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13. ABSTRACT *(Maximum 200 Words)*

Our goal to elucidate the molecular mechanisms of transcriptional regulation by the estrogen receptor alpha (ER) in breast cancer. ER is a hormone-dependent transcription factor involved in the regulation of both normal and malignant breast cell growth by controlling target genes and signaling pathways involved in cellular proliferation. ER signal transduction and transcriptional regulation is modulated by accessory proteins and through phosphorylation. The N-terminus of ER contains a transcriptional activation function, AF-1, that is phosphorylated at four major sites in cultured mammalian cells. Several kinases have been identified that phosphorylate ER in vitro at the identified sites. Of these, we have shown that the cyclin A/ cyclin-dependent kinase 2 complex (cyclinA/Cdk2) phosphorylate serine 104 (S104) and serine 106 (S106) and that the phosphorylation of these sites is important for ER function: serine to alanine mutations of S104 and S106 decrease ER transcriptional activation. In addition, we have identified the hsp90 associated cochaperone p23 as an important regulator of the ER signaling pathway. Finally, results from our lab have established the Rho GTPases as novel modulators of ER transcriptional activation.

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PI - Signature

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Date

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5. INTRODUCTION

The overall objective of this proposal is to elucidate the molecular mechanisms of transcriptional regulation by the estrogen receptor alpha (herein termed ER) in breast cancer. ER is a hormone-dependent transcription factor involved in the regulation of both normal and malignant breast cell growth by controlling target genes and signaling pathways involved in cellular proliferation. Although estrogens, such as 17-beta-estradiol, act as the primary signal in activating ER's transcriptional regulatory functions, ER-mediated transactivation is also controlled by phosphorylation and accessory proteins that modulate signal transduction and transcription regulation. The aims of this proposal are to 1) identify novel factors that affect ER function and 2) to define the effects of phosphorylation on ER function.

6. BODY

Task 1
Identification and characterization of non-receptor components involved in ER signal transduction

To identify proteins that affect ER function, we have carried out a dosage suppression screen in yeast. In this technique, a mutant ER protein, with a reduced ability to activate transcription, is used as a substrate to isolate yeast gene product(s) that are capable of overcoming this mutant phenotype by favoring the interaction between ER and these factors, thus reconstituting receptor transcriptional activity. The mutant ER used in our first dosage suppression screen contains a glycine to valine substitution at position 400 (G400V ER). This mutation is believed to alter the conformation of the ligand binding domain, which results in decreased hormone binding by the receptor, with a corresponding reduction in G400V ER's ability to activate transcription in response to estradiol. This mutant was selected as the substrate for the screen because it affects an early step in the ER signaling pathway, namely steroid binding, and therefore has the potential to result in the isolation of factors important for either steroid binding or transcriptional activation. We anticipate that characterization of these proteins will ultimately give rise to a more complete understanding of the ER signal transduction pathway.

Using G400V ER as our dosage suppression screen substrate, we have isolated a yeast gene that, when overexpressed, is capable of increasing both G400V and wild type (wt) ER's ability to activate transcription in response to estradiol. This gene product is the yeast homologue of the vertebrate p23 protein, a component of the Hsp90-based molecular chaperone complex associated with unliganded steroid receptors as part of the aporeceptor complex. We have examined the functional relationship between p23 and ER *in vivo* under a range of receptor, estradiol and p23 concentrations. Our findings suggest that p23 is an important regulator of the ER signaling pathway.

For complete record of the research finding see:
Knoblauch, R. and Garabedian, M.J., (1999) Role of Hsp-90-associated cochaperone p23 in estrogen receptor signal transduction, *Mol. Cell. Biol.* 19, 3738-3759.

In a separate dosage suppression screen, we used an ER phosphorylation site-deficient derivative that is defective in transcriptional activation as a substrate and identified RDI1, a Rho guanine nucleotide dissociation inhibitor (Rho GDI), as a positive regulator of ER transactivation. Overexpression of the human homologue of RDI1, Rho GDIα, increases ERα, ERβ, AR, and GR transcriptional activation in mammalian cells, but not activation by unrelated transcription factors SRF and Sp1. In contrast, expression of constitutively active forms of RhoA, Rac1, and Cdc42, decrease ER transcriptional activity, suggesting that Rho GDI increases ER transactivation by antagonizing Rho function. Inhibition of RhoA by expression of either the *Clostridium botulinum* C3 transferase or a dominant negative RhoA resulted in enhanced ER transcriptional activation, thus phenocopying the effect of Rho GDI expression on ER transactivation. Together, these findings establish the Rho GTPases as novel modulators of ER transcriptional activation. Since Rho GTPases regulate actin polymerization, our findings suggest a link between the major regulators of cellular architecture and steroid receptor transcriptional response.

For complete record of the research finding see:
Su, L.,F., Knoblauch, R. and Garabedian, M.J., Rho GTPases As Novel Modulators of the Estrogen Receptor Transcriptional Response *(JBC in press)*

Task 2

Studies on ER phosphorylation.

The N-terminus of ER contains a transcriptional activation function, AF-1, that is phosphorylated at four major sites in cultured mammalian cells. Several kinases have been identified that phosphorylate ER in vitro at the identified sites. Of these, we have shown that the cyclin A/ cyclin-dependent kinase 2 complex (cyclinA/Cdk2) phosphorylate serine 104 (S104) and serine 106 (S106) and that the phosphorylation of these sites is important for ER function: serine to alanine mutations of S104 and S106 decrease ER transcriptional activation. Thus, ER-mediated transcriptional activity is regulated positively by phosphorylation at S104 and S106.

For complete record of the research finding 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.

Rogatsky, I., Trowbridge, J.M. and Garabedian, M.J. (1999) Potentiation of human estrogen receptor alpha transcriptional activation through phosphorylation of serines 104 and 106 by the cyclin A/Cdk2 complex, *J. Biol. Chem.* 274, 22296-22302.

7. KEY RESEARCH ACCOMPLISHMENTS

- Identification of p23 as a regulator of ER signal transduction
- Identification of Rho GTPases as modulators of ER transcriptional activation
- Identification of cyclinA/Cdk2 as an ER kinase that phosphorylates serines 104 and 106, thereby increasing ER transcriptional activation.

8. REPORTABLE OUTCOMES

Manuscripts:

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.

Knoblauch, R. and Garabedian, M.J., (1999) Role of Hsp-90-associated cochaperone p23 in estrogen receptor signal transduction, *Mol. Cell. Biol.* 19, 3738-3759.

Rogatsky, I., Trowbridge, J.M. and Garabedian, M.J. (1999) Potentiation of human estrogen receptor alpha transcriptional activation through phosphorylation of serines 104 and 106 by the cyclin A/Cdk2 complex, *J. Biol. Chem.* 274, 22296-22302.

Su. L,F., Knoblauch, R. and Garabedian, M.J., Rho GTPases As Novel Modulators of the Estrogen Receptor Transcriptional Response (JBC in press)

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Degrees obtained that are supported by this award:

The following students received their Ph.D. and were supported in part by this award
Janet Trowbridge, M.D., Ph.D.
Inez Rogatsky, Ph.D.
Roland Knoblauch, M.D., Ph.D.

Development of cell lines-none

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P.I. Michael Garabedian, Ph.D.

Employment opportunities applied for:

Inez Rogatsky, Ph.D. is a post-doctoral fellow at UCSF in Keith Yamamoto's lab.
Janet Trowbridge, M.D., Ph.D., is currently a resident at UCSD.
Roland Knoblauch is completing his MD degree at NYU School of Medicine.

9. CONCLUSIONS

Our approach has uncovered novel signaling pathways that affect ER transcriptional activation and signal transduction, which can be used in the development of new therapies for breast cancer.

10 REFERENCES-detailed bibliographies can be found in the attached manuscripts.

11. APPENDICES

Enclosed Manuscripts/Reprints

12. FINAL REPORT

Bibliography

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.

Knoblauch, R. and Garabedian, M.J., (1999) Role of Hsp-90-associated cochaperone p23 in estrogen receptor signal transduction, *Mol. Cell. Biol.* 19, 3738-3759.

Rogatsky, I., Trowbridge, J.M. and Garabedian, M.J. (1999) Potentiation of human estrogen receptor alpha transcriptional activation through phosphorylation of serines 104 and 106 by the cyclin A/Cdk2 complex, *J. Biol. Chem.* 274, 22296-22302.

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

(Received for publication, April 26, 1999, and in revised form, May 27, 1999)

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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 $p90^{rsk1}$ *in vitro* and to regulate ER AF-1-dependent transcrip-

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‡ Both authors contributed equally to this work.

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[1] The abbreviations used are: ER, estrogen receptor; CDK, cyclin-dependent kinase; AF-1, activation function-1; wt, wild type; GST, glutathione *S*-transferase; E2, 17β-estradiol.

tional activation *in vivo* (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 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 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 reactions were 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 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 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 h 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 h 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 dishes in 250 μl of 1× reporter lysis buffer (Promega). Luciferase activity was quantified in a reaction mixture containing 25 mM glycylglycine, pH 7.8, 15 mM $MgSO_4$, 1 mM ATP, 0.1 mg/ml bovine serum albumin, 1 mM dithiothreitol. 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 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, 10% glycerol, 1% Triton X-100, 1 mM NaF, 25 μM $ZnCl_2$ 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 10 min at 4 °C), protein concentration in all samples was adjusted with the lysis buffer, and 200 μl of the whole cell extracts 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 (GST-ER_{121}) as described (24). 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 (10^7 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 (1×10^7 cells/100-mm dish) were infected with 0.5 ml (5×10^6 plaque-forming units) 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* 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 12CA5 (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 3 times in 1 ml of lysis buffer (described above), once with 1 ml of lysis buffer without Nonidet P-40, and once with DK buffer (50 mM potassium phosphate, pH 7.15, 10 mM $MgCl_2$, 5 mM NaF, 4.5 mM dithiothreitol) with protease inhibitors (described above). The wild type or mutant GST-ER_{121} substrates (approximately 10 μg in 100 μl) were added to the immobilized kinase complex, the kinase reaction was initialized by adding 25 μM ATP, 10 mM $MgCl_2$, 1 mM dithiothreitol, and [γ-^{32}P]ATP (100 μCi) 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-

buffered saline to remove unincorporated radioisotope, and the labeled GST-ER_{121} derivative was released by boiling at 100 °C for 3 min in an equal volume of 2× SDS sample buffer and fractionated on 10% SDS-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. 1*A*), 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. 1*B* 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. 1*B*, *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. 1*B*, *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 in Vitro Is Abolished in the ER Triple Mutant S104A/S106A/S118A—To examine whether Ser-104, Ser-106, and Ser-118 were indeed 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. 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. 3*A* 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

A

S104 leu-asn-ser-val-**ser(P)**-pro-ser-pro-leu

S106 asn-val-ser-pro-**ser(P)**-pro-leu-met-leu

S118 pro-pro-gln-leu-**ser(P)**-pro-phe-leu-gln

B

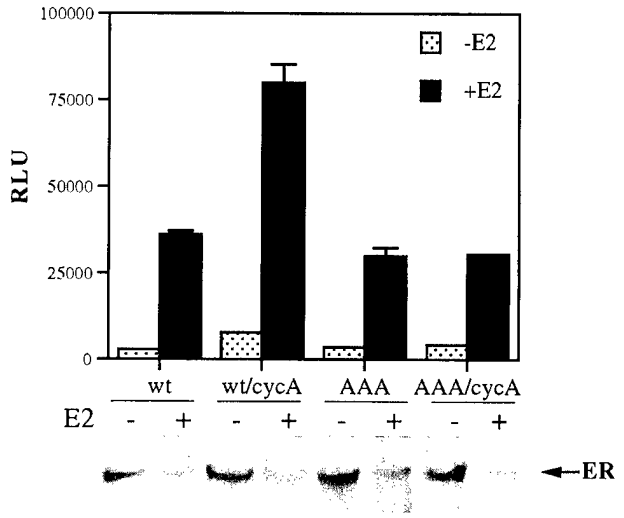

FIG. 1. **Replacement of ER N-terminal phosphorylation sites abolishes cyclin A-dependent induction of ER transcriptional enhancement in U-2 OS cells.** *A*, sequence context of Ser-Pro phosphorylation sites in ER AF-1. Shown are the amino acid residues surrounding the phosphorylation sites Ser-104, Ser-106, and S118 (in *bold*). Candidate kinases with the potential to modify these sites are CDK (consensus motif = Ser/Thr(P)-Pro-Lys/Arg), GSK-3 (consensus motif = Ser/Thr(P)-Pro-Xaa-Ser(P)), and mitogen-activated protein kinase (consensus motif = nonpolar-Xaa-Ser/Thr(P)-Pro), where Xaa is any amino acid. *B*, the ER derivative triple-mutated at Ser-104, Ser-106, and Ser-118 is not responsive to cyclin A overexpression. U-2 OS human osteosarcoma cells were transiently transfected via the calcium phosphate precipitation method with the full-length human ER (pcDNA3-ER, 1 μg/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 μg/60-mm dish), a pCMV-LacZ plasmid (0.5 μg/60-mm dish), and a pCMV-Myc-cycA plasmid (cycA, 3 μg/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 17β-estradiol (E2) was measured via luciferase assay 12 h after the addition of E2 to the medium, normalized to β-galactosidase activity, and expressed as relative luminescence units (*RLU*, *top panel*). The effect of cyclin A on the ER-responsive reporter did not reflect general activation of transcription, as it was dependent on ER (23), and pCMV-LacZ activity was not affected by cyclin A overexpression compared with empty vector-transfected cells. To verify equal expression of ER derivatives in the presence or absence of overexpressed cyclin A, whole cell extracts were prepared as described under "Experimental Procedures" from a set of identical dishes and the expression of the wt, and triple-mutated ER was analyzed by immunoblotting with ER-specific mouse monoclonal antibodies (Santa Cruz-787, *bottom panel*). Parental U-2 OS cells do not contain endogenous ER α based on immunoblotting as well as transcriptional activity assays (data not shown).

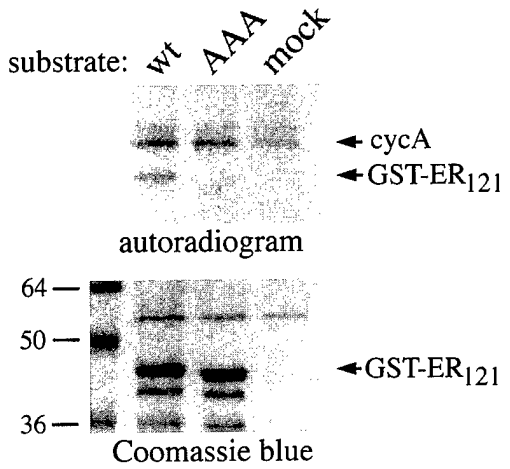

FIG. 2. **Cyclin A-CDK2 complex does not phosphorylate the ER S104A/S106A/S118A derivative *in vitro*.** GST-ER fusion proteins containing receptor amino acid residues 1 through 121 (GST-ER_{121}), either wt or containing three amino acid substitutions at receptor phosphorylation sites S104A/S106A/S118A (AAA), were expressed in *E. coli* and purified as described (24). The cyclin A-CDK2 complex was expressed in insect cells by baculovirus infection, immunopurified using anti-hemagglutinin mouse monoclonal antibodies as described under "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-polyacrylamide electrophoresis gels and stained with Coomassie Blue to visualize the substrate proteins (*bottom panel*), and autoradiography was performed (*top panel*).

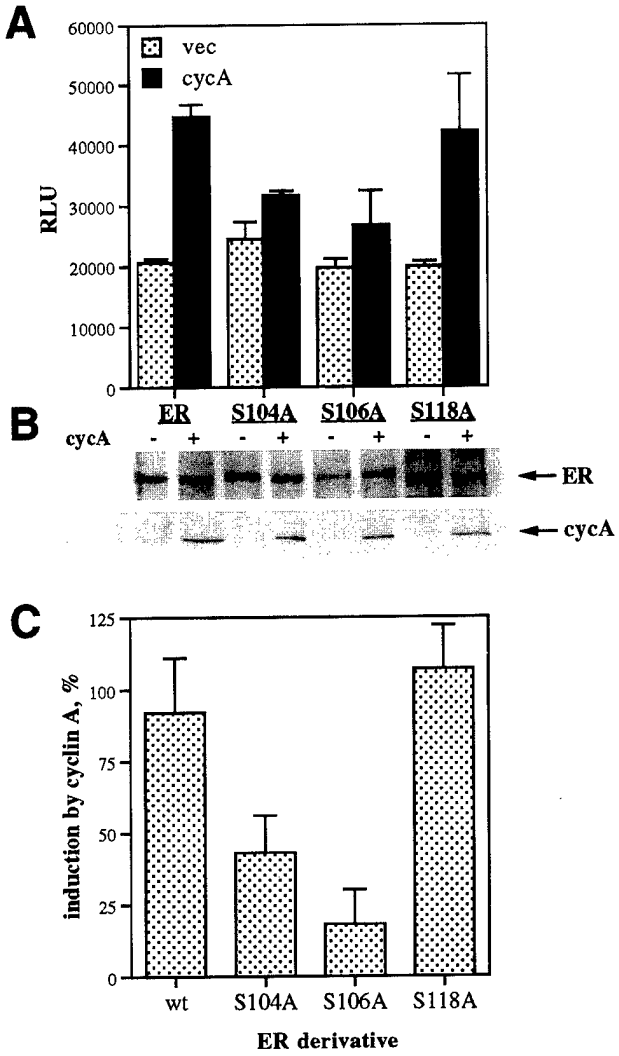

FIG. 3. **ER Ser-104 and Ser-106, but not Ser-118, are critical for cyclin A-mediated enhancement of ER transcriptional activation in U-2 OS cells.** *A*, S104A and S106A, but not the S118A mutation, abolish cyclin A-dependent induction of ER transactivation. U-2 OS cells were transfected as described in Fig. 1*B* with pcDNA3-ER (wt, S104A, S106A, or S118A, as indicated, 1 μg/60-mm dish), an XETL reporter plasmid (2 μg/60-mm dish), a pCMV-LacZ plasmid (0.5 μg/60-mm dish), and a pCMV-Myc-cycA plasmid (*cycA*, 3 μg/60-mm dish) or an empty pCMV vector (*vec*). ER transcriptional activation was assessed after a 12-h treatment with E2 via luciferase assay, normalized to the β-galactosidase activity, and expressed as relative luminescence units (*RLU*). *B*, ER expression level is not affected by cyclin A overexpression or point mutations at phosphorylation sites. Whole cell extracts were prepared from transfected cells as described under "Experimental Procedures," and the expression of ER derivatives and Myc-tagged transfected cyclin A was analyzed by Western blotting. *C*, differential enhancement of ER mutant 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 are the average and a S.E. of four independent experiments.

level in both the presence and absence of exogenous cyclin A (Fig. 3*B*). The results from four independent experiments (Fig. 3*C*) 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-ER_{121}. Fig. 4*A* 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 ER 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. 4*B*). 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. 4*C*). The ER S118A mutation also results in a decrease in ER phosphorylation by the cyclin A-CDK2 complex, albeit to a much smaller extent

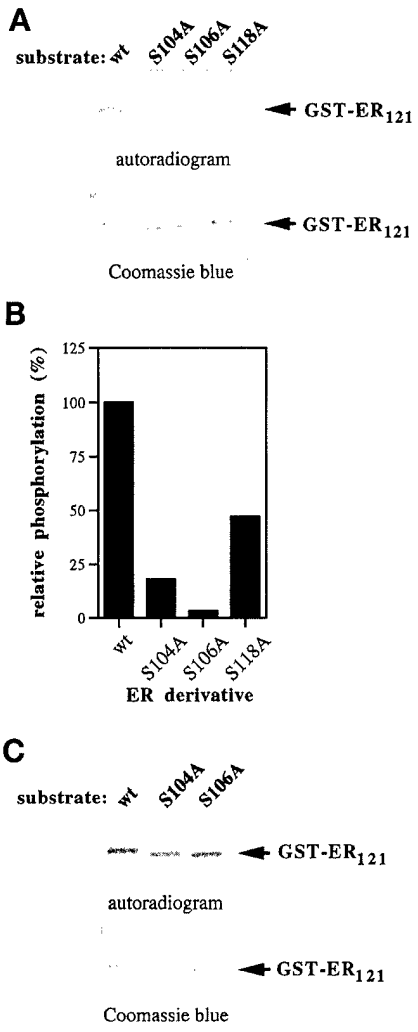

FIG. 4. **Individual mutations at ER N-terminal phosphorylation sites decrease ER phosphorylation by cyclin A-CDK2 complex *in vitro*.** GST-ER_{121} fusion proteins, either wt or containing single amino acid substitutions at receptor phosphorylation sites S104A, S106A, or S118A, were expressed in *E. 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-ER_{121} and cyclin A bands were subsequently excised from the gel and subjected to scintillation counting. ^{32}P 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-ER_{121} as a 100% (*B*). Note that each serine-to-alanine substitution decreases the amount of GST-ER_{121} phosphorylation; however, S104A and S106A do so to a greater extent than S118A.

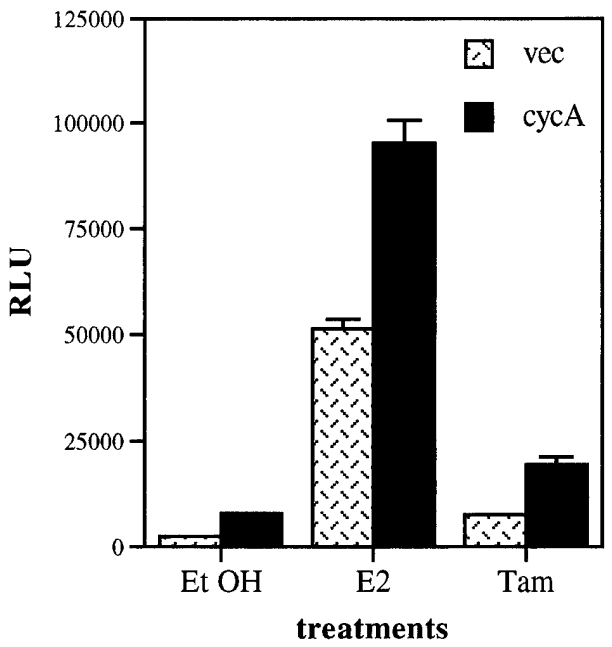

FIG. 5. **Cyclin A-mediated induction of ER transactivation is ligand-independent.** U-2 OS cells were transfected as described in Fig. 1, and ER transcriptional activation in the absence of ligand (*Et OH*), in the presence of 100 nM 17β-estradiol (*E2*), and in the presence of 1 μM 4-hydroxytamoxifen (*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.

than the S104A and S106A substitutions. Although the phosphorylation of all three mutant receptor derivatives by the cyclin A-CDK2 complex *in vitro* is reduced (rank order of ER phosphorylation by cyclin A-CDK2 *in vitro*: S106A < S104A < S118A < wt), S106A and S104A substitutions most profoundly affect phosphorylation by the cyclin A-CDK2 complex. Although Ser-118 appears to contribute to ER phosphorylation by the cyclin A-CDK2 complex *in vitro*, this may result from the artificial exposure of the Ser-118 site in the truncated GST-ER_{121} fusion protein. In contrast, in the context the full-length receptor expressed in mammalian cells, Ser-118 may not be accessible to the cyclin A-CDK2 complex or may be already phosphorylated by a different kinase (such as mitogen-activated protein kinase). 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. 1*B*). 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-

tivity, and ER AF-1 is sufficient to confer the receptor responsiveness to cyclin A.

DISCUSSION

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 *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-ER_{121} 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. 1*A*), 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.

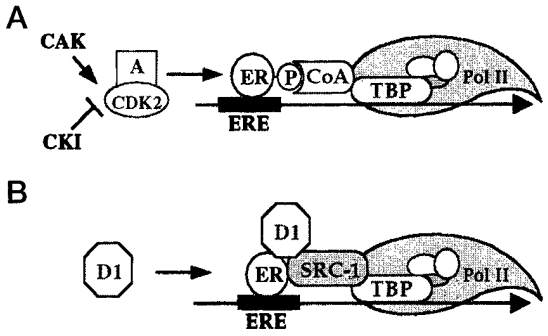

FIG. 6. **Potential mechanisms underlying ER transcriptional activation by cyclin A and cyclin D1.** *A*, a model for cyclin A-CDK2-dependent regulation of ER transcriptional activation. According to this scheme, increased expression of cyclin A leads to enhanced formation of active cyclin A-CDK2, which phosphorylates (*P*) ER at Ser-104 and Ser-106, thereby promoting the interaction between ER AF-1 and a putative co-activator (*CoA*) necessary for ER-dependent transcriptional activation. Alternatively, ER phosphorylation may dissociate a putative inhibitor (not shown). The model further envisions that increasing the concentration of positive regulators of the cyclin A-CDK2 complex, including CDK-activating complex (*CAK*) or negative regulators, for example CDK inhibitors (*CKI*) such as $p27^{KIP}$ will increase and decrease, respectively, the activity of cyclin A-CDK2, resulting in differential regulation of ER transcriptional activation. *B*, CDK-independent activation of ER by cyclin D1. Increased expression of cyclin D1 promotes the association of SRC-1 with the ER AF-2 and forms an ER-SRC-1-cyclin D1 ternary complex, thereby leading to enhanced ER transcriptional activation. *PolII*, polymerase II; *TBP*, TATA-binding protein.

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 the effect of D1 on ER transcriptional activity is dependent on AF-2 and does not involve ER phosphorylation (33). Recently, it has been suggested that cyclin D1 increases ER activity by acting as a bridge between AF-2 and the coactivator, SRC-1 (34). Thus, the mechanism of 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.

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Role for Hsp90-Associated Cochaperone p23 in Estrogen Receptor Signal Transduction

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The mechanism of signal transduction by the estrogen receptor (ER) is complex and not fully understood. In addition to the ER, a number of accessory proteins are apparently required to efficiently transduce the steroid hormone signal. In the absence of estradiol, the ER, like other steroid receptors, is complexed with Hsp90 and other molecular chaperone components, including an immunophilin, and p23. This Hsp90-based chaperone complex is thought to repress the ER's transcriptional regulatory activities while maintaining the receptor in a conformation that is competent for high-affinity steroid binding. However, a role for p23 in ER signal transduction has not been demonstrated. Using a mutant ER (G400V) with decreased hormone binding capacity as a substrate in a dosage suppression screen in yeast cells (*Saccharomyces cerevisiae*), we identified the yeast homologue of the human p23 protein (yhp23) as a positive regulator of ER function. Overexpression of yhp23 in yeast cells increases ER transcriptional activation by increasing estradiol binding in vivo. Importantly, the magnitude of the effect of yhp23 on ER transcriptional activation is inversely proportional to the concentration of both ER and estradiol in the cell. Under conditions of high ER expression, ER transcriptional activity is largely independent of yhp23, whereas at low levels of ER expression, ER transcriptional activation is primarily dependent on yhp23. The same relationship holds for estradiol levels. We further demonstrate that yhp23 colocalizes with the ER in vivo. Using a yhp23-green fluorescent protein fusion protein, we observed a redistribution of yhp23 from the cytoplasm to the nucleus upon coexpression with ER. This nuclear localization of yhp23 was reversed by the addition of estradiol, a finding consistent with yhp23's proposed role as part of the aporeceptor complex. Expression of human p23 in yeast partially complements the loss of yhp23 function with respect to ER signaling. Finally, ectopic expression of human p23 in MCF-7 breast cancer cells increases both hormone-dependent and hormone-independent transcriptional activation by the ER. Together, these results strongly suggest that p23 plays an important role in ER signal transduction.

Estrogen is a steroid hormone responsible for the proper function of a variety of mammalian physiological processes. In addition to its central role in reproduction (6, 33), estrogen also affects the cardiovascular (19), skeletal (47), immune (9), and nervous (57) systems and plays a role in the initiation and progression of breast cancer (58).

The estrogen signal is mediated by the estrogen receptor (ER), a ligand-inducible transcription factor. In the absence of estradiol, the ER is found predominantly in the nucleus (32, 56), as part of a multiprotein complex consisting of a dimer of Hsp90 (6), a p23 monomer (42), and one of several immunophilins, including Cyp-40 (49) and FKBP52 (48). It has been proposed that this Hsp90-based chaperone complex inactivates the ER's transcriptional regulatory capabilities and maintains the ER in a conformation competent for steroid binding (46). Upon binding estradiol, ER dissociates from this complex, dimerizes, and recognizes specific DNA sequences (35), termed estrogen-response elements (EREs), within the promoters of estrogen-responsive genes. Once bound to an ERE, ER is believed to modulate transcription of the linked gene through direct or indirect interactions with general transcriptional factors (15, 25).

Although many aspects of ER signaling are not yet understood, it appears that the proteins essential to ER function are conserved among eukaryotes to such an extent that introduction of ER into *Saccharomyces cerevisiae*, which lacks endogenous ER, is sufficient for the faithful reconstitution of estrogen signaling within these cells (18, 41). When expressed in yeast cells, the human ER will activate transcription from EREs located in reporter gene promoters in response to estradiol. This ability of ER to function within yeast cells allows a wide variety of genetic approaches to be taken toward defining the mechanisms of signal transduction and transcriptional regulation by the receptor.

To identify proteins that affect ER function, we have carried out a dosage suppression screen in yeast cells. In this technique, a mutant ER protein, with a reduced ability to activate transcription, is used as a substrate to isolate a yeast gene product(s) that is capable of overcoming this mutant phenotype by favoring the interaction between ER and these factors, thus reconstituting receptor transcriptional activity. The mutant ER used in our dosage suppression screen contains a glycine-to-valine substitution at position 400 (G400V ER). This mutation is believed to alter the conformation of the ligand-binding domain, resulting in decreased hormone binding by the receptor, with a corresponding reduction in G400V ER's ability to activate transcription in response to estradiol (54). This mutant was selected as the substrate for the screen because it affects an early step in the ER signaling pathway, namely steroid binding, and therefore has the potential to result in the isolation of factors important for either steroid binding or transcriptional activation. We anticipate that characterization of these proteins will ultimately lead to a more complete understanding of the ER signal transduction pathway.

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Using G400V ER as our dosage suppression screen substrate, we have isolated a yeast gene that, when overexpressed, is capable of increasing both G400V and wild-type (wt) ER's ability to activate transcription in response to estradiol. This gene product is the yeast homologue of the vertebrate p23 protein (yhp23) (28), a component of the Hsp90-based molecular chaperone complex associated with unliganded steroid receptors as part of the aporeceptor complex. We have examined the functional relationship between p23 and ER in vivo under a range of receptor, estradiol, and p23 concentrations. Our findings suggest that p23 plays a role in ER signal transduction.

MATERIALS AND METHODS

Yeast strains and growth conditions. The W303a (**a** *ade2 leu2 his3 trp1 ura3*) yeast strain was used in experiments where indicated. The yhp23/SBA1 knockout (KO) and parental (PA) strains are described elsewhere (2). Standard genetic methods were used for growth and manipulation. Cultures were propagated at 30°C in rich medium (yeast extract-peptone-dextrose) or selective minimal medium with 2% glucose (or raffinose or galactose) supplemented with amino acids. To induce gene expression from vectors containing the Gal1-10 inducible promoter, yeast cells were grown in selective medium containing either 2% galactose–1% raffinose or 2% raffinose.

Plasmid constructs. The YKL117w gene was cloned from the 4.3 library plasmid by PCR with the following primers: 5′-GAAGATCTCCACCATGTAC CCATACGATGTTCCTGACTATGCGTCCGATAAAGTTATTAACCCTC AAGTTGC-3′ (encoding a hemagglutin [HA] epitope and containing a *Bgl*II site) and 5′-CCCCATGGTTACTCATTCTAGCACTCCAGGTTGATTT-3′. The PCR product was gel purified and subcloned into the pGEM-T Easy vector (Promega). The entire fragment was sequenced to ensure the fidelity of the PCR product. HA-yhp23 was released from this vector by digesting it with *Bgl*II (within primer) and *Pst*I (within the polylinker of pGEM-T Easy) and then subcloned into *Bgl*II/*Pst*I-digested pCMV5. HA-yhp23 was released from pCMV5 by the *Bgl*II/*Bam*HI digest and subcloned into the *Bam*HI site of the yeast expression vectors containing the glycerol-phosphate dehydrogenase promoter (GPD) pRS314$_{GPD}$(Trp1) and PRS315$_{GPD}$(Leu2). The yhp23-green fluorescent protein (GFP) fusion protein was created by subcloning the *Hin*dIII/*Xba*I fragment of GFP from pEGFP-N3 (Clontech) into a *Bgl*II/*Hin*dIII fragment of HA-yhp23 in the pGEM-T Easy vector and placing the HA-yhp23-GFP fusion protein into pCMV5 at the *Bgl*II/*Xba*I sites. The HA-yhp23-GFP fusion protein was excised from pCMV5 by using *Bgl*II/*Bam*HI and then subcloned into the *Bam*HI site of the yeast expression vector pRS314$_{GPD}$. The ER$_{1-115}$–Lex DNA binding domain fusion protein was constructed by excising the *Eco*RI/*Pvu*II fragment of pGEX-ER$_{1-185}$ (a kind gift from P. Kushner) and inserting it into the *Eco*RI- and *Xho*I-digested pEG 202 two-hybrid vector (20). The *Xho*I sticky end was first blunted to make ligation with the *Pvu*II blunt end possible.

An HA epitope was also placed at the amino terminus of the human p23 cDNA by PCR by using the following primers: 5′-GCGGATCCACCATGTAC CCATACGATGTTCCTGACTATGCGCAGCCTGCTTCTGCAAAGTGGTAC GATCG-3′ (containing a *Bgl*II site and encoding an HA epitope) and 5′-CACCA CCCATGTTGTTCATCATCTCAGAG-3′. The same PCR and cloning strategy was used to create the HA-p23 yeast and mammalian expression vectors.

wt ER, G400V ER, and wt glucocorticoid receptor (GR) were expressed from vectors containing the Gal1-10 promoter, the 2μm plasmid replication origin from yeast cells, and either the *TRP1* (p2T-GAL) or *HIS3* (p2H-GAL) gene. Reporter plasmids ERE-CYC1-LacZ or GRE-CYC1-LacZ contain a single ERE or three glucocorticoid response elements (GREs) upstream of a truncated *CYC1* promoter linked to the β-galactosidase gene; these plasmids also contain the *URA3* gene as a selectable marker and the yeast 2μ plasmid replication origin. Full-length GRIP1 was expressed constitutively in yeast from the alcohol dehydrogenase promoter as described previously (21).

Transient transfections. MCF-7 cells were seeded onto 60-mm dishes at 2.5 × 10^5 cells/dish in phenol red-free Dulbecco modified Eagle medium supplemented with 10% charcoal-stripped fetal bovine serum. The following day the cells were transfected by the liposome-mediated method as described by the manufacturer (Trans IT-100; Pan Vera, Madison, Wis.) with 5 μg of ERE-thymidine kinase-luciferase reporter plasmid (XETL) and 2 μg of pCMV-HA-p23. At 12 h posttransfection, cells were refed with the same medium containing 0.1 nM 17-β-estradiol or ethanol vehicle. A luciferase assay was performed 24 h later as previously described (50).

β-Galactosidase assays. With yeast liquid cultures, quantitative β-galactosidase measurements were carried out as previously described (18). Cultures were grown overnight in selective media containing 2% glucose. Equal numbers of cells (as determined by measuring the optical density at 600 nm [OD_{600}]) were pelleted and washed in sterile water to remove glucose medium and subcultured into 2 ml of selective medium containing either 2% galactose–1% raffinose or 2% raffinose. Steroid hormones were added to the medium as a 1,000-fold dilution in ethanol. Cultures were incubated for 12 h at 30°C. One-half of the cells were pelleted, washed in 0.5 ml of LacZ buffer (5 mM KCl, 0.5 mM $MgSO_4 \cdot 7H_2O$, 60 mM $Na_2HPO_4 \cdot 7H_2O$, 60 mM $NaH_2PO_4 \cdot H_2O$ [pH 7.0], with 0.025% β-mercaptoethanol added freshly), and pelleted again. Cells were resuspended in 50 μl of LacZ buffer and permeabilized by adding 50 μl of $CHCl_3$–20 μl of 0.1% sodium dodecyl sulfate (SDS) and vortexing for 10 s. After 5 min, 0.5 ml of a 2-mg/ml concentration of *o*-nitrophenyl-β-D-galactopyranoside (ONPG) was added, and the reaction was allowed to continue for 1 to 10 min, after which the reaction was stopped by adding 0.5 ml of 1 M Na_2CO_3. Reactions were centrifuged for 1 min to pellet the cell debris, and levels of β-galactosidase activity were determined by measuring the OD_{420}. The cell number was determined by measuring the OD_{600} of the remaining cells. Receptor activity is expressed as β-galactosidase units, determined by using the following equation: β-galactosidase units = (1,000 × OD_{420})/(reaction volume [milliliters] × reaction time [minutes] × OD_{600}). All experiments were performed in triplicate, and the data shown are representative of multiple experiments. The experiments were performed in the linear range of the assay.

Plate assays were performed by replica plating colonies from glucose plates onto galactose X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) indicator plates containing 1 nM 17-β-estradiol.

Preparation of yeast extracts and immunoblotting. Yeast extracts were prepared from 5-ml cultures. Equal numbers of cells (as determined by measuring the OD_{600}) were pelleted and washed in 0.5 ml of 1× phosphate-buffered saline (PBS) supplemented with 3 mM dithiothreitol and protease inhibitors, including 1 mM phenylmethylsulfonyl fluoride and 1 μg each of aprotinin, pepstatin A, and leupeptin per ml. Subsequent steps were carried out at 4°C. Cells were again pelleted and resuspended in 200 μl of receptor buffer (10 mM Tris, pH 7.5; 1 mM EDTA; 50 mM NaCl; 20% glycerol) containing protease inhibitors. An equal volume of glass beads was added to each tube, and cells were lysed by shaking for 20 min in an Eppendorf horizontal shaker. Extracts were separated from glass beads by centrifuging the extracts through a small hole (made with a 20-gauge needle) at the bottom of the microcentrifuge tube. The lysates were subsequently cleared by centrifugation at 10,000 rpm for 15 min, after which the supernatants were transferred to a new tube. The protein concentration of each extract was measured by using the Bio-Rad Protein Assay and then standardized accordingly. For Western blotting, protein extracts (50 to 100 μg) were fractionated on SDS–10% polyacrylamide gels and transferred to Immobilon paper (Millipore). Blots were probed either with a combination of the ER monoclonal antibodies C311 and C314 (Santa Cruz) or with the anti-HA monoclonal antibody 12CA5 (Boehringer Mannheim). Rabbit polyclonal antiserum against endogenous yhp23 was the generous gift of B. Freeman (University of California at San Francisco). The blots were developed with horseradish peroxidase-coupled sheep anti-mouse or donkey anti-rabbit antibodies and enhanced chemiluminescence reagent (Amersham).

In vivo estradiol-binding assay. Ligand-binding assays were carried out as previously described (34). Duplicate cultures of yeast cells containing either G400V ER with or without HA-yhp23 or wt ER with or without HA-yhp23 were inoculated into selective liquid medium containing 2% galactose–1% raffinose and grown overnight at 30°C to induce receptor expression. Equal numbers of cells were pelleted and inoculated into 3 ml of 2% galactose–1% raffinose medium containing either 1 nM (G400V ER) or 0.1 nM (wt ER) of ^{3}H-labeled 17-β-estradiol (72 Ci/mmol) (NET317; NEN) such that the final OD_{600} was 2. Cells were incubated with the labeled estradiol for 1 h at 30°C, after which 1 ml of the cultures was pelleted and washed three times with 2% glucose in PBS, resuspended in 150 μl of the same solution, and then transferred to a scintillation vial. After the addition of scintillation fluid, the total ^{3}H-labeled 17-β-estradiol was measured by scintillation counting. To account for any nonspecific binding, ^{3}H-labeled 17-β-estradiol binding to a GR-containing strain was subtracted from the counts obtained with the ER-expressing strain. β-Galactosidase assays were carried out as described above with 1 ml of the remaining culture.

Subcellular localization of yhp23. W303a was transformed with the pRS314$_{GPD}$-yhp23-GFP expression vector; with the ERE-dependent β-galactosidase reporter plasmid; or with the p2H GAL expression vector containing G400V ER, wt ER, GR, or p2H GAL plasmid alone. Yeast strains were grown in 2 ml of 2% galactose–1% raffinose for 12 h. Cells were fixed by adding 240 μl of 37% formaldehyde to each 2-ml culture and incubated at 30°C for 90 min. Cultures were then pelleted by centrifugation and washed three times with 200 μl of solution A (1.2 M sorbitol, 50 mM KPO_4). Cell walls were digested by resuspending cells in 100 μl of solution A plus 0.1% β-mercaptoethanol, 0.02% Glusulase (Dupont), and 5 μg of Zymolase (U.S. Biological) per ml for 1 h at 37°C. Cells were then washed twice in 100 μl of PBS and once in 100 μl of PBS plus 0.1% Nonidet P-40 (NP-40) and then blocked for 2 h in 5% bovine serum albumin in Tris-buffered saline (pH 7.4) at room temperature. Cells were then incubated with 100 μl of the appropriate anti-steroid receptor primary antibody (for ER, a mixture of monoclonal antibodies C311 and C314 [Santa Cruz], and for GR, monoclonal antibody BuGr2 diluted in blocking solution) for 2 h at room temperature. Cells were washed once in 100 μl of PBS, once in 100 μl of PBS plus 0.1% NP-40, and once again in 100 μl of PBS, followed by incubation with goat anti-mouse rhodamine-conjugated secondary antibody (Vector Labs), diluted in blocking solution, for 4 h at room temperature. Secondary antibody was removed by washing the cells twice in 100 μl of PBS and then twice in 100 μl of PBS plus 0.1% NP-40. In order to visualize the nuclei, cells were then incubated

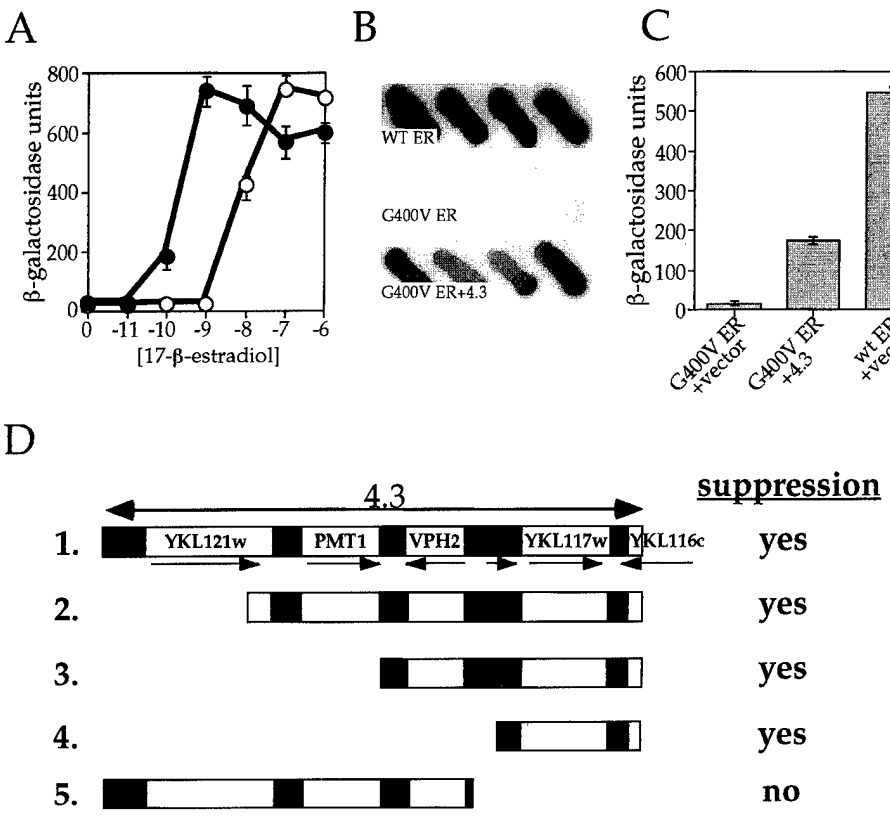

FIG. 1. Isolation of a yeast genomic fragment that suppresses the G400V ER phenotype. (A) Transcriptional activity of wt ER and G400V ER as a function of 17-β-estradiol concentration. The W303a yeast strain was transformed with a galactose-inducible expression vector containing either wt or G400V ER, along with an ERE-containing β-galactosidase reporter plasmid. Transcriptional activation by wt ER (solid circles) and G400V ER (open circles) in response to increasing 17-β-estradiol concentration was determined by liquid β-galactosidase assay as described in Materials and Methods. Note that G400V ER requires a 100-fold-higher estradiol concentration to induce transcriptional activation than wt ER. The dosage suppression screen was carried out in the presence of 1 nM 17-β-estradiol, the conditions under which the G400V ER phenotype is most pronounced. (B) The relative activity of wt ER, G400V ER, and G400V ER plus suppressor 4.3. Four independent colonies on X-Gal indicator plates containing 1 nM 17-β-estradiol are shown and represent wt ER plus empty library plasmid (wt ER), G400V ER plus empty library plasmid (G400V ER), or G400V ER plus suppressor plasmid 4.3 (G400V ER+4.3). (C) Transcriptional activity of wt ER, G400V ER, and G400V ER in the presence of suppressor 4.3. Liquid β-galactosidase assays were performed on yeast strains containing wt ER plus empty library plasmid, G400V ER plus empty library plasmid, or G400V ER plus 4.3 at 1 nM 17-β-estradiol. Suppressor 4.3 increases G400V ER activity 10-fold, bringing its activity to nearly one-third that of wt ER. (D) Identification of YKL117w as the G400V ER suppressor. The sequence of the yeast genomic fragment contained within the suppressor 4.3 was determined by aligning the 5′ and 3′ ends of the insert to the yeast genomic sequence database. Suppressor 4.3 contains an 8,147-bp fragment comprising four complete ORFs (YKL121w, PMT1, VPH2, and YKL117w), a partial ORF (YKL116w), and a tRNA-Ala gene (shaded box). The relative positions and orientations of the genes within the 4.3 fragment are shown schematically (fragment 1). Identification of the gene responsible for suppressing the G400V ER phenotype was accomplished by constructing 5′ and 3′ deletion derivatives of the 4.3 suppressor (fragments 2 to 5) and assaying their ability to increase G400V ER transcriptional activity at 1 nM 17 β-estradiol. "yes" indicates that the fragment was capable of suppressing the G400V ER phenotype; "no" indicates that the fragment failed to increase G400V ER transcriptional activity. YKL117w was present within the suppressing fragments (fragments 1 to 4) but was absent within the fragment that did not suppress (fragment 5), suggesting that its gene product is responsible for increasing G400V ER transcriptional activation. YKL117w encodes the yeast homologue of the human p23 protein (yhp23).

in 1 μg of Hoechst dye H334211 per ml for 10 min, followed by one wash with 100 μl of PBS. Cells were then resuspended in 30 μl of PBS, 5 μl of which was then plated onto a poly-D-lysine-treated microscope slide and allowed to settle for 10 min before excess fluid was removed by aspiration. Unattached cells were then removed by washing the slide with 20 μl of PBS. The cells were mounted by using 3 μl of Citifluor (Ted Pella, Reading, Calif.), and a coverslip was secured to the slide with rubber cement. GFP, rhodamine, and Hoechst signals were imaged and photographed by using a Zeiss Axioplan 2 microscope.

RESULTS

Yeast dosage suppression screen. To compare wt ER and G400V ER function in yeast cells, we constructed two strains containing a galactose-inducible expression vector, encoding either wt ER or G400V ER, and a reporter plasmid containing an ERE located upstream of the β-galactosidase gene. The transcriptional activities of wt ER and G400V ER, as a function of hormone concentration, were measured. Compared to wt ER, G400V ER requires a 100-fold increase in 17-β-estradiol concentration before receptor transcriptional activation is observed in our yeast assay (Fig. 1A). At saturating ligand concentrations, however, G400V ER is able to reach the same maximal activity as wt ER, suggesting that once the block to steroid binding is overcome, the receptor is able to act as efficiently as wt ER in entering the functional interactions downstream of estradiol binding, including both protein-protein and protein-DNA interactions, that are necessary for transcriptional activation.

The G400V ER phenotype is most apparent at a concentration of 1 nM 17-β-estradiol, where wt ER shows maximal transcriptional response, but G400V ER displays only minimal transcriptional activity (Fig. 1A). Exploiting this phenotypic difference, we carried out a dosage suppression screen to identify yeast proteins that, when overexpressed, would increase the transcriptional activity of G400V ER, thereby suppressing the mutant phenotype. The yeast strain containing G400V ER

and an estrogen-responsive β-galactosidase reporter gene was transformed with a high-copy-number yeast genomic library and assayed for G400V ER transcriptional activity on X-Gal indicator plates containing 1 nM 17-β-estradiol. Under these conditions, yeast colonies expressing the wt ER are blue, while the G400V ER-expressing yeast colonies appear white (Fig. 1B). Blue colonies were considered to be potential suppressor candidates. Five candidate suppressors of the G400V ER phenotype were isolated after screening ~6,000 colonies, which we estimate to represent about one-half of the yeast genome.

Identification of the yeast ORF YKL117w as a suppressor of the G400V ER phenotype. One high-copy-number suppressor plasmid, designated 4.3, was found to increase G400V ER transcriptional activity 10-fold, bringing G400V ER transcriptional activation to one-third the wt ER level at 1 nM 17-β-estradiol (Fig. 1B and C). The 5′ and 3′ ends of the insert of the library plasmid were sequenced and aligned with the yeast genome. In this manner, we were able to identify the suppressor DNA as an 8,147-bp genomic fragment of chromosome XI (26) containing multiple open reading frames (ORFs) (Fig. 1D). To identify the suppressing ORF, a series of deletions were constructed and analyzed for their ability to increase G400V ER transcriptional activity. As seen in Fig. 1D, the suppression of the G400V ER phenotype correlates with the presence of ORF YKL117w.

Possible role of YKL117w in ER signaling. A search of the Swissprot database revealed YKL117w to be the yeast homologue of the human p23 protein (yhp23) (28), a component of the Hsp90-based molecular chaperone complex. During completion of this study, two separate reports (2, 16) characterizing yeast strains with YKL117w deleted have called this gene *SBA1*, reflecting an increased susceptibility of steroid signaling to benzoquinone ansamycin antibiotics. Since the YKL117w gene product's homology to the vertebrate p23 protein was of considerable importance in investigating its role in ER function, we have chosen to refer to it as yhp23 to maintain this emphasis.

Although p23's specific function is not known, in vitro studies suggest that it is crucial to the stability of the aporeceptor complex of unliganded steroid receptors. Removal of p23 greatly reduces the formation of stable aporeceptor complexes of the GR (11, 12, 24) and the progesterone receptor (PR) (29, 30). In addition, in vitro studies have suggested that p23 possesses abilities typical of molecular chaperones, since it is capable of interacting with denatured β-galactosidase, suppressing its aggregation and maintaining it in an intermediate, folding-competent conformation (17). The relative importance of these two aspects of p23 function in ER signaling has not been determined.

Characterization of yhp23's role in ER signaling. Having identified yhp23 as the suppressing ORF, we cloned the YKL117w gene with an amino-terminal HA epitope tag (HA-yhp23) into a yeast expression vector containing a constitutively active GPD promoter. We then established a yeast strain that overexpresses HA-yhp23 in the presence of G400V ER, along with a reporter plasmid containing the β-galactosidase gene under control of an ERE. As seen in Fig. 2A, overexpression of HA-yhp23 increases G400V ER transcriptional activity by 10-fold. Extracts from these yeast strains were prepared and analyzed by immunoblotting with antibodies specific for ER and HA. Figure 2B demonstrates that the overexpression of yhp23 does not affect the level of G400V ER protein and, therefore, the increase in transcriptional activity is not a result of increased receptor expression. The HA-tagged yhp23 protein migrates on an SDS-polyacrylamide gel at approximately 34 kDa. The higher molecular mass of the yeast protein (34 kDa versus the 23-kDa human protein) is expected, as the yhp23 is larger (216 residues) than its human counterpart (160 residues). Further comparison of G400V ER activity in the presence or absence of yhp23 overexpression over a range of hormone concentrations revealed that G400V ER function was also enhanced at 10 nM 17-β-estradiol (Fig. 2C). The 30% increase in G400V ER activity seen at this level of hormone, however, is relatively small compared to the 10-fold increase observed at the 1 nM 17-β-estradiol concentration used in the screen. This pattern continues at even higher hormone concentrations, so that no increase of G400V ER activity by yhp23 is observed at 100 nM concentrations of 17-β-estradiol (Fig. 2C) and 1 mM 17-β-estradiol (data not shown). These findings suggest that yhp23's importance to G400V ER signaling decreases at high estradiol concentrations.

Given p23's proposed chaperone-like activities in vitro, it could be argued that yhp23's interaction with the mutant G400V ER might arise as a function of the receptor's misfolded steroid-binding domain rather than reflect a true role in the ER signaling pathway. To determine whether yhp23 overexpression affects wt ER activity, we constructed an additional yeast strain that overexpresses yhp23 in the presence of wt ER, along with an ERE-controlled β-galactosidase gene reporter plasmid. The effect of yhp23 overexpression upon ER activity was then assayed over a range of hormone concentrations (Fig. 2D). wt ER activity was affected in a manner similar to that of G400V ER, albeit less dramatically. At 0.1 nM 17-β-estradiol, wt ER activity is increased by approximately 70% in the presence of overexpressed yhp23, with no increase observed at the higher concentrations of 1 and 10 nM 17-β-estradiol. In contrast to G400V ER, however, an increase in wt ER activity was observed at 0.01 nM 17-β-estradiol. To more closely examine the effect of yhp23 on wt ER activity at this low hormone concentration, we compared wt ER ligand-independent activity to wt ER activity at 0.01 nM 17-β-estradiol. As seen in Fig. 2E, overexpression of yhp23 increases wt ER ligand-independent activity by approximately twofold and ligand-dependent activation by threefold in the presence of 0.01 nM 17-β-estradiol. These findings suggest that overexpression of yhp23 greatly increases wt ER's ability to respond to low levels of ligand. Without yhp23 overexpression, the fold induction of wt ER activity was only 50% upon administration of 0.01 nM 17-β-estradiol compared to a 250% induction of wt ER activity in the presence of overexpressed yhp23. These results suggest that increased levels of yhp23 can facilitate wt ER ligand-independent activity and potentiate ER signaling at low levels of hormone. Thus, the ability of yhp23 to functionally interact with wt ER and not just the G400V ER mutant strongly implicates yhp23 as a member of the ER signaling pathway in yeast cells.

Importantly, yhp23 does not appear to have any effect upon the activity of the ER AF-1 domain. When we fused the amino-terminal 115 residues of ER to a Lex DNA binding domain and expressed this construct in yeast cells, we observed constitutive activation of a Lex-responsive β-galactosidase reporter plasmid, a result consistent with earlier findings demonstrating that the AF-1 activity of ER is contained within this region of the receptor (39). As shown in Fig. 2F, this constitutive activation was not affected by yhp23 overexpression, suggesting that the increased function of both the mutant and the wt ER observed in Fig. 2C and D, respectively, does not appear to be mediated by an increase in AF-1 activity. This finding, taken together with the observed hormone concentration-dependent effect of yhp23 overexpression (Fig. 2C and D), suggests that yhp23 affects the ER signal transduction pathway at the step of ligand binding.

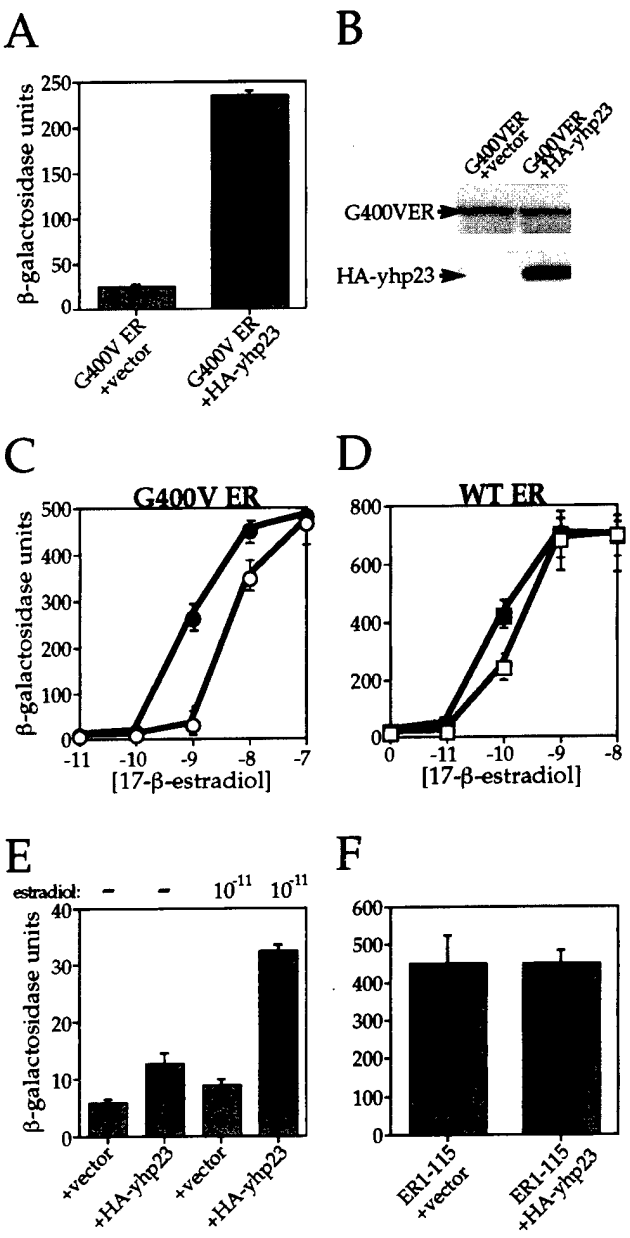

FIG. 2. Effect of yhp23 overexpression on G400V ER and wt ER transcriptional activity. An HA-tagged yhp23 gene was cloned into the yeast expression vector pRS314 downstream of the GPD promoter. Yeast strains were then constructed which coexpress G400V ER or wt ER with the HA-tagged yhp23 in the presence of a β-galactosidase reporter gene under the control of the appropriate hormone response element (ERE). (A) Overexpression of HA-yhp23 increases G400V ER transcriptional activation. The activity of G400V ER in the presence or absence of overexpressed yhp23 was determined by liquid β-galactosidase assay at a 1 nM concentration of 17-β-estradiol. Overexpression of yhp23 increased G400V ER activity approximately 10-fold. (B) Increased transcriptional activation of G400V ER by yhp23 is not a function of increased ER levels. Whole-cell lysates were prepared from the yeast strains described in panel A. Equal amounts of proteins were separated on an SDS–4 to 20% gradient polyacrylamide gel, transferred to Immobilon paper, and probed with an ER-specific monoclonal antiserum (top panel) or a monoclonal antibody directed against the HA epitope on yhp23 (bottom panel) and visualized by enhanced chemiluminescence. (C) Dose-response curves for G400V ER. The activity of G400V ER in the absence (open circles) or presence (solid circles) of overexpressed yhp23 at the indicated 17-β-estradiol concentrations is shown. The increased transcriptional activity displayed by G400V ER in the presence of HA-yhp23 overexpression is seen to be greatest at low hormone concentrations and is lost completely at the highest hormone concentrations assayed. (D) Dose-response curves for wt ER. The activity of wt ER in the absence (open squares) or presence (solid squares) of overexpressed HA-yhp23 at the indicated 17-β-estradiol concentrations is shown. The effect of HA-yhp23 overexpression is seen to be greatest at low hormone concentrations and is lost completely at the highest hormone concentrations assayed. (E) Overexpression of HA-yhp23 increases the ligand-independent activity of the ER. The activity of the ER in the absence or presence of 0.01 nM 17-β-estradiol is shown. Ligand-independent transcriptional activity by ER is approximately threefold higher in the presence of HA-yhp23 overexpression. The addition of 0.01 nM 17-β-estradiol resulted in a greater level of ER ligand-dependent activity in the HA-yhp23 overexpressing strain, suggesting that yhp23 overexpression increases the sensitivity of ER to low concentrations of 17-β-estradiol. (F) HA-yhp23 overexpression does not affect the AF-1 activity of ER. The amino-terminal 115 residues of ER, containing the AF-1 activity of the ER in yeast cells, were fused to a Lex DNA binding domain (see Materials and Methods). Constitutive AF-1 activity resulted in transcription of a reporter β-galactosidase gene downstream of a LexA binding site, which was unaffected by overexpression of HA-yhp23.

yhp23 overexpression increases ligand binding by both G400V ER and wt ER. Considering p23's proposed role in aporeceptor complex formation and given the nature of the G400V ER mutation, we examined whether the increase in G400V ER transcription in the presence of overexpressed yhp23 results from increased estradiol binding by the receptor. Estradiol binding by G400V ER and wt ER in the presence or absence of yhp23 overexpression was measured in vivo. The yeast strains were incubated for 1 h in medium containing ^{3}H-labeled 17-β-estradiol and washed three times to remove unbound steroid, and the amount of estradiol bound to G400V ER and wt ER was measured by quantifying the ^{3}H-labeled 17-β-estradiol content by liquid scintillation counting. β-Galactosidase assays were carried out in parallel on the same cells to correlate effects of ligand binding to transcriptional activation.

We first examined ligand binding by G400V ER at a 1 nM concentration of ^{3}H-labeled 17-β-estradiol. As seen in Fig. 3A, ligand binding by G400V ER was increased by approximately fivefold in the presence of overexpressed yhp23. This increase in ligand binding was found to correlate with a sevenfold increase in G400V ER transcriptional activity (Fig. 3B). Immunoblot analysis showed that the levels of G400V ER were unchanged by yhp23 overexpression (data not shown).

We then examined ligand binding by wt ER, which was also found to increase in the presence of yhp23 overexpression, although to a lesser extent. At a 0.1 nM concentration of ^{3}H-labeled 17-β-estradiol, wt ER ligand binding was increased by approximately 50% (Fig. 3C). β-Galactosidase assay of these cells demonstrated a corresponding 70% increase in transcriptional activity (Fig. 3D). Again, immunoblot analysis showed no difference in the level of ER expression exhibited by the two strains (data not shown). These results indicate that yhp23 overexpression increases ER transcriptional activity by increasing the total number of ligand-bound receptors.

The magnitude of yhp23's effect on wt ER signaling is a function of wt ER and estradiol concentrations. Having demonstrated that overexpression of yhp23 increases wt ER transcriptional activity, we next asked whether a decrease in yhp23 concentration would reduce wt ER activity. To this end, we compared wt ER transcriptional activity in the yhp23 knockout strain (KO) to that in the parental strain (PA) (2), as well as the parental strain overexpressing HA-yhp23 (PA+HA-yhp23 strain). In this way, we were able to assay wt ER function (i) in the absence of yhp23 (KO), (ii) in the presence of endogenous levels of yhp23 (PA), and (iii) in the presence of both endogenous yhp23 and overexpressed HA-yhp23 (PA+HA-yhp23) (Fig. 4B). A wt ER expression vector and an ERE–β-galactosidase reporter plasmid were introduced into these three yeast strains, and wt ER transcriptional activity was assayed at 0.1 nM 17-β-estradiol. The activity of wt ER within the three different strains at this hormone concentration is shown in Fig.

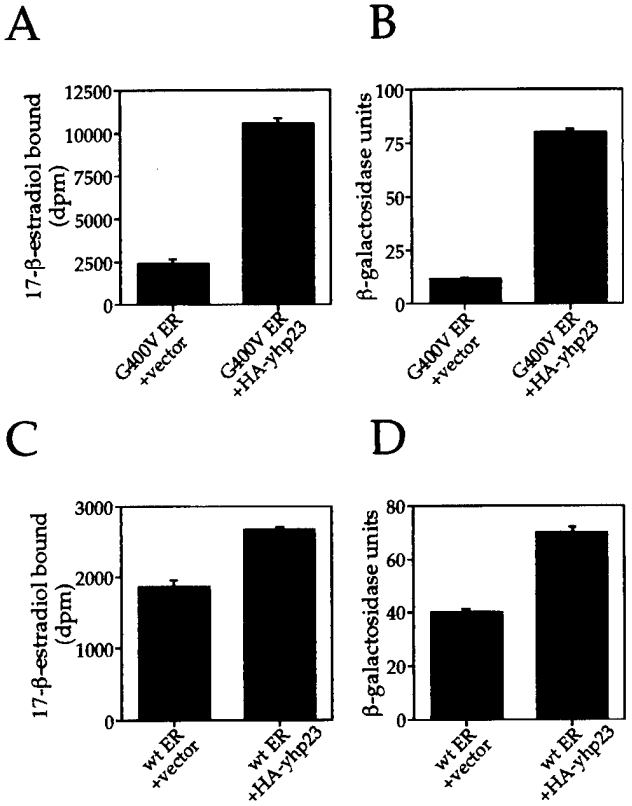

FIG. 3. Overexpression of yhp23 increases estradiol binding by G400V ER and wt ER in vivo. The total amount of estradiol bound by G400V ER and wt ER in the presence or absence of yhp23 overexpression was determined by an in vivo ligand-binding assay with the same yeast strains as described in Fig. 2. After a 1-h incubation in the presence of ^{3}H-labeled 17-β-estradiol, cells were washed to remove unbound ligand, and the amount of bound estradiol was determined by liquid scintillation counting. Liquid β-galactosidase assays were carried out, in parallel, on an aliquot of the same ^{3}H-labeled 17-β-estradiol-incubated cells in order to correlate the levels of ligand binding to the resulting levels of transcriptional activation. Whole-cell extracts from the assayed yeast strains were fractionated on SDS-polyacrylamide gel electrophoresis, and ER expression was examined by use of immunoblotting with an ER-specific rabbit polyclonal antiserum as described in Materials and Methods. No alteration in G400V ER or wt ER levels in response to increased HA-yhp23 expression were noted (data not shown). (A) HA-yhp23 overexpression increases ligand binding by G400V ER. Cells expressing G400V ER in the presence or absence of HA-yhp23 overexpression were assayed for ligand binding in the presence of 1 nM ^{3}H-labeled 17-β-estradiol. Ligand binding by G400V ER is seen to increase approximately fivefold in the presence of overexpressed HA-yhp23. (B) Increased ligand binding by G400V ER in the presence of HA-yhp23 overexpression correlates with an increase in transcriptional activation by the receptor. Liquid β-galactosidase assays, carried out on an aliquot of the cells described in panel A, demonstrate a corresponding sevenfold increase in transcriptional activity by G400V ER. (C) HA-yhp23 overexpression increases ligand binding by wt ER. Cells expressing wt ER in the presence or absence of HA-yhp23 overexpression were assayed for ligand binding in the presence of 0.1 nM ^{3}H-labeled 17-β-estradiol. Ligand binding by wt ER is seen to increase by approximately 50% in the presence of overexpressed HA-yhp23. (D) Increased ligand binding by wt ER in the presence of HA-yhp23 overexpression correlates with an increase in transcriptional activation by the receptor. Liquid β-galactosidase assays, carried out on an aliquot of the cells described in panel C, demonstrate a corresponding 70% increase in transcriptional activity by wt ER.

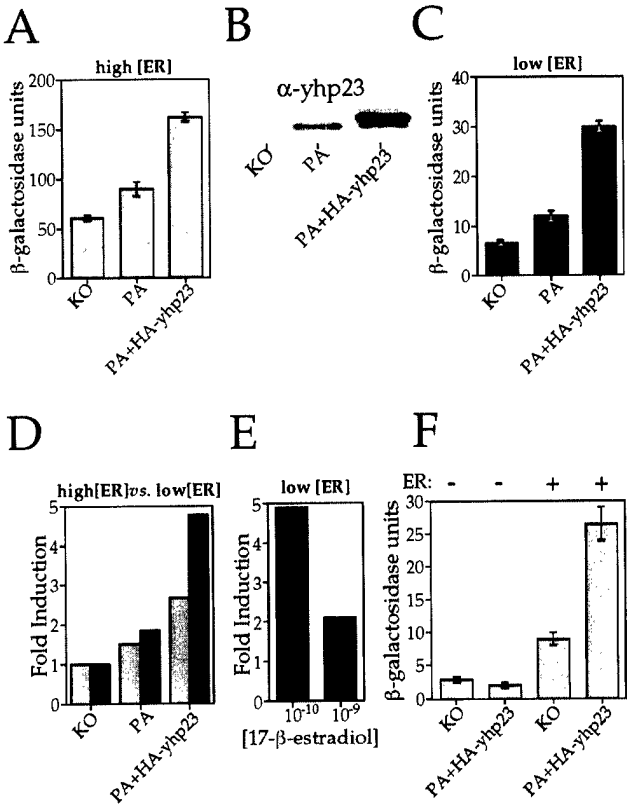

FIG. 4. Yhp23's effect on wt ER activity is a function of the yhp23, ER, and estradiol concentrations. wt ER transcriptional activation was compared in three yeast strains expressing yhp23 at different concentrations. The knockout strain (KO) is yhp23 deficient, the parental strain (PA) expresses yhp23 at endogenous levels, and the HA-yhp23-transformed parental strain (PA+HA-yhp23) expresses endogenous yhp23 and exogenous HA-yhp23. All three strains express wt ER under the control of a galactose-inducible promoter, along with an ERE–β-galactosidase reporter plasmid. wt ER activity in the KO and PA+HA-yhp23 strains was compared over a range of hormone concentrations as shown in Fig. 2D. The greatest effect of yhp23 levels was observed at 0.1 nM 17-β-estradiol. Data shown represent the results of β-galactosidase assays repeated with all three yeast strains (KO, PA, and PA+HA-yhp23) at this hormone concentration. (A) The effect of yhp23 on wt ER transcriptional activation at high ER concentrations. ER transcriptional activity as a function of yhp23 concentration was determined by liquid β-galactosidase assay in cells incubated in galactose-raffinose-supplemented medium containing 0.1 nM 17-β-estradiol. (B) KO, PA, and PA+HA-yhp23 strains express different levels of yhp23. Equal amounts of whole-cell lysates from the KO, PA, and PA+HA-yhp23 strains were analyzed by immunoblotting by using anti-yhp23 polyclonal antibody as described in Materials and Methods. The upper band seen in the PA+HA-yhp23 lane corresponds to the HA-tagged yhp23. (C) The effect of yhp23 on wt ER transcriptional activity at low wt ER concentration. ER transcriptional activity as a function of yhp23 concentration was determined by liquid β-galactosidase assay in cells incubated in raffinose-supplemented medium containing 0.1 nM 17-β-estradiol. Under these conditions ER expression is 10-fold lower than with cells grown in the presence of galactose (not shown). (D) yhp23 induction of wt ER transcriptional activity is inversely proportional to wt ER concentration. The fold induction of yhp23 on ER transcriptional activity at 0.1 nM 17-β-estradiol under conditions of high ER expression (galactose; shaded columns) or low ER expression (raffinose; solid columns) is standardized to the ER activity in the KO strain. (E) The effect of yhp23 on wt ER function is inversely proportional to the ligand concentration. The fold induction of ER transcriptional activation in the PA+HA-yhp23 versus KO strains at 0.1 and 1 nM 17-β-estradiol was determined under conditions of low ER expression (raffinose medium). The results indicate that the fold induction of ER transcriptional activation in the presence of yhp23 overexpression is significantly greater at 0.1 nM 17-β-estradiol than at 1 nM 17-β-estradiol. (F) yhp23 overexpression increases estradiol-independent wt ER transcriptional activation. The effect of yhp23 overexpression on ERE-dependent transcriptional activation in the presence or absence of ER is shown. KO and PA+HA-yhp23 strains containing the ERE reporter construct were transformed with either the empty expression vector or with the ER-containing expression vector and assayed for β-galactosidase activity in galactose-raffinose medium without estradiol.

4A. In the absence of yhp23 (KO), wt ER signaling still occurs, demonstrating that wt ER is capable of functioning in a yhp23-independent manner. Endogenous levels of yhp23 (PA) result in a 50% increase in wt ER activity relative to the KO strain. The PA+HA-yhp23 strain, which contains the highest yhp23 levels (Fig. 4B), exhibits an even higher level of wt ER induction (2.7-fold) than that seen in the KO strain. This increase in

wt ER activity is not a function of increased receptor levels, since wt ER levels were unchanged in the absence or presence of yhp23 (data not shown). Thus, wt ER transcriptional activity increases in direct proportion to the concentration of yhp23.

Having shown that wt ER activity increases as a function of yhp23 concentration, we next asked if the effect of yhp23 upon ER signaling is also a function of wt ER concentration. This was accomplished by repeating the above experiment in medium containing only raffinose as a carbon source, resulting in low levels of wt ER expression. When wt ER transcriptional activity was assayed under these conditions, a similar pattern of yhp23-dependent ER activation was observed: wt ER functions in the KO strain, and this activity increases as a function of yhp23 concentration (Fig. 4C). Importantly, the magnitude of the effect of yhp23 on wt ER transcriptional activation is greater at the lower wt ER concentration, such that overexpression of the yhp23 in the PA+HA-yhp23 strain results in an almost fivefold increase in wt ER transcriptional activity relative to the KO strain, thereby doubling the induction seen at high wt ER concentrations (Fig. 4D). Thus, the magnitude of yhp23's effect on wt ER transcriptional activity is greater at low, rather than high, wt ER concentrations.

Our earlier studies of yhp23 induction of G400V ER and wt ER activity (Fig. 2C and D) suggested that the importance of yhp23 to wt ER function correlates inversely to hormone concentration. This idea was further confirmed with the KO and PA+HA-yhp23 strains, when the activity of wt ER within these strains was assayed at both 0.1 and 1 nM 17-β-estradiol under conditions of low wt ER expression. As seen in Fig. 4E, the fivefold induction of wt ER transcriptional activity observed within the PA+HA-yhp23 strain at 0.1 nM 17-β-estradiol decreases to only a twofold induction at the higher concentration of 1 nM 17-β-estradiol. Again this trend continues such that, when activities are compared at yet higher hormone concentrations, no difference in activity is observed between the KO and PA+HA-yhp23 strains at 10 nM 17-β-estradiol (data not shown). Thus, the effect of yhp23 overexpression on wt ER transcriptional activation is inversely proportional to the hormone concentration.

Comparison of wt ER activity in the KO and PA+HA-yhp23 strains in the absence of estradiol again confirmed a role for yhp23 in wt ER ligand-independent transcriptional activation. As shown in Fig. 4F, yhp23 is not essential to wt ER-dependent activation in the absence of ligand. Interestingly, as was observed with ligand-dependent activity, wt ER function in the absence of ligand increases with yhp23 overexpression. This effect on transcription is not observed in the absence of wt ER expression, suggesting that yhp23 is operating via wt ER to induce estradiol-independent transcriptional activation.

Thus, the relationship of yhp23 to wt ER signal transduction is dependent not only on concentrations of yhp23 but also upon the levels of wt ER and estradiols. Our data demonstrate that the effect of yhp23 upon wt ER function is most pronounced when wt ER activity is examined at low estradiol and wt ER concentrations.

yhp23 is not essential for the formation of a functional ER-GRIP1 complex. The ER activates transcription in mammalian cells through two transcriptional activation domains, termed AF-1 and AF-2 (36). The AF-1 domain, located within the amino terminus of the receptor, does not require steroid binding to achieve an active conformation (39). In contrast, the AF-2 domain lies within the steroid-binding domain and is dependent upon estradiol binding for its activity (55). The ER AF-2 region has been shown to activate transcription in yeast cells and in cultured mammalian cells through interaction with coactivator proteins, including GRIP1/TIF2 (21) and SRC1

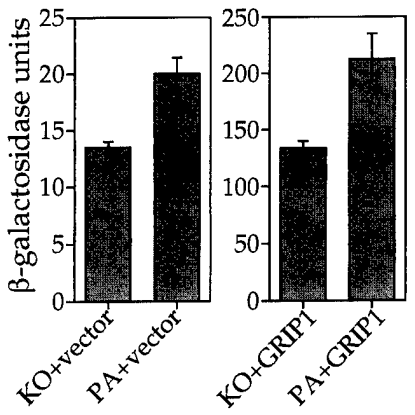

FIG. 5. yhp23 is not essential for the functional interaction between ER and the coactivator GRIP1. yhp23 KO and PA strains were constructed that express wt ER both in the presence or in the absence of the coactivator GRIP1. ER activity was assayed in the four strains after incubation in raffinose medium containing 0.1 nM 17-β-estradiol. Data were collected in the same experiment but are displayed with separate *y* axes to more clearly demonstrate GRIP1 induction of ER activity within each strain. Coexpression of GRIP1 increases ER transcriptional activation in both KO and PA strains approximately 10-fold, an effect that is independent of yhp23 expression. Note that yhp23 increased ER transcriptional activation to the same extent in the absence or presence of GRIP1.

(43). Although AF-2 activity is observed in some promoter contexts in yeast cells (40), the AF-2 coactivators have no yeast homologues, and thus ER transcriptional activity in yeast cells is greatly potentiated in the presence of ectopically expressed mammalian coactivators (22). We therefore sought to more specifically determine whether yhp23 might play a role in AF-2–coactivator interactions.

The recent determination of the structure of the ER ligand-binding domain bound to estradiol has suggested that proper folding of this region around the steroid hormone is crucial to the formation of an AF-2 domain competent for interaction with coactivators (3). Since p23 has been proposed to have chaperone-like activities (17), we hypothesized that it may play a role in the proper folding of the AF-2 domain around estradiol during the process of steroid binding. To determine whether yhp23 facilitates wt ER AF-2–coactivator interactions, we introduced the mammalian coactivator GRIP1 into yhp23 KO and PA yeast strains expressing wt ER (KO+GRIP1 and PA+GRIP1, respectively). Coexpression of GRIP1 should activate AF-2, thereby increasing wt ER transcriptional activation relative to the control strains lacking GRIP1 (22) and allowing us to compare GRIP1 induction of wt ER activity in the presence or absence of yhp23. If yhp23 were important for AF-2 interaction with GRIP1, then GRIP1-dependent ER transcriptional activation would be reduced in the KO versus the PA strain. However, as shown in Fig. 5, this is not the case. wt ER transcriptional activation in the PA and KO strains is enhanced to similar extents (approximately 10-fold) compared to the corresponding control strains lacking GRIP1 (Fig. 5). Thus, GRIP1 induction of wt ER transcriptional activation is not altered by yhp23 expression, suggesting that yhp23 is not required for the formation of a functional ER-GRIP1 complex.

ER and yhp23 colocalize within the nucleus of yeast cells. Prior genetic and biochemical studies have demonstrated that aporeceptor complex formation is conserved in yeast cells (5). Hsp82, the yeast homologue of Hsp90, has been shown to associate with hormone-free ER and GR in yeast cells. In addition, genetic studies indicate that ER and GR signaling is

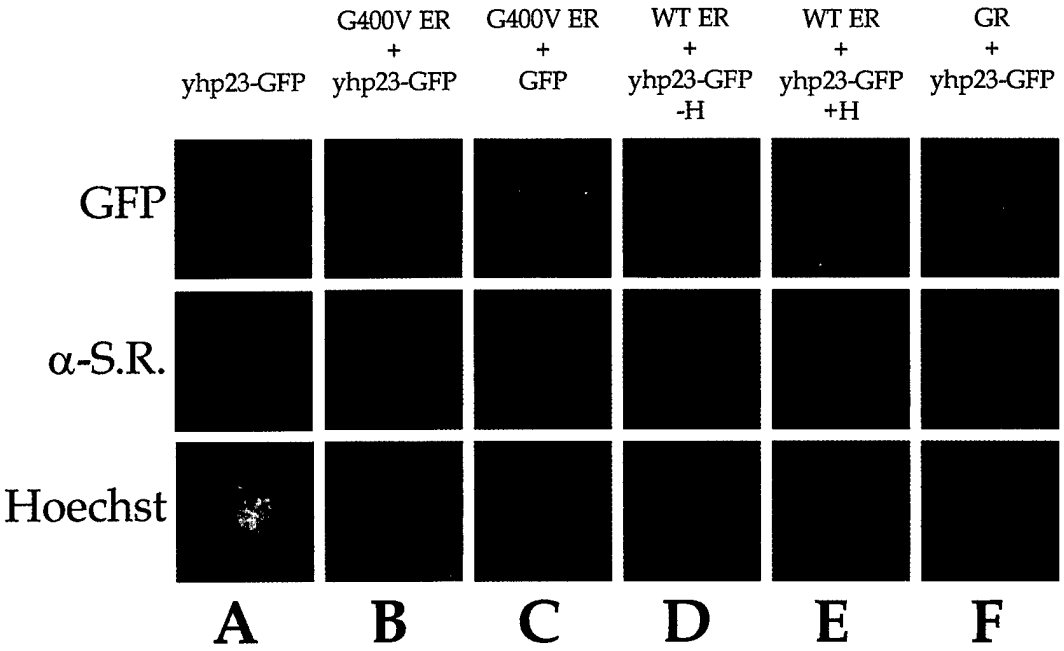

FIG. 6. ER and yhp23-GFP colocalize within the nucleus of yeast cells. To examine yhp23 subcellular localization in yeast cells, a yhp23-GFP fusion protein was constructed. Yeast strains were created that express yhp23-GFP (A) or GFP (C) either alone or in combination with G400V ER (B and C), wt ER (D and E), or GR (F). Cells were grown in galactose-raffinose-containing medium in the absence or the presence of 17-β-estradiol. Cells were fixed, permeabilized, and incubated with the appropriate receptor primary antibody, a corresponding Texas-red-conjugated secondary antibody, and the DNA in the nucleus was stained with Hoechst dye H334211. The GFP, Texas red, and Hoechst fluorescent signals were visualized by using a Zeiss Axioplan 2 fluorescence microscope. Note that yhp23-GFP is expressed throughout the cytoplasm in the absence of ER expression. In the presence of coexpressed G400V ER as well as wt ER, yhp23-GFP becomes localized to the nucleus. Incubation of the wt ER strain in 1 μM 17-β-estradiol results in the redistribution of yhp23-GFP from the nucleus to the cytoplasm, thereby reversing the ER-yhp23-GFP colocalization observed in the absence of estradiol. α-S.R., anti-steroid receptor primary antibody.

reduced in yeast strains expressing only 5% of the wt level of Hsp82 (44). Compelling genetic evidence also exists for the role of the yeast Hsp70 (31), p60 (8), Hsp40 (31), and immunophilin (14) homologues in steroid signaling in yeast cells, with the majority of these proteins having been shown to associate with GR in the absence of hormone (2, 7).

Given human p23's presence in the aporeceptor complexes of PR (28) and GR (11) and having shown that yhp23 affects ER function, we proceeded to determine whether yhp23 and ER colocalize in vivo. To determine the cellular distribution of yhp23 in yeast cells, we created a yhp23-GFP fusion protein by subcloning GFP at the carboxy terminus of the yhp23 protein. Expression of the fusion protein was confirmed by Western blotting. Importantly, the yhp23-GFP fusion protein is also able to suppress the G400V ER phenotype (not shown), proving that addition of the GFP moiety does not eliminate yhp23's ability to functionally interact with G400V ER.

We constructed several yeast strains that express yhp23-GFP either alone or in combination with G400V ER, wt ER, or wt GR. Figure 6A demonstrates that the distribution of yhp23-GFP in the absence of steroid receptor expression is largely cytoplasmic, though a small proportion of signal corresponding to the nucleus is also evident. This pattern is consistent with the expression pattern described for the human p23 protein within mammalian cells (28). Strikingly, upon coexpression of G400V ER, yhp23-GFP becomes predominantly limited to the nucleus, thus colocalizing with G400V ER (Fig. 6B). Recall that ER is a steroid receptor that resides in the nucleus in the absence of hormone. Importantly, this pattern of nuclear localization was not seen when G400V ER was coexpressed with just the GFP protein, indicating that yhp23 is responsible for the localization of the fusion protein to the nucleus (Fig. 6C). Nuclear localization of yhp23-GFP was also observed when it was coexpressed with wt ER (Fig. 6D). Thus, the ability of G400V ER to colocalize yhp23-GFP in the same manner as wt ER suggests that the G400V ER phenotype is not a result of deficient aporeceptor complex formation. Additionally, when cells coexpressing wt ER and yhp23-GFP were incubated in 17-β-estradiol, yhp23-GFP redistributed to the cytoplasm, reestablishing the pattern seen in yeast cells lacking ER expression (Fig. 6A). As a final control, yhp23-GFP coexpressed with GR, a steroid receptor that exists outside the nucleus in the steroid-free state, did not localize to the nucleus (Fig. 6F) but instead showed a cytoplasmic distribution similar to that of GR.

The colocalization of yhp23 and ER is consistent with the proposed role of yhp23 as a member of the ER aporeceptor complex. Upon coexpression with ER, aporeceptor complex formation causes yhp23, presumably through an interaction with Hsp82 (the yeast homologue of Hsp90), to become localized to the nucleus. The addition of hormone appears to result in the dissociation of the aporeceptor complex, allowing yhp23 to redistribute throughout the cell.

Complementation of yhp23 by human p23 in yeast cells. We next examined whether human p23, when ectopically expressed in yeast cells, functions like yhp23 to suppress the G400V ER phenotype. We established yeast strains that express G400V ER and an ERE-responsive reporter plasmid in the presence of HA-tagged human p23 (HA-p23) or HA-yhp23. A third strain containing the expression vector without an insert (vector) was used as a negative control. As shown in Fig. 7A, human p23 is capable of increasing hormone-dependent G400V ER transcriptional activation in yeast cells, although to a lesser degree than yhp23 (4-fold and 13-fold, respectively). The reduced G400V ER transcriptional activity is not a function of reduced human p23 expression relative to yhp23, since immunoblot analysis with an antibody directed against the HA

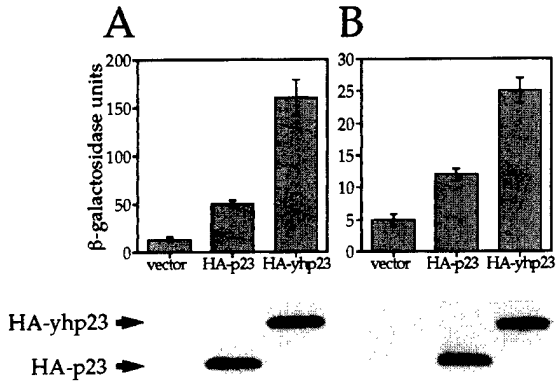

FIG. 7. Complementation of yhp23 by human p23 in yeast cells. (A) Overexpression of HA-p23 suppresses the G400V ER phenotype. An HA epitope-tagged human p23 (HA-p23) was subcloned into the yeast expression vector $pRS316_{GPD}$. The W303a yeast strain expressing G400V ER and an ERE–β-galactosidase reporter gene was transformed with expression vectors containing no insert (vector), HA-p23, or HA-yhp23. G400V ER transcriptional activity was measured by liquid β-galactosidase assay in galactose-containing medium with 1 nM 17-β-estradiol. Equal amounts of whole-cell lysates from the strain containing vector, HA-p23, and HA-yhp23 were analyzed by immunoblotting by using anti-HA antibody as described in Materials and Methods (bottom panel). (B) Human p23 partially complements the loss of yhp23 with respect to ER signaling. yhp23 KO yeast strain expressing wt ER and an ERE-responsive β-galactosidase reporter gene were transformed with expression vectors containing no insert (vector), HA-p23, or HA-yhp23. ER transcriptional activity was determined by liquid β-galactosidase assay in raffinose medium containing 0.1 nM 17-β-estradiol. Immunoblot analysis for HA-p23 and HA-yhp23 was performed as in panel A and demonstrates that HA-p23 and HA-yhp23 are expressed at similar levels (bottom panel).

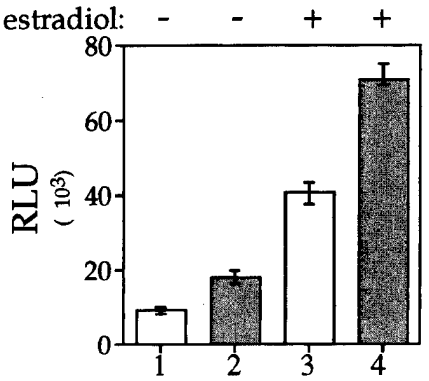

FIG. 8. Activation of ER transcriptional enhancement by p23 overexpression. ER-containing MCF-7 cells (2.5×10^5 cells/6-cm dish) were transiently transfected by using the lipid Trans-IT 100 with 5 μg of the ERE-containing luciferase reporter plasmid and 2 μg of a pCMV expression vector (open columns) or pCMV-HA-p23 expression vector (shaded columns). Cells were incubated for 24 h in the presence of 0.01 nM 17-β-estradiol or ethanol vehicle and then harvested. ER transcriptional activation was measured by using a luciferase assay, normalized to total protein concentration in each sample, and expressed as relative luminescence units (RLU). The data represent the mean of an experiment done in triplicate, which was repeated four times.

epitope present on both proteins shows equal expression levels in yeast cells (Fig. 7A, bottom panel). In addition, G400V ER expression is unaffected by yeast or human p23 coexpression (data not shown). These findings suggest that human p23 can function like yhp23 in yeast cells, albeit less potently, to increase G400V ER transcriptional activity.

We next evaluated whether human p23 could function in yeast cells to increase ER transcriptional activation in the absence of endogenous yhp23. The KO strain expressing wt ER and an ERE-responsive promoter were transformed with the empty expression vector, HA-p23, or HA-yhp23. When assayed at a 0.1 nM concentration of 17-β-estradiol, expression of human p23 increases ER transcriptional activity compared to the use of the vector only (Fig. 7B), but to a lesser extent than with yhp23 (twofold and fivefold, respectively). Again, the reduced activity of ER in the human p23-expressing strain is not a function of reduced p23 levels relative to yhp23 (Fig. 7B, bottom panel). These findings indicate that human p23 can partially complement the loss of yhp23 function in yeast cells with respect to ER signaling, thus suggesting that yhp23 and p23 are functional homologues.

Increased ER transcriptional activation by human p23 overexpression in MCF-7 cells. To establish whether p23 affects ER signal transduction in mammalian cells, we examined the ability of human p23 to increase ER-mediated transcriptional enhancement when overexpressed in cultured mammalian cells. ER-containing MCF-7 cells were transfected with the reporter plasmid ERE-thymidine kinase-luciferase and a plasmid encoding human p23. Transfected cells were treated with 0.1 nM 17-β-estradiol or ethanol vehicle for 24 h, and the transcriptional activity was quantified by measuring the luciferase activity. As shown in Fig. 8, both hormone-dependent and hormone-independent ER transcriptional activity are increased roughly twofold when p23 is overexpressed. This likely represents an underestimate of p23's importance to ER function, since these results are obtained in a cell line that contains endogenous p23 (not shown). These findings suggest that p23 is a limiting factor for ER signal transduction and therefore subsequent ER-mediated transcriptional enhancement.

DISCUSSION

Using dosage suppression analysis in yeast cells to isolate factors involved in ER signal transduction, we have identified the yeast homologue of the human p23 (yhp23) as a protein that, when overexpressed, results in a 10-fold increase in G400V ER transcriptional activation. In vivo estradiol-binding assays suggest that yhp23 overexpression increases G400V ER transcriptional activity by increasing the number of estradiol-bound receptors. The effect of yhp23 overexpression was not limited to G400V ER, as it also increases both ligand binding and transcriptional activation by wt ER. No effect of yhp23 overexpression was observed on the constitutive activity of ER_{1-115}, thereby demonstrating that yhp23 does not affect AF-1 activity per se. We therefore conclude that yhp23 is a member of the reconstituted steroid receptor signaling pathway in yeast cells, acting at the step of ligand binding by the receptor. This role is consistent with the currently proposed function of human p23 in steroid receptor-aporeceptor complex formation.

By using a yeast strain deficient in yhp23 expression (KO), analyses of wt ER signaling as a function of yhp23, wt ER, and estradiol concentrations were carried out. Our studies demonstrate that the magnitude of the effect of yhp23 on wt ER transcriptional activation is inversely proportional to the concentration of both wt ER and estradiol. Thus, at low, subsaturating concentrations of estradiol, yhp23 overexpression markedly increases wt ER transcriptional activation. In contrast, at saturating concentrations of estradiol, the effect of yhp23 overexpression on wt ER transcriptional activation is comparatively small. Furthermore, the magnitude of the effect of yhp23 on wt ER transcriptional activation is greater at low, rather than high, wt ER expression levels. Taken together, our findings indicate that the effect of yhp23 on wt ER signaling varies depending on yhp23, wt ER, and estradiol concentrations.

Subcellular localization studies with a yhp23-GFP fusion protein indicate that yhp23 is largely cytoplasmic in the ab-

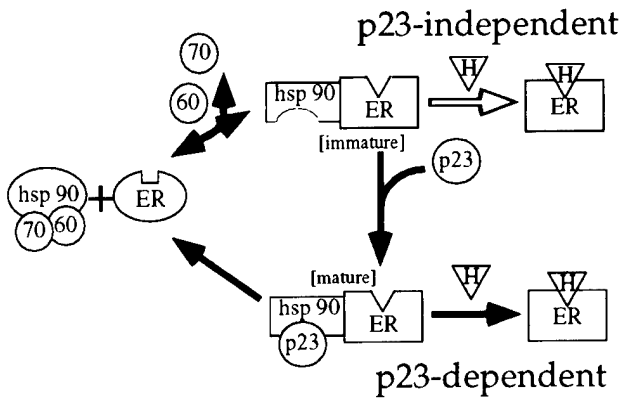

FIG. 9. A model for p23-dependent and -independent ER signal transduction. A simplified version of the current model of aporeceptor complex formation as deduced from in vitro studies is indicated by the black arrows. Hsp90 refers to the Hsp90 dimer, 70 refers to Hsp70, 60 refers to p60, p23 refers to the mammalian p23 protein, ER refers to the estrogen receptor, and H refers to hormone. The immunophilins, which do not appear to be essential to aporeceptor complex formation but are isolated with the complex in vivo have been excluded from the model for simplicity. According to the model, p60, Hsp70 (and possibly Hsp40), and a dimer of Hsp90 preassociate to form the "foldosome" complex. The foldosome binds to the steroid-binding domain of the native receptor. Hsp70 and p60 are released from the complex in a process that requires ATP and a monovalent cation, while Hsp90 and the receptor undergo conformational changes, such that the receptor assumes a conformation with high affinity for steroid. This new complex (labeled "immature" in this figure) is inherently unstable and quickly disassociates unless p23 binds to Hsp90, thereby stabilizing the aporeceptor complex (labeled "mature" in this figure). Our data, however, suggests that a second, p23-independent pathway to ER ligand binding exists in vivo, occurring when ligand binds directly to the immature aporeceptor complex (open arrow), which is favored at high ER and/or estradiol concentrations. We suggest that the G400V ER mutation renders the receptor less capable of participating in the p23-independent pathway, thereby functionally uncoupling the p23-independent and -dependent pathways.

sence of ER expression. When coexpressed with either the mutant or wt ER, yhp23 colocalizes with the receptor to the nucleus in the absence of estradiol. This colocalization is reversed upon estradiol treatment, such that yhp23 is released into the cytoplasm. From these observations, we conclude that yhp23 is part of the ER aporeceptor complex in yeast cells and that the distribution of yhp23 within the cell is dynamic and affected both by ER expression and estradiol binding.

It has been proposed that the function of p23 in steroid receptor signaling is to promote, through its interaction with Hsp90, the maturation or stabilization of the aporeceptor complex (24, 30). This model, derived largely from in vitro experiments (45, 46), proposes that the heat shock proteins Hsp90, p60, Hsp70, and possibly Hsp40 (10) form a complex termed a "foldosome" (23), within which Hsp90 exists in a conformation incompatible with p23 binding (53) (Fig. 9). The foldosome binds to the free receptor, which exists in a conformation with low affinity for the ligand. In a process that requires ATP and monovalent cations, the Hsp90 component of the foldosome and the receptor undergo conformational changes (13), such that Hsp90 is now capable of binding p23 (53), and the receptor exhibits high-affinity steroid binding. p23 binding to Hsp90 appears to stabilize this immature aporeceptor complex in vitro. In the absence of p23, the Hsp90-receptor complex is inherently unstable and rapidly dissociates (13).

The ability of yhp23 to increase ER transcriptional activation might be expected if yhp23 is limiting for the formation of mature aporeceptor complexes. Although we cannot exclude the possibility that yhp23 overexpression increases the ratio of yhp23 binding to each molecule of ER, this idea runs contrary to the current model of p23 function. Increasing the concentration of yhp23, therefore, would be expected to result in a greater number of mature ER aporeceptor complexes in the cell. As a result, the total estradiol binding will increase, which is consistent with our in vivo estradiol binding assays (Fig. 3). Thus, overexpression of yhp23, by increasing the number of mature aporeceptor complexes, will manifest itself as an increase in transcriptional activation by ER at a given hormone concentration (Fig. 2 and 4). Thus, our in vivo findings are consistent with the current model of p23 function as derived from in vitro experiments.

Our results indicating that wt ER activity is detectable in the absence of yhp23 (Fig. 4), however, suggest that in addition to the p23-dependent pathway, there must also exist a p23-independent pathway leading to estradiol binding and signaling by the ER. We propose that p23-independent activation of the ER in vivo occurs through estradiol binding directly to the immature (p23-deficient) aporeceptor complex (Fig. 9). This hypothesis is consistent with in vitro observations that in the absence of p23, the foldosome proteins Hsp90, Hsp70, and p60 are sufficient to induce the hormone-binding conformation of steroid receptors (13). We therefore propose that estradiol binding by the ER is a composite of both the p23-independent and p23-dependent pathways. The relative contribution of each pathway to ER activation is dependent upon the concentration of p23, ER, and estradiol (see below).

One prediction of our model is that yhp23 becomes less relevant to ER activation as the ratio of immature to mature aporeceptor complexes increases. The ratio of the two types of aporeceptor complexes is, in turn, a reflection of both ER and yhp23 concentrations. Increasing ER expression when p23 levels are constant results in a greater number of the immature aporeceptor complexes, which favors hormone binding through the p23-independent pathway. Conversely, increasing yhp23 levels facilitates the formation of mature aporeceptor complexes and therefore the p23-dependent pathway. This model of ER signal transduction is consistent with our in vivo findings that indicate that the magnitude of the effect of yhp23 on ER transcriptional activation is inversely proportional to the concentration of ER (Fig. 4D).

The proposed model further envisions that the concentration of estradiol also affects the relative contribution of the p23-dependent and p23-independent steroid binding pathways. As suggested by Fig. 9, free steroid can be considered to be competing with yhp23 for binding to the immature aporeceptor complex. As a result, the p23-independent pathway becomes more prominent as estradiol concentrations rise or, conversely, as yhp23 levels fall. Consistent with this notion, our data demonstrate that the magnitude of yhp23's effect on ER signaling is greatest at low subsaturating, rather than high saturating, estradiol concentrations (Fig. 4E). Thus, our model predicts that the balance among yhp23, ER, and estradiol, ultimately determines the relative contributions of the p23-dependent and p23-independent pathways to ER signal transduction.

Our model also provides insight into the observation that yhp23 overexpression induces G400V ER activity to a greater extent than that observed for wt ER (Fig. 2). We suggest that by altering the steroid-binding domain conformation, the G400V mutation largely eliminates the p23-independent pathway. G400V ER is temperature sensitive, relative to the wt ER, for estradiol binding in vitro, displaying reduced estradiol binding at 25°C but not at 4°C (54). This suggests that the G400V ER mutation destabilizes the conformation of the steroid-binding domain, such that the receptor is unable to bind steroid with high affinity at 25°C. This mutation does not inhibit G400V ER's interaction with the aporeceptor complex since G400V ER has been reported to be complexed with Hsp90 (1)

and since our subcellular localization studies suggest that G400V ER associates with yhp23 as efficiently as does wt ER. We therefore propose that the G400V mutation, by altering the structure of the steroid-binding domain, largely inhibits estradiol binding to the transient, immature aporeceptor complex, thereby diminishing estradiol binding through the p23-independent pathway. As a result, yhp23 competes more effectively with estradiol for binding to the complex, favoring the p23-dependent estradiol-binding pathway. The stability gained through yhp23 binding to the Hsp90-chaperone machinery, in turn, facilitates steroid binding by G400V ER.

It has been suggested by others that wt ER does not stably interact with Hsp90. This conclusion is based, in part, on studies with a VP16-Gal-ER_{LBD} fusion protein (1, 37). Although these constructs continue to exhibit ligand-dependent activation, association of this construct with Hsp90 could not be demonstrated. While there are a number of possible reasons for this discrepancy, we feel that the most probable explanation is a difference in the inherent characteristics of the wt and fusion proteins (1). In addition, there are several reports that support a role for Hsp90 in the process of ligand binding by ER. (i) ER has been isolated with members of the aporeceptor complex from MCF-7 cells (52) and bovine uterus (49). (ii) Hsp90 has been demonstrated to colocalize with ER in the nucleus (38). (iii) ER function has been found to be affected by mutant Hsp90 molecules (44). (iv) ER function is also affected by the Hsp90-specific inhibitor geldanamycin (51). Although these studies do not exclude the possibility of additional transcriptional repressors of wt ER, these findings, along with our results, strongly support a role for the components of the Hsp90-based chaperone complex in ER signal transduction.

Elegant genetic studies of yhp23 function in yeast cells by Bohen (2) and Fang et al. (16) demonstrate that yhp23 associates with Hsp90 and is a part of the GR aporeceptor complex in yeast cells. In contrast to our findings with ER, analysis of androgen receptor (AR) signaling in yeast cells suggests that it is largely p23 independent. This may reflect inherent differences in the mechanism of signal transduction employed by the receptors. Alternatively, we would suggest that, although the analysis of AR signaling was performed under a range of steroid concentrations, the levels of AR expression used may have favored the p23-independent pathway. It would be interesting to reevaluate AR signaling as a function of yhp23 at both low AR and testosterone concentrations, conditions that would favor the p23-dependent pathway.

The partial complementation of human p23 in yeast cells lacking yhp23 strongly suggests that yhp23 functions as the p23 homologue with respect to ER signaling (Fig. 7). Although the yeast and human p23 proteins have regions of identity, significant sequence differences between the proteins also exist (2). We speculate that this reflects species-specific differences in p23-Hsp90 association and might therefore explain the inability of human p23 to fully complement the loss of yhp23 function. It would be interesting to examine whether yeast cells expressing human Hsp90 (44) and p23 increase ER activity to the same extent as their yeast counterparts.

Finally, our studies also provide insight into the possible mechanisms by which the ER communicates with other signaling pathways. Unexpectedly, a significant increase in estradiol-independent activation of ER was observed as a result of yhp23 overexpression (Fig. 4F). This estradiol-independent activation of wt ER was also observed upon p23 overexpression in MCF-7 cells (Fig. 8). Previous studies have proposed that estradiol-independent transcriptional activation by wt ER results, in part, from ER phosphorylation through an epidermal growth factor-dependent pathway (4, 27). Thus, the most direct interpretation of our data suggests that maintenance of ER within the aporeceptor complex facilitates (but is not essential to) estradiol-independent activity, perhaps by maintaining ER in a conformation amenable to phosphorylation.

Besides ER, several other signaling molecules (including c-Src and c-Raf) are dependent upon chaperone complexes for their function (45). Yeast cells deficient in the DnaJ homologue YDJ1, for instance, display both altered steroid receptor and Src kinase activity (31). Thus, molecular chaperones link diverse signaling pathways. Additional insight into the mechanism of this cross talk comes from our subcellular localization studies that reveal a striking colocalization of ER and yhp23 within the nucleus. Estradiol treatment was shown to liberate yhp23 (and presumably other chaperone proteins) from the nucleus, allowing it to redistribute throughout the cytoplasm, where it can potentially interact with other signaling proteins. Although our colocalization studies were carried out under conditions of overexpression, we speculate that estradiol activation of ER may, through the release of chaperone components, modulate the activity of a variety of chaperone-dependent pathways. In light of p23's role in stabilizing chaperone complexes, it is likely to play a key regulatory role in any such "chaperone signaling."

In conclusion, we have provided evidence that yhp23 is a member of the ER signaling pathway and a positive regulator of ER function. We also suggest that at high ER and/or estradiol concentrations, conditions often present in the yeast system or during transient overexpression of ER in cultured mammalian cells, ER signaling occurs largely through a p23-independent pathway. Under low physiological concentrations of ER and estradiol, p23 is likely to be an important contributor to ER signaling. Our results also indicate that alterations in the level or subcellular distribution of p23 are potential mechanisms for modulating estradiol-dependent and -independent ER transcriptional activation. We are currently examining whether the p23 levels, the subcellular distribution, or the modification state fluctuates between normal and tumor cells, during cellular proliferation, during differentiation, or upon growth factor treatment. As aporeceptor complex formation is also believed to be important for ligand binding by GR, PR, and AR, p23 likely plays an important role in these pathways as well.

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Biochemistry

Regulation of estrogen receptor transcriptional enhancement by the cyclin A/Cdk2 complex

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ABSTRACT **We have found that ectopic expression of cyclin A increases hormone-dependent and hormone-independent transcriptional activation by the estrogen receptor *in vivo* in a number of cell lines, including HeLa cells, U-2 OS osteosarcoma cells and Hs 578Bst breast epithelial cells. This effect can be further enhanced in HeLa cells by the concurrent expression of the cyclin-dependent kinase activator, cyclin H, and cdk7, and abolished by expression of the cdk inhibitor, $p27^{KIP1}$, or by the expression of a dominant negative catalytically inactive cdk2 mutant. ER is phosphorylated between amino acids 82 and 121 *in vitro* by the cyclin A/cdk2 complex and incorporation of phosphate into ER is stimulated by ectopic expression of cyclin A *in vivo*. Together, these results strongly suggest a direct role for the cyclin A/cdk2 complex in phosphorylating ER and regulating its transcriptional activity.**

The estrogen receptor (ER) is a ligand-dependent transcriptional regulatory protein that controls the genetic programs affecting many aspects of cell growth and differentiation. In addition to ligand binding, phosphorylation plays an important role in regulating ER function. The receptor contains sites for both constitutive and ligand-dependent phosphorylation. Three serine residues (amino acids 104, 106, and 118) located within the N-terminal activation domain (AF-1) and one residue in the hinge region, S294, match the consensus sequence recognized by a family of serine/threonine proline-directed kinases that includes cyclin-dependent kinases (cdk), mitogen-activated protein kinases and glycogen synthase kinase-3. Ser-104, -106, and -118 are phosphorylated upon hormone treatment; serine to alanine mutations at these positions decrease ligand-dependent transcriptional activity (1–3). Accumulating evidence suggests that mitogen-activated protein kinase can phosphorylate Ser-118 and that this may lead to estradiol-independent ER activation or, alternatively, an increase in ligand-dependent ER activation. However, whether cdks target ER as a substrate for phosphorylation and affect its transcriptional activity remains unclear. Recently, a cdk-independent effect of cyclin D1 upon ER-dependent transcriptional activity was reported in T-47D breast cancer cells (4). A link between cdk enhancement of ER function and attendant receptor phosphorylation has not yet been demonstrated.

Cdks are a family of proteins composed of a regulatory cyclin subunit associated with a catalytic kinase subunit. The cyclin subunit appears to regulate subcellular localization and timing of activation as well as substrate specificity of the kinase complex. Cdk complexes regulate the activity of target molecules, including transcriptional regulatory proteins, by phosphorylation. Regulation of cdk activity is accomplished by proteins that activate (cdk activators or CAKs), or inhibit (cdk inhibitors or CDIs), kinase function (5–8). Because cdks control cell division, the dysregulation of cyclins, their kinase partners, and/or the upstream regulatory CAKs and CDIs, have been implicated in the initiation and promotion of hyperplasia and oncogenesis. In fact, the overexpression of the regulatory cyclin subunit and the dysregulation of the catalytic cdk subunit have been identified in a number of solid tumors, leukemias, and tumor-derived cell lines (9–18).

This study examines the effects of the cyclin A/cdk2 complex on ER transcriptional activation. We chose to focus on cyclin A for several reasons: (*i*) Cyclin A plays a multifaceted role in cell cycle progression and is a key regulator of cdk2, a serine/threonine proline-directed kinase with the potential to phosphorylate ER. (*ii*) Cyclin A expression is cell adhesion-dependent, such that its overexpression can lead to adhesion-independent cell growth, a hallmark of cellular transformation (19, 20). (*iii*) The synthesis and degradation of cyclin A are tightly regulated, suggesting that its aberrant expression could seriously jeopardize the control of cell growth (21, 22). (*iv*) Cyclin A overexpression has been implicated as an important indicator of oncogenesis in several contexts including human breast tumor-derived cell lines and a mouse mammary tumor virus breast cancer model (11, 13, 23–25). (*v*) Cyclin A shares several features with the protooncogene cyclin D1 including the ability to bind to and phosphorylate the retinoblastoma protein, such that inappropriate cyclin A expression leads to perturbations in the regulation of the G1 to S transition (26–28). (*vi*) Recent reports have also linked the degradation of $p27^{KIP1}$ (hereafter referred to as p27), an inhibitor of cyclin A/cdk2 activity, and aggressive breast and colorectal cancers (15, 17, 18). Together, these findings suggest that cyclin A may function as a protooncogene. To determine whether the cyclin A/cdk2 complex can affect ER function, we have examined the consequences of activation or inhibition of the cyclin A/cdk2 pathway on ER-dependent transcriptional activation.

MATERIALS AND METHODS

Plasmids. A FLAG epitope was added to the N terminus of the full-length wild-type ER cDNA. This construct was inserted into the pCMV-Neo^r (Invitrogen) expression vector; 0.5 μg DNA per 60-mm dish was used in the transfections. The vector pCDLSRα296 was used to express cyclin A, cyclin H, cdk7, cdk2, or the dominant negative mutant, cdk2TS. The pCMV5 plasmid expressed p27. Two micrograms of cyclin or cdk DNA was used for each 60-mm transfection plate, except in Fig. 2*C* where the amount of cyclin A-encoding plasmid was varied from 0.5 to 10.0 μg per dish. The ΔETCO reporter plasmid contained one estrogen response element upstream of the thymidine kinase promoter (-109) driving the expression of the chloramphenicol acetyltransferase (CAT) gene. This

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Abbreviations: ER, estrogen receptor; cdk, cyclin-dependent kinase; CAK, cyclin-dependent kinase activator; CDI, cyclin-dependent kinase inhibitor; CAT, chloramphenicol acetyltransferase; β-gal, β-galactosidase; GST, glutathione *S*-transferase.

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reporter lacks a nearby activator protein-1 binding site to ensure that the results obtained are not influenced by other regulatory elements in the plasmid. The vector pCMV-lacZ was used as an internal control to measure the efficiency of each transfection. The reporter- and β-galactosidase (β-gal)-encoding vectors were used at 2.0 and 0.5 μg DNA per 60-mm dish, respectively.

Mammalian Cell Culture and Treatments. The cell lines used in these studies were obtained from the American Type Culture Collection and maintained in DMEM (GIBCO/BRL) supplemented with 10% fetal bovine serum (HyClone), 50 units/ml each of penicillin and streptomycin (GIBCO/BRL), and 2 mM L-glutamine (GIBCO/BRL). Transfections were performed in phenol red-free DMEM supplemented with 10% charcoal-stripped fetal bovine serum. For transfections, cells were seeded into 60-mm dishes at 2×10^5 cells per dish and transfected the following day by either the calcium phosphate precipitation or the liposome-mediated (Lipofectamine, GIBCO/BRL) methods (29). At 12–16 h posttransfection, cells were rinsed twice with PBS and refed with phenol red-free DMEM supplemented with 10% charcoal-stripped fetal bovine serum containing either 100 nM 17β-estradiol or the ethanol vehicle. CAT and β-gal assays were performed 24 h later as described (30). Protein expression was monitored by preparing whole cell extracts. Cells were lysed for 30 min on ice in 200 μl of high salt lysis buffer [400 mM NaCl/50 mM Tris·HCl, pH 8.0/0.5% Nonidet P-40/1 mM EDTA/1 mM DTT with protease inhibitors (1 μg/ml aprotinin/1 μg/ml leupeptin/1 μg/ml pepstatin A/1 mM phenylmethylsulfonyl fluoride)] and phosphatase inhibitors (1.0 mM NaF/10 mM β-glycerophosphate/1.0 mM sodium orthovanadate). Whole cell extract (100 μg) was separated by SDS/10% polyacrylamide gel and transferred to Immobilon paper (Millipore).

Glutathione *S*-Transferase (GST)-Protein Expression and Purification. Truncated versions of the human ER cDNA coding for amino acids 1–82, 1–115, and amino acids 1–121 were cloned into pGEX-5T-1 (Pharmacia). GST fusion proteins were expressed and purified as described (31).

Insect Cell Culture and Baculovirus Methods. High Five insect cells were maintained in Ex-Cell 405 Insect Culture Media (JRH Biosciences, Lenexa, KS) at 27°C. Baculovirus vectors (10^{-7} plaque-forming units) engineered to express human cyclin A or an hemagglutinin-tagged human cdk2 were used separately or in combination to infect cells. Cells (1×10^7cells per 100-mm dish) were infected with 0.5 ml of virus in a final volume of 3.0 ml for 2 h at 27°C and refed with 10 ml of Ex-Cell medium. Two days postinfection, cells were lysed on ice for 30 min 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 with protease and phosphatase inhibitors as described above.

Immunoprecipitations. Insect cell immunoprecipitations were performed using ≈100 μg of extract and 5 μg of the mAb 12CA5 (Boehringer Mannheim) directed against the cdk hemagglutinin-epitope, or 5 μg of a human cyclin A-specific polyclonal antibody (#06–138, Upstate Biotechnology, Lake Placid, NY). Immune complexes were immobilized on protein A/G agarose beads (Santa Cruz Biotechnology), washed four times in 0.5 ml of lysis buffer and used in the *in vitro* kinase assay.

***In Vitro* Kinase Assays.** The GST-ER substrate (10 μg), ER 1–82, ER 1–115, or ER 1–121, was absorbed to 100 μl of a 50% slurry of glutathione-Sepharose 4B beads (Pharmacia) for 20 min at room temperature and washed twice with kinase buffer (50 mM potassium phosphate, pH 7.15/10 mM $MgCl_2$/5 mM NaF/4.5 mM DTT/1 mM phenylmethylsulfonyl fluoride). The immobilized substrate was added to the immunopurified kinase subunit(s) and incubated on ice for 5 min prior to the initiation of the kinase reaction in a final volume of 150 μl as described (31). The reaction products were separated by 12.5% SDS/PAGE, stained with Coomassie blue to visualize the receptor band, and autoradiography was performed from 5 to 30 min at room temperature. Aliquots of the reaction mixtures were also separated by SDS/PAGE and subjected to Western blot analysis to determine the levels of ER, cyclin A, and cdk2.

***In Vivo* Metabolic Labeling.** HeLa cells (1×10^6 cells per 100-mm dish) were transiently transfected with FLAG-ER and/or cyclin A and metabolically labeled with 1 mCi/ml (1 Ci = 37 GBq) of [^{32}P]orthophosphate in 2 ml of phosphate-free DMEM for 2 h at 37°C in the absence or presence of 100 nM 17β-estradiol. Cells were washed twice with PBS, placed on ice, and lysed directly on the plate by adding 200 μl of high salt lysis buffer. The *in vivo* labeled FLAG-tagged ER was immunopurified using 5 μg of the monoclonal anti-FLAG antibody (M2, Eastman Kodak). The ER protein recovered by immunoprecipitation was resolved on SDS/10% polyacrylamide electrophoresis gel, silver-stained, and dried. Autoradiography was performed for 12 h at room temperature to visualize the radiolabeled ER. The incorporated radioactivity was quantified using the National Institutes of Health IMAGE program to analyze the scanned autoradiogram and a digitized version of the silver stained gel.

RESULTS

Increased ER Transcriptional Enhancement by Ectopic Cyclin A Expression. To establish whether ectopic expression of cyclin A affects ER-dependent activation, we examined the ability of cyclin A to increase ER-mediated transcriptional enhancement. ER-deficient HeLa cells were transfected with an expression vector for the full-length human ER containing a FLAG epitope at its N terminus, the reporter plasmid estrogen response element-thymidine kinase-CAT, plasmids encoding human cyclin A and a constitutive β-gal expression vector as an internal transfection standard. Transfected cells were treated with 17β-estradiol or the ethanol vehicle for 24 h. Transcriptional activity was measured by CAT assay and normalized to β-gal activity. As shown in Fig. 1*A*, both hormone-dependent and hormone-independent ER transcriptional activity were increased roughly 3-fold when cyclin A is overexpressed. No effect of cyclin A on reporter gene activity was observed in the absence of ER (not shown). To ensure that this increased transcriptional activity was not a result of additional ER protein production, we monitored protein expression in whole cell extracts using Western blot analysis. As Fig. 1*B* illustrates, ER levels are not increased by cyclin A coexpression (compare lanes 5 and 6 to lanes 7 and 8). In addition, cyclin A is expressed above endogenous levels as a result of our transient transfection scheme and estradiol treatment does not alter cyclin A expression (Fig. 1*B*, compare lanes 1 and 2 to lanes 3 and 4). By increasing the amount of cyclin A used in these transfections, we were able to observe a concomitant increase in ER transcriptional activation (Fig. 1*C*). Coexpression of cyclin A and cdk2 also results in an increased ER-dependent transcriptional activity slightly above that of cyclin A alone. Expression of cdk2 alone, on the other hand, did not significantly alter the ER-dependent transcriptional activity (not shown). These findings suggest that cyclin A is a limiting factor for full hormone-dependent ER-mediated transcriptional enhancement, presumably by favoring the formation of active cyclin/cdk complexes from endogenous cdk2 subunits. Thus, cyclin A expression greatly magnifies the characteristic hormone-dependent ER transcriptional response, which suggests that this cyclin/cdk complex can act as an effector of the ER signaling pathway.

Reciprocal Effects of cdk Activators and Inhibitors on ER Transcriptional Enhancement. To further demonstrate that alterations in cyclin A/cdk2 activity can modify ER transcriptional enhancement, we used two classes of cdk regulatory proteins, CAK, which is composed of cyclin H and cdk7, and the CDI, p27. p27 inhibits many cyclin/cdk complexes, includ-

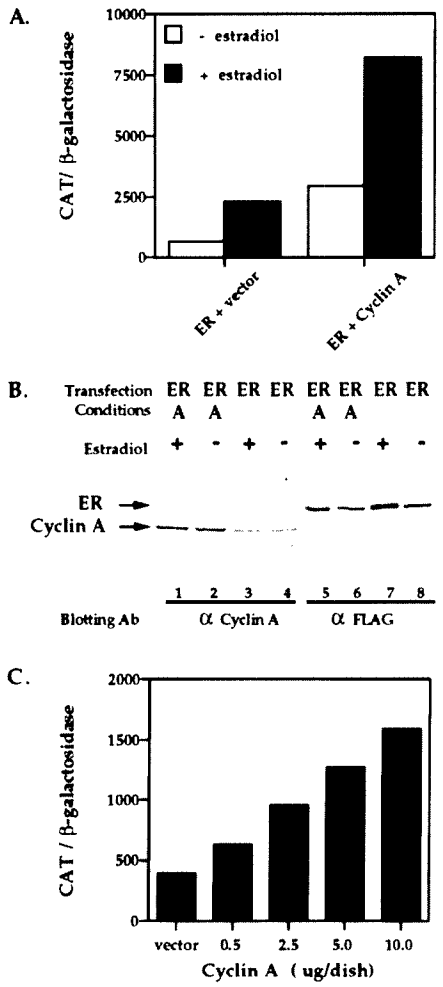

FIG. 1. Activation of ER transcriptional enhancement by ectopic cyclin A expression. (*A*) ER-deficient HeLa cells (2×10^5 cells per 60-mm dish) were transiently transfected using Lipofectamine with 2 μg of the estrogen response element containing reporter plasmid, possessing a single estrogen response element upstream of the thymidine kinase promoter fused to the CAT gene (ΔETCO), and 0.5 μg of the ER expression vector and 4 μg of the expression vector SRα296 (ER + vector) or 0.5 μg of the ER expression vector and 4 μg of SRα296-cyclin A (ER + cyclin A), along with 0.5 μg of pCMV-lacZ as an internal standard for transfection efficiency. Cells were incubated with 100 nM 17β-estradiol or the ethanol vehicle for 24 h as indicated, harvested and assayed for CAT and β-gal activity. (*B*) ER and cyclin A expression in transfected HeLa cells. Whole cell extracts were prepared from a parallel set of transfected cells. Equal amounts of protein (100 μg per lane) were separated by SDS/10% polyacrylamide gel, transferred to Immobilon paper, probed with the M2 monoclonal antibody directed against the FLAG-epitope on ER or a polyclonal antibody against human cyclin A, and visualized with an alkaline phosphatase-conjugated goat secondary antibody. (*C*) Increasing amounts of cyclin A lead to increased ER transcriptional activity. Using the calcium phosphate procedure, HeLa cells were transiently transfected with increasing amounts of cyclin A (0.5 μg to 10.0 μg) with a constant amount of ER expression and reporter plasmids, and CAT activity was measured in the presence of 17β-estradiol. For transfection experiments, data represent the mean of at least two experiments done in duplicate with <10% variation.

ing cyclin A/cdk2, cyclin E/cdk2, and cyclin D/cdk4 (32, 33). We were particularly interested in studying p27 in light of recent reports (15, 17, 18) linking its premature or excessive degradation to aggressive breast and colorectal cancers.

Fig. 2*A* illustrates that cdk activation by expression of cyclin A/cdk2 or CAK (cyclin H and cdk7) leads to a greater than 2-fold increase in both hormone-dependent and hormone-independent ER transcriptional activity. The coexpression of

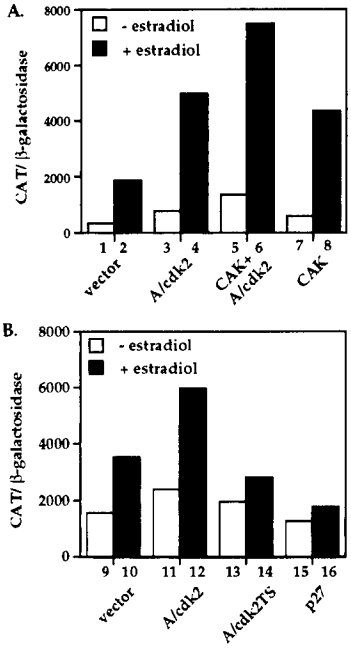

FIG. 2. Effects of cdk activators and inhibitors on ER transcriptional enhancement. (*A*) Effects of CAK (cyclin H/cdk7) on ER transcriptional activation. HeLa cells were transiently transfected using the calcium phosphate procedure with paired ER expression and reporter plasmids as described in Fig. 1, along with a control empty expression vector, (lanes 1 and 2); or expression vectors for cyclin A/cdk2 (lanes 3 and 4); cyclin A/cdk2 + CAK (lanes 5 and 6) and CAK alone (lanes 7 and 8). (*B*) Effects of cdk inhibitors on ER transcriptional activation. HeLa cells were transiently transfected with ER expression and reporter constructs along with an empty expression vector (lanes 9 and 10); or expression vectors for cyclin A/cdk2 (lanes 11 and 12); cyclin A/cdk2TS (dominant negative) (lanes 13 and 14) and p27 (lanes 15 and 16). Hormone treatment and activity assays were performed as described in Fig. 1. Data represent the mean of two experiments done in duplicate with <10% error.

all four proteins, the cyclin/cdk complex as well as the CAK complex, further augments (4-fold) this response and lends further support for cyclin/cdk involvement in the regulation of ER-dependent transcriptional activity.

We next asked if a decrease in cdk activity would reduce ER-dependent transcriptional activation. We chose two means of inhibiting cdk2 activity. Initially, the CDI, p27, was ectopically expressed in HeLa cells and ER-dependent transcriptional enhancement was measured. Ligand-dependent and, to a lesser degree, ligand-independent transcriptional activation by ER was reduced by p27 expression (Fig. 2*B*). This effect is noted in either the presence or absence of ectopically expressed cyclin A. Therefore, reducing cdk activity leads to impaired ER transcriptional activity.

At this point, we could not discriminate between an effect of p27 upon cdc2, cdk2, or cdk4, since p27 can inhibit all of these kinases. Therefore, we sought another means of reducing cdk2 activity by using a catalytically inactive cdk2 mutant to specifically block endogenous cdk2 activity. This cdk derivative, designated cdk2TS, is competent for cyclin A binding, but it cannot bind to ATP due to two consecutive amino acid changes in the ATP-binding site (Lys-33 and -34 are replaced by threonine and serine, respectively). This mutant acts as a dominant negative by sequestering cyclin A, thereby preventing it from binding and activating endogenous wild-type cdk2.

By expressing the dominant negative cdk2 mutant, we were able to reduce significantly the ER response to ligand treatment (Fig. 2*B*). Ectopic expression of a dominant negative cdc2 mutant had little effect on ER activity (not shown). These results strongly argue that the observed decrease of ER

transcriptional activity by p27 is due to inactivation of cdk2 and further suggests the importance of cyclin A/cdk2 enzymatic activity for hormone-dependent transcriptional enhancement by ER. It appears then, that the balance among the cdk regulatory proteins, cyclins, CAK, and CDIs, is critical in determining ER transcriptional activity.

Phosphorylation of ER by the Cyclin A/cdk2 Complex. Next, we investigated whether the cyclin A/cdk2 complex can phosphorylate ER. To determine if ectopic expression of cyclin A increased the amount of phosphate incorporated into ER *in vivo*, HeLa cells were transfected with ER alone or in combination with cyclin A and cells were metabolically labeled with [^{32}P]orthophosphate for 2 h in the presence or absence of 17β-estradiol. For each sample, the total amount of ER visualized by silver staining was used to standardize the amount of incorporated ^{32}P. The untreated ER condition was arbitrarily set as 1. As shown in Fig. 3*A*, ER phosphorylation is increased by ectopic expression of cyclin A in both the absence (3×) and presence (3.7×) of hormone. Thus, the presence of cyclin A increases incorporation of phosphate into ER by activating endogenous cdks.

To further investigate the effect of cyclin A/cdk2-dependent phosphorylation of ER we performed *in vitro* kinase assays. Three ER derivatives, containing amino acids 1–82, 1–115 or 1–121 were bacterially expressed and purified as GST-fusion proteins and used as substrates for phosphorylation by immunopurified baculovirus-expressed cyclin A and cdk2. These particular derivatives were chosen since the ER 1–121 derivative contains three serine-proline motifs at Ser-104, -106, and -118, whereas ER 1–115 contains only Ser-104 and -106. ER 1–82 lacks all of the putative serine-proline phosphorylation sites and thus serves as a negative control. Fig. 3*B* demonstrates that both ER 1–121 and ER 1–115 were phosphorylated by the cyclin A/cdk2 complex but not by either subunit alone. On the other hand, ER 1–82 was not phosphorylated by the cyclin A/cdk2 complex. In each reaction, expression of the ER substrate and the kinase subunits was verified by Western blotting and found to be identical (not shown). The fact that ER 1–121 and ER 1–115 derivatives were phosphorylated while ER 1–82 was not, strongly suggests that the residues contained in the region comprised by amino acids 83–121 comprise a motif targeted by the cyclin A/cdk2 complex. This data provide *in vitro* biochemical evidence that ER is a substrate for cyclin A/cdk2-dependent phosphorylation.

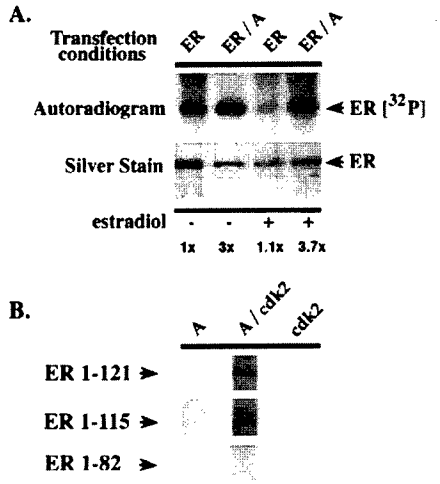

FIG. 3. ER phosphorylation by cyclin A/cdk2. (*A*) Phosphorylation of ER *in vivo* in the absence and presence of cyclin A. HeLa cells were transfected with FLAG-ER in the absence and presence of cyclin A expression vector using the Lipofectamine method as described in Fig. 1 and metabolically labeled with [^{32}P]orthophosphate as described in *Materials and Methods*. Whole cell extracts were prepared and ER was immunoprecipitated using the FLAG-mAb, M2. ER immunoprecipitates were separated by SDS/10% polyacrylamide gel, silver-stained (*Lower*), and exposed to film to visualize the phosphorylated receptor (*Upper*). The incorporated radioactivity was normalized to the amount of ER immunoprecipitated in each condition. The value of the untreated ER was arbitrarily set as 1. (*B*) Phosphorylation of ER *in vitro* by the cyclin A/cdk2 complex. Bacterially expressed GST ER 1–82, 1–115, and 1–121 derivatives were absorbed onto glutathione agarose beads and used as substrates for *in vitro* kinase assays. Cyclin A and cdk2 were produced in 5B insect cells, separately or in combination, purified by immunoprecipitation, and used in the kinase assays as described in *Materials and Methods*. The proteins were separated by SDS/10% polyacrylamide gel and the phosphorylated products were visualized by autoradiography.

Increased ER Transcriptional Enhancement in Response to Ectopic Cyclin A Expression in Multiple Cell Lines. To test our hypothesis that the regulation of ER-dependent transcriptional activity by the cyclin A/cdk2 complex is not specific to HeLa cells but rather reflects a general mode of regulation, we repeated our transcriptional activity assay in a variety of cell lines. We tested Hs 578Bst cells derived from breast tissue peripheral to an infiltrating ductal carcinoma and an ER-negative osteosarcoma cell line, U-2 OS. Cells were transiently transfected as described above, treated with 17β-estradiol or the ethanol vehicle for 24 h and transcriptional activity was measured. In the three cell types utilized, we observed a significant increase in ER ligand-dependent and -independent transcriptional activation (Fig. 4). These data imply that the ability of a cyclin A/cdk2 complex to enhance ER ligand-dependent transcription is conserved across multiple cell types.

DISCUSSION

We have examined the effects of cyclin A/cdk2 activation and inhibition on ER-dependent transcriptional enhancement. Here, we provide evidence that alterations in the regulation of the cyclin A/cdk2 complex lead to changes in both hormone-independent and hormone-dependent ER transcriptional enhancement. Our findings indicate that the ectopic expression of cyclin A elevates ER transcriptional activity and that this effect is not restricted to a single cell type. Our results likely represent an underestimate of the full impact of cyclin A on ER transcriptional activity, since these findings are obtained in cells that contain endogenous cyclin A and cdk2. Consistent with this view, ER transcriptional activity in both the presence and absence of hormone is virtually abolished under conditions where cyclin A/cdk2 activity is suppressed by the kinase inhibitor p27, or by a dominant negative cdk2 mutant. Thus it appears that cyclin A, the regulatory subunit of cdk2, is a limiting cofactor in the regulation of ER-dependent transcriptional activation in the cells examined.

Our findings also demonstrate that ER is a substrate for cyclin A/cdk2. ER is phosphorylated *in vitro* by cyclin A/cdk2 complexes and incorporation of phosphate into ER is stimulated by cyclin A expression *in vivo*. Importantly, our biochemical data demonstrate that the presence of three putative cdk target sites (Ser-104, Ser-106, Ser-118) is correlated with cyclin A/cdk2-dependent phosphorylation of the ER substrate *in vitro*. Work is ongoing to identify the precise residue(s) on the receptor phosphorylated by the cyclin A/cdk2 complex. Together, these data suggest that the cyclin A/cdk2 complex directly phosphorylates ER and that this modification serves to increase the receptor's transcriptional regulatory properties.

Zwijsen *et al.* (4) observed that overexpression of cyclin D1 increased ER-dependent transcriptional activation in T-47D cells without direct ER phosphorylation by cyclin D1/cdk4. Cyclin D1 appears to act indirectly as an ER cofactor, perhaps by tethering or phosphorylating other regulatory proteins that effect downstream signaling by ER. In contrast, we observed the cyclin A/cdk2 complex directly acting upon the receptor leading to its phosphorylation and a marked increase in

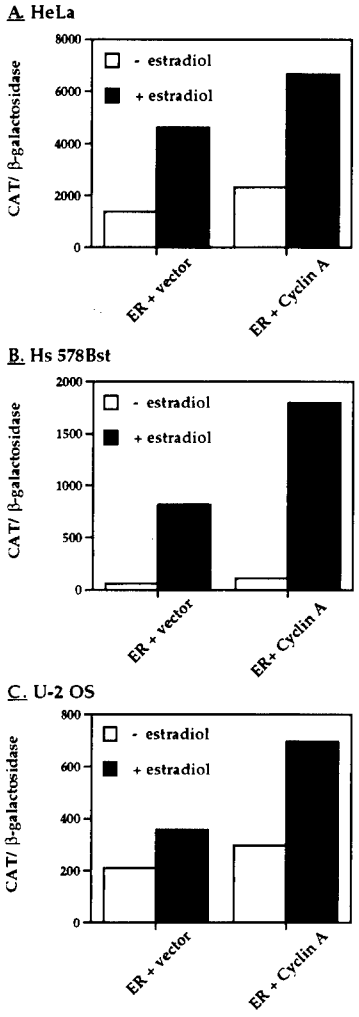

FIG. 4. Cyclin A enhances ER-dependent transcriptional activation in multiple human cell lines. Three ER-negative cell lines, (*A*) HeLa cells derived from a human cervical carcinoma, (*B*) Hs 578Bst, a human cell line derived from normal breast tissue, and (*C*) U-2 OS, a human osteosarcoma cell line, were transfected with ER expression and reporter plasmids as described in Fig. 1. Cells were also cotransfected with the empty expression vector or the expression vector encoding cyclin A. Hormone treatment and activity assays were performed as described in Fig. 1. Results shown represent a single experiment done in duplicate whose error was <10%. This experiment was repeated twice more with similar results.

ER-dependent transcriptional activity. Zwijsen *et al.* (4) did not observe an effect of cyclin A overexpression on ER-dependent transcriptional activation in T-47D cells and conversely, we failed to detect an increase in ER activity when cyclin D1 was ectopically expressed in either HeLa or ER-expressing U-2 OS human osteosarcoma cell line (J.M.T. and M.J.G., unpublished data). One factor contributing to the observed differences may lie in the cell types used in these studies. Recent reports and our own observations suggest that the level of expression of CDIs, such as p27, differ dramatically among cell types (34). Given that these proteins function as kinase inhibitors and as recently recognized cyclin D/cdk4 assembly factors, differences in CDI expression might significantly alter cdk signaling (34, 35). Among several breast cancer cell lines tested, T-47D cells were found to express high levels of p27 (34). This finding may account for the lack of a cyclin A effect in these cells, since the resulting cyclin A/cdk2 complex will be inhibited by endogenous p27. In contrast, HeLa cells used in this study express comparatively low amounts of p27, making ER-dependent transcription more sensitive to ectopic cyclin A expression. The cell-specific differences in the level of endogenous p27 may also help explain the ability of cyclin D1 to activate ER in T-47D cells, but not in HeLa cells, since abundant p27 may favor the formation of a cyclin D1/cdk4 complex, which may in turn phosphorylate an ER coactivator, or facilitate complex formation between ER and a receptor cofactor. Examination of the consequences of ectopic cyclin A expression in several breast cell lines has revealed an inverse correlation between cyclin A activation of ER transcriptional enhancement and the level of endogenous p27 (J.M.T. and M.J.G., unpublished data). Thus, the level of endogenous CDI may determine which cyclin isotypes will affect ER transcriptional activity, and may account for the observed differences between our findings and that of the Zwijsen *et al* (4).

Based on our findings, we propose a model for ER regulation by the cyclin A/cdk2 complex (Fig. 5). The cyclin A/cdk2 complex directly phosphorylates the receptor and in doing so, facilitates its interaction with the basal transcriptional machinery or an ER coactivator, which increases the receptor's ability to activate transcription. Inhibition of cdk activity by CDIs, such as p27, or through a reduction in cyclin or cdk expression, would decrease receptor phosphorylation, weakening these putative ER-transcription factor contacts, thus leading to decreased receptor transcriptional activity. We further envision that the expression of the CAK complex, cyclin H and cdk7, enhances ER transcriptional activation by increasing the activity of the endogenous cyclin A/cdk2 pool. Since cyclin H and cdk7 are also components of TFIIH (5, 36–38), we cannot exclude the possibility that this complex may be acting at the level of TFIIH to increase its catalytic activity, which in turn, increases ER transcriptional activity. Together, these data suggest that the cyclin A/cdk2 complex directly influences ER's transcriptional regulatory properties. We conclude that ultimately the balance of these cdk regulatory proteins determines kinase activity, which in this case translates into differential transcriptional activation by ER.

A complex picture of signal transduction by ER is emerging that appears to rely on the collaboration of multiple factors for its regulation, with each event in the pathway vulnerable to subversion. This subversion may take the form of aberrant expression of cyclin or cdk subunits, or CDIs, leading to alterations in receptor phosphorylation and activity that might contribute to uncontrolled cell proliferation. Clearly, the involvement of cyclins, cdks, CAKs, and CDIs in ER-mediated

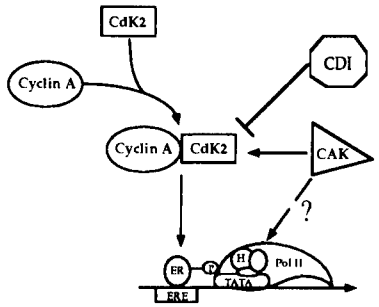

FIG. 5. Model for regulation of ER-dependent transcriptional activation by the cyclin A/cdk2 complex. The cyclin A/cdk2 complex phosphorylates ER which increases the receptor's ability to activate transcription by facilitating its interaction with the basal transcriptional machinery or an ER coactivator. Inhibiting cdk activity by CDIs has the opposite effect, resulting in reduced ER phosphorylation and decreased receptor transcriptional activity. Expression of the CAK complex, cyclin H, and cdk7, enhances ER-dependent transcription by increasing the activity of the endogenous cyclin A/cdk2 pool. It is also conceivable that the CAK complex may be acting at the level of TFIIH to increase ER transcriptional enhancement. We conclude that it is the balance among the cyclins, cdks, and their regulatory proteins that will ultimately determine ER transcriptional activity.

transcriptional regulation is complex and will require further investigation. It is likely, that phosphorylation events mediated by the cyclin/cdk pathway will emerge as a general mechanism of controlling steroid hormone action (31, 39).

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Rho GTPases As Novel Modulators of the Estrogen Receptor Transcriptional Response

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SUMMARY

The estrogen receptor α (ER) is a ligand-dependent transcription factor that plays a critical role in the development and progression of breast cancer, in part, by regulating target genes involved in cellular proliferation. To identify novel components that affect the ER transcriptional response, we performed a genetic screen in yeast and identified RDI1, a Rho guanine nucleotide dissociation inhibitor (Rho GDI), as a positive regulator of ER transactivation. Overexpression of the human homologue of RDI1, Rho GDIα, increases ERα, ERβ, AR, and GR transcriptional activation in mammalian cells, but not activation by unrelated transcription factors SRF and Sp1. In contrast, expression of constitutively active forms of RhoA, Rac1, and Cdc42, decrease ER transcriptional activity, suggesting that Rho GDI increases ER transactivation by antagonizing Rho function. Inhibition of RhoA by expression of either the *Clostridium botulinum* C3 transferase or a dominant negative RhoA resulted in enhanced ER transcriptional activation, thus phenocopying the effect of Rho GDI expression on ER transactivation. Together, these findings establish the Rho GTPases as novel modulators of ER transcriptional activation. Since Rho GTPases regulate actin polymerization, our findings suggest a link between the major regulators of cellular architecture and steroid receptor transcriptional response.

INTRODUCTION

The estrogen receptor α (ER) is a ligand-dependent transcription factor that transduces the estrogen signal (1). Activation of ER is responsible for female sexual development and maintenance of bone density (2,3). In addition, ER plays a critical role in the development and progression of breast cancer by regulating genes and signaling pathways involved in cellular proliferation (4). Regulation of gene expression by the ER requires the coordinate activity of ligand binding, phosphorylation, and cofactor interactions, with particular combinations likely resulting in the tissue-specific responses elicited by the receptor (5-7). However, the extracellular cues and intracellular signaling pathways modulating these components and regulating ER transcriptional activation are not fully understood.

To identify novel components that modulate ER transcriptional activation, we performed a genetic screen in yeast for yeast factors that when overexpressed would increase the transcriptional activity of an ER derivative defective in transactivation by virtue of serine to alanine mutations in the three major N-terminal phosphorylation sites, serines 104, 106, and 118 (ER_{AAA}). This approach, given the ease of genetic manipulation and simplicity of gene identification in yeast, has proven successful for investigating various aspects of ER signal transduction (8). We expect to isolate factors that enhance ER transcriptional activity and, importantly, have functional homologues that affect ER transcriptional response in mammalian cells. Using this system, we have isolated RDI1, the yeast Rho guanine nucleotide dissociation inhibitor (Rho GDI), as a gene product that is capable of increasing both ER_{AAA} and wt ER transcriptional activation when overexpressed. This gene product is the yeast homologue of the mammalian Rho GDIα, a cytoplasmic protein originally identified as a negative regulator of the Rho family of GTP-binding proteins (9-12). The Rho family of GTPases, which include RhoA,

Rac1, and Cdc42, are best known for their ability to regulate actin cytoskeletal remodeling in response to extracellular signals, thereby promoting changes in cell morphology, adhesion and motility (13). In addition, by affecting multiple signaling pathways, Rho family members regulate gene transcription, cell cycle progression and have been implicated in cellular transformation in cooperation with Ras (14-18). The Rho family members possess intrinsic GTPase activity, and cycle between the inactive cytoplasmic GDP-bound, and the active membrane-associated GTP-bound state. The exchange of GDP for GTP induces a conformational change in the G protein that allows effector molecules, such as kinases, to bind and initiate downstream signaling events. This GTP/GDP cycle is tightly regulated in response to extracellular signals by three different classes of proteins. Guanine nucleotide exchange factors (GEFs) catalyze the exchange of GDP for GTP, GTPase-activating proteins (GAPs) accelerate the intrinsic GTPase activity of the Rho GTPases and guanine nucleotide dissociation inhibitors (GDIs) antagonize their activity by blocking GEFs and GAPs (9,19). In addition, Rho GDI controls the subcellular localization of the GTPases, stimulating their release from the plasma membrane (9,20). However, since the cytoplasmic GDP-bound Rho GTPases predominate under physiological conditions, Rho GDI acts as a negative regulator of Rho GTPases mainly by blocking the dissociation of GDP.

We have examined the effect of human Rho GDIα as well as the Rho GTPases, RhoA, Rac1, Cdc42, on transcriptional activation by ER in mammalian cells. Our findings indicate that Rho GDIα specifically increases the transcriptional activity of ER α and β as well as the glucocorticoid receptor (GR) and androgen receptor (AR), but not of unrelated transcription factors serum response factor (SRF) and Sp1, and that this activation is mediated via repression

of Rho GTPases. These results establish Rho-mediated signaling pathway as a novel regulator of ER, GR and AR transcriptional activity.

EXPERIMENTAL PROCEDURES

Plasmids

Yeast - The reporter plasmid ERE-CYC1-LacZ contains a single estrogen response element (ERE) upstream of a truncated *CYC1* promoter linked to the β-galactosidase gene. The yeast high copy genomic was made by subcloning Sau3A partially digested yeast genomic DNA into BamHI site of the YEP351 plasmid. Wt ER and ER_{AAA} were expressed from the Gal1-10 promoter in *Trp1*, 2 μM plasmid (p2T-GAL). p2T-GAL-ER_{AAA} was constructed by subcloning the BamHI fragment containing ER_{AAA} sequence from pcDNA3 (21) plasmid into p2T-GAL.

Mammalian cells- The ER reporter plasmid contains one ERE upstream of the herpes simplex virus thymidine kinase (tk) promoter (-109) linked to the firefly luciferase coding sequence (XETL). The GR reporter plasmid is identical to XETL, except 2 consensus GREs are substituted for the ERE ($XG_{46}TL$). The steroid receptor expression plasmids are: pcDNA3-human ERα (21), pCMV5-human ERβ (provided by Jan-Ake Gustafsson, Karolinska, Sweden), pcDNA3-rat GR (22), and pcDNA3-human AR (provided by Roger Miesfeld, University of Arizona Medical School) . Rho GDIα is in pcDNA3 and is a gift from Mark Philips, New York University School of Medicine. The N-terminally myc-tagged Rac1.L61 and Cdc42.L61 were generously provided by Alan Hall, University College London, UK. pRK5-myc-RhoA.V14 was subcloned as an EcoRI fragment containing myc-tagged RhoA.V14 from EXV plasmid. Both EXV.RhoA.V14 and EFC3- expressing myc-tagged C3 transferase under EF1α promoter are gifts from Richard Treisman, Imperial Cancer Research Fund, UK. The dominant negative form of RhoA, RhoA.N19, was made by site-directed mutatgenesis using the Quick Change Mutagenesis Kit (Stratagene) with pRK5-myc-RhoA as the template. The entire RhoA.N19

coding region was sequenced to verify the base changed and to insure that no other mutations were introduced. The Sp1 reporter contains six Sp1 binding sites upstream of the adenovirus major late promoter (from Naoko Tanese, New York University School of Medicine) and SRF reporter contains a fragment of the c-Fos promoter upstream of luciferase (provided by Susan Logan, New York University School of Medicine).

Yeast strains, growth conditions, and β-galactosidase assay

The yeast strain W303a (**a** *ade2 leu2 his3 trp1 ura3*) was used to screen for ER activators. Yeast transformation was performed by the standard lithium acetate/ polyethylene glycol method (8). To assay ER transcriptional activation, cells were cultured overnight in the appropriate selective media containing 2% glucose and subcultured 1:20 in selective minimal media containing 2% galactose-1% raffinose to induce receptor expression and treated with 17β-estradiol for 12 hr. and quantitative liquid β-galactosidase assays were performed as described (8). Plate assays were performed by replica plating colonies from glucose plates onto galactose X-Gal indicator plates containing 0.1 nM 17β-estradiol.

Cell culture, transfection, and luciferase assays

Human osteosarcoma cell line U2OS was obtained from American Type Culture Collection (ATCC HTB 96) and maintained in DMEM (GIBCO/BRL) supplemented with 10% fetal bovine serum (HyClone), 10 units/ml each of penicillin and streptomycin (Cellgro), and 2 mM L-glutamine (Cellgro). Between 1.2-1.3 X 10^5 cells were seeded onto 35-mm plates in phenol red-free DMEM supplemented with 10% charcoal-stripped fetal bovine serum and 2 mM L-glutamine. Transfections using Lipofectamine Plus reagent (GIBCO/BRL) were performed

according to manufacturer's recommendation. Cells were treated with hormone agonists (100 nM 17β-estradiol, 100 nM dexamethasone, and 100 nM R1881 for ER, GR and AR, respectively) or ethanol vehicle 12 hours post-transfection for 24 hours. Transfected cells were then washed once in phosphate-buffered saline and harvested in 1X reporter lysis buffer (Promega) as per the manufacturer's instructions. 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 BSA, 1 mM DTT, using a Lumen LB 9507 luminometer (EG&G Berthold) and 1 mM D-luciferin as substrate. The steroid receptor transcriptional activity as reported are normalized to reporter activity in the absence of transfected steroid receptors, and to protein concentration as determined by the Bradford protein assay (Bio-Rad).

Immunoblotting

To prepare protein extracts from transfected cells, whole cell extracts prepared for luciferase assay in 1X reporter lysis buffer were normalized for total protein and boiled for 3 minutes with SDS sample buffer. Protein extracts were then fractionated by 12% SDS-polyacrylamide gel electrophoresis, transferred to Immobilon membrane (Millipore), and probed with anti-ERα polyclonal antibody (HC-20, Santa Cruz Biotechnology), anti-Rho GDI polyclonal antibody (A-20, Santa Cruz Biotechnology), or anti-c-Myc monoclonal antibody (9E10, Santa Cruz Biotechnology). 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).

RESULTS

A Genetic Screen for Activators of ERα Transcriptional Enhancement

Concomitant serine to alanine mutations at N-terminal phosphorylation sites 104, 106, and 118 (ER_{AAA}) result in a ~50% reduction in ER transcriptional activity in mammalian cells (23). To determine if the transcriptional activity of ER_{AAA} is also reduced in yeast, strains were constructed containing a galactose-inducible expression vector encoding either wt ER or ER_{AAA}, and an ER-responsive reporter plasmid. The transcriptional activities of wt ER and ER_{AAA} were measured as a function of hormone concentration. Compared to wt ER, ER_{AAA} exhibited ~40% reduction of transcriptional activity at all hormone concentrations tested, suggesting that the ER_{AAA} is less efficient at engaging in the interactions necessary for transcriptional activation (Fig. 1A).

The ER_{AAA} phenotype is most striking at 0.1 nM 17β-estradiol. Under these conditions, yeast colonies expressing wt ER are blue, while ER_{AAA}-expressing colonies appear white (Fig. 1B). To screen for ER activators, yeast expressing ER_{AAA}, along with an estrogen-responsive reporter gene, were transformed with a high copy yeast genomic library and assayed for receptor transcriptional activation on X-Gal indicator plates containing 0.1 nM 17β-estradiol. Candidate high copy suppressors changing the ER_{AAA}-expressing yeast from white to blue were selected for further analysis (Fig 1B). Of the 29,000 colonies screened, which represents approximately three times the size of the yeast genome, we identified seven yeast open reading frames (ORFs) that enhance ER transcriptional activation (Table 1).

A search of the yeast genome database revealed that three of the candidate suppressors were yeast homologues of mammalian proteins previously shown to affect ER transactivation. These include: 1) CKA1, a homologue of the mammalian alpha subunit of casein kinase II that

phosphorylates serine 167 *in vitro* (24); 2) CAD1, a member of the Jun transcription factor family that synergizes with ER in mammalian cells (25); and 3) YAK1, the catalytic subunit of cAMP-dependent protein kinase whose mammalian homologue has been shown to phosphorylate ER at serine 236 and regulate receptor dimerization (26). In addition to genes known to regulate ER activity, several genes not known to affect ER were identified. IME2, a kinase with homology to CDC28, which controls yeast entry into meiosis, was isolated five times. In addition, MCK1, an upstream regulator of IME2 that shares sequence homology with glycogen synthase kinase-3, was identified once. Additionally, we have also isolated RDI1, the yeast Rho guanine nucleotide dissociation inhibitor (Rho GDI), three times, and LRG1, a yeast protein of unknown function that contains a GTPase activating protein (GAP) homology domain, once. Although LRG1 is presently linked to GAP merely through sequence homology, it is interesting to note that GAP and RDI1 are both negative regulators of Rho GTPases. The recovery of known ER regulators together with the repeated isolation of certain genes indicates that the approach was sound and that the library was likely screened to saturation. Since Rho GDI negatively regulates Rho GTPases, this result suggested that the Rho GTPases may modulate ER transcriptional activation and is the focus of this report.

Rho GDI expression increases ER transactivation

Among the human Rho GDIs, RDI1 is most similar to human Rho GDIα, which negatively regulates the best studied Rho GTPases, RhoA, Rac1, and Cdc42. To examine whether the mammalian Rho GDI affects ER transcription in mammalian cells, we tested the ability of human Rho GDIα to enhance ER transcriptional activity in the human osteosarcoma cell line U2OS. ER negative U2OS cells were transiently transfected with ERα, an ER-

responsive reporter plasmid, along with increasing amount of Rho GDIα. As shown in Figure 2A, Rho GDIα stimulates ER transactivation in a dose-dependent manner. Enhancement of ER transcriptional activation by Rho GDIα was also observed for ER_{AAA} mutant (not shown). To ensure that this enhanced transcriptional activity was not a result of increased ER protein production, we monitored protein expression and found that ER levels decreased in the presence of Rho GDI (Fig. 2B), indicating that the effect of Rho GDIα on ER activity is greater than that observed. This effect is not restricted to single cell type, since Rho GDIα also enhanced ER transactivation in the ER-positive breast cancer cell line MCF7 (not shown). Thus, Rho GDIα can act as a positive regulator of ER-dependent transcriptional activation in mammalian cells.

Rho GDI specifically activates steroid hormone receptors

We next tested the ability of Rho GDIα to affect transactivation by other members of the steroid receptor family, ERβ, GR and AR, using transient transfection assays. Our results indicate that Rho GDIα also increased the transcriptional activity of ERβ, GR, and AR in a dose-dependent manner (Fig. 3A-C). To determine if Rho GDI-mediated activation is specific to steroid receptors, we tested the effect of Rho GDIα on Sp1 and SRF dependent transactivation. Rho GTPase signaling has been previously shown to enhance transcriptional activation by SRF (15), thus we would expect Rho GDIα, as a negative regulator of Rho GTPases, to decrease SRF transcriptional activity. Consistent with this idea, Rho GDIα expression decreased SRF activity from a reporter plasmid containing the *c-fos* SRF element (Fig. 4A). Similarly, Sp1 transcriptional activity using a Sp1-responsive reporter also decreased in response to Rho GDI overexpression (Fig. 4B). Taken together, these results strongly suggest that Rho GDI

specifically increases transactivation by steroid hormone receptors, perhaps through a mechanism involving suppression of Rho GTPase signaling.

Rho GTPases inhibit ER transactivation

The GTPases known to interact with Rho GDIα include RhoA, Rac1, and Cdc42. To determine whether Rho GDI increases ER transactivation by inhibiting the Rho GTPases, we expressed constitutively active forms of Rho GTPases (RhoA.V14, Rac1.L61, and Cdc42.L61) in U2OS cells and examined ER transcriptional activation. As shown in Figure 5, expression of RhoA.V14, Rac1.L61, and Cdc42.L61 decreased ER transcriptional enhancement, consistent with the model that Rho GDI activates ER transcriptional enhancement by antagonizing Rho GTPases.

As an independent means of examining the effect of RhoA inhibition on ER transcriptional activation, we ectopically expressed the *Clostridium botulinum* C3 transferase, a protein toxin that ADP-ribosylates and inhibits RhoA, but not Rac1 or Cdc42 (27,28). As with Rho GDI, expression of C3 transferase results in an enhancement of ER transcriptional activity, but decreases SRF transcriptional activation (Fig. 6A and not shown). Inhibition of ER transcriptional activation by activated RhoA, but not Rac1 or Cdc42, was also relieved by C3 coexpression (not shown). Likewise, inhibition of endogenous RhoA by expression of a dominant negative form of RhoA (RhoA.N19) also results in greater ER transactivation (Fig. 6B). Thus, inhibition of RhoA results in enhanced ER transcriptional activation, indicating that Rho-mediated signaling events suppress ER transactivation.

DISCUSSION

We have demonstrated that Rho GDIα enhances the transcriptional activity of the ERα as well as ERβ, GR, and AR, but not SRF or Sp1. We also show that activated mutant forms of RhoA, Rac1, and Cdc42 decrease, whereas inhibition of endogenous RhoA by C3 transferase or dominant negative RhoA increase ER transcriptional activation. From these results, we conclude that the enhanced ER transactivation observed upon Rho GDIα overexpression is mediated by antagonism of Rho GTPases and implicates the Rho family proteins RhoA, Rac1 and Cdc42 in signaling to ER.

What is the mechanism underlying the modulation of ER transactivation by RhoA? Since Rho GTPases mediate actin cytoskeleton reorganization, as well as the activation of multiple signaling pathways, including those regulating transcriptional activation, Rho-mediated inhibition of ER may result from either of these events. Recently, it has been shown that changes in the actin cytoskeleton can affect transcriptional activation by SRF (29). Our results also suggest that RhoA, Rac1 and Cdc42-mediated signaling to ER may converge at some common point through a shared signaling molecule. An attractive candidate for such a common regulatory molecule affecting the actin cytoskeleton is LIM kinase (30). The GTP-bound forms of RhoA and Rac1/Cdc42 activate LIM kinase via phosphorylation through effector kinases ROCK and Pak, respectively (31,32). The activated LIM kinase phosphorylates cofilin, an actin-binding protein, thereby inhibiting its actin-depolymerizing activity and leading to the accumulation of actin filaments. In a model reminiscent to that proposed for regulation of SRF by actin (29), we speculate that suppression of ER transactivation could result either from releasing an ER corepressor that is associated with free G-actin, or from binding a coactivator to

actin filaments, thus preventing its interaction with the ER (Fig. 7). Although we cannot rule out that Rho GTPases are affecting ER activity by altering ligand binding, we think this is unlikely since ER ligand-independent and ligand-dependent transcriptional activation are both affected by Rho GDI signaling. Rather, we favor the notion that other steps required for ER transactivation, such as cofactor recruitment, are being affected. It is also likely that the regulatory cofactor(s) affected by Rho signaling would be conserved between yeast and mammalian cells. One example of such a conserved regulatory factor is the SWI/SNF complex (33). While the effect of actin cytoskeletal changes on ER remains unknown, actin dynamics may provide a means of modulating ER transcriptional activity during normal development or in pathological settings, such as tumor progression, when cells undergo extensive actin reorganization.

Alternatively, changes in ER transcriptional regulatory properties may result from the activation of signal transduction pathways by Rho GTPases. For example, RhoA, Rac1 and Cdc42 activate NF-κB, which has been shown to inhibit steroid receptor transactivation by forming inhibitory heterocomplexes (34,35). It is tempting to speculate that the inhibition of ER by the Rho GTPases is mediated by NF-κB. However, our preliminary findings suggest that inhibition of NF-κB by overexpressing IκB, does not relieve the repressive effects of Rho GTPases on ER transactivation (not shown), suggesting that Rho GTPases regulate ER independent of NF-κB.

Recently, an ER-interacting protein, termed Brx, was identified and shown to contain a domain virtually identical to the Rho GEF Lbc, although its enzymatic activity has not been demonstrated (36). Overexpression of Brx in Ishikawa cells increases ER transcriptional activation, and a dominant negative form of Cdc42, but not RhoA or Rac1, reduces its coactivator function. This apparent discrepancy between our result, where Rho GTPases repress

ER transactivation, and the ER activating function of Brx may be attributed to cell line-specific differences. Alternatively, since Brx coactivator function is likely mediated by direct ER binding, the inhibition of ER activity by the dominant negative form of Cdc42 may have resulted from competition between ER and dominant negative Cdc42 for Brx binding, rather than from blocking the signaling pathway downstream of Brx. While Brx and Rho GTPases may modulate ER activity through distinct mechanisms, the identification of different components in the Rho signaling pathway as modulators of ER transactivation underscores their importance in the receptor regulation.

Our findings suggest that the Rho GTPases decrease transcriptional activation by ERα, thus establishing a novel pathway of crosstalk between cell surface receptors that regulate Rho GTPase signaling and steroid receptor transcriptional activation. Another example of cross talk between the cell surface and ER is the modulation of ER ligand-independent transcriptional activation by the epidermal growth factor (EGF) /Ras/MAPK signaling pathway (37,38). It has been shown that treatment of cells with EGF results in ER ligand-independent activation and phosphorylation by the MAPK, Erk1 (39,40). Although the mechanism of this increased ER transcriptional activation remains to be elucidated, it likely involves phosphorylation-dependent cofactor recruitment (41). Thus, Ras acts as a positive regulator of ER transcriptional enhancement (40) (L. Su, unpublished observation), whereas Rho GTPases suppress receptor transactivation. We speculate that the opposing actions of Ras and Rho GTPases on ER-mediated transcriptional activation provide a means of fine tuning the ER transcriptional response to changes in the extracelluar environment (Fig. 7).

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FIGURE LEGENDS

Figure 1. Isolation of yeast factors that increase transcriptional activation by ERα.

(A) Transcriptional activation of wt ER and ER_{AAA} as a function of 17β-estradiol concentration. Yeast strains were transformed with either a galactose-inducible wt ER or ER_{AAA}, along with an ERE-containing β-galactosidase reporter plasmid. Transcriptional activation by the wt ER (dotted line) and ER_{AAA} (solid line) in response to increasing 17β-estradiol concentration was determined by liquid β-galactosidase assay as described in the "Experimental Procedures". Note that the ER_{AAA} in yeast exhibits approximately 40% of the wt ER transcriptional activity at each estradiol concentration tested. The dosage suppression screen was carried out in the presence of $1x10^{-10}$ M 17β-estradiol, conditions under which the ER_{AAA} phenotype is the most pronounced. (B) The relative activity of wt ER, ER_{AAA} and ER_{AAA} with an ER activator. Three independent colonies on X-gal indicator plates in the presence of $1x10^{-10}$ M 17β-estradiol are shown and represent wt ER with an empty expression vector (wt ER), ER_{AAA} plus an empty expression vector ($ER_{AAA)}$ and ER_{AAA} plus the RDI1 suppressor plasmid (ER_{AAA}+ ER activator).

Figure 2. Enhancement of ERα activation transcriptional by overexpression of Rho GDIα.

(A) ER-deficient U2OS cells ($1.2 x10^5$ cells/35 mm dish) were transiently transfected using Lipofectamine Plus reagent with 0.1 μg of ERα expression construct or empty vector, 0.2 μg of the ERE-containing reporter gene XETL and increasing amount of Rho GDIα, as indicated. Twelve hours after the transfection, cells were treated with 100 nM 17β-estradiol (dark bars) or the ethanol vehicle (light bars) for 24 hr, harvested, and assayed for luciferase activity. ERα transcriptional activity is normalized to XETL reporter activity in the absence of ER. The data represent the mean of an experiment done in duplicate, which was repeated three times. B)

Expression of ERα is reduced by Rho GDIα coexpression. Whole cell extracts were prepared from transfected cells as described in the "Experimental Procedures", and the expression of ERα and Rho GDIα was analyzed by Western blotting.

Figure 3. Rho GDIα enhances the transcriptional activation by ERβ, GR, and AR.
U2OS cells were transfected as described in Figure 2 with paired expression and reporter plasmids for A) ER β + XETL, B) GR + $XG_{46}TL$ or C) AR + $XG_{46}TL$, and along with the indicated amount of Rho GDIα, treated with 100 nM 17β-estradiol (E2), dexamethasone (Dex) and R1881, respectively, and harvested. In each case, receptor transcriptional activity shown is normalized to reporter activity in the absence of the receptor. The data shown represent experiments done in duplicate that have been repeated at least twice with similar results.

Figure 4. Rho GDI inhibits transcriptional activation by SRF and Sp1.
U2OS cells transfected as in Figure 2 with 0.4 μg of Rho GDIα together with 0.2 μg of A) SRF reporter or B) Sp1 reporter, harvested after 24 hr later and assayed for luciferase activity. Results shown represent an experiment done in duplicate and repeated twice.

Figure 5. The Rho GTPases, RhoA, Rac1 and Cdc42, inhibit ER transcriptional activation.
U2OS cells were transfected as in Figure 2 with the indicated amount of constitutively active forms of the Rho GTPases, RhoA.V14, Rac1.L61, and Cdc42.L61, along with 0.1 μg of ERα and 0.2 μg of XETL. Cells were treated with 100 nM 17β-estradiol 12 hr post-transfection, and harvested after 24 hr of estradiol treatment. ER transcriptional activity as depicted is normalized

to reporter activity in the absence of ER. Results shown represent an experiment done in duplicate and repeated twice.

Figure 6. Inhibition of endogenous RhoA by C3 transferase and dominant negative RhoA potentiates ER transactivation.

U2OS cells were transfected as in Figure 2 with the indicated amount of A) C3 expression vector or B) dominant negative form of RhoA (RhoA. N19) along with 0.1 μg of ERα and 0.2 μg XETL. Cells were treated as described in Figure 2 and ER transcriptional activation was measured. Shown is a representative experiment performed in duplicate.

Figure 7. Model for ER regulation by Ras and Rho GTPases.

In the model shown, RhoA, Rac1 and Cdc42 activation by extracellular signals, such as growth factor receptor (GFR) activation via PDGF, induction of G-protein coupled receptors (GPR) by lysophosphatidic acid (LPA), or the clustering of integrins through interaction with the extracellular matrix (ECM), might attenuate the ER transcriptional via the activation of a common signaling molecule, LIM kinase (LIMK), which phosphorylates cofilin and leads to the accumulation of actin filaments. We propose that Rho GTPases inhibit ER activity by either sequestering a putative coactivator (CoA) via actin filaments (F-actin) or through a corepressor (CoR) associated with free actin (G-actin) that is released during actin polymerization. In contrast, ER function would be enhanced by activation of the Ras pathway, likely through phosphorylation of ER by the MAPK, Erk1. We propose that the opposing actions of Ras and Rho GTPases on ER-mediated transcriptional activation refines the receptor transcriptional response to fit particular cellular needs.

Table 1. Yeast Genes that Enhance ER Transactivation

Top: yeast homologues of previously known mammalian regulators that affect ER function.

Bottom: factors that appear to link ER transcriptional activation to signal transduction pathways previously not known to affect ER function.

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Table 1. Yeast Genes that Enhance ER Transactivation

Gene	Function	Mammalian homologue	Effect on ER
CKA	alpha subunit of casein kinase II	casein kinase II	phosphorylates ER S167 *in vitro*
CAD1	yeast Jun-family	c-Jun	potentiates ER transactivation
YAK1	catalytic subunit of cAMP-dependent kinase	PKA	phosphorylates ER S236, regulates dimerization
IME2	serine/threonine kinase, a positive regulator of meiosis	rat MAK	enhances ER transactivation
MCK1	positive regulator of IME2	GSK-3	enhances ER transactivation
RDI1	Rho GDP dissociation inhibitor	Rho GDI	enhances ER transactivation
LRG1	similar to LIM domain proteins and Rho GTPase activating proteins	unknown	enhances ER transactivation

Figure 1

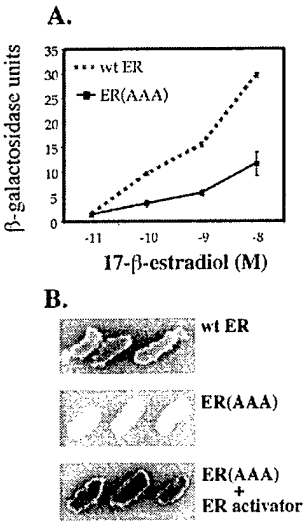

A.
β-galactosidase units
35
30
25
20
15
10
5
0
wt ER
ER(AAA)
-11
-10
-9
-8
17-β-estradiol (M)
B.
wt ER
ER(AAA)
ER(AAA)
+
ER activator

Figure 2

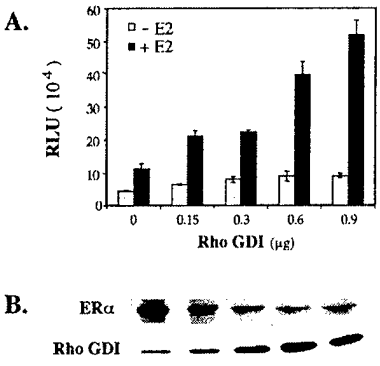

A.
□ - E2
■ + E2
RLU (10^{-4})
60
50
40
30
20
10
0
0
0.15
0.3
0.6
0.9
Rho GDI (μg)
B.
ERα
Rho GDI

Figure 3

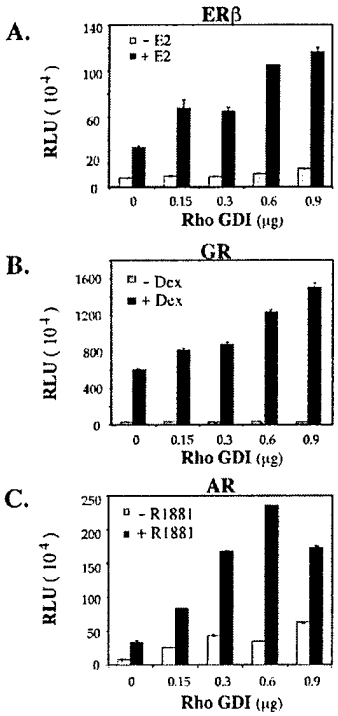

A.
ERβ
- E2
+ E2
RLU (10^{-4})
140
100
60
20
0
0
0.15
0.3
0.6
0.9
Rho GDI (μg)
B.
GR
- Dex
+ Dex
RLU (10^{-4})
1600
1200
800
400
0
0
0.15
0.3
0.6
0.9
Rho GDI (μg)
C.
AR
- R1881
+ R1881
RLU (10^{-4})
250
200
150
100
50
0
0
0.15
0.3
0.6
0.9
Rho GDI (μg)

Figure 4

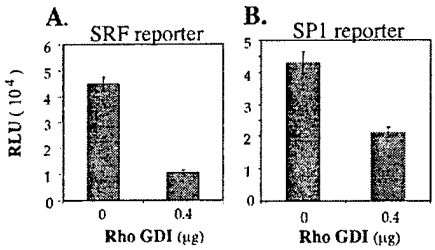

A.
SRF reporter
RLU (10^4)
6
5
4
3
2
1
0
0
0.4
Rho GDI (μg)
B.
SP1 reporter
5
4
3
2
1
0
0
0.4
Rho GDI (μg)

Figure 5

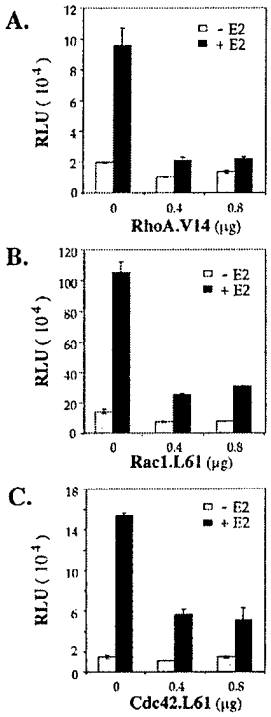

A.
RLU (10^{-4})
- E2
+ E2
0
0.4
0.8
RhoA.V14 (μg)
B.
RLU (10^{-4})
- E2
+ E2
0
0.4
0.8
Rac1.L61 (μg)
C.
RLU (10^{-4})
- E2
+ E2
0
0.4
0.8
Cdc42.L61 (μg)

Figure 6

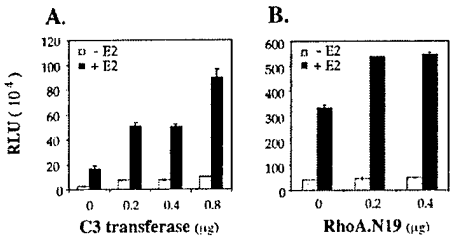
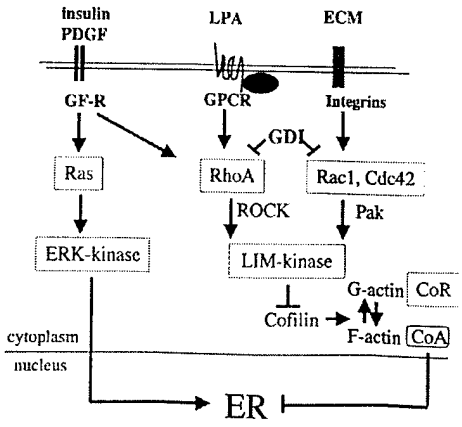

A.
B.
- E2
+ E2
RLU (10^4)
120
100
80
60
40
20
0
0
0.2
0.4
0.8
C3 transferase (μg)
600
500
400
300
200
100
0
0
0.2
0.4
RhoA.N19 (μg)

Figure 7


insulin
PDGF
LPA
ECM
GF-R
GPCR
Integrins
GDI
Ras
RhoA
Rac1, Cdc42
ROCK
Pak
ERK-kinase
LIM-kinase
G-actin
CoR
Cofilin
F-actin
CoA
cytoplasm
nucleus
ER