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p300 is a component of an estrogen receptor coactivator complex

(transcription/steroid hormone/estrogen receptor-associated protein)

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ABSTRACT **The estrogen receptor (ER) is a ligand-dependent transcription factor that regulates expression of target genes in response to estrogen in concert with other cellular signaling pathways. This suggests that the mechanism by which ER transmits an activating signal to the general transcription machinery may include factors that integrate these diverse signals. We have previously characterized the estrogen receptor-associated protein, ERAP160, as a factor that complexes with ER in an agonist-dependent manner. We have now found that the transcriptional coactivator p300 associates with agonist bound ER and augments ligand-dependent activation by ER. Our studies show that an ER coactivator complex involves a direct hormone-dependent interaction between ER and ERAP160, resulting in the recruitment of p300. In addition, antibodies directed against the cloned steroid receptor coactivator 1 (SRC1) recognize ERAP160. The known role of p300 in multiple signal transduction pathways, including those involving the second messenger cAMP, suggests p300 functions as a point of integration between ER and these other pathways.**

The estrogen receptor (ER) is a member of the nuclear receptor superfamily that regulates target genes in response to estrogen and with input from other transduced signals (for review, see refs. 1 and 2). It does so by binding as a homodimer to a conserved estrogen response element in the regulatory region of target genes and via two discrete transactivating domains. The N-terminal transactivating region (AF1) is capable of activating transcription in a hormone-independent manner, and has recently been shown to be a target of the mitogen-activating protein kinase signaling pathway (3, 4). The AF2 domain overlaps the C-terminal hormone binding domain and activates transcription in response to estrogen or synthetic agonists. While the mechanisms by which the AF2 domain transmits hormone binding signals to basal transcription machinery remain poorly understood, several groups have identified proteins that interact with the AF2 domain of various nuclear receptors in a hormone-dependent manner. Several lines of biochemical evidence have focused attention upon two nuclear receptor interacting proteins of approximately 160 and 140 kDa. These proteins were initially identified based upon their ability to interact with agonist bound estrogen receptor in an AF2-dependent manner (5, 6). Other studies demonstrate that similar factors interact with several other nuclear receptors, and at least one of these factors, the steroid receptor coactivator 1 (SRC1), has been cloned and shown to potentiate nuclear receptor signaling (7). The full-length SRC1 encodes a protein similar in size to the biochemically defined ERAP160 (8, 25). Several other factors including TRIP1 (9), as well as TIF1 (10) and RIP140 (11), have been cloned based upon their ability to interact with various nuclear receptors in response to activating ligands, suggesting possible roles in coactivation.

Studies of ER-mediated transcription have demonstrated that compounds such as forskolin and other activators of the protein kinase A pathway can cooperate with the effects of estrogen; however, the molecular mechanisms that underlie this cooperativity have not been elucidated (12, 13). cAMP is capable of eliciting a transcriptional response through the activation of protein kinase A and the subsequent phosphorylation of the cAMP response element binding protein (CREB). This phosphorylation has been shown to result in the interaction between CREB and the CREB-binding protein (CBP) (14, 15). Furthermore, this interaction has been demonstrated to be required for transcriptional activation of cAMP response element-containing promoters, implying that CBP functions as a coactivator of CREB-mediated signaling. The closely related factor, p300, is also capable of similar coactivation, and has been reported to interact with components of the basal transcription machinery (16, 17). These studies demonstrate that CBP and p300 are coactivators of CREB, and suggest that they may be targets of other signaling pathways.

In this paper, we report that p300 associates with ER in an agonist-dependent manner, and that this association involves the previously defined ERAP160. We also show that the interaction between ERAP160 and p300 is direct and exists *in vivo*. Furthermore, we show that ERAP160 is recognized by antibodies raised against the cloned coactivator SRC1. Finally, we report that overexpression of p300 was sufficient to augment estrogen-dependent transcriptional activation. Taken together, our studies suggest that in response to estrogen binding, ER becomes associated with a complex containing SRC1 and p300, and that this complex functions to coactivate ER-mediated transcriptional regulation.

MATERIALS AND METHODS

Cells and Cell Culture. Cell lines used were obtained from American Type Culture Collection and include the human estrogen receptor positive breast cancer cell line MCF-7, the human embryonic kidney cell line 293, and the African green monkey kidney cell line CV-1. Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (vol/vol) (Sigma) at 37°C and 5% CO_2/95% air.

Abbreviations: ER, estrogen receptor; ERAP, estrogen receptor-associated protein; GST, glutathione *S*-transferase; SRC1, steroid receptor coactivator 1; CREB, cAMP response element binding protein; CBP, CREB-binding protein; AF2, activating function domain 2; HBD, hormone binding domain; E2, estradiol; WCE, whole cell extract; CMV, cytomegalovirus; β-gal, β-galactosidase.

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Metabolic Labeling and Protein–Protein Interaction Assay. Before the metabolic labeling, cells were preincubated with methionine-free DMEM for 10–20 min. Confluent 150-mm diameter dishes were labeled with 1–3 mCi (1 Ci = 37 GBq) [^{35}S]methionine (NEN) for 4 hr in methionine-free DMEM. After labeling, cells were washed extensively with ice-cold phosphate-buffered saline (PBS) and lysed in 1 ml of buffer A (150 mM NaCl/50 mM Tris 7.4/5 mM EDTA/0.5% Nonidet P-40). After 30 min of rotation at 4°C, cell extracts were clarified by centrifugation at 12,000 rpm and the supernatant was collected in a fresh tube. Lysates containing 2.5×10^7 cpm were then incubated with a glutathione *S*-transferase (GST) fusion protein containing the hormone binding domain of the human ER (GST–HBD) immobilized on 50 μl of glutathione-Sepharose beads in the presence or absence of the appropriate ligand in buffer B (150 mM NaCl/50 mM Tris 7.4/5 mM EDTA) as previously described (5). After washing the beads three times in 1 ml of buffer B and once in 1 ml of buffer A, proteins were eluted in SDS/sample buffer and resolved on 7.5% SDS/PAGE. Gels were fixed in 35% methanol/10% glacial acetic acid, fluorographed in Enhance solution (NEN), and dried before exposure to film.

Transient Transfection and Mammalian Two-Hybrid Assays. Full-length ER was fused to the bacterial tetracycline repressor (pTetR-ER), which serves as a tet-operator specific DNA binding domain. A plasmid containing the bacterial tet operator sequence upstream of a minimal cytomegalovirus (CMV) TATA element and firefly luciferase gene (pTetO-luc) was used as reporter (18). Expression plasmids encoding either a fusion protein of p300 and the acidic activation domain of VP-16 (pCMV–p300–VP16) (19) or wild-type p300 fused at its carboxyl terminus to a hemagglutinin tag (pCMV–p300 CHA) were cotransfected in the amounts indicated in the figure legends.

Transfections of CV-1 cells were performed by calcium phosphate precipitation. Subconfluent CV-1 cells were transferred to phenol red-free DMEM containing 15% charcoal-treated serum 48 hr before transfection. Duplicate 35-mm plates were transfected with 1 μg of pTetO-luc, 25 ng of pTetR–ER, 100 ng of pCMV–β-gal (β-gal, β-galactosidase), and pCMV–p300–VP16 or pCMV–p300 CHA at a constant final DNA concentration as indicated in the figure legends. Twenty-four hours after transfection, cells were washed in PBS and refed with the same medium in the absence or presence of 10 nM estradiol (E2) or 100 nM 4-OH-Tamoxifen. Forty-eight hours posttransfection, cell lysates were prepared and assayed for luciferase activity. Results are corrected for transfection efficiency by normalizing to the level of β-gal activity.

***In Vitro* Transcription and Translation.** Recombinant p300 cDNAs in pBluescript were transcribed and translated in TNT-T3 coupled rabbit reticulocyte lysates (Promega) in the presence of [^{35}S]methionine from the T3 promoter following the manufacturer's guidelines.

Immunoprecipitations, Western Blotting, and Far Western Blotting. For hormonal stimulation, MCF-7 cells were

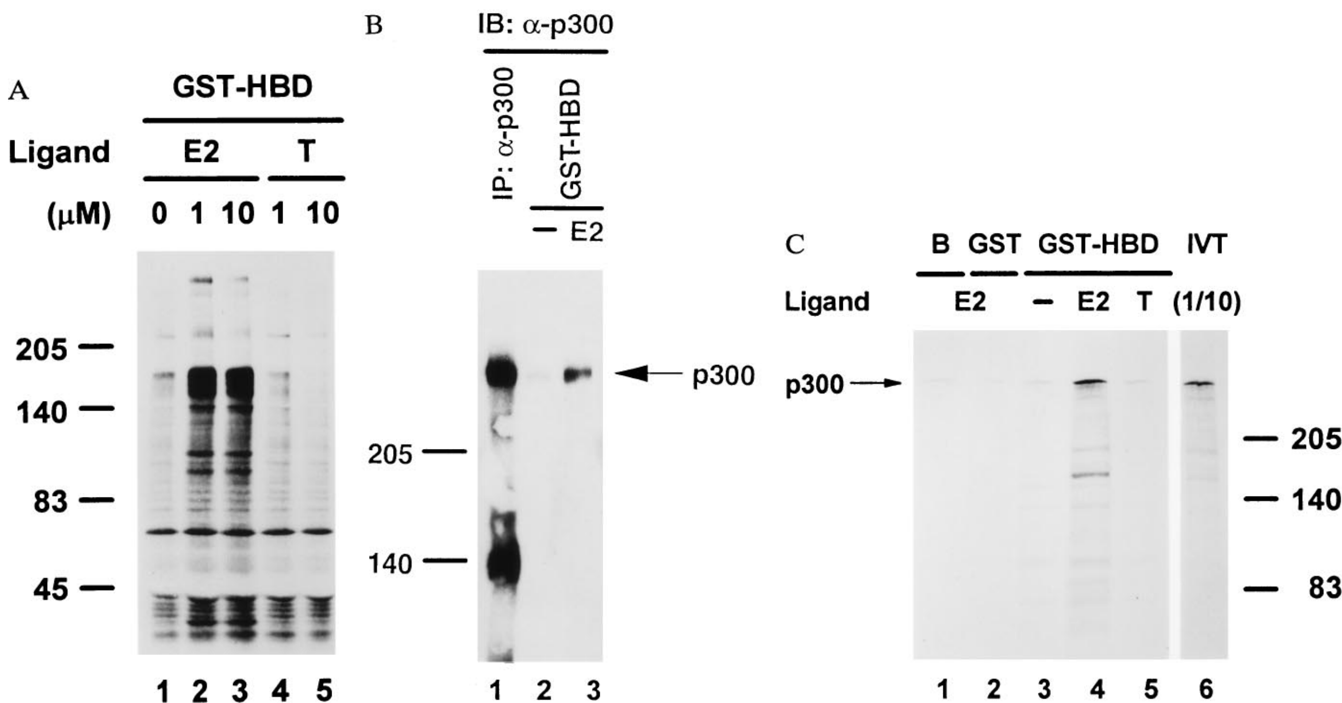

FIG. 1. The ER HBD interacts with a complex containing p300 *in vitro*. (*A*) Multiple proteins associate *in vitro* with the ER HBD in a ligand-dependent manner. WCEs of MCF-7 cells metabolically labeled with [^{35}S]methionine were bound to the GST–HBD fusion immobilized on glutathione-Sepharose in the presence (lanes 2 and 3) or absence (lane 1) of 17-β-estradiol (E2) or tamoxifen (T) (lanes 4 and 5) at the concentrations indicated. Proteins were eluted in SDS/sample buffer and were resolved on 7.5% SDS/PAGE. Several proteins ranging in size from 30 to 300 kDa were observed to interact with GST–HBD in an estrogen-dependent manner. (*B*) The transcriptional coactivator p300 associates with GST–HBD in an estrogen-dependent manner. WCEs from the human embryonic kidney cell line, 293, were incubated with GST–HBD immobilized on glutathione-Sepharose in the absence (lane 2) or presence of 1μM E2 (lane 3). Specifically associated proteins were recovered by boiling in SDS/sample buffer, resolved on 7.5% SDS/PAGE, and transferred to nitrocellulose. Filters were probed with a p300 monoclonal antibody. Immunoprecipitated p300 was run as a positive control (lane 1). (*C*) The ER HBD specifically associates with recombinant p300 in response to estrogen but not tamoxifen. Recombinant ^{35}S-labeled p300 produced by *in vitro* transcription/translation in a rabbit reticulocyte lysate was bound to GST–HBD immobilized on glutathione-linked Sepharose in the presence of 1 μM E2 (lane 4), 1 μM 4-hydroxytamoxifen (T) (lane 5), or the absence of hormone (lane 3) or to negative control samples containing only glutathione-Sepharose (lane 1) or GST immobilized on glutathione-Sepharose (lane 2). The translation product (15 μl) was incubated with the different affinity matrices that had been blocked with 3% bovine serum albumin under the indicated conditions at 4°C for 30 min. After extensive washing, bound proteins were eluted in 20 mM reduced glutathione-containing elution buffer (120 mM NaCl/100 mM Tris·HCl, pH 8.0) and separated by 7.5% SDS/PAGE. Gels were treated with a fluorophore, dried, and visualized by autoradiography. Lane 6 contains 10% of the input ^{35}S-labeled p300 used in lanes 1–5.

grown overnight in phenol red-free DMEM supplemented with 15% charcoal-treated fetal bovine serum. After stimulation with 100 nM E2 for 3 hr or without hormonal stimulation, protein extracts were prepared using buffer A. Protein content was determined by Bradford assay (Bio-Rad). Proteins were either resolved directly in SDS/polyacrylamide gels after boiling in SDS sample buffer or subjected to immunoprecipitations with the indicated antibodies. For immunoprecipitations, 2.5 mg of cellular proteins were incubated with the indicated antibodies for 2 hr at 4°C. Immune complexes were collected by adding 70 μl of a 50% slurry of protein-A Sepharose in buffer B for 1 hr at 4°C. After two washes in buffer A, protein complexes were released from beads by boiling in SDS/sample buffer for 3 min. Proteins were resolved by SDS/PAGE and transferred to nitrocellulose (20). Immunodetection was performed after blocking the membranes overnight at 4°C in 20 mM Tris·HCl (pH 7.5), 137 mM NaCl, 0.05% Tween 20, and 5% powdered milk by incubating membranes with the indicated antibodies for 2 hr at room temperature. Specifically bound primary antibodies were detected with peroxidase coupled secondary antibody and chemiluminescence.

For Far Western blot analysis, *in vitro* translated p300 proteins, immune complexes containing p300, or whole cell extracts (WCEs) from MCF-7 cells that were previously incubated with GST–HBD in the presence or absence of E2, were separated by 7.5% SDS/PAGE and electroblotted onto nitrocellulose in transfer buffer (25 mM Tris/192 mM glycine/20% methanol). After denaturation and renaturation of proteins, the blots were incubated with ^{32}P-labeled GST–HBD probe as previously described (5) in the presence of 1 μM E2. The filters were then washed, dried, and exposed for autoradiography.

RESULTS

The mechanism of hormone-dependent transcriptional regulation by ER was addressed by affinity purification of ER-associated proteins (ERAPs). The HBD of the human ER was fused to GST and used to purify a group of proteins ranging in size from 30 kDa to ≈300 kDa from metabolically labeled MCF-7 breast cancer cells. These proteins bound GST–HBD in the presence of E2 (Fig. 1*A*, lanes 2 and 3) but not in its absence (lane 1) nor in the presence of the ER antagonist tamoxifen (lanes 4 and 5). In addition to the previously reported ERAP160 and ERAP140 (5, 6), less stringent wash conditions allowed the detection of proteins of approximately 300 kDa, 100 kDa, 90 kDa, and 30 kDa. The agonist-dependence of these interactions suggests a role in transcriptional activation by ER.

Studies of ER-mediated transcription have demonstrated that compounds such as forskolin and other activators of the protein kinase A pathway can cooperate with the effects of estrogen (12, 13). These studies suggested to us the possibility that the protein(s) of ≈300 kDa might be related to the previously described coactivators of cAMP signaling, p300 (21, 22) and phospho-CBP (14, 15). WCEs from the human embryonic kidney cell line 293 (Fig. 1*B*) or MCF-7 (data not shown) were bound to the GST–HBD affinity matrix in the absence (lane 2) or presence (lane 3) of 1 μM E2. Specifically bound proteins were resolved by SDS/PAGE and transferred to nitrocellulose. Immunoprecipitated p300 was run as a positive control (lane 1). Immunoblotting with a monoclonal antibody directed against human p300 (21) revealed significant p300 binding only in the presence of E2 (Fig. 1*B*). Similar results were obtained using an antibody directed against the closely related transcriptional coactivator CBP (data not shown). To confirm the association, we tested the ability of recombinant p300 to interact with ER. *In vitro* translated ^{35}S-labeled p300 (Fig. 1*C*, lane 6) associated specifically with GST–HBD only in the presence of E2 (lane 4) supporting a role for p300 in hormone-dependent transactivation by ER.

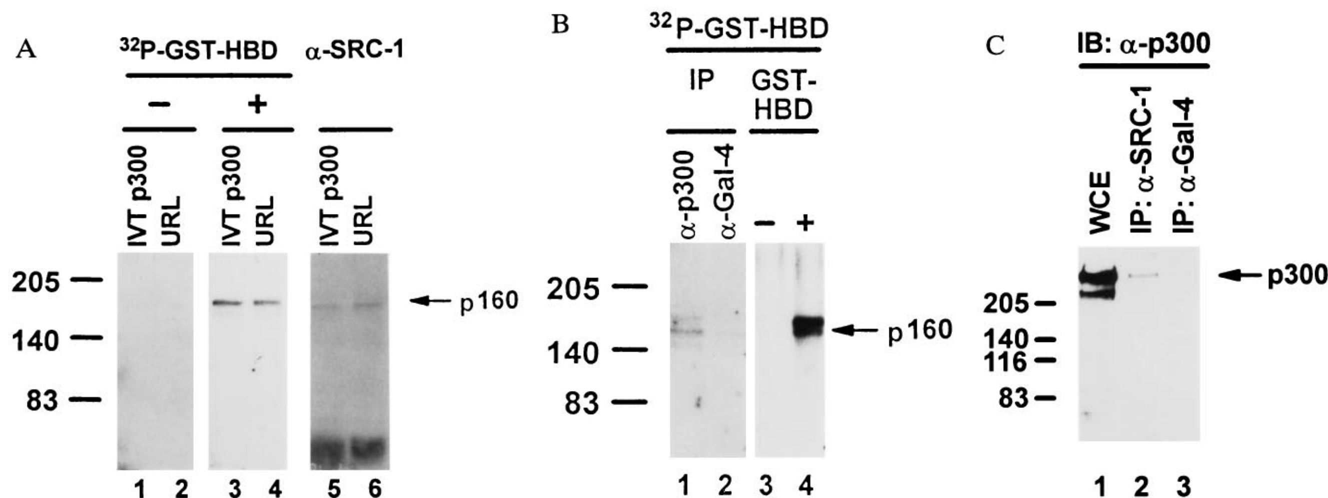

FIG. 2. ERAP160 directly interacts with the ER HBD and associates with p300 *in vivo*. (*A*) ERAP160 present in rabbit reticulocyte lysate interacts directly with ER HBD and is recognized by anti-SRC1 antiserum. Rabbit reticulocyte lysates that were programmed to express recombinant p300 (lanes 1, 3, and 5) or unprogrammed (URL) (lanes 2, 4, and 6) were resolved on 7.5% SDS/PAGE and transferred to nitrocellulose. The filter was subjected to denaturation and renaturation in guanidine hydrochloride as previously described (5) and was probed with ^{32}P-labeled GST–HBD either in the absence (lanes 1 and 2) or presence of 1 μM E2 (lanes 3 and 4). The filter was washed and exposed to film. Following autoradiography, the filter was stripped and immunoblotted with an antiserum directed against SRC1 (lanes 5 and 6). Specifically bound antibody was detected using chemiluminescence as previously described. (*B*) ERAP160 associates with p300 *in vivo*. Immunoprecipitations using a p300 monoclonal antibody (lane 1) or a control Gal4-DBD monoclonal antibody (lane 2) were performed on WCEs from MCF-7 cells. Immunoprecipitated proteins were resolved by SDS/PAGE, transferred to nitrocellulose, and, after renaturation, incubated with ^{32}P-labeled GST–HBD in the presence of 1 μM E2. In parallel, MCF-7 WCE was incubated with GST–HBD-bound glutathione-Sepharose beads in the absence (lane 3) or presence of 1 μM E2 (lane 4) as described in Fig. 1, followed by Far Western blot analysis with ^{32}P-labeled GST–HBD. Exposure times were 48 hr for lanes 1 and 2 and 12 hr for lanes 3 and 4. C. SRC1 and p300 form a complex *in vivo*. Immunoprecipitation using antiserum directed against SRC1 (lane 2) or control Gal4-DBD monoclonal antibody (lane 3) were performed on WCE from MCF-7 cells. Immunoprecipitated proteins were resolved by SDS/PAGE and transferred to nitrocellulose and probed with a p300 monoclonal antibody. WCE was run as a positive control (lane 1). Specifically bound antibody was detected using chemiluminescence as previously described.

To determine whether the ligand-dependent interaction between ER and p300 is direct or involves an intermediary factor, Far Western blot analyses were performed using the ^{32}P-labeled GST–HBD fusion protein as a probe either in the presence of E2 (Fig. 2*A*, lanes 3 and 4) or in the absence of E2 (Fig. 2*A*, lanes 1 and 2). Such an analysis of either p300 programmed or unprogrammed rabbit reticulocyte lysate reveals the presence of endogenous ERAP160 (Fig. 2*A*, compare lanes 3 and 4 to lanes 1 and 2), raising the possibility that the complex between p300 and ER formed *in vitro* might include ERAP160.

To test whether ERAP160 and p300 can associate *in vivo*, p300-containing complexes were immunoprecipitated from MCF-7 cells with a p300-specific monoclonal antibody, and analyzed by Far Western blotting using E2-liganded ^{32}P-labeled GST–HBD probe. A 160-kDa protein present in anti-p300 immunoprecipitates was detected by this probe, identifying this protein as ERAP160 (Fig. 2*B*, lane 1). Furthermore, this protein comigrates with ERAP160 affinity-purified with GST–HBD (lane 4). This protein was not detected in immunoprecipitates using a control anti-Gal4 monoclonal antibody (lane 2). While these studies do not rule out a direct interaction between ER and p300, they demonstrate that ERAP160 and p300 can associate *in vivo* supporting a possible role for ERAP160 as an intermediary factor in the interaction between ER and p300.

Interestingly, a yeast interaction trap for proteins interacting with p300 isolated a cDNA for SRC1 (25), a protein first cloned as a coactivator for steroid receptors including ER (7). The full-length SRC1 cDNA encodes a protein similar in size to the biochemically defined ERAP160 (8, 25). In immunoblots, antiserum raised against recombinant SRC1 detects a protein that comigrates with ERAP160 visualized by Far Western blot analysis (Fig. 2*A*, lanes 5 and 6), suggesting that SRC1 and ERAP160 are related. Furthermore, incubation of GST–SRC1 with metabolically labeled MCF-7 breast cancer cells specifically purifies a protein of 300 kDa (data not shown). More significantly, immunoprecipitation of SRC1 from MCF-7 cell extracts followed by anti-p300 Western blotting provides evidence for the *in vivo* association of SRC1 and p300 (Fig. 2*C*, lane 2).

A physiologic role for p300 as a coactivator involved in ER signaling would require the formation of an estrogen-dependent complex *in vivo* containing endogenous ER and p300 (Fig. 3*A*). To demonstrate the existence of such a complex, ER was immunoprecipitated from MCF-7 cells treated for 3 hr in the presence or absence of E2. Immune complexes were resolved by SDS/PAGE and then immunoblotted with p300 antibody. E2 dramatically stimulated the *in vivo* association of p300 with ER (Fig. 3*A*, lane 3). Immunoblotting demonstrated that p300 expression is not altered under these hormonal conditions (data not shown). These results demonstrate the presence of an endogenous hormone-dependent complex containing both p300 and ER.

To provide further evidence for an agonist-dependent *in vivo* association between ER and p300, we used a mammalian

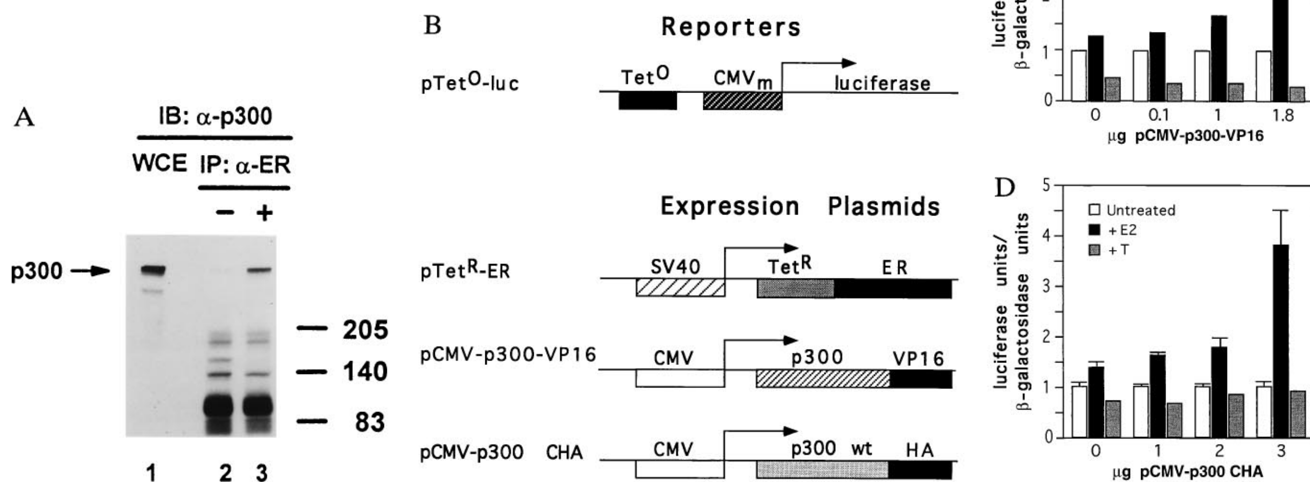

FIG. 3. p300 is recruited to ER *in vivo* in the presence of ligand and functions as an ER coactivator. (*A*) p300 coimmunoprecipitates with ER in an E2-dependent manner. MCF-7 cells were grown overnight in phenol red-free DMEM supplemented with 15% charcoal-treated fetal bovine serum. Cells were left untreated (lane 2) or stimulated for 3 hr with 100 nM E2 (lane 3) before lysis. Immunoprecipitations were performed on equal protein amounts using a monoclonal anti-ER antibody (AER 314, Neomarkers, Fremont, CA). After addition of a polyclonal rabbit anti-mouse IgG antiserum, immune complexes were collected on protein-A Sepharose beads, eluted in SDS/sample buffer, and subjected to SDS/PAGE. After transfer to nitrocellulose filters, Western blot analysis was performed using a p300 monoclonal antibody as described in Fig. 1*B*. Crude WCE (50 μg) was run as a positive control (lane 1). (*B*) Schematic representation of the pTetO-luc reporter and the expression plasmids encoding pTetR–ER, CMV–p300–VP16, and CMV–p300 CHA (wild type). (*C*) A mammalian two-hybrid system confirms the agonist-dependent association of p300 and ER *in vivo*. Transfection experiments in CV-1 cells were performed using an expression vector encoding a full-length ER fused to the bacterial tetracycline repressor DNA binding domain (pTetR–ER) and a reporter plasmid consisting of a luciferase gene, under the control of the bacterial tetracycline operon, linked to a cytomegalovirus minimal promoter (pTetO-luc) (22). The expression plasmid encoding the fusion protein of p300 and the acidic activation domain of VP16 has been previously described (19) and was transfected at the indicated concentrations. An internal control plasmid (CMV–β-gal) was cotransfected to correct for transfection efficiency. Data were normalized as the ratio of raw light units to β-gal units. Results are expressed as fold stimulation above untreated samples. The data presented are from one of three representative experiments. (*D*) Wild-type p300 acts as a coactivator of ligand-dependent ER-mediated transactivation. Transfection experiments were performed as in *C*, replacing the CMV–p300–VP16 plasmid with the wild-type CMV–p300 CHA counterpart. Data are expressed as described in *C* and represent the mean of three independent experiments ± SEM.

two-hybrid system (Fig. 3*B*) in which a tetR–ER fusion expression vector was cotransfected with the ptetO-luciferase reporter (18). When these plasmids were cotransfected with increasing levels of a p300–VP16 expression plasmid, enhancement of the level of activation was seen with the tetR-ER fusion only in the presence of E2 (Fig. 3*C*). Significantly, 4-hydroxytamoxifen was able to block the interaction even at the highest level of cotransfected p300–VP16. These results confirm the agonist-dependent *in vivo* association of p300 and ER.

The ability of p300 and ER to associate *in vivo* coupled with previous studies showing that p300 can function as a CREB coactivator (23) led us to test directly whether p300 could serve to augment ligand-dependent transcriptional activation by ER. In these experiments, the ptetO-luciferase reporter was cotransfected with subthreshold levels of the tetR–ER expression plasmid in the presence of increasing amounts of a p300 expression vector (Fig. 3*D*). Under these conditions, p300 significantly increased E2-induced activation by ER, suggesting that the association of p300 with the agonist bound ER complex serves to potentiate the estrogen signal.

DISCUSSION

The data presented here support a model in which ER transmits agonist binding signals through a complex of proteins including p300/CBP. Far Western blot analysis revealed the presence of ERAP160 as an intermediary factor in this interaction. In addition, antiserum raised against the cloned coactivator SRC1 recognizes ERAP160. The presence of a constitutive, *in vivo* interaction between p300 and ERAP160/SRC1 was demonstrated by immunoprecipitation of p300 followed by ER Far Western blot as well as by the immunoprecipitation of SRC1 followed by anti-p300 Western blot. The *in vivo* association between ER- and p300-containing complexes was confirmed using a mammalian two-hybrid system. Finally, overexpression of p300 augmented the ability of ER to activate a reporter in response to E2 in a cotransfection assay. These results suggest hormone-stimulated activation by the estrogen receptor involves recruitment of a coactivator complex that includes ERAP160/SRC1 and p300/CBP. In addition p300/CBP functions as a coactivator for a number of transcription factors regulated by discrete cellular signaling pathways including the 12-*O*-tetradecanoylphorbol 13-acetate (TPA)-regulated factor AP-1 (24) and the cAMP-regulated factor CREB (23). Also, p300 has been shown to interact with the general transcription machinery perhaps as part of the RNA polymerase II holoenzyme (16, 17). Taken together these results suggest a model (Fig. 4) in which p300/CBP functions as an integrator of transcriptional regulatory signals from multiple pathways including estrogen receptor.

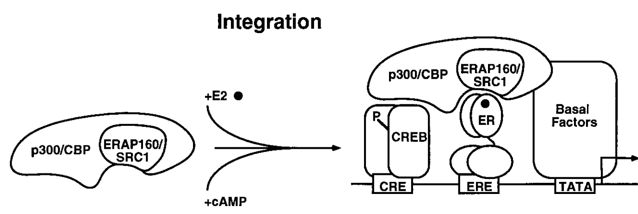

FIG. 4. Model of the potential role of p300/CBP and ERAP160/SRC1 in the integration of steroid hormone signals with other pathways. ERAP160 is in a complex with p300 in the absence of exogenous signals. Binding of E2 to ER promotes the recruitment of this complex through a direct interaction with ERAP160/SRC1 and perhaps p300. p300 directly interacts with transcription factors that are activated by diverse cellular signaling pathways such as the cAMP signals mediated by phospho-CREB (23) and with components of the basal transcription machinery (16, 17). Thus, p300 may play a role as an integrator of signals from multiple pathways including ER.

Recently, Kamei *et al.* (8) reported studies that independently reach similar conclusions to those presented here. Interestingly, these investigators found that p300/CBP interacts both directly with the nuclear receptors and indirectly through ERAP160/SRC1. While our studies do not rule out a direct interaction between p300 and ER, our data support a model in which ER interacts directly with ERAP160/SRC1, which exists as part of a preformed complex that includes p300/CBP. Future studies will determine the relative importance of direct versus indirect interactions between ER and p300/CBP in mediating ligand-dependent activation.

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