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CDK-Independent Activation of Estrogen Receptor by Cyclin D1

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Summary

Both cyclin D1 and estrogens have an essential role in regulating proliferation of breast epithelial cells. We show here a novel role for cyclin D1 in growth regulation of estrogen-responsive tissues by potentiating transcription of estrogen receptor–regulated genes. Cyclin D1 mediates this activation independent of complex formation to a CDK partner. Cyclin D1 activates estrogen receptor–mediated transcription in the absence of estrogen and enhances transcription in its presence. The activation of estrogen receptor by cyclin D1 is not inhibited by anti-estrogens. A direct physical binding of cyclin D1 to the hormone binding domain of the estrogen receptor results in an increased binding of the receptor to estrogen response element sequences, and upregulates estrogen receptor–mediated transcription. These results highlight a novel role for cyclin D1 as a CDK-independent activator of the estrogen receptor.

Introduction

Cyclins have been identified as positive regulatory subunits of a class of protein kinases termed cyclin-dependent kinases (CDKs). These protein kinases have been shown to be important regulators of major cell cycle transitions in a number of diverse eukaryotic systems. In mammalian cells, at least six different cyclin types have been identified, which act at specific stages of the cell cycle. A transient accumulation of cyclin proteins results in activation of their CDK partners and subsequently in phosphorylation of target proteins (Resnitzky and Reed, 1995; Weinberg, 1995). These protein phosphorylation cascades are thought to play a crucial role in the various cell cycle transitions.

D-type cyclins are strongly implicated in controlling progression through the G1 phase of the cell cycle. Three closely related human D-type cyclins have been identified (Inaba et al., 1992; Xiong et al., 1992), all of which interact with and activate CDK4 and CDK6, although they have specialized functions in distinct cell types (Matsushime et al., 1991; Motokura et al., 1992). Among the D-type cyclins, cyclin D1 has been implicated most strongly in oncogenesis. Amplification and overexpression of the gene encoding cyclin D1 was frequently observed in several types of human neoplasia, in particular breast cancer (Lammie et al., 1991; Schuuring et al., 1992; Bartkova et al., 1994, 1995). We and others demonstrated that cyclin D1 overexpression reduces the growth requirement for mitogens in estrogen-responsive breast tumor cells (Musgrove et al., 1994; Reeves et al., 1995; Zwijsen et al., 1996). Consistent with this result, overexpression of cyclin D1 in mammary cells of transgenic mice predisposes them to breast cancer (Wang et al., 1994). Moreover, it was shown recently that cyclin D1–deficient mice have a defect in proliferation of the estrogen-responsive breast epithelium during pregnancy (Fantl et al., 1995; Sicinski et al., 1995). Taken together, these results suggest a critical role for cyclin D1 in proliferation of breast epithelium.

Estrogens act via binding to a specific estrogen receptor (ER), which belongs to the conserved family of steroid and thyroid hormone receptors. The receptor is a 66 kDa nuclear regulatory protein that functions as a hormone-activated transcription factor. Receptor activation is thought to be a consequence of ligand-induced conformational changes in the structure of the ER (Kumar and Chambon, 1988; Tsai et al., 1988). Extracellular 17β-estradiol freely diffuses across the cell membrane and binds ER, leading to ER dimerization and translocation to the nucleus. The estrogen–ER complex binds with high affinity to a well-defined 13 bp palindromic sequence, i.e., the estrogen response element (ERE) (Evans, 1988; Kumar and Chambon, 1988; Beato et al., 1995). The enhancer-like ERE sequences are located near or within estrogen-responsive genes (Beato, 1991). After ERE binding, the liganded ER activates transcription by an as yet unknown mechanism(s) resulting in stimulation of proliferation (Dubik and Shiu, 1988; Weisz and Bresciani, 1988). A direct link between 17β–estradiol-mediated proliferation and ER-mediated transcription is strongly supported by in vivo and in vitro studies using anti-estrogens (DeFriend et al., 1994). Anti-estrogens inhibit expression of estrogen-regulated genes and reduce proliferation of ER-positive breast cells, whereas 17β-estradiol is a mitogen for breast epithelium (Dubik and Shiu, 1988; Weisz and Bresciani, 1988; DeFriend et al., 1994). Here we report an unexpected relationship between cyclin D1 and ER, which has important implications for our understanding of proliferation of normal breast epithelium and its deregulation in breast cancer.

Results

Cyclin D1 Enhances Transcription of ERE-Responsive Genes Independent of a CDK Partner

Activation of ERE-containing genes can be elicited in ER-positive cells by treatment with 17β-estradiol and can be measured by means of a chloramphenicol acetyl transferase (CAT) reporter construct with an upstream ERE site in these cells (Klein-Hitpass et al., 1986, 1988). Using this system, we have investigated the effect of cyclin D1 in combination with its associated kinase CDK4 on ERE-responsive transcription in the well-differentiated and estrogen-responsive T47D breast cancer

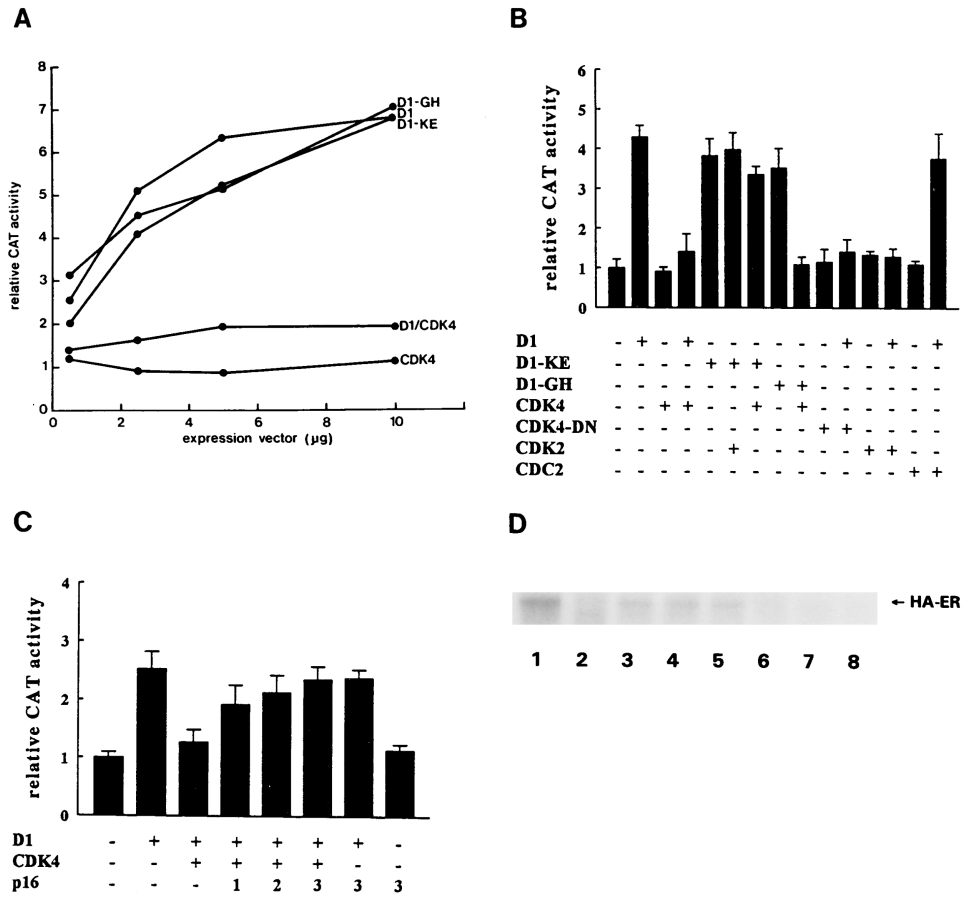

Figure 1. Cyclin D1 Potentiates ERE-Responsive Gene Transcription in the Presence of 17β-estradiol

(A) The effect of cyclin D1 and its associated kinase activity on ERE-mediated transcription. An ERE-reporter construct was used in transient transfections of T47D breast cancer cells (Klein-Hitpass et al., 1986, 1988) together with wild-type cyclin D1, CDK4, or with mutants of cyclin D1, which are unable to bind to CDK4 (cyclin D1-KE), or with retinoblastoma protein pRb (cyclin D1-GH) or a catalytic inactive mutant of CDK4 (CDK4-DN). pCMV-luciferase (0.5 μg) served as an internal control. The relative CAT activity was calculated by normalizing to the luciferase activity and was divided by the ERE-CAT activity in the presence of 17β-estradiol (10 nM).

(B) The effects of CDKs on regulation of ERE-mediated transcription by cyclin D1. Cyclin D1 (mutants) in combination with CDKs (2.5 μg each) were cotransfected with the ERE-reporter construct in T47D cells. The relative CAT activity was calculated by normalizing to the luciferase activity and was divided by the ERE-CAT activity in the presence of 17β-estradiol (10 nM).

(C) The effect of $p16^{INK4}$ in ectopic cyclin D1/CDK4 expressing cells on ERE-mediated transcription. One microgram cyclin D1 and CDK4 expression vector was used in an ERE-dependent system, and for $p16^{INK4}$ the amount was variable: 1 = 0.5 μg; 2 = 1.0 μg; 3 = 5.0 μg. CAT activities were quantified by a phase separation assay (Seed and Sheen, 1988) and represent an average of three independent transfection studies. The relative CAT activity was calculated by normalizing to the luciferase activity, and was divided by the ERE-CAT activity in the presence of 17β-estradiol (10 nM).

(D) Cyclin D1 does not affect phosphorylation status of ER. ER-negative U2-OS cells were transfected with k-Ras (lane 1), control plasmid (lane 2), HA-epitope tagged ER (lane 3 and 6), HA-ER and cyclin D1 (lane 4 and 7), and HA-ER and dominant negative CDK4 (lane 5 and 8) in the presence (lanes 1–5) and absence (lanes 6–8) of 17β-estradiol. After transfection, cells were in vivo labeled with [^{32}P]orthophosphate and immunoprecipitated with a monoclonal antibody directed against HA-tag (12CA5).

cell line. Figure 1 shows that cyclin D1 is a strong enhancer of ERE-responsive gene transcription in the presence of 17β-estradiol (10 nM). The activity of the ERE-containing reporter construct increased when cotransfected with increasing amounts of cyclin D1 expression vector. Up to 7-fold potentiation was seen as compared to its basal level of 17β–estradiol-activated transcription, which was arbitrarily set at 1 (Figure 1A). The importance of the ERE in this effect is supported by the fact that cyclin D1 did not stimulate the activity of a reporter construct that lacked an ERE (Figure 3A).

Cyclin D1 in combination with its kinase partner CDK4 did not enhance transcription of ERE-CAT, nor did CDK4 alone (Figure 1A). To verify that a CDK was not required in this process, a mutant of cyclin D1 (cyclin D1-KE) was used, which carries a mutation in the cyclin box. This mutant cyclin D1 fails to bind CDKs and as a result is unable to mediate phosphorylation of pocket proteins (Hinds et al., 1994; Beijersbergen et al., 1995). Like wild-type cyclin D1, the cyclin D1-KE mutant potentiated transcriptional activation of ER in a dose-dependent manner. Cotransfection of CDK4 and cyclin D1-KE did not influence the cyclin D1 effect on transactivation (Figure 1B). This finding supports the notion that the effect

of cyclin D1 on ER is independent of its ability to activate a CDK partner. Consistent with this notion, binding of cyclin D1 to a CDK that lacks intrinsic kinase activity, such as the dominant negative CDK4 mutant (CDK4 DN) or CDK2 (which is not activated by cyclin D1; Ewen et al., 1993), prevented the stimulatory effect of cyclin D1. In addition, a CDK that cannot bind to cyclin D1, CDC-2, was ineffective in preventing transactivation by cyclin D1 (Figure 1B). These data strongly suggest that the kinase activity of any cyclin D1–CDK complex is irrelevant in activating ERE-mediated transcription. Significantly, cotransfection of cyclin D1 and CDK4 with $p16^{INK4}$, a CDK inhibitor that competes with cyclin D1 for binding to CDK4 (Parry et al., 1995), enhanced transcription to a level comparable with that induced by cyclin D1 alone (Figure 1C). Taken together, these data indicate that "free" cyclin D1 can enhance ERE-mediated transcription, whereas CDK-bound cyclin D1 is inactive.

It has recently been shown that ER can be activated by phosphorylation induced by mitogens and/or oncogenes (Kato et al., 1995). To investigate the ability of cyclin D1 to induce phosphorylation of ER, ER-negative cells were transfected with HA–epitope-tagged ER in combination with cyclin D1 or dominant negative CDK4. Transiently transfected cells were labeled with [^{32}P]-orthophosphate, and lysates were subjected to immunoprecipitation with a monoclonal antibody directed against the HA-tag. As expected, expression of k-Ras resulted in phosphorylation of ER (Figure 1D) (Kato et al., 1995). The phosphorylation status of ER, however, was not altered in cells cotransfected with ER and cyclin D1 or ER and CDK4 DN in the presence and absence of hormone (Figure 1D). Thus, alterations in phosphorylation of ER are not responsible for the observed activation of ERE-responsive transcription by cyclin D1. To investigate whether the motif in cyclin D1 that mediates binding to the retinoblastoma protein (pRB) was required for activation of ER, another cyclin D1 mutant (cyclin D1-GH) was used. Cyclin D1-GH carries a mutation in the LXCXE motif, and this mutation renders the protein unable to bind to pocket proteins, such as pRB (Dowdy et al., 1993; Hinds et al., 1994). Figure 1A shows that this cyclin D1 mutant enhanced the expression of the ERE-CAT reporter construct to the same extent as wild-type cyclin D1, indicating that this enhancement of transcription was independent of an intact pRB-binding motif in cyclin D1.

The uniqueness of cyclin D1 among the cyclins in the activation of ERE-mediated transcription is illustrated in Figure 2. Cyclin D1 potentiated ER-mediated transcription, whereas the other D type cyclins (which were expressed at comparable levels, data not shown) did not. The specific effect of cyclin D1 on ERE-dependent transcription was further demonstrated by the inability of cyclin E, cyclin A, cyclin B1, and cyclin B2 to activate ER-mediated transactivation. To study whether cyclin D1 activates transcription mediated by other steroid receptors, we tested the effect of cyclin D1 on the progesterone receptor (PR) using a progesterone response element (PRE)-containing reporter construct (Figure 2B). As expected, transcription of the PRE-reporter construct was induced by a progestin, Org 2058. Cyclin D1 failed, however, to enhance PRE-mediated transcription

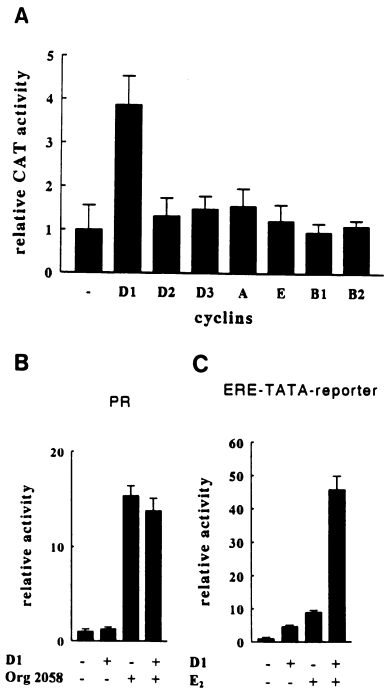

Figure 2. The Specific Action of Cyclin D1 on ERE-Responsive Transcription

(A) The specificity of cyclin D1 in activation of ERE-mediated transcription. The effect of wild-type cyclins (2.5 μg) was tested using an ERE-reporter construct in transient transfection studies of T47D breast cancer cells in the presence of 17β-estradiol (10 nM). The relative CAT activity was calculated by normalizing to the internal control and was divided by ERE-CAT activity in the presence of 17β-estradiol.

(B) The effect of cyclin D1 on PRE-mediated transcription. A PRE-reporter construct was used in transient transfections of T47D cells together with cyclin D1 (2.5 μg) in the presence or absence of 10 nM progestin Org 2058. The relative CAT activity was calculated by normalizing to the internal control and was divided by PRE-luciferase activity in the absence of progestin.

(C) The effect of cyclin D1 on ERE-TATA-reporter construct. An ERE-TATA-luciferase construct was used in transient transfection studies with T47D cells in combination of cyclin D1. The relative luciferase activity was calculated by normalizing to the internal control and was divided by ERE-luciferase activity in the absence of estrogen.

both in the presence and absence of Org 2058. In addition, cyclin D1 did not affect glucocorticoid receptor-mediated transcription (data not shown). Thus, cyclin D1-mediated activation is not general among steroid receptors. To ask whether the cyclin D1 activation of ER depends on the promoter context, we generated a second reporter construct in which the ERE is upstream of a core promoter (TATA box only: ERE-TATA-reporter). As shown in Figure 2C, cyclin D1 gave similar levels of transactivation in the presence and absence of 17β-estradiol when tested on a construct containing ERE sequence linked to a TATA promoter. This result suggests that the cyclin D1 action is not promoter specific.

The Effect of Cyclin D1 on ERE-Responsive Transcription Is Hormone Independent

To study the contribution of estrogen to the observed enhanced transcription of ERE-containing genes by

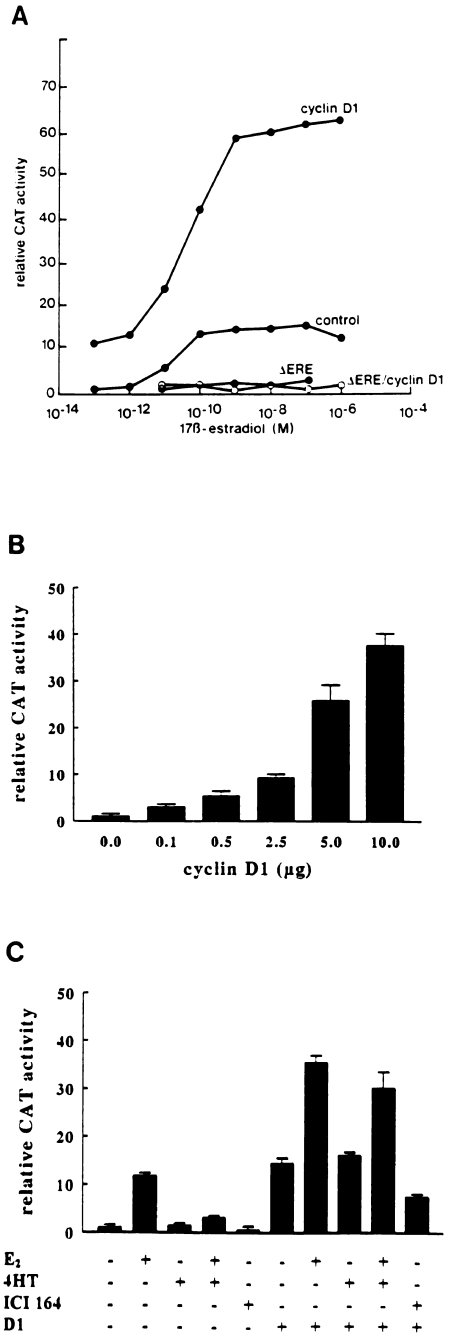

Figure 3. Cyclin D1 Enhances ERE-Responsive Gene Transcription Independently of Ligand Activation of ER

(A) The effect of estrogen and cyclin D1 on ERE-responsive transcription activity. T47D cells were maintained in medium containing various concentrations of 17β-estradiol, and were transfected with an ERE-reporter construct or a reporter construct that lacked ERE (ΔERE) in concert with cyclin D1 expression vector.

(B) Cyclin D1 activates ERE-CAT transcription in 17β-estradiol free conditions. T47D cells were transfected with various amounts of cyclin D1 constructs in the absence of ligand.

(C) Cyclin D1 induces ERE-gene expression by a hormone-independent mechanism. The effect of cyclin D1 on ERE-responsive transcription was examined in T47D cells treated with 17β-estradiol and/or the antiestrogens 4-hydroxytamoxifen and ICI 164,384.

T47D cells were transfected with ERE-reporter plasmid, cyclin D1 expression vector, and the internal control pCMV-luciferase construct, as described in Experimental Procedures. The amount of cyclin D1 plasmid used for transient transfections was 5 μg per 10 cm plate in (A) and (C), and variable in (B). During the experiment, cells were maintained in phenol red–free DMEM supplemented with insulin (10 μg/ml) and transferrin (10 μg/ml); cells were treated with 17β-estradiol (0.1 nM) and/or antiestrogen (100 nM) as indicated. After 40 hr, cells were lysed and assayed for both CAT activity and luciferase activity (Promega, Luciferase system). Data are expressed as relative CAT activity compared with basal CAT level in the absence of 17β-estradiol: E2, 17β-estradiol; D1, cyclin D1; 4HT, *trans*-4-hydroxytamoxifen; ΔERE, pCAT lacking ERE.

cyclin D1, T47D cells were transiently transfected with cyclin D1 and the ERE-reporter construct, and were maintained in medium with various concentrations of 17β-estradiol. Figure 3A demonstrates that the activity of the ERE-CAT reporter construct was dramatically enhanced in T47D cells by 17β-estradiol, as expected. The activity of the ERE-CAT reporter construct, however, was enhanced substantially by cotransfection of cyclin D1 expression vector at all concentrations of 17β-estradiol tested. In the absence of ligand, cyclin D1 was able to restore the transcriptional activity to levels similar or even higher than obtained with 17β-estradiol alone (Figure 3B). These data indicate that cyclin D1 and estrogen are synergistic in stimulating estrogen-regulated transcription, and that cyclin D1 can also act independently of estrogen in activating ERE-responsive transcription. Therefore, cyclin D1 can substitute for estrogen in activating ER. This may contribute to the mitogen-independent proliferation in cyclin D1–overexpressing cells, which is frequently found in breast tumors (Schuuring et al., 1992; Bartkova et al., 1994; Michalides et al., 1996). A hormone-independent mechanism of action of cyclin D1 is further supported by experiments with the anti-estrogen 4-hydroxytamoxifen, which binds to the estrogen receptor in a manner that is competitive with estrogen but fails to activate gene transcription (Berry et al., 1990). Figure 3C shows that 4-hydroxytamoxifen antagonized the effect of 17β-estradiol on transcriptional activation, but did not prevent transcriptional potentiation by cyclin D1. Also, a pure anti-estrogen ICI 164,384, which blocks both activation functions of ER (AF-1 and AF-2), did not prevent cyclin D1 transactivation. These data indicate that cyclin D1 was effective in activating transcription of ERE-containing genes in cells harboring ERs occupied with 17β-estradiol, as well as in cells with unliganded ERs.

Cyclin D1 Action Is ER-Mediated

Because estrogen was not required for this cyclin D1 action, the question arose whether ER itself was required for the effect of cyclin D1 on ERE-responsive transcription. Therefore, we used the human osteosarcoma cell line U2-OS and cervical carcinoma cell line HeLa, both of which have negligible levels of ER. Cotransfection of cyclin D1 and the ERE-reporter construct in these cells did not increase ERE-responsive transcription, whereas cotransfection of these genes with human ER expression vector led to activation of the ERE-CAT (Figure 4A). As in T47D cells, cyclin D1 stimulated transcription both in the presence and absence of 17β-estradiol in ER-transfected cells. Thus, cyclin D1 acts

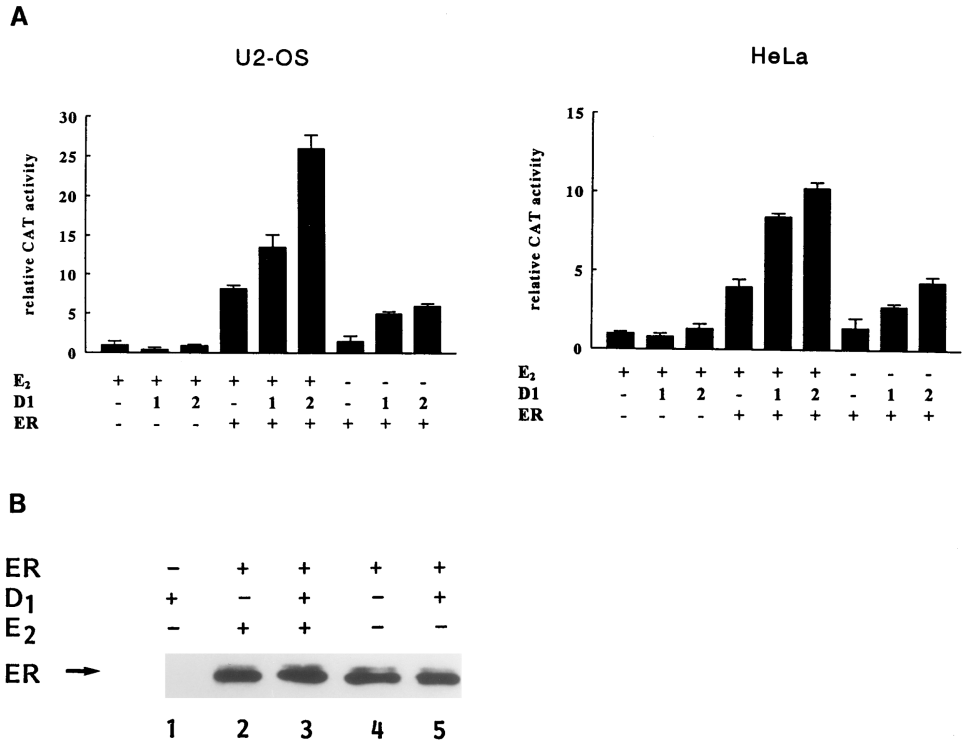

Figure 4. The Potentiation of Gene Activation by Cyclin D1 Is ER Mediated

(A) ER is involved in modulation of ERE-responsive gene transcription triggered by cyclin D1. The human ER-negative U2-OS and HeLa cell lines were used to investigate the role of ER in potentiation of ERE-CAT transcription activity by cyclin D1. Together with ERE-CAT and pCMV-luciferase plasmids, cells were transfected with cyclin D1 in combination with human ER and tested for transcription of the CAT reporter construct. The CAT activity was normalized to luciferase activity and was divided by the basal CAT activity of cells lacking ER in order to obtain the "relative CAT activity" as indicated: E_2, 17β-estradiol; D1, cyclin D1: 1 = 0.25 μg, 2 = 1.0 μg; ER, estrogen receptor.

(B) Cyclin D1 does not modulate ER protein synthesis. U2-OS cells transfected with ER and/or cyclin D1 were subjected to immunoblotting using an antibody directed against ER.

through the ER to enhance transcription of ERE-containing genes. The ER protein levels in U2-OS cells containing transiently expressed cyclin D1 were similar to those seen in cells without cotransfected cyclin D1 (Figure 4B). Therefore, the enhancement of ERE-responsive gene transcription by cyclin D1 was not due to a modulation of ER protein levels.

Cyclin D1 Binds to (Un)liganded ER In Vivo and In Vitro

Cyclin D1 could well activate ER-mediated transcription through direct binding to ER. When we mixed in vitro–translated ^{35}S–methionine labeled human ER with GST-cyclin D1 protein in an in vitro binding assay, we did indeed observe specific binding of ER to GST-cyclin D1 (Figure 5A). Binding was observed both with liganded as well as with unliganded receptor (lanes 2 and 3). To investigate whether cyclin D1 also binds to both liganded ER and unliganded ER in vivo, we used a mammalian two-hybrid assay. To test the in vivo binding between cyclin D1 and liganded ER, a chimeric expression vector harboring cyclin D1 fused to a GAL4 DNA binding domain (GAL4-D1) was generated and cotransfected with ER and a GAL4-site containing reporter plasmid into U2-OS cells. Transcription of the reporter construct should be stimulated in the presence of 17β-estradiol if a complex is formed between GAL4-D1 and ER, where the transactivational activity in this complex is provided by the hormone-dependent activation domain AF-2 in ER. GAL4-dependent CAT transcription was minimally stimulated when either GAL4-D1 or ER were transfected separately, as is shown in Figure 5B. CAT activity was significantly increased, however, by GAL4-D1 in combination with ER, suggesting an interaction in vivo between cyclin D1 and liganded ER. Consistent with the data obtained in the ERE-CAT assays (Figure 1), CDK4 and progesterone receptor (PR-B) act in this in vivo binding assay as a competitor and as a negative control, respectively. To investigate an in vivo binding between cyclin D1 and unliganded ER, we next generated an ER fusion protein containing a herpes simplex virus VP16 activation domain (VP16-ER). Cotransfection of two chimeric expression vectors, GAL4-D1 and VP16-ER, in combination with a GAL4-dependent reporter construct resulted in a significant increase in transcription of the reporter construct (Figure 5C). This result suggests that cyclin D1 interacts with unliganded ER in vivo.

To get an impression of the relative strength of the ER–cyclin D1 and cyclin D1–CDK4 interactions, we performed transfection studies with GAL4-D1 in combination with VP16-CDK4 (Figure 5C). Using the mammalian two-hybrid system, the relative activity of GAL4-D1/VP16-CDK4 was increased 3-fold, as compared with

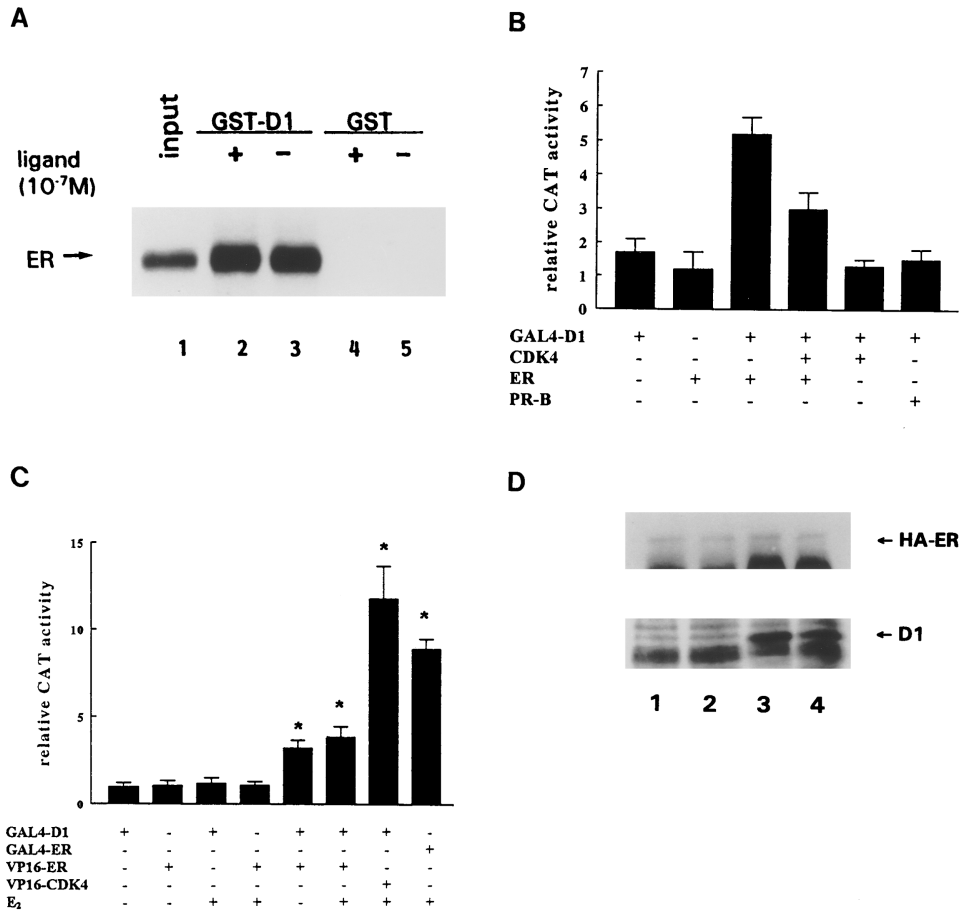

Figure 5. Cyclin D1 Interacts with (Un)liganded ER

(A) The in vitro interaction between cyclin D1 and ER. The in vitro interaction between cyclin D1 fused to glutathione-S-transferase (GST) and ^{35}S-labeled ER was studied both in the presence and absence of 17β-estradiol. GST-cyclin D1 or control GST protein were mixed with ^{35}S-labeled ER and immobilized on glutathion-agarose beads to establish in vitro binding. Lane 1 represents 10% of ER input, lanes 2 and 3 represent the amount of ER bound to GST-cyclin D1, and lanes 4 and 5 represent the amount of ER bound to GST protein.

(B) The in vivo interaction between cyclin D1 and ER in the presence of 17β-estradiol. The in vivo interaction between cyclin D1 and liganded ER was studied using a mammalian two hybrid assay with a chimeric protein GAL4-D1, which contains the GAL4 DNA binding domain (amino acid 1–147) fused to cyclin D1, in combination with ER that acts as a hormone-activated transcription factor. As a result of protein–protein interaction, hybrid proteins are able to stimulate transcription of a GAL4-dependent reporter construct (G5BCAT). This reporter construct, G5BCAT, contains five GAL4 binding sites 5′ to the E1B TATA sequence (Kim et al., 1992). The human steroid progesterone receptor (PR-B) served as negative control. The unliganded ER also did not stimulate CAT transcription (data not shown).

(C) The in vivo interaction between cyclin D1 and ER in the presence and absence of 17β-estradiol. The in vivo interaction between unliganded ER and cyclin D1 was studied using a mammalian two-hybrid assay with two chimeric proteins, VP16-ER (which contains the herpes simplex virus VP16 activation domain fused to ER) and GAL4-D1, in the GAL4 dependent reporter system as described in Figure 5B. GAL4-D1 in combination with VP16-CDK4 and dimerized ER served as positive controls. The results are expressed as average of at least three separate experiments ± SD, and related to CAT activity of a transfection using vector containing GAL4 DNA binding domain in combination of G5BCAT reporter vector in the presence of 10 nM ligand (17β-estradiol or Org 2058) for panel B and in the absence of ligand for panel C: E_2, 17β-estradiol; D1, cyclin D1; ER, estrogen receptor; (asterisks)$P \leq 0.05$.

(D) In vivo interaction between cyclin D1 and ER using immunoprecipitation. HA-tagged ER and cyclin D1 were transfected in U2-OS cells and immunoprecipitated using an antibody against HA-epitope tag (12CA5). The HA-ER associated proteins were tested for cyclin D1 by Western blot analysis. The upper part shows HA-tagged ER and (part of) IgH chains, and the lower part shows cyclin D1 protein. Lanes 1–4 of the Western blot represent HA-tagged ER-transfected cells in combination with cyclin D1 (lanes 3 and 4) in the presence of 17β-estradiol (lane 1 and 3) or absence of 17β-estradiol (lane 2 and 4).

GAL4-D1/VP16-ER. This finding suggests that, besides the binding of cyclin D1 to its kinase partner CDK4, the cyclin D1–ER interaction is of physiological importance.

Immunoprecipitation studies support the in vivo interaction between cyclin D1 and (un)liganded ER (Figure 5D). HA-tagged ER constructs were transfected together with cyclin D1 into U2-OS cells and maintained in 17β–estradiol-enriched or -free conditions. HA-ER fusion protein was immunoprecipitated and analyzed for cyclin D1 binding by Western blot analysis. Cotransfection with cyclin D1 resulted in coimmunoprecipitation of cyclin D1 with liganded as well as with unliganded ER. Thus, cyclin D1 physically interacts both with liganded and unliganded ER in vivo.

Cyclin D1 Acts through the EF Region of ER

In order to localize the ER domain that responds to cyclin D1, we have used chimeras in which either regions

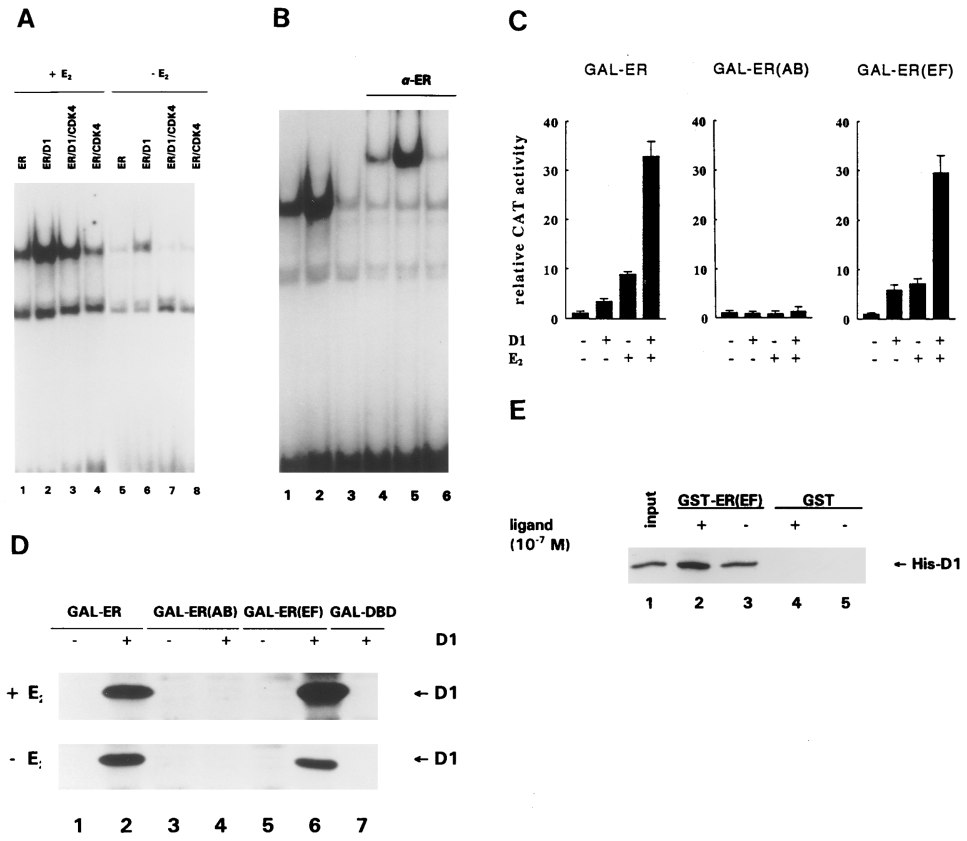

Figure 6. Involvement of Cyclin D1 in Binding of ER to ERE Sequences

(A) Analysis of protein-ERE complexes in response to cyclin D1 and 17β-estradiol. Extracts were prepared of U2-OS cells transfected with human ER in combination with cyclin D1, and subjected to mobility shift analysis as described in Experimental Procedures. Lanes 1 and 5 show the effect on ERE binding of transfected U2-OS cells with ER, lanes 2 and 6 represent ER and cyclin D1 cotransfectants, lanes 3 and 7 represent ER, cyclin D1, and CDK4 cotransfectants, and lanes 4 and 8 represent ER and CDK4 cotransfectants. Cell extracts were maintained in 10 nM 17β-estradiol (lanes 1–4) or in 17β-estradiol free conditions (lanes 5–8).

(B) ER identification of ERE-associated protein complex. Protein identification of ERE-associated complexes was tested by antibody-dependent supershifts using antibodies directed against ER. Lanes 1 and 4 represent ER transfectants, lanes 2, 3, 5, and 6 represent ER and cyclin D1 transfectants in the presence of 17β-estradiol (lanes 1, 2, 4, and 5) or absence of ligand (lanes 3 and 6). The transfectants were subjected to mobility shift analysis in the presence of ER antibody (lanes 3–6).

(C) Activation of ER by cyclin D1 does not require sequence- specific DNA binding, and cyclin D1 specifically activates the EF region of ER. U2-OS cells were transfected with 1.5 μg of a vector encoding full-length ER, AB region of ER or EF region of ER fused to GAL4 DNA binding domain yielding GAL-ER, GAL-ER(AB), and GAL-ER(EF), respectively. Transfection studies were performed together with cyclin D1 expression vector (1.5 μg) and G5BCAT reporter vector (3 μg), and cells were kept in the presence or absence of 10 nM 17β-estradiol. The results are expressed as relative CAT activity compared with basal CAT level in the absence of ligand.

(D) In vivo binding between cyclin D1 and GAL-ER fusion proteins. Constructs encoding GAL-ER fusion proteins and cyclin D1 were transfected in U2-OS cells in the presence and absence of 17β-estradiol (E_2), and immunoprecipitated using antibody directed against GAL-tag (sc 577, Santa Cruz). The GAL-ER (full length), GAL-ER(AB), and GAL-ER (EF) associated proteins were tested for cyclin D1 binding by Western blot analysis as indicated. Lanes 2, 4, 6 and 7 represent cyclin D1 transfected cells. Transfection with a construct encoding the GAL4 DNA binding domain (GAL-DBD) together with cyclin D1 served as negative control (lane 7).

(E) Direct binding between cyclin D1 and EF-region of ER. Bacterially produced and purified GST-ER(EF) or control GST protein and his-tagged cyclin D1 (His-D1) were mixed and immobilized on glutathion-agarose beads. Direct in vitro binding was analyzed by Western blotting using monoclonal antibody directed against cyclin D1. Lane 1 represent 1% of input, lanes 2 and 3 represent the amount of His-D1 bound to GST-ER(EF) and lanes 4 and 5 represent the amount of His-D1 bound to GST protein.

A/B (amino acids 1–184) or E/F (amino acids 282–595) were fused to the GAL4 DNA binding domain, yielding GAL-ER(AB) (Ali et al., 1993) and GAL-ER(EF) (Webster et al., 1988). Using these constructs in a GAL4-dependent reporter system in combination with cyclin D1, we found that the GAL-ER(AB) construct did not respond to cyclin D1 (Figure 6C). In contrast, cyclin D1 was able to stimulate transactivation by both GAL-ER(EF) and GAL-ER wild-type to the same extent (Figure 6C). To ask whether cyclin D1-mediated transactivation of ER correlated with binding to ER, we performed transient transfection experiments (Figure 6D). Immunoprecipitation of GAL fusion proteins, which contain the AB or EF region of ER, from transiently transfected U2-OS cell extracts, followed by Western blot analysis using a monoclonal antibody directed against cyclin D1, revealed that cyclin D1 predominantly binds to the EF region and not, or at least with lower affinity, to the AB region of ER. This binding between cyclin D1 and the C-terminal domain of ER occurred both in the presence

as well as in the absence of 17β-estradiol. The interaction between the carboxy-terminal region of ER and cyclin D1 was also confirmed in a GST pulldown experiment using bacterially produced and purified His-cyclin D1 and GST-ER(EF), containing amino acids 340–595 (Figure 6E). Since no other mammalian proteins were present in this experiment, these data indicate that cyclin D1 acts through direct binding to the C-terminal EF region of ER.

Cyclin D1 Enhances Binding of ER to ERE Sequence

To investigate whether the cyclin D1 action on transcription was due to enhanced ER binding to ERE, we analyzed the binding of ER-ERE complexes in response to cyclin D1 using a gel retardation assay. When transiently transfected into U2-OS cells and maintained in 17β–estradiol enriched medium, cyclin D1 increased the ER binding to ERE sequence 3-fold (Figure 6A). Consistent with data obtained from ERE-CAT and interaction assays, cyclin D1 in combination with CDK4 was less effective in stimulating ER binding to ERE sequence. Analysis of DNA binding complexes in response to hormone deprivation revealed that cyclin D1 stimulated ER binding to ERE, which was absent in cells lacking ectopic cyclin D1 (Figure 6A). Antibody supershift experiments of ERE-associated complexes revealed that ER is present in these ERE-associated complexes (Figure 6B). Thus, cyclin D1 triggers binding of liganded and unliganded ER to ERE sequences.

To investigate whether cyclin D1 only acts on ER specifically bound to an ERE sequence, we used an ER fused to GAL4 DNA binding domain in a GAL4-dependent reporter system. In this assay, cyclin D1 enhanced GAL4-dependent transcription in a ligand-independent manner (Figure 6C), indicating that cyclin D1 does not require sequence-specific DNA binding by ER for activation. These data therefore indicate that cyclin D1 also affects ER activity by enhancing transactivation by ER. In conclusion, cyclin D1 acts by both enhancing the DNA binding and transactivation capacity of ER.

Discussion

The present study establishes a new role for cyclin D1 as a positive regulator of ER-mediated transcription. The transcriptional activation was specific in that of all cyclins tested only cyclin D1 could enhance ER-mediated transactivation. Furthermore, cyclin D1 could only stimulate transactivation by ER and not the PR. Importantly, activation of ER by cyclin D1 was entirely independent of CDK binding and did not require occupancy by cognate steroid of the receptor. In normal breast epithelial cells, estrogen receptors are thought to be transcriptionally inactive within the cell before binding to hormone. Occupancy by hormone results in conformational change in its cognate receptor protein, which leads to intranuclear translocation and allows association with specific DNA sequence in the regulatory region of target genes. As a result of ER-ERE binding, transcription of *cis*-linked target genes will be stimulated. We demonstrated that ER transactivation by cyclin D1 is associated with a direct physical interaction between cyclin D1 and ER. Cyclin D1 enhances binding of both liganded as well as unliganded ER to ERE-sequences. These data support a model in which cyclin D1-ER interaction triggers ER to undergo an activation step by means of a conformational change. Cyclin D1 interaction increases binding of ER to ERE sequences and enhances transactivation of ER. Together, this activity results in a dramatic enhancement of transcription of ERE-responsive genes.

In contrast to the current view that receptor activation is dependent on binding its cognate ligand, the present study shows that cyclin D1 also activates ER-mediated transcription in the absence of ligand. In addition, it was recently shown that EGF, TGFα, and dopamine also elicit ER-dependent transcription of an ERE-containing gene, which was not dependent on estrogens (Ignar-Trowbridge et al., 1993; O'Malley et al., 1995). One possible mechanism for estrogen receptor activation could be phosphorylation of the receptor (Zhang et al., 1994; Kato et al., 1995). Transcriptional activation by ER can be induced by growth factors through the membrane-associated receptor tyrosine kinase-Ras-Raf-MAPK cascade, which enhances the activity of AF-1 site in ER molecule via phosphorylation of the Ser-118 residue (Kato et al., 1995). The present study clearly demonstrates that cyclin D1 does not enhance phosphorylation of ER. Instead, our data indicate that cyclin D1 acts through direct physical association to activate ER.

Until now, cyclin D1 has been regarded only as a CDK regulator. Via CDK activation, it regulates progression of cells through the G1 phase of the cell cycle. Overexpression of cyclin D1 causes several effects on the cell cycle, including a shortening of the G1 interval, an increase of G0/G1 transition, and a decrease of G1/G0 transition (Resnitzky et al., 1994; Resnitzky and Reed, 1995; Zwijsen et al., 1996). The dual activity of cyclin D1 as activator of ER and mediator of cell cycle progression via cdk kinase activity is thus far unique among the cyclins. Overexpression of cyclin E also shortens G1, as does overexpression of cyclin D1 (Resnitzky et al., 1994; Resnitzky and Reed, 1995), but does not affect ER activation. This finding indicates that perturbed G1 progression and premature S-phase entry on their own do not account for activation of ER, and highlights that cyclin D1 acts in this respect via two different mechanisms.

In breast tumor cells, several ER variants have been identified (Zhang et al., 1993; Wiltschke and Fuqua, 1995). Many of these variants are the results of alternative mRNA splicing and of point mutations, which usually yield truncated products (Wiltschke and Fuqua, 1995). The ER variants are coexpressed with, and often in excess of, wild-type ER. In the present study, we have used the breast tumor T47D cell line in which small levels of ER variants with exon 2, 3, 5, and 7 deletions are identified (Wang and Miksicek, 1991; Zhang et al., 1993). It is very unlikely that the cyclin D1 action is attributed to one of the ER variants in these cells, since cyclin D1 was effective in the ER-negative U2-OS cells transfected with wild-type ER cDNA expression vector. Furthermore, immunofluoresence studies revealed that these ectopic ER proteins in U2-OS cells are predominately

located in the nucleus, and that cyclin D1 did not modulate the subcellular localization of ER (data not shown). Thus, it appears that the activation of ER by cyclin D1 is not due to a change in ER translocation to the nucleus.

We have determined that the site of cyclin D1 action is located in the E/F region of liganded as well as unliganded ER. Binding of cyclin D1 to this region could modulate the binding of accessory protein(s) of ER, including Hsp90, Hsp70, p60, p23, and proteins of the transcriptional machinary (Pratt, 1993). It is conceivable that they assist in the activation of the ER by cyclin D1. For instance, Hsp90 promotes a receptor structure that is capable of ligand binding. Ligand binding could induce a conformational change in the receptor, which in turn could prevent rebinding of Hsp90, while simultaneously allowing receptor dimerization. This Hsp, however, is an unlikely candidate to assist cyclin D1 in ER activation, because (i) it is dissociated from the ER-complex after ligand binding, whereas cyclin D1 also activates the liganded complex; (ii) the subcellular localization differs between Hsp90 and cyclin D1 (cytoplasm versus nucleus); and (iii) Hsp90 acts on all members of the steroid receptor family, whereas the cyclin D1 action is restricted to ER.

Our data suggest that cyclin D1 is involved in activation of ERE-containing gene transcription. Cyclin D1 may therefore contribute to a potentiation of estrogen-mediated cell proliferation in hormone-responsive tissues, such as breast epithelium. Consistent with this is the recent finding that cyclin D1-deficient mice have a defect in hormone-responsive proliferation of breast epithelium (Fantl et al., 1995; Sicinski et al., 1995). These defects in lobuloalveolar development in cyclin D1-deficient animals were caused by a diminished hormone responsiveness of the breast tissue and are reminiscent of those observed in mice lacking the PR (Lydon et al., 1995). Because the PR gene is an ER target gene, these data support the novel mechanism of cyclin D1 proposed in this study, and strengthen its physiological relevance.

Recently, it was shown that overexpression of cyclin D1 renders breast cancer cells less mitogen dependent (Musgrove et al., 1994; Zwijsen et al., 1996). In this context, the present study clearly shows that cyclin D1 can also enhance transcription of ER-responsive genes without assistance of estrogens by stimulating ER binding to ERE-sequences. This cyclin D1 action enables cells to bypass the requirement for estrogens and therefore provides a mechanism for estrogen-independent growth of cyclin D1-overexpressing breast tumor cells (Schuuring et al., 1992; Bartkova et al., 1994; Michalides et al., 1996). Our finding that activation of ER by cyclin D1 cannot be inhibited by the anti-estrogen tamoxifen raises the possibility that tamoxifen treatment is ineffective in cyclin D1-amplified breast cancers. We are currently performing a retrospective clinical study on breast cancer biopsies to investigate this possibility in detail.

Experimental Procedures

Cell Lines

Human T47D, HeLa, and U2-OS cells were cultured in Dulbecco's modified Eagle medium (DMEM) with 10% fetal calf serum. During the transfection, cells were maintained in phenol red-free DMEM containing insulin/transferrin (10 μg/ml each) or 10 nM ligand (the estrogen 17β-estradiol or the progestin Org 2058) as indicated.

Transfection Studies

The reporter plasmids, ERE-TK-CAT (3 μg) containing the ERE from the *vitellogenin A2* gene or $(PRE)_2$-TK-luc, were transiently transfected into subconfluent T47D cells using the calcium phosphate precipitation technique (Graham and van der Eb, 1973). The expression vectors used in the transient transfection studies were pCMV cyclin D1, cyclin D2, cyclin D3, cyclin A, cyclin E, cyclin B1, cyclin B2, CDC2, CDK2, CDK4, CDK4-DN, cyclin D1-GH, and cyclin D1-KE, which have been described elsewhere (Hinds et al., 1992, 1994). ER-negative cells were transfected with 0.4 μg human ER construct (HEGO; Migliaccio et al., 1991). After 40 hr, cells were harvested and resuspended in 0.1 M Tris-HCl (pH 8.0), and assayed for luciferase activity (Promega, Luciferase system), β-galactosidase activity (Tropix, galactolight assay), and/or CAT activity (Seed and Sheen, 1988).

Phosphate Labeling and Immunoprecipitation

U2-OS cells were transfected in the presence or absence of serum. After 24 hr, cells were incubated for 1 hr in phosphate-free DMEM, followed by incubation in phosphate-free DMEM supplemented with 1.0 mCi/ml [^{32}P]orthophosphate for 4 hr in the absence of mitogens or in the presence of 17β-estradiol (10 nM) and 10% dialyzed serum. After labeling, cells were lysed in RIPA buffer^{+} containing 150 mM NaCl, 1% NP-40, 50 mM Tris (pH 7.8), 10 mM EDTA, 0.5% deoxycholate, 0.1% SDS, 1 mM PMSF, 5 μg/ml aprotinin, 10 μg/ml trypsin inhibitor, 10 μg/ml leupeptin, and 10 mM β-glycerophosphate. The lysate was precleared three times with 5 μl of normal mouse serum coupled to protein A-Sepharose beads. For immunoprecipitations, the supernatant was incubated with 100 μl of 12CA5 hybridoma supernatant, which was coupled to protein A sepharose beads at 4°C. After 1 hr, beads were washed in RIPA buffer, boiled in Laemmli-buffer and separated on a 10% SDS-polyacrylamide gel, dried, and subjected to autoradiography.

Western Blot Analysis

U2-OS cells transfected with ER (0.4 μg), together with cyclin D1 (1 μg), were lysed in extraction buffer (50 mM Tris [pH 7.4], 150 mM KCl, 15 mM NaCl, 30 mM MgCl2, 10 mM EGTA, 0.5% NP-40, 1 mM PMSF, 5 μg/ml aprotinin, 10 μg/ml trypsin inhibitor, and 10 μg/ml leupeptin). Equal amounts of total cell extracts were separated by SDS-polyacrylamide gel electrophoresis and blotted to a cellulose nitrate membrane. Immunoblot analysis was performed with anti-cyclin D1 (DCS-6, Progen) and anti-ER (LH2, Novacastra) monoclonal antibodies, and immunodetection was performed using the enhanced chemiluminescence system.

In Vitro Binding Assay

Total bacterial extracts expressing cyclin D1 protein fused to glutathione-S-transferase (GST) or GST protein alone were generated and purified on gluthathione agarose beads. ^{35}S–methionine labeled ER protein was prepared by in vitro transcription/translation and incubated with 1 μg GST-cyclin D1 in ELB (250 mM NaCl, 0.1% NP-40, 50 mM Hepes [pH 7.0], and 5 mM EDTA) with 0.5 mM DTT, 1 mM PMSF, 10 μg/ml aprotinin, and 10 μg/ml leupeptin, bound to gluthathione-agarose beads, washed in ELB, eluted, and fractionated by SDS-polyacrylamide gel electrophoresis.

GAL4-Dependent Reporter System

To generate chimeric proteins, A/B region of ER (amino acid 1–184) or E/F region of ER (282–595), or full-length of ER or cyclin D1 cDNAs, were inserted in frame downstream of the GAL4 (1–147) DNA binding domain fragment or sequence coding for the acidic activation domain of the Herpes Simplex virus VP16 protein. U2-OS cells were transfected with 1.5 μg GAL4-D1, ER, PR-B, and CDK4 expression vectors in combination with 10 μg G5B-CAT reporter constructs. As an internal control, 0.5 μg of pCMV-luciferase plasmid was used in the assays. The CAT activity was corrected for differences in transfection efficiency by normalizing to luciferase activity.

Gel Retardation Assay

A 31-base oligonucleotide ERE probe (5′-GATCCGTCAGGTCACAG TGACCTGATGGATC-3′) was used. Equimolar amounts of both strands were annealed in buffer (10 mM Tris, 1 mM EDTA [pH 8.0]) and end-labeled using [γ-^{32}P]ATP (Amersham) and T4 polynucleotide kinase (Boehringer Mannheim). U2-0S cell were transfected with ER in combination with cyclin D1 (2.5 μg each) in 10 cm tissue culture dishes. For the preparation of cell extract, cells were washed with cold PBS, collected, and resuspended in 100 μl ice cold extraction buffer (0.4 M KCl, 10 mM Tris [pH 7.4], 1 mM EDTA, 2 mM DTT, and 10% glycerol [v/v]). The cells were kept on ice for 20 min and spun for 10 min at 14,000 rpm and 4°C. The amount of cell extract used for gel shift assays was adjusted for protein content and transfection efficiency. Ten micrograms of cell extract was added to incubation buffer (10 mM Tris [pH 7.5], 50 mM NaCl, 0.5 mM EDTA, 0.5 mM DTT, 1 mM $MgCl_2$, 4% glycerol [v/v], 1 μg poly [dI.dC], and 1 μg ssDNA) and placed on ice. After 15 min, 0.5 ng ^{32}P-labeled ERE oligonucleotide was added, and the reaction was incubated for 20 min at room temperature. Where indicated, 2 μl antibody directed against human ER (SRA-1010, Stress Gen) was added to the reaction mixture for an additional 10 min. The protein-ERE complexes were separated on a 5% polyacrylamide gel in 0.5X TBE at 200 V for 3 hr, vacuum dried, and subjected to autoradiography.

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