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13. ABSTRACT (Maximum 200 words) The overall goal of our laboratory is to define the mechanism of signal transduction and transcriptional regulation by the estrogen receptor (ER), a transcription factor that plays a critical role in the development, progression and hormone responsiveness of breast cancer cells. Our approach combines genetic, molecular and biochemical strategies to identify and characterize molecules that affect the ER signaling pathway. We have identified the Hsp90-associated co-chaperone, p23, as a key regulator of ER action and have also determined that ER is regulated by phosphorylation via the cyclin A/Cdk2 complex. Understanding of the communication between ER and these regulator factors is fundamental to understanding the mechanism of ER-regulated gene expression and may reveal novel points of intervention to be exploited in the development of new therapies for ER-dependent malignancies, such as breast cancer.				
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FOREWORD

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Introduction

The long-term objective of this proposal is to understand the mechanism of signal transduction by the estrogen receptor (ER), a transcriptional regulatory protein implicated in the initiation and maintenance of many human breast tumors (1,2). ER plays a critical role in the development, progression and hormone responsiveness of breast cancer cells. The growth of nearly half of all human breast cancers is dependent upon the presence of an active estrogen-ER complex. The treatment of estrogen-dependent breast cancer as well as prevention of the disease has been mostly based on the functional inactivation of ER by anti-estrogen hormonal agents such as tamoxifen(3). However, not all breast cancer cells containing ER respond to anti-estrogen therapy and many tumors that initially respond to the growth suppressing properties of tamoxifen become resistant to the drug over time, suggesting the existence of a compensatory mechanism. Thus, the isolation and characterization of non-receptor factors that influence ER function is not only fundamental to understanding the mechanism of estrogen-regulated gene expression, but represents likely points of intervention to be exploited in the development of new therapies for breast cancer, particularly the ones that no longer respond to steroid treatment alone.

To further define the mechanism of signal transduction and transcriptional regulation by the ER, genetic strategies have been used to isolate molecules that modulate ER activity. Our goal is to genetically mark the signaling pathway of the ER in yeast by isolating genes that influence its function and thus identify factors with which the receptor functionally interacts. Using dosage suppression analysis, we have isolated the Hsp90 associated co-chaperone p23 as a factor that increases both hormone-dependent and hormone-independent activity of ER (*see Knoblauch, R. and Garabedian, M.J., Role of Hsp-90-associated cochaperone p23 in estrogen receptor signal transduction, Mol. Cell. Biol. in press*). In addition to identifying factors with which the receptor functionally interacts, we have also defined the effects of phosphorylation by the cyclin A/Cdk2 complex on ER function (*see Trowbridge, J.M., Rogatsky, I.,*

and Garabedian, M.J. (1997) Regulation of estrogen receptor transcriptional enhancement by the cyclin A/Cdk2 complex, Proc. Natl. Acad. Sci. USA 94, 10132-10137 and below).

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

Although ligand binding is considered essential for the full activation of ER, it has long been recognized that the receptor is subjected to post-translational alterations, such as phosphorylation, which also regulate its activity(6). The phosphorylation of three N-terminally-located residues, serines 104, 106, and 118, which are the focus of our current studies, are phosphorylated in response to ligand treatment and impairment of this modification decreases receptor-dependent transcriptional enhancement(7,8). 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 this level of complexity and flexibility is not surprising.

Much work has been directed towards understanding which sites of the receptor are phosphorylated and under what circumstances. A number of potential phosphorylation sites have been identified, however, the kinases that modify not these residues have yet to be identified. 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 (S104, S106, S118, and S167) are clustered in the N-terminus within AF-1 of the receptor. Based upon the sequence context surrounding each of the putative serine phosphorylation sites, the identities of the kinases acting upon individual sites are believed, for serines 104, 106, and 118 reside to be targeted by members of the serine/proline-directed protein kinase family including mitogen-activated protein kinases (MAPK), glycogen synthase kinase-3 (GSK-3) and the cyclin-dependent kinases (CDK). Indeed, S118 has been shown to be phosphorylated by the MAPK family member, ERK-1, *in vitro*, although recent findings cast doubt on whether this MAPK is the kinase that phosphorylates ER at S118 *in vivo*(9-11). Serine 167 has been shown to be phosphorylated by p90^{RSK}; 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(12-14). As mentioned above, 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 in response to ligand treatment. 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 81 and 121 *in vitro* and that ligand-independent hyperphosphorylation of the receptor results from overexpression of cyclin A *in vivo*. This mechanism of regulation has also been described for three other members of the nuclear hormone receptor family: The glucocorticoid receptor is phosphorylated by two cyclin/CDK complexes, A/CDK2 and E/CDK2(15). The progesterone 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 enhancement(16,17).

In order to identify which residue(s) of ER are 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 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*.

Body--Experimental Procedures

Plasmids and generation of ER phosphorylation site mutants.

Phosphorylation site mutants were generated via a two-step PCR process wherein overlapping primers (a "top" strand and a "bottom" strand; Genelink, Thornwood, NY) bearing the mutation of interest were mixed and amplified. The reactions were carried out on a Perkin Elmer GeneAmp 2400 System using Perkin Elmer reagents and Taq DNA Polymerase. Intermediate PCR products were separated from excess primer and template using the Quiagen PCR Purification Kit (Quiagen). A double mutant, pCMV5-ER S104A/S106A, was kindly provided by B. Katzenellenbogen (University of Illinois, Urbana). Triple phosphorylation site mutants in the context of pGex4T-1 (Pharmacia) 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, USB).

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 ER transcriptional activity. pCMV-MycA plasmid expressed Myc-tagged cyclin A. A pCMV empty vector was used to equalize the total amount of DNA transfected in each experiment. pCMV-LacZ plasmid produced β -galactosidase (β -Gal).

Cell culture, transient transfections and ER activity assays.

U-2 OS human osteosarcoma cells (ATCC HTB 96) were maintained in Dulbecco's modified Eagle medium (DMEM; GIBCO/BRL) supplemented with 10% fetal bovine serum (FBS; HyClone), 50 units/ml of each Penicillin and Streptomycin, and 2 mM L-Glutamine (GIBCO/BRL).

For transient transfections, U-2 OS cells were seeded into 60 mm dishes (120,000 cells per dish) in DMEM-10% FBS. One hour prior to transfection, cells were re-fed with phenol red-free DMEM supplemented with 10%

charcoal-stripped FBS and transfected with indicated plasmids via the calcium phosphate precipitation method as described elsewhere. Five hours post-transfection, cells were washed three times with phosphate buffered saline (PBS) to remove calcium phosphate precipitates, allowed to recover overnight in phenol red-free DMEM-10% stripped FBS and incubated with fresh medium containing 100 nM 17 β -estradiol (E2, resuspended in 100% ethanol) or 1 mM 4-Hydroxy-Tamoxifen (Calbiochem-Novabiochem Corporation, La Jolla, CA; resuspended in 100% ethanol), where indicated, for an additional 12 hours.

Transfected cells were washed twice with PBS and lysed directly on the dishes in 250 μ l of 1x Reporter Lysis buffer (Promega). Luciferase activity was quantified in a reaction mixture containing 25 mM glycylglycine, pH 7.8, 15 mM MgSO₄, 1 mM ATP, 0.1 mg/ml bovine serum albumin, 1 mM DTT. A Lumat LB 9507 luminometer (EG&G Berthold) was used with 1 mM D-luciferin (Analytical Luminescence Laboratory) as substrate. Luciferase assays were performed, normalized to b-Gal activity and expressed as Relative Luminescence Units (RLU).

Immunoblotting.

To prepare protein extracts from transfected cells, U-2 OS cells were washed twice with PBS and lysed directly on the plates in 200 μ l of ice-cold lysis buffer [150 mM NaCl/50 mM Hepes pH 7.5/1 mM EDTA/1 mM EGTA/10% glycerol/1% Triton X-100/1 mM NaF/25 mM ZnCl₂, supplemented with protease inhibitors (1 μ g/ml aprotinin, 1 μ g/ml leupeptin, 1 μ g/ml pepstatin A, 1 mM phenylmethylsulfonyl fluoride (PMSF)] and a phosphatase inhibitor, 1 mM sodium orthovanadate. The lysates were collected, incubated on ice for 15 min and precleared by centrifugation (10,000 x g for 10 min at 4°C), protein concentration in all samples was adjusted with the lysis buffer and 200 μ l of the whole cell extracts (WCE) was boiled for 3 min with 50 μ l of 5xSDS sample buffer. For immunoblotting, protein extracts were fractionated by 10% SDS-PAGE, transferred to Immobilon membrane and probed with the Myc-specific mouse monoclonal antibody,

9E10, to detect transfected Myc-tagged cyclin A or with anti-ER rabbit polyclonal antiserum (Santa Cruz Biotechnology, Inc. cat# and , 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).

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 (Pharmacia) and expressed in *E. coli* as glutathione S-transferase (GST) -fusion proteins (GST-ER₁₂₁) as described. The most concentrated fractions (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 (10⁻⁷pfus) engineered to express human cyclin A or an HA-tagged human CDK2 were used separately or in combination to infect cells. Cells (1x10⁷ cells per 100 mm dish) were infected with 0.5 ml of each virus in a final volume of 2.5 ml for 3 hours at 27°C and re-fed with 10 ml of Ex-Cell medium. Two days post-infection, cells were lysed on ice for 1 hour 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 DTT supplemented with protease inhibitors (described above) and phosphatase inhibitors (1.0 mM NaF, 10 mM b-glycerophosphate, 1.0 mM sodium orthovanadate). Lysates were cleared by centrifugation at 12,000 x g 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 hour on ice with 5 µg of the

monoclonal antibody 12CA5 (Boehringer Mannheim), directed against the HA-epitope on CDK2. Immune complexes were immobilized on Protein A/G agarose beads (Santa Cruz Biotechnology) for 1.5 hours at 4°C, washed 3 times in 1 ml of lysis buffer (described above), once with 1 ml of lysis buffer without NP-40 and once with DK buffer (50 mM potassium phosphate/pH 7.15/10 mM MgCl₂/5 mM NaF/4.5 mM DTT) with protease inhibitors (described above). The wild type or mutant GST-ER substrates (approximately 10 µg in 100 ml) were added to the immobilized kinase complex, the kinase reaction was initialized by adding 25 mM ATP, 10 mM MgCl₂, 1 mM DTT and [γ -³²P]ATP (100 mCi) in a total volume of 300 µl and allowed to proceed for 30 min at RT with continuous shaking. Reaction mixtures containing the immobilized receptor 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 PBS to remove unincorporated radioisotope, the labeled GST-ER derivative was released by boiling for 3 min in an equal volume of 2xSDS sample buffer and fractionated on 10% SDS-PAGE. The gels were stained with Coomassie blue to visualize the receptor protein, dried and phosphorylation of substrates was examined by autoradiography at RT. To quantitate the amount of ³²P incorporated into each ER derivative, the receptor bands were excised out of the gel, immersed in scintillation fluor and quantitated using a scintillation counter.

Results

Enhancement of ER transcriptional activation by cyclin A 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 the three potential CDK phosphorylation sites in AF-1, S104, S106 and S118, we have constructed a triple mutant ER S104A/S106A/S118A in the context of the full length human receptor and compared the transcriptional responses of the wt vs. triple mutant ER with respect to cyclin A overexpression in ER-deficient U-2 OS human osteosarcoma cells. Figure 1 demonstrates overexpression cyclin A results in a two-fold increase of the wt ER transcriptional enhancement.

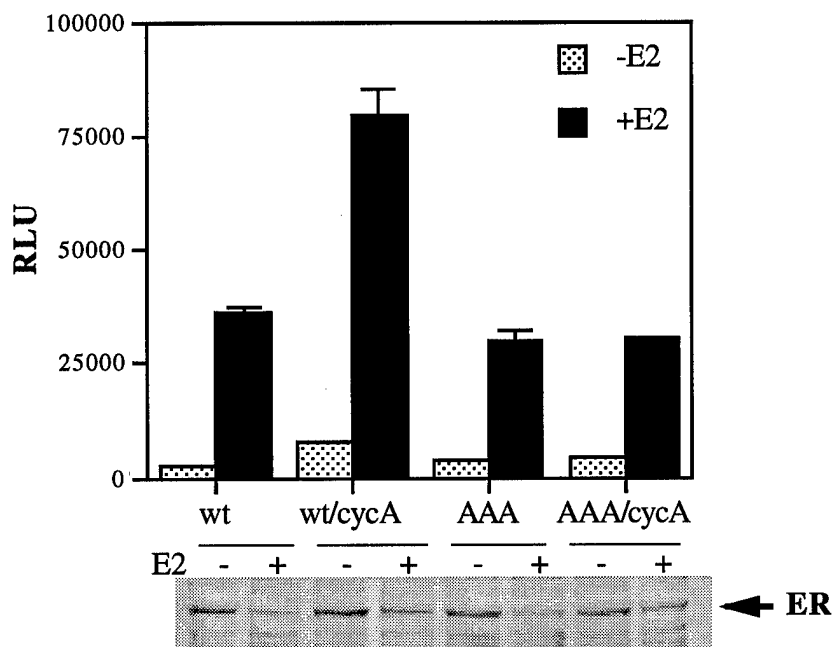


Figure 1. Replacement of ER N-terminal phosphorylation sites abolishes cyclin A-dependent induction of ER transcriptional enhancement in U 2-OS cells.

U-2 OS human osteosarcoma cells were transiently transfected via the calcium phosphate precipitation method with the full length human ER (pcDNA3-ER, 1 μ g per 60 mm dish), either wild type (wt) or a S104A/S106A/S118A triple mutant (AAA), an XETL reporter plasmid containing a single consensus ERE upstream of a luciferase gene (2 μ g per 60 mm dish), a

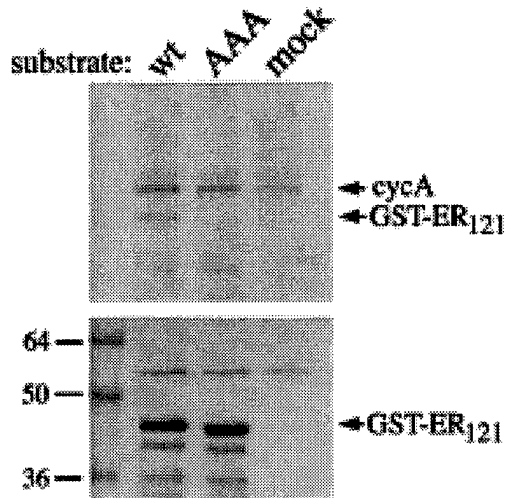
pCMV-LacZ plasmid (0.5 µg per 60 mm dish), and a pCMV-MycA plasmid (3 µg per 60 mm dish), expressing Myc-tagged full length human cyclin A, where indicated. The total amount of DNA transfected per dish was equalized with a pCMV empty expression vector. Receptor transcriptional activity in the absence or presence of 17-β-estradiol (E2) was measured via luciferase assay 12 hours after the addition of E2 to the medium, normalized to β-Galactosidase activity and expressed as relative luminescence units (RLU, top panel). To verify equal expression of ER derivatives in the presence or absence of overexpressed cyclin A, whole cell extracts (WCE) were prepared as described in Experimental Procedures from a set of identical dishes and the expression of wt and triple mutant ER was analyzed by immunoblotting with ER-specific rabbit polyclonal antiserum (bottom panel).

In contrast, the ER triple mutant S104A/S106A/S118A (AAA mutant) completely abolished receptor response to cyclin A. 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 (Figure 1). These results suggest that the effect of cyclin A on ER transcriptional activation is not a function of alterations in expression of ER or cyclin A, but rather is mediated, individually or collectively, through serines 104, 106, and/or 118.

Phosphorylation of ER by the cyclin A/CDK2 *in vitro* is abolished in the ER triple mutant S104A/S106A/S118A.

To examine whether S104, S106 and S118 were potential sites for cyclin A/CDK2 phosphorylation, 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 *in vitro*. 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. Cyclin A/CDK2 complex was purified from baculovirus infected insect cells by immunoprecipitation using antibody directed against an HA-epitope present on CDK2 subunit of the complex. As shown in Figure 2 (top panel), immunopurified cyclin

A/CDK2 complex phosphorylates the wt ER₁₂₁ GST derivative, but not the AAA mutant, *in vitro*. These results suggest that the cyclin A/CDK2 complex



directly phosphorylates one or more of the serines residues, 104, 106, or 118 *in vitro*.

Figure 2. The ER S104A/S106A/S118A derivative is not phosphorylated by immuno-purified cyclin A/CDK2 complex *in vitro*.

GST-ER fusion proteins containing receptor amino acid residues 1 through 121 (GST-ER₁₂₁), either wt or containing three amino acid substitutions at receptor phosphorylation sites S104A/S106A/S118A (AAA) were expressed in *E. coli* and purified as described []. The Cyclin A/CDK2 complex was expressed in insect cells by baculovirus infection, immuno-purified using anti-HA mouse monoclonal antibodies as described in Experimental Procedures and added to the wt or AAA substrate for the kinase reactions. Immunopurified kinase complex without added ER substrate (mock) was used as negative control. The reaction products were separated on 10% SDS-PAGE, stained with Coomassie blue to visualize the substrate proteins (bottom panel) and autoradiography was performed (top panel).

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* indicates three candidate target sites for the cyclin

A/CDK2-mediated phosphorylation, S104, S106 and S118, 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.

Figure 3A demonstrates that the ER S104A and S106A mutations, but not the S118A substitution, partially suppressed the effect of cyclin A enhancement on ER transcriptional activation. Relative the wt ER, transcriptional enhancement in response to cyclin A for the S104A and S106A ER derivatives was reduced by 55% and 75%, respectively. These differences in ER transcriptional activity are not a reflection of alterations in the level of ER protein synthesized, since the all derivatives were expressed at a comparable level in both the presence and absence of exogenous cyclin A (Figure 3B). A compilation of several such experiments comparing the effect of cyclin A overexpression on the transcriptional activation of the serine to alanine mutants *versus* wt ER, suggest that both residues 104 and 106 but not 118 are responsible for the observed cyclin A-dependent enhancement of ER transcriptional activity in cultured mammalian cells (Figure 3). Interestingly, neither the S104A nor the S106A mutations completely eradicate cyclin A enhancement of ER activity suggesting that both residues participate in the observed effect. 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 inhibition phosphorylation of the adjacent site.

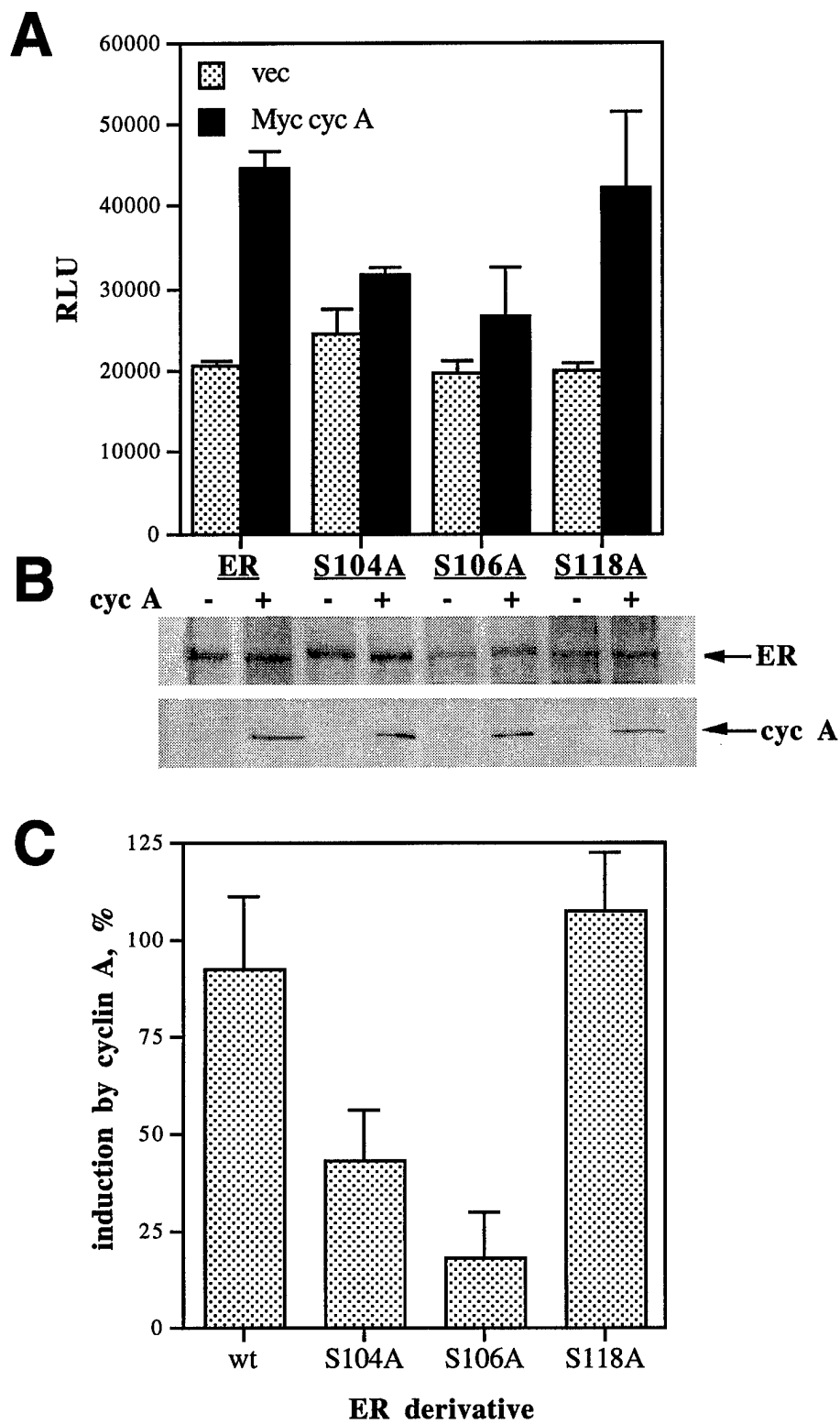


Figure 3. ER S104 and S106, but not S118, are critical for cyclin A-mediated enhancement of ER transcriptional activation in U-2 OS cells.

A) S104A and S106A, but not the S118A mutation, abolish cyclin A-dependent induction of ER transactivation. U-2 OS cells were transfected as described in Figure 1 with pcDNA3-ER (wt, S104A, S106A or S118A, as indicated, 1 μ g per 60 mm dish), an XETL reporter plasmid (0.5 μ g per 60 mm dish), a pCMV-LacZ plasmid (0.5 μ g per 60 mm dish), and a pCMV-MycA plasmid (0.5 μ g per 60 mm dish) or an empty pCMV vector. ER transcriptional activation was assessed after a 12-hour treatment with E2 via luciferase assay, normalized to the β -Gal activity and expressed as RLU. B) ER expression level is not affected by cyclin A overexpression or point mutations at phosphorylation sites. WCE were prepared from transfected cells as described in Experimental Procedures and the expression of ER derivatives and Myc-tagged transfected cyclin A was analyzed by Western blotting. C) Differential enhancement of ER mutants transcriptional activation by overexpressed cyclin A. The average induction of transcriptional activation displayed by the wt ER and ER phosphorylation site mutants was expressed as "% enhancement", over the activity of each mutant in the absence of overexpressed cyclin A, which was arbitrarily set as 100%. Shown is the average and a standard error of four independent experiments.

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 tested whether individual serine to alanine mutations of ER (S104A, S106A and S118A), in the context of residues 1-121 fused to GST, could be phosphorylated by the cyclin A/CDK2 complex *in vitro*. Figure 4A demonstrates that phosphorylation of each of the serine to alanine ER mutants, S104A, S106A, and S118A, is reduced as compared to the wt ER. The lower panel is the Coomassie stained gel demonstrating that in each case the receptor is expressed at comparable levels. In order to quantify the amount of phosphate incorporated into each receptor derivative, the bands were excised from the gel and the amount of 32 P incorporated in each derivative was determined by liquid scintillation counting. Phosphate incorporation into the S104A derivative by cyclin A/CDK2 is decreased by over 80%, relative to the wt ER, whereas phosphorylation is virtually abolished when the S106A derivative is used as the substrate, reducing the amount of phosphorylation by over 95% compared to the wt ER (Figure 4B). To establish that the integrity of the S106A derivative is preserved, we tested it as a substrate for MAPK

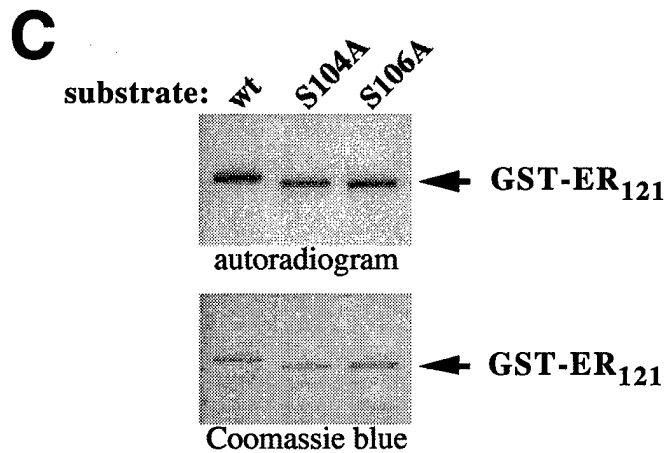
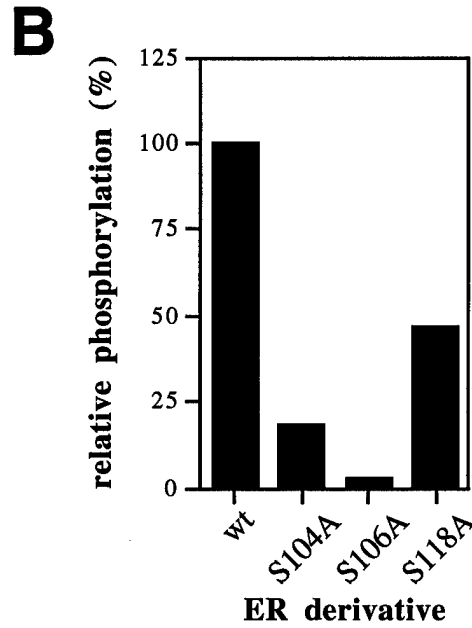
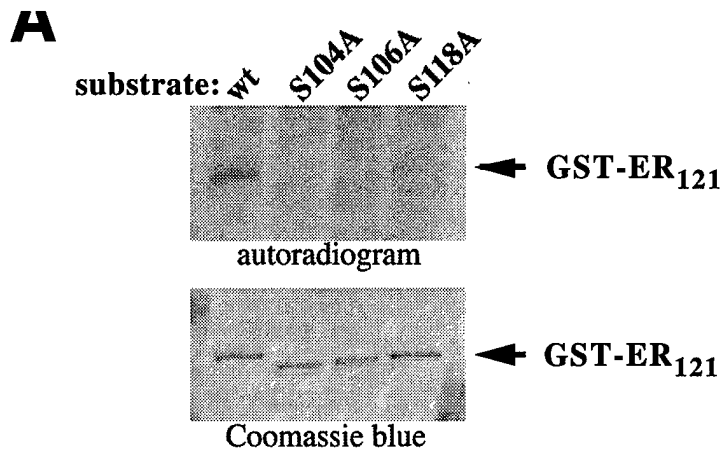


Figure 4. Individual mutations at ER N-terminal phosphorylation sites decrease ER phosphorylation by immuno-purified cyclin A/CDK2 complex *in vitro*.

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 immuno-purified as described in Figure 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-PAGE, stained with Coomassie blue to visualize the substrate proteins (A and C, bottom panels) and exposed to film (A and C, top panels). The ER bands were subsequently excised from the gel and subjected to scintillation counting. Relative efficiency of phosphorylation was calculated for each ER mutant by setting CPM 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. GST-ER fusion proteins were phosphorylated by the purified ERK-1.

(extracellular signal regulated kinase, ERK-2) which utilizes S118 as a target phosphorylation site. MAPK readily phosphorylates S106A, suggesting that the inability of cyclin A/CDK2 to phosphorylate S106 does not result from changes in protein conformation as a result of the mutation, but rather reflects the specificity of the kinase with respect to the particular substrates site (Figure 4C). The ER S118A mutation also exhibits a decrease in phosphorylation by the cyclin A/CDK2 complex, albeit to a much smaller extent than the S104A and S106A derivatives. Although the phosphorylation of all three receptor derivatives by the cyclin A/CDK2 complex *in vitro* are reduced (rank order of ER phosphorylation by cyclin A/CDK2 *in vitro*: S106<S104<S118<wt) the effect of S106A and S104A substitutions most profoundly affect phosphorylation by the cyclin A/CDK2 complex *in vitro*. These results suggest that the ER residues S104 and S106 are *bona fide* cyclin A/CDK2 targets, which is supported by our transcriptional activity assays in mammalian cells.

Cyclin A-dependent enhancement of ER transcriptional activation is AF-2-independent.

In the course of our studies, we have consistently observed that cyclin A overexpression enhances the transcriptional activity of the ER in cultured mammalian cells both in the presence and in the absence of estradiol. Thus, the effect of cyclin A overexpression and the activation of the ER by the cyclin A/CDK2 complex appeared independent of ligand binding suggesting the involvement of AF-1, but not AF-2. To 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, which prevents the productive interaction of the ER with co-activator protein(s) necessary for transcriptional activation via AF-2. U-2 OS cells were transiently transfected with the ER as well as the reporter constructs and treated with either the ethanol vehicle, estradiol or 4-hydroxy-tamoxifen, a mixed agonist and antagonist agent currently used in the treatment of ER-positive breast cancers. For each treatment ER transcriptional enhancement was assayed in the absence and presence of cyclin A overexpression. Importantly, the fold-induction of ER-dependent transcriptional activation by cyclin A in response to tamoxifen treatment is comparable to that observed with estradiol (Figure 5).

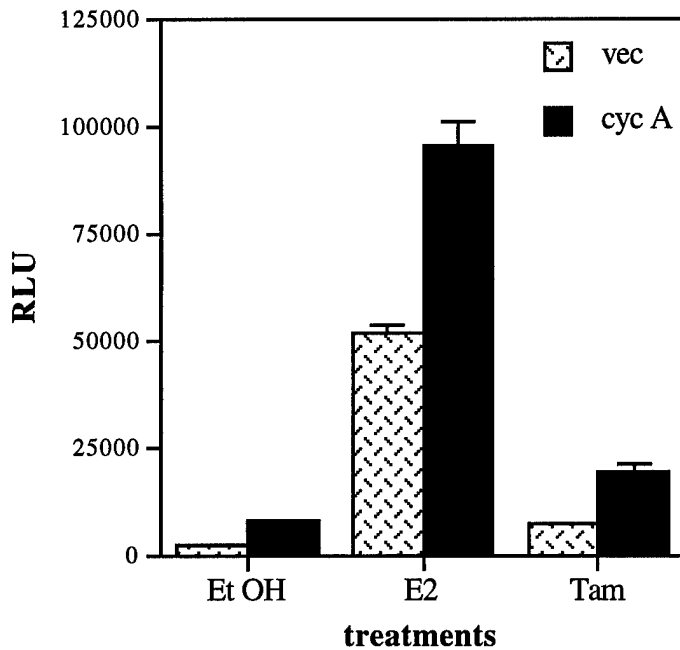


Figure 5. Cyclin A-mediated induction of ER transactivation is ligand independent.

U-2 OS cells were transfected via the calcium phosphate precipitation method with pcDNA3-ER (1 μ g per 60 mm dish), an XETL reporter plasmid (2 μ g per 60 mm dish), a pCMV-LacZ plasmid (0.5 μ g per 60 mm dish), and either a pCMV-MycA plasmid (3 μ g per 60 mm dish) or a pCMV empty expression vector (3 μ g per 60 mm dish), as indicated. ER transcriptional activation in the absence of ER ligand (Et OH), in the presence of 100 nM 17 β -estradiol (E2) and in the presence of 1 μ M 4-hydroxy-tamoxifen (Tam) was assessed via a luciferase assay, normalized to β -Gal activity and expressed as RLU. Shown is one of two independent experiments, performed in duplicates, with similar results. Note that 2-3 fold induction of ER transcriptional activation by cyclin A occurs in either of the three conditions used.

Thus, the recruitment of co-activator proteins to AF-2 is dispensable for the cyclin A-mediated enhancement of ER activity and that ER AF-1 is sufficient to confer the receptor responsiveness to cyclin A.

Conclusions

These results indicate that the enhancement of ER transcriptional activation by the cyclin A/CDK2 complex is mediated through the phosphorylation of serines 104 and 106. We propose that these residues control ER transcriptional activity in response to a variety of physiological signals that affect cyclinA/CDK activity. This work highlights the effect of the cyclinA/CDK2 complex on ER function and in view of increasing clinical data linking CDK dysregulation to a variety of human cancers, notably breast cancer, we believe that the subversion of this pathway might account for a subpopulation of breast hyperplasias and/or tumors.

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