalog #SC-040, SC-787 and SC-543, respectively). The blots were developed using horseradish peroxidase-coupled sheep anti-mouse or goat anti-rabbit antibodies and the Enhanced Chemiluminescence (ECL) substrate as per the manufacturer’s instructions (Amersham Pharmacia Biotech).

Purification of ER Derivatives as GST Fusion Proteins and Generation of Cyclin A-CDK2 Complexes in Baculovirus Expression System—Human ER derivatives containing N-terminal amino acids 1 through 121, either wild type (wt) or containing single S104A, S106A, S118A or triple S104A/S106A/S118A amino acid substitutions were subcloned into the pGex4T-1 vector (Amersham Pharmacia Biotech) and expressed in Escherichia coli as glutathione S-transferase (GST) fusion proteins (29). The most potent kinase reactions (1 mg/ml) were used as substrates for the in vitro kinase assays.

High Five insect cells were maintained in Ex-Cell 405 insect culture media (JRH Biosciences) at 27 °C. Baculovirus vectors (107 plaque-forming units/ml) engineered to express human cyclin A or a hemagglutinin-tagged human CDK2 were used separately or in combination to infect cells. Cells (1 × 107 cells/100-mm dish) were infected with 0.5 plaque-forming unit of each virus in a final volume of 2.5 ml for 3 h at 27 °C and re-fed with 10 ml of Ex-Cell medium. Two days post-infection, cells were lysed on ice for 1 h in 0.5 ml of 120 mM NaCl, 50 mM Tris-HCl, pH 8.0, 0.5% Nonidet P-40, 1 mM EDTA, 1 mM dithiothreitol supplemented with protease inhibitors (described above) and phosphatase inhibitors (1 mM NaF, 10 mM β-glycerophosphate, 1 mM sodium orthovanadate). Lysates were cleared by centrifugation at 12,000 g × 10 min for 10 min at 4 °C, frozen on dry ice and stored at −80 °C.

In Vitro Kinase Assays—The cyclin A-CDK2 complex was immunoprecipitated from approximately 100 μg of insect cell extract for 1 h on ice with 5 μg of the monoclonal antibody 12A5 (Roche Molecular Biochemicals) directed against the hemagglutinin epitope on CDK2. Immune complexes were immobilized on protein A/G-agarose beads (Santa Cruz Biotechnology) for 1.5 h at 4 °C, washed 5 times in 1 ml of lysis buffer (described above), once with 1 ml of lysis buffer without Nonidet P-40, and once with 10 ml of 50 mM potassium phosphate, pH 7.15, 10 mM MgCl2, 5 mM NaF, 4.5 mM dithiothreitol) with protease inhibitors (described above). The wild type or mutant GST-ER S121 substrates (approximately 10 μg in 100 μl) were added to the immobilized kinase complex, the kinase reaction was initiated by adding 25 mM ATP, 10 mM MgCl2, 1 mM dithiothreitol, and [γ-32P]ATP (10 μCi/ml) in a total volume of 300 μl and allowed to proceed for 30 min at room temperature with continuous shaking. Reaction mixtures containing the immobilized receptor on glutathione beads and recombinant purified ERK-2 (New England Biolabs) were set up according to the manufacturer’s instructions. The beads containing the kinase complex and the bound substrate were then washed 3 times with 1 ml of phosphate.
Phosphorylation of Substrates Was Examined by Autoradiography at Room Temperature

Polyacrylamide electrophoresis gels. The gels were stained with Coomassie Blue to visualize the receptor protein and dried, and the phosphorylation of substrates was examined by autoradiography at room temperature. To quantitate the amount of \( ^{32}P \) incorporated into each ER derivative, the receptor bands were excised from the gel, immersed in scintillation fluor, and quantitated using a scintillation counter.

**RESULTS**

Enhancement of ER Transcriptional Activation by Cyclin A Overexpression Is Abolished in the ER Triple Mutant S104A/S106A/S118A—We have previously demonstrated that overexpression of cyclin A in mammalian cells enhances ER transcriptional activation. To determine whether the effect of cyclin A is mediated through one or more of the three potential CDK phosphorylation sites in AF-1, Ser-104, Ser-106, and Ser-118 (Fig. 1A), we have substituted these serines with alanines (S104A/S106A/S118A) in the context of the full-length human receptor and compared the effect of cyclin A overexpression on the transcriptional response of the wt versus triple mutant ER in ER-deficient U-2 OS human osteosarcoma cells. Fig. 1B demonstrates that overexpression of cyclin A results in a 2-fold increase of wt ER transcriptional enhancement. The ER triple mutation S104A/S106A/S118A (AAA mutant) completely abolished the receptor response to cyclin A (Fig. 1B, top panel). Importantly, the ER AAA mutant was expressed at the same level as the wt ER, and the expression of either derivative was not affected by the exogenously transfected cyclin A (Fig. 1B, bottom panel). These results suggest that the effect of cyclin A on ER transcriptional activation is not a function of alterations in expression of ER but rather is mediated, individually or collectively, through serines 104, 106, and/or 118.

Phosphorylation of ER by the Cyclin A-CDK2 complex was purified cyclin A-CDK2 phosphorylation sites, we have examined whether purified cyclin A-CDK2 could phosphorylate an ER derivative containing receptor amino acid residues 1 through 121 using an immune complex kinase assay. Both the wt ER and an ER containing the three amino acid substitutions S104A/S106A/S118A (AAA) were fused to GST, expressed in E. coli, and purified by glutathione affinity chromatography. The cyclin A-CDK2 complex was purified from baculovirus-infected insect cells by immunoprecipitation using antibody directed against an hemagglutinin epitope present on the CDK2 subunit of the complex. As shown in Fig. 2 (top panel), immunopurified cyclin A-CDK2 complex phosphorylates the wt GST-ER\(_{121}\) derivative, but not the AAA mutant, in vitro. These results suggest that the cyclin A-CDK2 complex directly phosphorylates one or more of the serine residues, 104, 106, or 118, in vitro.

Serines 104 and 106, but Not 118, Mediate Cyclin A-dependent Enhancement of ER Transcriptional Activation in Mammalian Cells—ER responsiveness to cyclin A overexpression as well as ER phosphorylation in vitro suggests three candidate target sites for the cyclin A-CDK2-mediated phosphorylation, Ser-104, Ser-106, and Ser-118, all of which lie within the serine-proline consensus motif, potentially modified by CDKs. To determine which of these serine residues are required for the cyclin A-mediated induction of ER transcriptional activation in mammalian cells, we constructed a series of full-length ER derivatives bearing individual serine-to-alanine substitutions, S104A, S106A, and S118A. These constructs were expressed in U-2 OS cells and assayed for ER-dependent transcriptional activation under conditions of cyclin A overexpression.

Fig. 3A demonstrates that the ER S104A and S106A mutations, but not the S118A substitution, partially suppress the effect of cyclin A on ER transcriptional activation relative to the wt ER. These differences in ER transcriptional activity are not a reflection of alterations in the level of ER protein synthesized, since all derivatives were expressed at a comparable
level in both the presence and absence of exogenous cyclin A (Fig. 3B). The results from four independent experiments (Fig. 3C) demonstrate that S118A mutant is fully responsive to cyclin A, whereas both S104A and S106A are reduced in their response, with the average induction by cyclin A 43 and 18%, respectively. Thus, residues 104 and 106, but not 118, are responsible for the observed cyclin A-dependent enhancement of ER transcriptional activity in cultured mammalian cells. Interestingly, neither the S104A nor the S106A mutations completely eradicate cyclin A enhancement of ER activity, suggesting that both residues participate in the observed regulation. In addition, since either mutation results in more than 50% reduction of ER transcriptional enhancement, phosphorylation at these two sites is likely cooperative, such that replacement of either serine 104 or 106 with alanine partially inhibits phosphorylation of the adjacent site.

**Individual Serine to Alanine Substitutions at ER Residues 104, 106, and 118 Are Differentially Phosphorylated by the Cyclin A-CDK2 Complex in Vitro**—We next assessed the ability of the cyclin A-CDK2 complex to phosphorylate individual serine-to-alanine ER mutants (S104A, S106A, and S118A) in vitro in the context GST-ER121. Fig. 4A illustrates that phosphorylation of each mutant, S104A, S106A, and S118A, is reduced compared with the wt ER. The lower panel is the Coomassie Blue-stained gel demonstrating that all receptor derivatives are expressed at comparable levels. To quantify the amount of phosphate incorporated into each mutant, the receptor and cyclin A bands were excised from the gel and subjected to liquid scintillation counting; ER phosphorylation was normalized to the amount of cyclin A immunoprecipitated and phosphorylated in each condition. Phosphate incorporation into the S104A derivative by cyclin A-CDK2 is decreased by more than 80%, relative to the wt ER (set as a 100%), whereas phosphorylation is virtually abolished when the S106A derivative is used as the substrate, reducing the amount of phosphorylation by more than 95% compared with the wt ER (Fig. 4B). To establish that the integrity of the S106A derivative is preserved, we tested it as a substrate for mitogen-activated protein kinase (ERK-2), which utilizes Ser-118 as a target phosphorylation site. ERK-2 readily phosphorylates S106A, suggesting that the inability of cyclin A-CDK2 to phosphorylate S106A does not result from potential changes in protein conformation induced by the mutation but rather reflects the specificity of the kinase with respect to the particular substrate site (Fig. 4C). The ER S118A mutation also results in a decrease in ER phosphorylation by the cyclin A-CDK2 complex, albeit to a much smaller extent.
FIG. 4. Individual mutations at ER N-terminal phosphorylation sites decrease ER phosphorylation by cyclin A-CDK2 complex in vitro. GST-ER121 fusion proteins, either wt or containing single amino acid substitutions at receptor phosphorylation sites S104A, S106A, or S118A, were expressed in E. coli and purified as described above. The cyclin A-CDK2 complex was expressed and immunopurified as described in Fig. 2. Purified cyclin A-CDK2 complex (A) or purified recombinant ERK-2 (C) was added to the wt or mutant ER substrates for the kinase reactions. The reaction products were separated on 10% SDS-polyacrylamide electrophoresis gels, stained with Coomassie Blue to visualize the substrate proteins (A and C, bottom panels), and exposed to film (A and C, top panels). The GST-ER121 and cyclin A bands were subsequently excised from the gel and subjected to scintillation counting. 32P incorporation into each ER derivative was normalized to the phosphorylation of cyclin A, immunoprecipitated in each condition. Relative efficiency of phosphorylation was calculated for each ER mutant by setting counts/min of the wt GST-ER121 as a 100% (B). Note that each serine-to-alanine substitution decreases the amount of GST-ER121 phosphorylation; however, S104A and S106A do so to a greater extent than S118A.

FIG. 5. Cyclin A-mediated induction of ER transactivation is ligand-independent. U-2 OS cells were transfected as described in Fig. 1, and ER transcriptional activation in the absence of ligand (Et OH), in the presence of 100 nM 17β-estradiol (E2), and in the presence of 1 μM 4-hydroxytamoxifen (Tam) was assessed via a luciferase assay, normalized to β-galactosidase activity, and expressed as relative luminescence units (RLU). The experiment was performed in duplicate, two times, with similar results. Note that 2–3-fold induction of ER transcriptional activation by cyclin A occurs in each of the three conditions used.

Phosphorylation of ER by Cyclin A-CDK2

Combined, our results argue that the ER residues Ser-104 and Ser-106 are bona fide cyclin A-CDK2 targets, which is supported by our transcriptional activity assays in mammalian cells.

Cyclin A-mediated Enhancement of ER Transcriptional Activation Is AF-2-independent—Cyclin A overexpression enhances the transcriptional activity of the ER in cultured mammalian cells both in the presence and in the absence of estradiol (Fig. 1B). Thus, the effect of cyclin A overexpression and the activation of the ER by the cyclin A-CDK2 complex appear to be independent of ligand binding, suggesting the involvement of AF-1 but not AF-2. To further evaluate the importance of AF-2 for the enhanced ER-dependent transcriptional activation in response to cyclin A overexpression, we used a pharmacological approach and employed the ligand tamoxifen, a mixed agonist/antagonist currently used in the treatment of ER-positive breast cancers. Tamoxifen prevents the productive interaction of the ER with co-activator protein(s) necessary for transcriptional activation via AF-2, thus allowing for the assessment of changes in AF-1 activity as a function of cyclin A concentration (8, 29). U-2 OS cells were transiently transfected with the ER as well as the reporter constructs described above and treated with the ethanol vehicle, estradiol or 4-hydroxytamoxifen. For each treatment, ER transcriptional enhancement was assayed in the absence and presence of cyclin A overexpression. Consistent with our previous findings, a 2-fold increase in ER-dependent transcription was observed upon cyclin A overexpression in the absence or presence of estradiol (Fig. 5). Importantly, the magnitude of induction of ER-dependent transcriptional activation by cyclin A in response to tamoxifen treatment is comparable to that observed with estradiol (Fig. 5). Thus, the recruitment of co-activator proteins to AF-2 is dispensable for the cyclin A-mediated enhancement of ER ac-
Phosphorylation of ER by Cyclin A-CDK2

We have identified serines 104 and 106 of the human ER as the likely targets of cyclin A-CDK2-dependent phosphorylation. A triple serine-to-alanine mutation at residues 104, 106, and 118 abolishes both the cyclin A-CDK2-dependent increase of ER transcriptional activation in U-2 OS cells and ER phosphorylation by the cyclin A-CDK2 complex in vitro. Individual S104A and S106A mutations reduce the cyclin A-CDK2-dependent enhancement of ER-dependent transcriptional activation. In contrast, the S118A mutant responds like wt ER to cyclin A overexpression in mammalian cells. Similarly, phosphorylation of an ER N-terminal derivative by the cyclin A-CDK2 complex in vitro is significantly reduced in the S104A and S106A mutants, relative to the wt ER. Although the ER S118A mutant also exhibits decreased phosphorylation by the cyclin A-CDK2 complex in vitro, this reduction is much smaller than that exhibited by either the S104A or the S106A derivatives; in addition, this site may be artificially exposed to the purified kinase in the context of the GST-ER121 fusion protein. These in vitro findings are consistent with our results in mammalian cells, where the cyclin A-CDK2-dependent enhancement of ER transcriptional activation is reduced in ER derivatives bearing serine-to-alanine mutations at 104 and 106 but not 118. These data suggest that Ser-118 is a poor target for cyclin A-CDK2 phosphorylation in vitro and in vivo. The inability of Ser-118 to serve as a substrate for cyclin A-CDK2 is also in agreement with previous reports proposing that Ser-118 is a substrate for epidermal growth factor-activated mitogen-activated protein kinase in the absence of estradiol as well as a target for another as yet unidentified kinase(s) in the presence of estradiol (18–20). Together, these results suggest that ER is a substrate for the cyclin A-CDK2 complex, with the predominant sites of phosphorylation being Ser-104 and Ser-106. Given the close proximity of Ser-104 and Ser-106, cooperativity between the sites such that the same kinase complex modifies them and phosphorylation of one site promotes phosphorylation of the other appears likely.

It is noteworthy that the ER sites phosphorylated by the cyclin A-CDK2 complex, Ser-104 and Ser-106, reside within sequence contexts that are noncanonical CDK phosphorylation targets (Fig. 1A), as determined by a systematic evaluation of a panel of substrates phosphorylated in vitro by cyclin A-CDK2 (30). It is likely that multiple factors confer specificity and efficiency to cyclin A-CDK2-mediated phosphorylation of a given target site. For example, a noncanonical site might fold in such a way that the target is presented to the kinase in a favorable manner. Furthermore, recent findings by Schulman et al. (31) show that a conserved hydrophobic patch on the surface of cyclin A is involved in substrate recognition through a RXL motif on the substrate and that this binding is important for phosphorylation of a subset of proteins by cyclin A-CDK2 (31). Interestingly, human ER-α contains three RXL motifs; one such motif is located in the N terminus (amino acids 37–39), whereas two others reside in the C-terminal ligand binding domain, at residues 352–354 and 477–479, respectively. In each case, these motifs are preserved among ERs from distinct species, including human, rat, mouse, sheep, pig, and chicken, suggesting a conservation of function. It is conceivable that one or more of these motifs serve as potential docking sites for the cyclin A-CDK2 complex and facilitate ER phosphorylation at Ser-104/Ser-106 by increasing the local concentration of the substrate.

The enhancement of ER transcriptional activation by cyclin A overexpression occurs not only in the absence and presence of estradiol but is also observed when the receptor is activated by tamoxifen. Since tamoxifen induces a receptor conformation that is incompatible with coactivator binding to AF-2 (8, 29), these results suggest that cyclin A-CDK2 enhances ER transcriptional activity through AF-1 and not AF-2.

We propose that ER phosphorylation at Ser-104/Ser-106 by the cyclin A-CDK2 complex provides sites that either recruit or prevent additional proteins from binding to ER AF-1. Although the p160 class of coactivators has recently been shown to interact with ER AF-1 and increase ER AF-1-dependent transcriptional activation, this effect is not dependent upon receptor phosphorylation at Ser-104, Ser-106, or Ser-118 (32). We further hypothesize that alterations in the level or activity of the cyclin A-CDK2 complex modulates ER activity by increasing or decreasing receptor phosphorylation, which in turn, affects the interaction of ER with accessory proteins involved in transcriptional regulation. This mechanism of cyclin A-CDK2 regulation of ER transcriptional activity through direct receptor phosphorylation and co-factor binding differs from that of cyclin D1-mediated enhancement of ER transcriptional activity (Fig. 6). The effect of cyclin A on ER transcriptional activation requires the kinase activity of the CDK2, whereas the effect of cyclin D1 on ER is CDK-independent (33). In addition, the enhancement of ER transactivation by cyclin A-CDK2 is achieved through phosphorylation of ER AF-1, whereas cyclin D1 enhancement of ER transcriptional activity appears to be through coactivator recruitment to AF-2. Although the means whereby cyclin D1 and cyclin A augment ER transcriptional activation differs, the result is the same; that is, an increase in ER transcriptional activation either through direct coactivator recruitment to AF-2 in the case of cyclin D1, or indirectly through AF-1 phosphorylation by cyclin A-CDK2 and subsequent cofactor interaction (Fig. 6). In view of increasing clinical data linking CDK...
dysregulation to a variety of human cancers, notably breast cancer (35–39), we believe that the subversion of either the cyclin D1 or the cyclin A-CDK2 pathway might account for a subpopulation of breast hyperplasias and/or tumors.

Acknowledgments—We are grateful to Benita Katzenellenbogen and Didier Picard for critically reading the manuscript.

REFERENCES

1. Blobel, G. A., and Orkin, S. H. (1996) Mol. Cell. Biol. 16, 1687–1694.
2. Gauth, M. P., Bellard, M., Schauer, I., Chambon, P., and Sassone-Corsi, P. (1990) Cell 63, 1267–1276.
3. Kanelo, K. J., Gelinas, C., and Gorski, J. (1993) Biochemistry 32, 8348–8359.
4. Weiss, A., and Rosales, R. (1990) Nucleic Acids Res. 18, 5097–5106.
5. Kumar, V., Green, S., Stack, G., Berry, M., Jin, J. R., and Chambon, P. (1987) Cell 51, 941–951.
6. Kraus, W. L., McInerney, E. M., and Katzenellenbogen, B. S. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 12314–12318.
7. McInerney, E. M., Tsai, M. J., O’Malley, B. W., and Katzenellenbogen, B. S. (1996) Proc. Natl Acad. Sci. U. S. A. 93, 10069–10073.
8. Shiau, A. K., Barstad, D., Loria, P. M., Cheng, L., Kushner, P. J., Agard, D. A., and Greene, G. L. (1996) Proc. Natl Acad. Sci. U. S. A. 93, 10069–10073.
9. McInerney, E. M., and Katzenellenbogen, B. S. (1996) J. Biol. Chem. 271, 24172–24178.
10. Garabedian, M. J., Rogatsky, I., Hittelman, A., Knoblauch, R., Trowbridge, J. M., and Krstic, M. D. (1998) in Molecular Biology of Steroid and Nuclear Hormone Receptors (Freedman, L. P., ed) pp. 237–260, Birkhaeuser Boston, Cambridge, MA.
11. Le Goff, P., Montano, M. M., Scho din, D. J., and Katzenellenbogen, B. S. (1994) J. Biol. Chem. 269, 4458–4466.
12. Ali, S., Metzger, D., Bornert, J. M., and Chambon, P. (1993) EMBO J. 12, 1153–1160.
13. Blobel, G. A., Steif, C. A., and Orkin, S. H. (1995) Mol. Cell. Biol. 15, 3147–3153.
14. Levenson, A. S., and Jordan, V. C. (1994) J. Steroid Biochem. Mol. Biol. 51, 229–239.
15. Ma, Z. Q., Spreafico, E., Pollin, G., Santagati, S., Conti, E., Cattaneo, E., and Maggi, G. (1993) Proc. Natl Acad. Sci. U. S. A. 90, 3740–3744.
16. Weiss, A., Viotti, L., Pratesi, E., Scalona, M., and Bresciani, P. (1990) Mol. Endocrinol. 1, 1041–1050.
17. von Lindern, M., Boer, L., Wellessy, O., Parker, M., and Beug, H. (1998) Mol. Endocrinol. 12, 265–277.
18. Bumene, G., Brind, P.-A., Mikesiek, R. J., and Picard, D. (1996) EMBO J. 15, 2174–2183.