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This project examines the mechanism of signal transduction by the estrogen receptor (ER), a hormone dependent transcriptional regulator involved in many human breast tumors. We have found that ectopic expression of the cyclin A/cdk2 kinase complex increases hormone-dependent transcriptional activity by the estrogen receptor in vivo. This effect can be further enhanced by the concurrent expression of cyclin-dependent kinase activators, CAKs, and abolished by coexpression of the cdk inhibitor (CDI), p27. Likewise, ER-dependent transcriptional activation is reduced by the expression of a dominant negative cdk2 mutant in mammalian cells or by deletion of a potential cyclin A homologue, Clb4, in yeast. ER is also phosphorylated in vitro by cyclin A/cdk2 and incorporation of phosphate into ER is stimulated by the ectopic expression of cyclin A in vivo. Together, these results strongly suggest a role for cyclin-dependent kinases in the regulation of ER transcriptional enhancement.

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INTRODUCTION

Nature of the problem

The long-term objective of this proposal is to understand the mechanism of signal transduction by the estrogen receptor (ER), a transcriptional regulatory protein implicated in the initiation and maintenance of many human breast tumors. This year we have focused on one parameter involved in signal transduction by the receptor, namely phosphorylation, a modification that may play an important role in modulating ER activity or responsiveness to extracellular signals.

The question we asked is the following: Does phosphorylation by the cyclin-dependent kinases affect ER's ability to control gene expression and cell proliferation?

Background

Estrogen and other steroid hormones regulate the genetic programs that affect many aspects of cell metabolism, growth and differentiation [1]. Moreover, clinical evidence has implicated estrogen as the hormone primarily involved in breast cancer progression [2, 3].

Estrogen is produced in the granulosa cells of the ovary as well as in extraovarian tissues including the nervous system, adipocytes, hepatocytes and mammary cells. The hormone binds an intracellular receptor protein that transduces the steroid signal into changes in gene expression. Thus, the estrogen receptor (ER) is a hormone-dependent transcriptional regulator. In the absence of hormone, ER is an inactive aporeceptor found in an oligomeric complex with heat shock proteins [4, 5]. Following hormone binding, the complex dissociates, allowing dimerization of the receptor and formation of a functional hormone-receptor complex. This complex binds specific DNA sequences and modulates transcription from nearby promoters [6].

By transducing growth regulatory signals, the ER controls cell cycle progression. However, the mechanism of signal transduction and receptor-induced cell proliferation are not well understood. The conventional view of steroid signaling is that the sole ligand for ER is its cognate steroid hormone. However, the fact that cells can undergo either differentiation or proliferation in response to ER activation suggests that its activity is affected by more than just steroid binding. Recent findings from several laboratories, including our

own, suggest that in addition to steroid ligands, ER activity can be modulated by phosphorylation. However, the mechanism by which phosphorylation regulates estrogen receptor function as well as the kinases target the receptor *in vivo* remain unknown.

ER is a phosphoprotein. Despite the prevalence of phosphorylation among eukaryotic signaling factors and transcriptional regulators, no simple rules have emerged for predicting the mechanism by which this class of modifications will alter protein function. In the few cases in which cellular processes affected by phosphorylation have been determined, the functional targets appear to be numerous [7]. For example, phosphorylation of the yeast transcription factor SWI 5 triggers its nuclear localization [8]; phosphorylation of mammalian serum response factor (SRF) alters its DNA binding kinetics[9]; phosphorylation of mammalian cAMP-response element binding protein (CREB) stimulates its association with a protein cofactor, CREB-binding protein (CBP), thus regulating its transcriptional activity [7]. With respect to steroid receptors, it has been suggested that phosphorylation might modulate nucleocytoplasmic shuttling of the proteins or affect their transcriptional regulatory functions [10]. However, the relationship of ER phosphorylation to function is not well understood. Clearly, a fuller understanding of the regulatory roles of ER phosphorylation will require the identification of both the phosphorylation sites and the modifying enzymes themselves. Inspection of the sequence context of the putative phosphorylation sites (Ser 104, Ser 106 , Ser 118 and Ser 294) reveals that these sites are potential targets for the cyclin-dependent kinases (cdks) [11, 12]. However, neither the ability of the cdks to phosphorylate ER nor their impact on ER transcriptional enhancement has been examined.

Purpose of the present work

Our present work examines the ability of the cdks to phosphorylate ER *in vitro* and affect receptor transcriptional enhancement *in vivo*. Cyclin dependent kinases are a family of proteins which are composed of a regulatory cyclin subunit associated with a catalytic kinase subunit. The cyclin subunit appears to regulate subcellular localization, timing of activation as well as substrate specificity of the kinase complex. Cyclin-cdk complexes regulate the activity of target molecules, including transcriptional regulatory proteins, by phosphorylation. Cyclin-kinase complexes have been mainly

characterized by their key role as regulators of cellular proliferation. Because these kinases control cell division, the dysregulation of cyclins and/or their kinase partners has been implicated in the initiation and/or promotion of hyperplasia and oncogenesis. The archetype of cyclin-Cdk activity is the relationship between cyclin D1, cdk4, and the retinoblastoma (Rb) tumor suppressor protein. Cyclin D1 tethers cdk4 to Rb. When upstream signaling pathways sense stimuli favoring cell division (e.g. mitogens), cdk4 phosphorylates Rb thereby releasing the Rb-dependent repression of E2F transcriptional activation. Cyclin-cdk complexes are not equivalent in their ability to bind to and phosphorylate Rb. In addition, different cyclin-cdk combinations are sequentially activated at different stages of the cell cycle, suggesting that specific cyclins and cdks perform discrete functions. For example, cyclin A pairs with cdc2 or cdk2 at G2 and G1/S respectively, and gives rise to distinct kinase activities therein. Regulation of the cyclin-cdk activity is accomplished by proteins that activate (cyclin-dependent kinases activators or CAKs), or inhibit (cyclin-dependent kinase inhibitors or CDIs), kinase function. Furthermore, recent evidence suggests that a hierarchy exists in which cyclin-kinase pairs may regulate other, downstream cyclin-kinase complexes. Interestingly, cyclin D, E and A overexpression as well as inappropriate or deranged cyclin expression patterns occur in a variety of breast cancer tumors and cell lines. Moreover, targeted mammary epithelial cell overexpression of cyclin D1 and A results in mammary carcinogenesis in transgenic mice. This evidence provides the basis for investigating the effects of cyclins and cdks on ER activity.

Methods of approach

To examine whether receptor transcriptional enhancement is affected by cyclin-dependent kinases *in vivo*, paired expression and reporter plasmids for ER will be introduced into a HeLa cells, along with cyclins, cdks or cyclin-dependent kinase inhibitors, such as $p27^{KIP}$. Receptor variants with phosphorylation sites substitutions will then be used to test whether the effects on kinase activation (or inhibition) operate through a particular phosphorylated residue.

We will test the ability of Cdk to phosphorylate ER *in vitro*. The immunopurified receptor produced in ER-baculovirus infected insect cells will be *in vitro* phosphorylated by various cyclin/cdks complexes. Cyclin-cdk

combinations will also be produced and immunopurified from baculovirus. The cdk kinase series will include cyclins D1, D2, D3 paired with the catalytic partner cdk4; cyclins A, E paired with cdk2 and cyclins A and B paired with cdc2. We have obtained baculovirus strains for cyclin D1, D2, D3 and Cdk 4 from C. Sheer (University of Memphis) and baculovirus strains containing cyclin B, A, Cdk2 and Cdc2 from D. Morgan (U.C.S.F.). As a control for kinase activity and specificity, receptor phosphorylation will be compared to that of established cdk substrates; histone H1, for cyclins A/B and cdk2/cdc2; the retinoblastoma protein (Rb), for cyclins D1,2,3/E and cdk4/cdk2; and myelin basic protein for MAPK[13, 14]. Cyclin-cdk pairs that efficiently phosphorylate the receptor, relative to control substrates, will be further analyzed by two-dimensional peptide mapping. We will compare not only the phosphopeptides generated by candidate kinases to one another, but will also determine their relationship to the receptor phosphopeptides observed *in vivo*. A similar phosphopeptide pattern observed *in vitro* and *in vivo* will implicate the receptor as a substrate for the kinase. If the receptor phosphorylation patterns observed *in vitro* and *in vivo* are different, this may suggest, this may suggest that ER phosphorylation may not be affected by these kinases. If the phosphorylation pattern is conserved *in vitro* and *in vivo*, then receptor variants with phosphorylation sites substitutions will also be tested as substrates for candidate cyclin-cdk complexes to help identify the precise residues that are phosphorylated.

BODY

To establish whether ectopic expression of a cyclin/cdk complex affects estrogen-dependent activation, we examined the ability of cyclin A, alone or in combination with cdk2, to increase ER-mediated transcriptional enhancement. Estrogen receptor-deficient HeLa cells were transfected with an expression vector for the human ER containing a FLAG epitope at its N-terminus, the reporter plasmid ERE-tk-CAT, plasmids encoding human cyclin A or cdk2, and a constitutive β-galactosidase expression vector as an internal transfection standard. Transfected cells were treated with 17-β-estradiol or the ethanol vehicle for 24 hours. Transcriptional activity was measured by CAT assay and normalized to β-galactosidase activity. Figure 1A shows an increase in ER-dependent transcriptional enhancement when cyclin A is expressed ectopically. No effect of cyclin A on reporter gene activity was

observed in the absence of ER (data not shown). To ensure that this increased transcriptional activity was not a result of additional ER protein production, an indirect mechanism to account for cyclin A effects upon ER activity, we monitored protein expression in whole cell extracts using Western Blot analysis. As shown in Figure 1B, ER levels are not increased by cyclin A co-expression. In addition, cyclin A is expressed above endogenous levels as a result our transient transfection scheme and estradiol treatment does not alter cyclin expression. Thus, cyclin A expression greatly magnifies the characteristic hormone-dependent ER transcriptional response, which suggests that cyclin-cdk complexes can act as effectors of the estrogen receptor signaling pathway.

Co-expression 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 alter the ER-dependent transcriptional activity (data not shown). By increasing the amount of cyclin A used in these transfections (0.5-10.0 μg/60 mm dish), we were able to further enhance ER transcriptional activation (Figure 1C). This suggests that cyclin A is a limiting factor for full hormone-dependent ER-mediated transcriptional enhancement.

To further demonstrate that alterations in cyclin A/cdk2 kinase activity can modify ER activity, we used two classes of cdk regulatory proteins, cyclin-dependent kinase activators (CAKs) and cyclin-dependent kinase inhibitors (CDIs). CAKs act as upstream regulators of cyclin-cdk complexes by phosphorylating the kinase subunit at a conserved threonine residue. The addition of this phosphate moiety along with cyclin binding and a dephosphorylation event at Thr14/Tyr15 are all required for full kinase activity. The CAK complex is composed of a regulatory subunit, cyclin H, and a catalytic subunit, cdk7. Cyclin-dependent kinase inhibitors (CDIs) bind to the kinase subunit of the cyclin-cdk complex and may exert their inhibitory action by: (1) blocking kinase activation by CAK phosphorylation; (2) blocking cdc25 mediated cdk dephosphorylation or (3) blocking the cyclin-cdk complex access to potential substrates. $p27^{KIP1}$(hereafter referred to as p27), the CDI used in our studies, inhibits many cyclin-cdk complexes, including cyclin A/cdk2, cyclin E/cdk2, and cyclin D2/cdk4.

Transient transfections were performed in HeLa cells as described previously. Figure 2A illustrates that cdk activation by expression of cyclin

A/cdk2 or CAK leads to a 2 fold increase in ER transcriptional enhancement. The co-expression of 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 have also expressed cyclin H and cdk7 individually and in combination with cyclin A. All combinations tested increased ER activity above the level of control plasmids (data not shown), presumably by favoring the formation of active cyclin-cdk complexes from the pool of endogenous cyclin A and cdk2 subunits.

We next asked if a decrease in cyclin-cdk kinase activity would reduce estrogen receptor-dependent transcriptional activation. We chose two means of inhibiting cdk2 kinase activity. Initially, the cyclin-dependent kinase inhibitor, p27, was ectopically expressed in HeLa cell and ER-dependent transcriptional enhancement measured. Ligand-dependent transcriptional activation by ER was greatly reduced by p27 expression (Fig. 2B). Therefore, reducing cdk activity leads to impaired ER transcriptional activity.

At this point in our studies, 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 impairing cdk2 activity. We used 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 (lysines 33 and 34 are replaced by threonine and serine, respectively). This mutant acts as a dominant negative by binding to and 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. 2B). These results strongly argue that the observed decrease of ER transcriptional activity by p27 (Fig. 2A) is due to inactivation of cdk2 and further suggests the importance of cyclin A/cdk2 kinase activity for hormone-dependent transcriptional enhancement by ER. It appears then, that the *balance* between the cdk regulatory proteins, CAKs and CDIs, is a critical step in determining ER transcriptional activity.

Next, we investigated whether the cyclin A/cdk2 complex can phosphorylate the estrogen receptor protein. The full length human estrogen

receptor was expressed in 5B insect cells using a baculovirus expression system. The protein was immunoprecipitated, immobilized on Protein A/G-agarose beads, and used in an *in vitro* kinase assay. Extracts from insect cells infected with cyclin A and cdk2, separately or in combination, were added to the immunoprecipitated ER protein and the kinase reaction was initiated by the addition of ATP. After a 30 minute incubation in the presence of ^{32}P-γ-ATP, the kinase reactions were stopped and the reaction products were resolved on SDS gels. The proteins were visualized by Coomassie Blue staining and autoradiography was performed. In parallel reactions, Histone H1, a known substrate for the cyclin A/cdk2 complex, was used to control for kinase efficiency. Figure 3A shows that ER is most heavily phosphorylated in the presence of both cyclin A and cdk2. Expression of cyclin A alone also led to some receptor phosphorylation presumably via activation of endogenous cdks present in the crude insect cell lysate. These results were consistent with those seen for the control substrate, Histone H1 (Fig. 3A). Cdk2 alone did not phosphorylate Histone H1, suggesting that in the absence of cyclin A the kinase complex is not active. The degree of ER phosphorylation seen with cdk2 alone probably reflects endogenous kinases present in the insect cell extracts. The higher apparent level of Histone H1 phosphorylation relative to ER represents the greater number of ser/thr-pro consensus sites present on H1. Nevertheless, it appears that cyclin A/cdk2 complexes can efficiently phosphorylate ER *in vitro*.

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 with a combination of ER and cyclin A. Cells were metabolically labeled with ^{32}P-orthophosphate for 2 hours in the presence or absence of 17-β-estradiol. Based upon our earlier findings (see Fig. 1A and 1B) that cyclin A is the limiting subunit of the cyclin-cdk complex, we sought to activate the endogenous cdk by overexpressing the cyclin. As shown in Figure 3B, ER phosphorylation is greatly increased by ectopic expression of cyclin A in both the absence and presence of hormone. Thus, the presence of cyclin A increases incorporation of phosphate into ER by activating endogenous cdks. Together with our *in vitro* findings, these results suggest that ER is a direct substrate of cyclin A/cdk2 complex.

In addition to their role in regulating the activity of the kinase, cyclins are believed to tether cdks to appropriate substrates. To examine whether ER

and cyclin A interact, baculovirus-expressed proteins were also examined for protein-protein interactions. A FLAG-tagged version of the full-length ER was expressed either independently or in combination with cyclin A. At two days post-infection, cells were treated with 17-β-estradiol or the ethanol vehicle for one hour, lysed, and cell extracts immunoprecipitated with anti-FLAG or anti-cyclin A antibodies. Figure 3C demonstrates that ER and cyclin A interact in our *in vivo* model. The cyclin A antibody immunoprecipitated ER in both the absence and presence of hormone, although more ER is associated with cyclin A when hormone is present (compare lanes 3 and 8, upper panel). The reciprocal is also true; monoclonal antibodies directed against the N-terminal FLAG-epitope on ER immunoprecipitates cyclin A from coinfected insect cell lysates (compare lanes 2 and 7, lower panel). Importantly, no cross-reacting proteins were immunoprecipitated from insect cells infected with the each virus separately or from wild-type virus using these antibodies. Based on these findings, we propose that cyclin A physically interacts with ER and may act to tether the cyclin-cdk complex to the receptor.

We next attempted to model an additional *in vivo* system to test transcriptional activity of ER in cells lacking particular cyclins. We have taken advantage of the finding that steroid hormone receptor signal transduction, gene activation function and phosphorylation is conserved between yeast and mammalian cells [15]. We have exploited this species conservation to ask what effect deletions of specific cyclin genes have on ER hormone-dependent transactivation. The Clb family of yeast cyclins are functionally equivalent to mammalian S and G2 cyclins (cyclins A and B) in both their temporal expression in the cell cycle and in their importance in S/G2 transition. A wild-type strain and a set of isogenic strains deleted of the yeast cyclins Clb2, Clb3 or Clb4 were transformed with an ER expression plasmid. An ERE-CYC1-LacZ reporter gene containing a single receptor binding site was also included in these strains. Cells were treated with increasing doses of 17-β-estradiol and β-galactosidase activity was measured. Receptor-dependent transcriptional activity was reduced up to 5 fold in yeast strains lacking Clb4 (Fig. 4). In contrast, deletion of Clb2 or Clb3 had little effect on ER-dependent transcriptional enhancement relative to the wild-type strain (Fig. 4). The observation that increasing amounts of hormone can partially overcome impaired ER-dependent transcription in the Clb4-deleted strain may indicate that at sufficiently high ligand concentrations, ER can be

driven into a productive interaction with other cyclins whose functions can partially complement those of Clb4 (Fig. 4). Interestingly, the temporal expression of Clb4 is identical to that of mammalian cyclin A which is expressed in late G1 and throughout S phase. Thus, a yeast strain deleted of a cyclin that bears a striking resemblance to cyclin A, also exhibits reduced receptor-dependent transcriptional enhancement. These findings are consistent with our observations in mammalian cells that inhibition of cyclin A/cdk activity decreases receptor transcriptional activity. In addition, these results suggest that only certain cyclin-cdk complexes are required for full receptor-dependent transcriptional enhancement *in vivo*..

CONCLUSIONS

We have shown that cyclin A, the regulatory subunit of cdk2, is a limiting cofactor in the regulation of ER-dependent transcriptional activation. We have further demonstrated that expression of cyclin-dependent kinase activators (CAKs) enhance the effect of cyclin A/cdk2 on ER transcriptional activation. Conversely, inhibition of cyclin-dependent kinase activity by the CDI, p27, or by a dominant negative cdk2 mutant impair ER transcriptional activation. Likewise, deletion of the yeast cyclin Clb4, whose timing of expression matches those of mammalian cyclin A, reduces ER-dependent transcriptional activation. Finally, ER is phosphorylated *in vitro* and *in vivo* by cyclin A/cdk complexes. Together, these data suggest that the cyclin A/cdk2 kinase complex directly phosphorylates ER and increases the receptor's transcriptional regulatory properties.

Work is ongoing to identify the precise residue(s) on the receptor phosphorylated by the cyclinA/cdk2 complex. At this point we can eliminate Ser 118 as a major site of phosphorylation by this kinase, since cyclin A/cdk2-induced ER transcriptional enhancement is unaffected in the receptor's serine 118 to alanine mutant. We are left with the possibility that other ser-pro sites in ER, (Ser 104, Ser 106 and Ser 294) are potential targets for phosphorylation by cyclin A/cdk2 complex. We are currently altering these residues to examine their significance for cyclin A-mediated activation of ER.

Based on our findings and upon those of others, we appreciate the complexity of the ER signal transduction pathway and the multiplicity of events in this pathway that are vulnerable to subversion. (1) Aberrant expression cyclin and cdk subunits can lead to an increase in receptor

phosphorylation, perhaps contributing to uncontrolled cell proliferation. (2) Lack of cyclin-dependent kinase inhibitors due to mutation may leave cdks in a constitutively active state, such that receptor phosphorylation events are unrestricted, with respect to specific stages of the cell cycle, for example. (3) Alterations in cdk substrates may render them immune to cdk regulation altogether, resulting in constitutively active or inactive transcriptional activators or repressors.

The critical link between cyclin-cdk regulation and tumor suppressor and oncogene function has provided us with a partial explanation as to how these molecular signaling mechanisms, when perturbed, can lead to clinical manifestations such as hyperplasias, dysplasias and ultimately carcinomas. Interestingly, cyclin overexpression as well as inappropriate cyclin expression patterns occur in a variety of breast cancer tumors and cell lines. It is likely, that phosphorylation events mediated by cyclin-cdk pathway will emerge as a general mechanism of controlling steroid hormone action.

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Figure Legends

Figure 1. Ectopic cyclin A expression increases ER transcriptional activity in HeLa cells. Receptor negative HeLa cells were transiently transfected with the receptor expression plasmid pCMV-FLAG-ER, a reporter gene (ΔETCO), and pCMV-β-galactosidase as an internal control for transfection efficiency. Expression vectors for cyclin A and cdk2 were transfected as indicated. The empty vector was included as a control. Cells were incubated for 24 hours with the 100 nM 17-β-estradiol or the ethanol vehicle as indicated, harvested and assayed for CAT and β-galactosidase activity. (A) The activity of ER transcriptional response in the absence (vector) and presence of cyclin A. (B) Ectopic expression of cyclin A does not affect ER protein levels. Whole cell lysates were prepared from cells transfected with ER and/or cyclin A. Fifty micrograms of total protein was separated by 10% SDS-PAGE, transferred to Immobilon paper and probed with the M2 monoclonal antibody against the FLAG epitope on ER and a polyclonal antibody against cyclin A, and visualized with an alkaline phosphate-conjugated goat secondary antibody. (C) Cyclin A is limiting for ER hormone-dependent transcriptional enhancement. Increasing amounts of the cyclin A expression plasmid (0.5 - 10.0 μg) were used in the transfection scheme. In all cases, the total amount of transfected DNA was held constant by the addition of empty expression vector. Normalized CAT activity is shown in the absence (-estradiol) and presence of hormone (+estradiol). Shown is a representative experiment using duplicate samples that varied less than 5%.

Figure 2. ER ligand-dependent transcriptional activation in response to activators and inhibitors of cyclin/cdk complexes. A) Effects of cyclin-dependent kinase activators cyclin H/cdk7 on ER-dependent transcriptional enhancement. Hela cells were transfected with ER expression and reporter plasmids as described in Fig. 1, along with the expression vectors encoding the following proteins: cyclin A/cdk 2; cyclin A/cdk2/cyclin H/cdk7 and cyclin H/cdk7. B) Effects of cyclin-

dependent kinase inhibitor p27 and a dominant negative cdk2 (cdk2TS) on ER-dependent transcriptional enhancement. HeLa cells were transfected with ER expression and reporter plasmids along with the expression vectors for p27 and cdk2TS. Data represent the mean of two determinations done in duplicate which varied less than 5%.

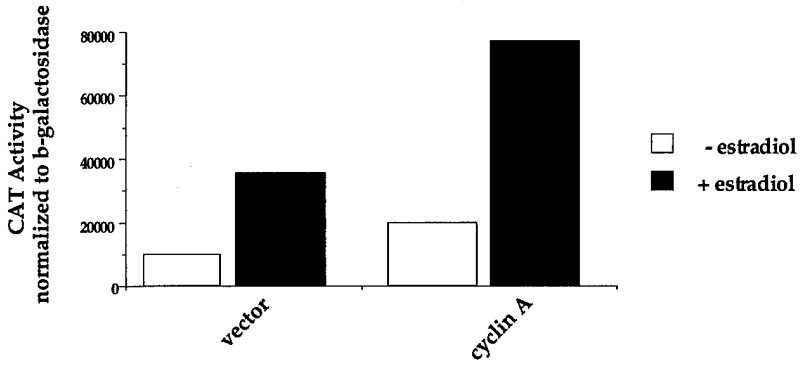
Figure. 3. ER phosphorylation by cyclin A/cdk2. A) *In vitro* phosphorylation by cyclin A/cdk2. Baculovirus-expressed full length ER was immunoprecipitated with an ER-specific monoclonal antibody. The immune complexes were collected on Protein A/G-agarose beads and used in an *in vitro* kinase assay. Cyclin A and cdk2 were produced in 5B insect cells, separately or in combination, and added to the kinase reaction mixture as crude cell lysates along with ^{32}P-γ-ATP. Histone H1 was used as a known cyclin A/cdk2 substrate. The products of the reaction mixture were separated by 10% SDS-PAGE, stained with Coomassie blue and the phosphorylated products were visualized by autoradiography (5 minute exposure shown). For *in vivo* labeling, HeLa cells were transfected as detailed in Fig. 1 with indicated plasmids. Cells were metabolically labeled with 1 mCi/ml of ^{32}P-orthophosphate for 2 hours at 37°C in the presence of 100 nM 17-β-estradiol or the ethanol vehicle. Cells were lysed, receptor immunoprecipitated, purified on 10% SDS-PAGE and the silver stained gel (bottom panel) was subjected to autoradiography (top panel). C). Interaction of ER and cyclin A. *In vivo* protein-protein interactions were examined as follows: Baculovirus expression vectors encoding a full length FLAG-tagged ER or cyclin A were used individually or in combination to infect 5B insect cells. Two days after infection, cell lysates were prepared and immunoprecipitated with monoclonal antibodies directed against either the FLAG-epitope of ER or cyclin A. Immune complexes were collected on Protein A/G-agarose beads, washed and

associated proteins separated by SDS-PAGE and probed for ER (top panel) or cyclin A (bottom panel).

Figure. 4. Receptor-dependent transcriptional enhancement in strains deleted of yeast cyclins. A wild-type strain (squares) and a set isogenic strains deleted of the yeast cyclins Clb2 (diamonds), Clb3 (circles) and Clb4 (triangles) were transformed with an ER expression plasmid. An ERE-CYC1-LacZ reporter gene containing a single receptor binding site was also included. Cells were treated with the indicated amount of hormone, and grown for an additional 6 hours when β-galactosidase activity was assayed and normalized to cell density. These data represent the mean of two independent assays with a variation of <10%.

FIGURE 1

A.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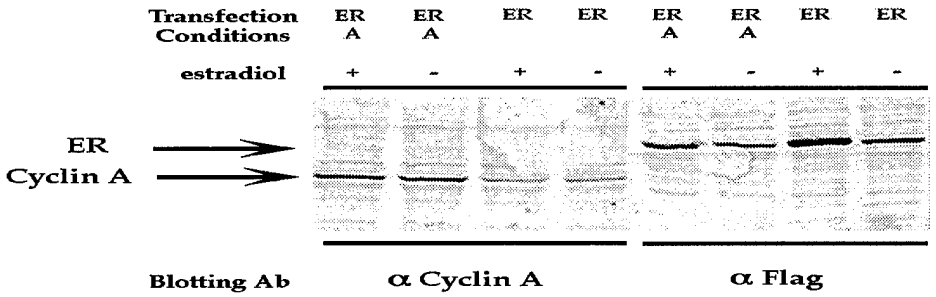
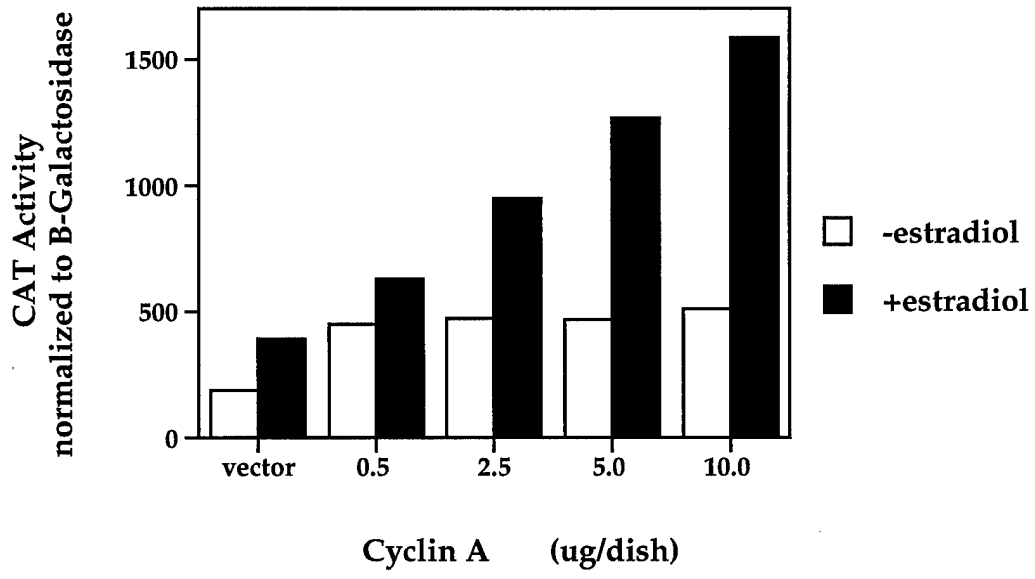
+ estradiol

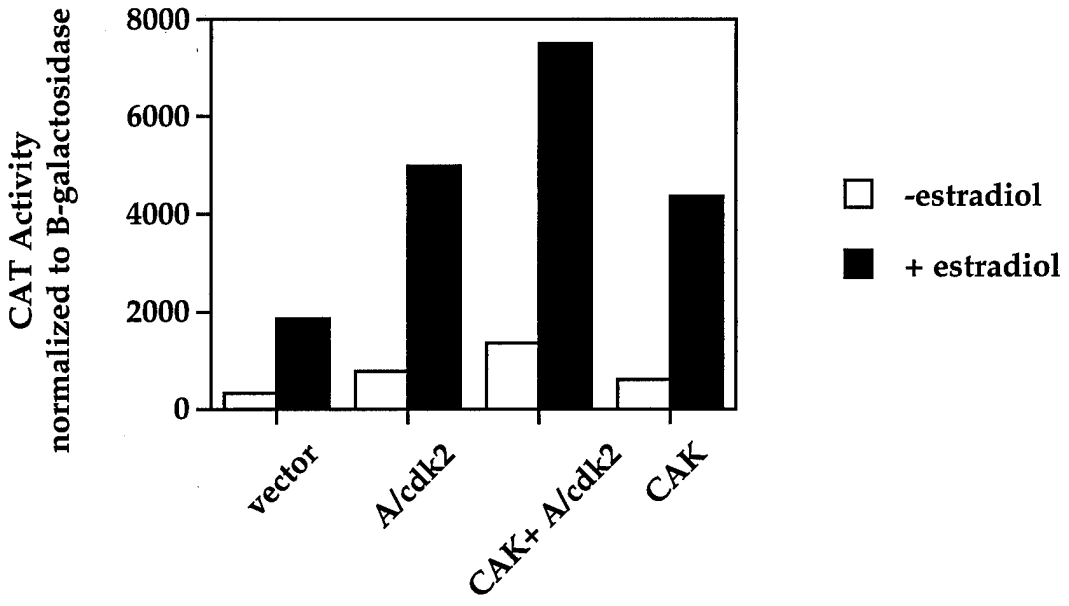
B.


Transfection Conditions
ER A
ER A
ER
ER
ER A
ER A
ER
ER
estradiol
+ - + - + - + -
ER
Cyclin A
Blotting Ab
α Cyclin A
α Flag

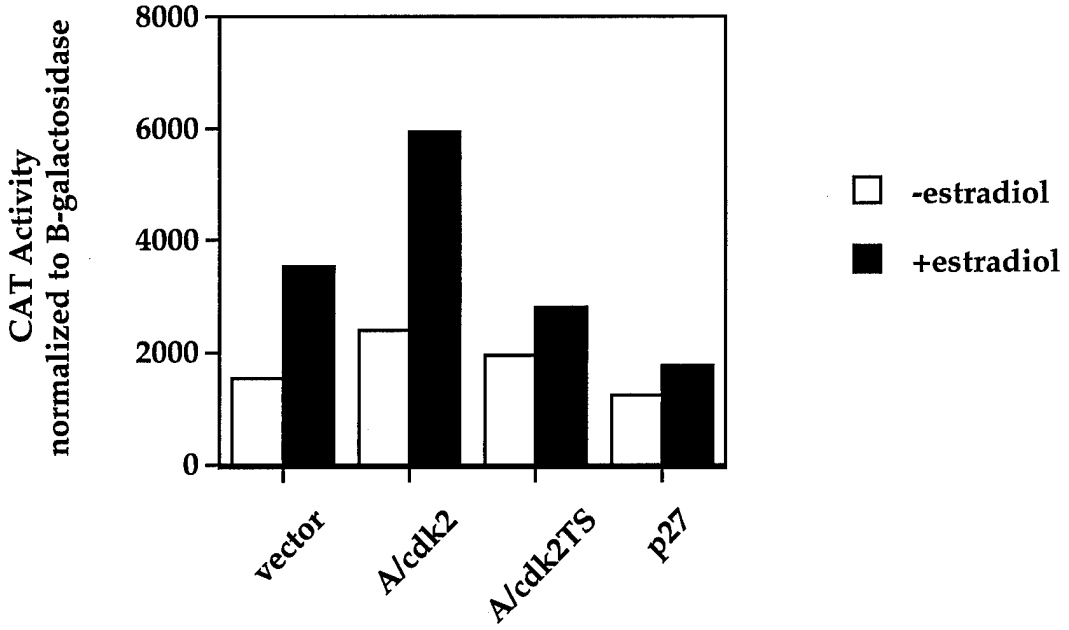
C.


CAT Activity
normalized to B-Galactosidase
1500
1000
500
0
vector
0.5
2.5
5.0
10.0
Cyclin A (ug/dish)
-estradiol
+estradiol

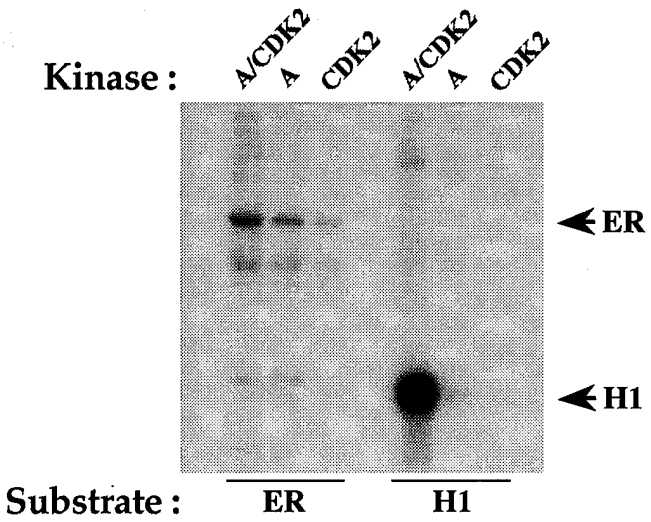
A.


CAT Activity
normalized to B-galactosidase
8000
6000
4000
2000
0
vector
A/cdk2
CAK+ A/cdk2
CAK
-estradiol
+ estradiol

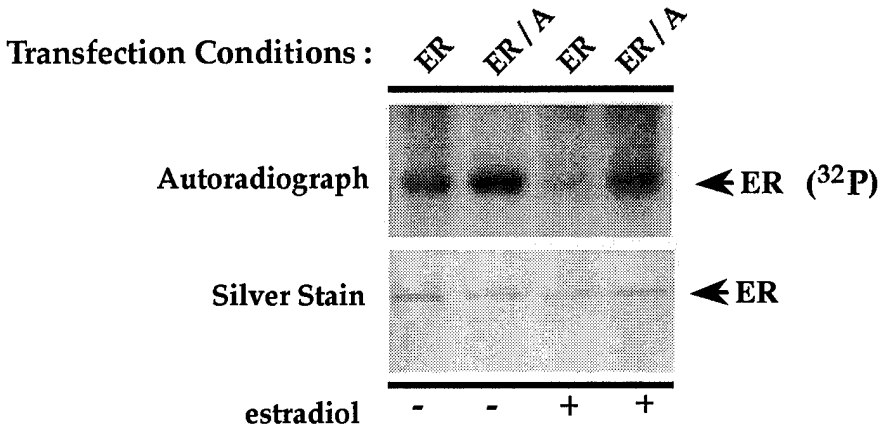
B.


CAT Activity
normalized to B-galactosidase
8000
6000
4000
2000
0
vector
A/cdk2
A/cdk2TS
p27
-estradiol
+estradiol

A.


Kinase :
A/CDK2
A
CDK2
A/CDK2
A
CDK2
ER
H1
Substrate :
ER
H1

B.


Transfection Conditions :
ER
ER / A
ER
ER / A
Autoradiograph
ER (^{32}P)
Silver Stain
ER
estradiol
- - + +

C.

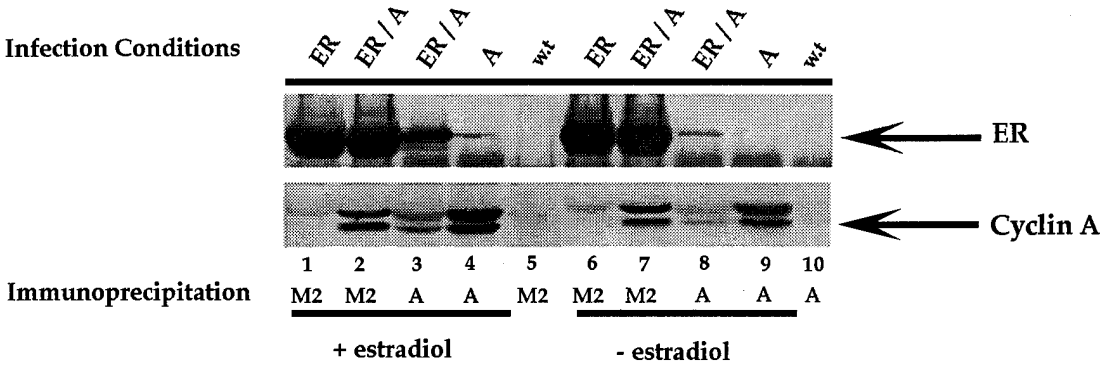
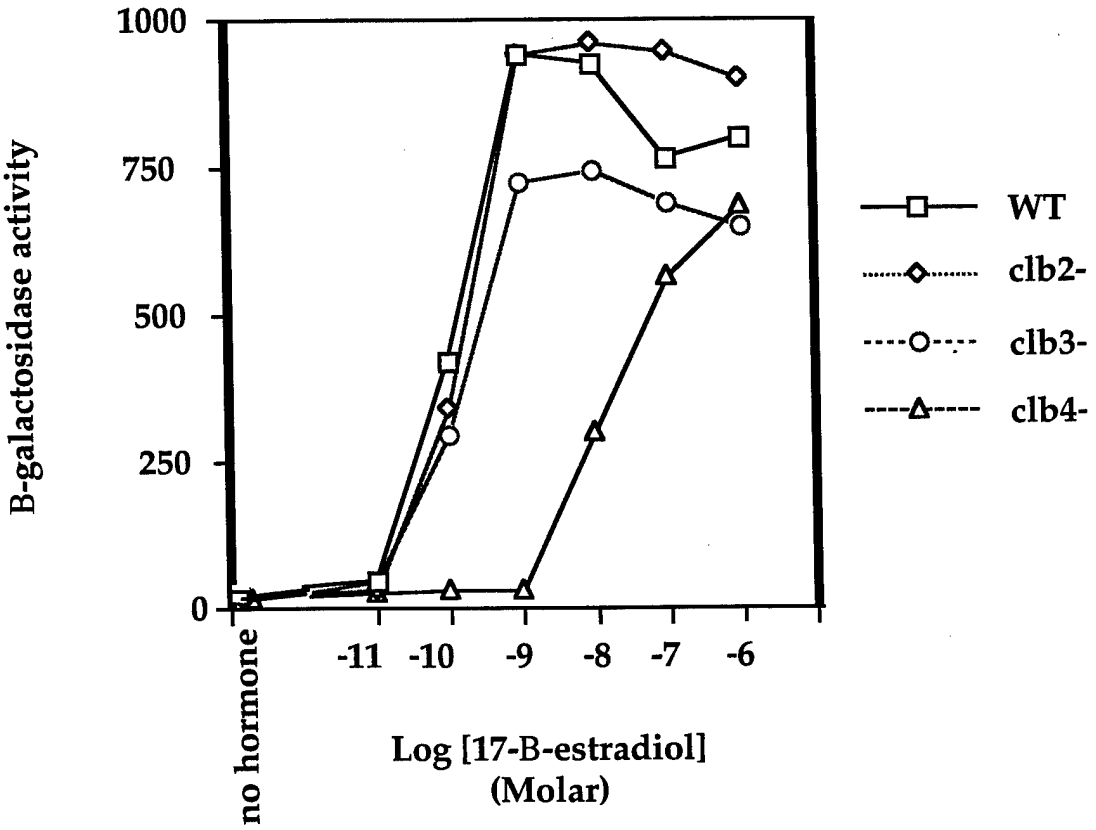

Infection Conditions
ER
ER / A
ER / A
A
wt
ER
ER / A
ER / A
A
wt
ER
Cyclin A
1 2 3 4 5 6 7 8 9 10
Immunoprecipitation
M2 M2 A A M2 M2 M2 A A A
+ estradiol
- estradiol

FIGURE 4


1000
750
500
250
0
B-galactosidase activity
no hormone
-11
-10
-9
-8
-7
-6
Log [17-B-estradiol]
(Molar)
WT
clb2-
clb3-
clb4-