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PRINCIPAL INVESTIGATOR: David Lonard, Ph.D.
B. O'Malley, Ph.D.

CONTRACTING ORGANIZATION: Baylor College of Medicine
Houston, Texas 77030-3498

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<td>Breast cancer patients who possess cancers that are estrogen-dependent usually respond well initially to the antiestrogen, tamoxifen. However, the cancer subsequently becomes resistant to tamoxifen, possibly through increases in cAMP and protein kinase A or through stimulation of the MAP kinase pathway, which have been associated with the conversion of the tamoxifen metabolite, 4-hydroxytamoxifen (4HT), into an estrogen receptor-α (ER) agonist. Tamoxifen resistance may also occur through cellular alterations in the balance of coactivators and corepressors whose protein levels may be regulated by the ubiquitin-proteasome protein degradation pathway. The relationship between phosphorylation of ER, its response to 4HT and its proteasome-mediated degradation was examined in transiently transfected immortalized cell lines. An inhibitor of the proteasome, MG132, interferes with ER-mediated transcription and blocks the agonist activity of 4HT. MG132 also stabilizes ER and prevents its ligand-mediated down-regulation. Phosphorylation-defective ER mutants were found to be more stable than the wild-type receptor suggesting that receptor phosphorylation plays a role in regulating its stability which has been observed for other transcription factors. Mutation of coactivator-binding surfaces in the AF-2 of the receptor also influenced the stability of the receptor, suggesting that a coactivator(s) promotes ER degradation through their association with the liganded receptor. These results provide evidence that the ubiquitin-proteasome protein degradation pathway plays an important role in ER function and its response to tamoxifen.</td>
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INTRODUCTION

Roughly two-thirds of breast cancer patients possess cancers that are estrogen-dependent and respond well to the antiestrogen, tamoxifen, at least initially. However, tumor resistance to tamoxifen occurs over time, nullifying the effectiveness of one of the most common forms of treatment for estrogen-dependent breast cancer. The purpose of this research is to elucidate the mechanism through which phosphorylation of the estrogen receptor-α (ER), coactivator association with the receptor, and the ubiquitin-proteasome pathway impinge upon ER function and its response to agonist and antagonist ligands. Evidence suggests that stimulation of the cAMP/protein kinase A and growth factor/MAP kinase pathways may contribute to the conversion of tamoxifen from an antagonist into an agonist. Additionally, the balance of coactivators and corepressors might contribute to the receptor’s response to 4-hydroxytamoxifen (4HT) in cell culture. Based upon results which were described in previous annual reports involving the identification of the ubiquitin-protein ligase E6-AP as a nuclear hormone receptor coactivator, the potential for the ubiquitin-proteasome protein degradation pathway to play a role ER function and the receptor’s response to 4HT has been examined in greater detail. The ubiquitin-proteasome system was shown to affect the qualitative response to tamoxifen, abrogating its partial agonist activity. Furthermore, phosphorylation of the receptor appears to contribute to its ligand-mediated down-regulation as mutation of ligand-dependent serine phosphorylation sites in the N-terminus of ER block its ligand-mediated down-regulation. New data discussed in this final report identifies the contribution that the C-terminal ligand-binding domain of ER plays in its ligand-mediated down-regulation. This data suggests that coactivator binding to the receptor promotes ligand-mediated down-regulation. Research performed under this fellowship has allowed for a better understanding of how the ubiquitin-proteasome protein degradation system impinges upon the estrogen receptor and contributes to the receptor’s ability to direct expression of estrogen-responsive genes.
Phosphorylation of ER has been implicated in its ligand-independent activation of estrogen responsive genes and in the conversion of 4HT from an antagonist into an agonist. Data presented in this final report expands upon these observations to further characterize how the ubiquitin-proteasome protein degradation pathway contributes to coactivator function and the ER response to 4HT. It is known that phosphorylation is often linked to protein degradation and that ER is hyperphosphorylated in the presence of estradiol concomitant with its preferentially degradation in the presence of estradiol. The identification of the ubiquitin-protein ligase, E6-AP, as a coactivator for nuclear hormone receptors, suggested that ubiquitination and protein degradation might represent a novel mechanism to modulate steroid receptor function and the response to estradiol and 4HT. Data presented in previous reports indicates that the ubiquitin-proteasome protein degradation pathway has an impact on both receptor coactivation by E6-AP and SRC-1 and for the qualitative response of the receptor to 4HT. A detailed account of the characterization of E6-AP as a nuclear hormone receptor coactivator is provided in the Nawaz et al. (1999a) article appended to this report.

The E6-associated protein (E6-AP) which was previously characterized as a ubiquitin-protein ligase, was identified to be a nuclear hormone receptor coactivator in our laboratory. This led to the investigation of how ubiquitin-proteasome mediated protein degradation might impact the coactivation function of E6-AP. However, a mutant form of E6-AP which lacks ubiquitin-protein ligase activity was still able to function as a coactivator of ER and other nuclear hormone receptors. In spite of this, inhibition of the proteasome did have an impact on coactivator function and was also able to interfere with ER transactivation. In HepG2 cells, the effect of proteasome inhibitors on ER’s response to estradiol and 4HT was examined and it was found that MG132 preferentially interfered with the agonist activity of 4HT. This behavior was similar to that observed for phosphorylation-defective mutants of ER which were also devoid of 4HT agonist activity.

Data presented in previous reports expanded upon these findings. To confirm that the ubiquitin-proteasome pathway is involved in receptor down-regulation, ER-transfected HeLa cells were treated with estradiol or its control vehicle in the absence or presence of MG132 for 24 hours. Western analysis revealed that MG132 was able to stabilize ER. Without MG132, ER was preferentially degraded in the presence of estradiol while treatment with MG132 was able to block ligand-mediated degradation. A detailed account of this data is provided in the Nawaz et al. (1999b) article appended to this report. In spite of the fact that ER is stabilized in MG132-treated cells and is thus present at higher steady-state levels, ER-mediated transcription is impaired. It is possible that ER degradation is coupled to its ability to stimulate transcription or that a transcriptional repressor must be degraded for ER-mediated transcription to proceed.

To assess what role phosphorylation might have on receptor stability, vectors for phosphorylation-defective ER mutants were transfected into HeLa cells and the cells were treated with estradiol and MG132. A number of mutant ER’s were tested, ER
S118A, ER S167A and ER S104A/S106A/S118A in which serine residues at the indicated ligand-dependent serine phosphorylation sites have been converted to the non-phosphorylatable residue, alanine. Estradiol treatment was unable to down-regulate these mutant receptors. Furthermore, the amount of receptor present in MG132 treated and untreated cells were essentially equal, indicating that these mutants are not as susceptible to proteasome-mediated degradation as the wild-type receptor.

In an effort to identify which regions of ER are targeted by the ubiquitin-proteasome protein degradation pathway, a number of truncated forms of ER were expressed in HeLa cells and the protein level of each truncated species in the presence and absence of MG132 was compared to determine their relative stability. An ER mutant expressing the first 282 amino acids which includes the AF-1 and DNA-binding domain of the receptor possessed a stability similar to the full-length receptor, although its degradation was not influenced by estradiol. Truncated forms of the receptor lacking the first 86 or 108 amino acids from the N-terminus also possessed a similar stability as the wild-type receptor. However, a truncation lacking the first 178 amino acids in which the entire region of the receptor upstream from the DNA-binding domain has been removed was very unstable in HeLa cells and MG132 was able to dramatically stabilize this truncated receptor. This region includes the major ligand-dependent phosphorylation sites at positions 104 and/or 106, 118, and 167. These data suggest that the region of the receptor between amino acids 87 and 179 are important for conferring stability to ER and that phosphorylation of serine residues in this region disrupts the protective function imparted by this region. This also implies that phosphorylation of serine residues in this region are not serving to directly recruit components of the ubiquitin-proteasome protein degradation machinery to the receptor, otherwise deletion of this phosphorylated region would have rendered the truncated receptor more stable.

In a previous report, it was indicated that MG132 treatment disrupted ER transactivation. To further characterize the role the proteasome may be playing in transcription, a number of experiments were performed. First, to assess whether MG132 affected transcription factors which are not part of the nuclear hormone receptor superfamily, the ability of the constitutive transcription factors Sp1 and E2F to activate transcription from a reporter construct in the absence or presence of MG132 were assessed. Transcription mediated though Sp1 and E2F was moderately stimulated in the presence of MG132. Possibly, MG132 is stabilizing a component of the transcription machinery utilized by these transcription factors which augments their ability to mediate transcription or is stabilizing the transcription factors themselves. The effect of MG132 on CMV and RSV promoters was also tested by assessing the expression of β-galactosidase or luciferase driven from these promoters. MG132 resulted in a modest 10 to 20% increase in their activity, indicating that CMV and RSV promoter are not greatly affected by disruption of proteasome function.

Because of the inhibitory effect MG132 had on ER-mediated transcription, it was reasoned that this may be due to increased stability of nuclear hormone receptor corepressors which might interfere with the ability of ER to stimulate transcription. It has been shown that N-CoR is stabilized in the presence of MG132 and can enhance
repression by unliganded RAR and TR. When a FLAG-tagged N-CoR was overexpressed in HeLa cells, MG132 treatment was able to greatly stabilize this protein, suggesting that its accumulation could interfere with ER-mediated transcription.

The observation that MG132 is able to preferentially abolish the agonist activity of 4HT in HepG2 cells suggests that proteasome inhibitors may be of clinical value in reversing tamoxifen antagonist/agonist switching. The ability of another proteasome inhibitor, PS-341, which is currently being tested in phase I clinical trials as a chemotherapeutic agent for a variety of cancers, was tested for its ability to also block tamoxifen antagonist/agonist switching. PS-341 was able to selectively block 4HT agonist activity in HepG2 cells like MG132. PS-341 was also able to stabilize ER and N-CoR. These results suggest that PS-341 may possess potential value as a chemotherapeutic agent for abrogating tamoxifen agonist activity in women receiving long-term tamoxifen therapy.

Results presented in last year’s report indicate that phosphorylation of ER is linked with its ligand-mediated degradation by the ubiquitin-proteasome protein degradation pathway, and that receptor phosphorylation, protein degradation and tamoxifen antagonist/agonist switching are interconnected. Further work remains to be done to address whether degradation of ER itself is necessary for transcription to occur. It is possible that after ER recruits other components of the preinitiation complex, the receptor or other members of this complex must be degraded in order to release the RNA polymerase from the promoter for the subsequent RNA elongation step to occur.

In this final report, the contribution of the C-terminus of ER to its ligand-mediated down-regulation is discussed. Deletion of the last 61 amino acids of ER, including residues that form helix 12 of the ligand binding domain, abolishes ligand-mediated down-regulation of the receptor. Point mutations to critical coactivator binding residues located in helix 3, 5, or 12 of the ligand-binding domain also abolished ligand-mediated down-regulation, suggesting that coactivator binding to the ligand-binding domain of the receptor is critical for ligand-mediated down-regulation of the receptor to occur. Binding of ER to DNA is unnecessary for ligand-mediated down-regulation, suggesting that coactivator binding to the receptor is sufficient for it to be down-regulated. The impact of the ubiquitin-proteasome pathway on ER and other nuclear hormone receptors’ ability to stimulate transcription was also explored in more detail. Transcription mediated through the progesterone receptor, thyroid hormone receptor and estrogen receptor-β was attenuated in the presence of MG132 while the human glucocorticoid receptor was unaffected. The coactivators SRC-1, TIF2, RAC3, and CBP were also identified as targets of the proteasome as the steady state level of these proteins was shown to be up-regulated in the presence of MG132. Interestingly, the intrinsic transcriptional activity of each of these coactivators was not impaired in the presence of MG132. A detailed account of this work is provided in the Lonard et al. (2000) article appended to this report.
KEY RESEARCH ACCOMPLISHMENTS

- identification of a role for the proteasome in ER-mediated transcription

- ligand-mediated degradation of ER is mediated through the proteasome

- phosphorylation-defective mutant forms of ER are more stable, indicating that phosphorylation influences receptor stability

- Both the N- and C-terminal portions of ER are subject to proteasome-mediated degradation

- The region of the ER between amino acids 87 and 179 which encompasses the major ligand-dependent phosphorylation sites confers stability to the receptor

- PS-341, a proteasome inhibitor which is currently in phase I clinical trials for the treatment of a variety of cancers, can also preferentially abolish the agonist activity of 4-hydroxytamoxifen in HepG2 cells

- Coactivator-binding surfaces in the C-terminus of ER are critical for ligand-mediated down-regulation of the receptor

- Coactivators are also targets of the proteasome, although their intrinsic transcriptional activity is not impaired when the proteasome is disabled
REPORTABLE OUTCOMES

Published manuscripts:


Abstracts:


CONCLUSIONS

Data presented here implicates the ubiquitin-proteasome protein degradation system in ER-mediated transcription and the control of the receptor's steady state level. A number of enzymatic activities have already been associated with nuclear hormone receptor-mediated transcription, including histone acetyltransferase and kinase activities. The identification of members of the ubiquitin-proteasome protein degradation system such as E6-AP, RPF1, TRIP1/Sug1 and UBC9 as receptor-interacting proteins has suggested that protease activity is also involved in nuclear hormone receptor function. Research conducted during this fellowship has led to a better characterization of how ubiquitin-proteasome mediated protein degradation impinges upon ER-mediated transcription and what events in the activation of the receptor by ligand contribute to its estradiol-mediated down-regulation. The mechanism through which the proteasome is involved in ER-mediated transcription is unknown and will require further research. One possibility is that proteasome-mediated degradation of ER or coactivators is required for an exchange with other coactivator or coactivator complexes which must occur for efficient ER-mediated transcription. Alternatively, the degradation of coregulators may be necessary for release of RNA polymerase from the pre-initiation complex. Data presented in this report suggests that coactivator binding to the ligand-binding domain of ER promotes the receptor’s down-regulation. It is possible that the responsible protein may be one of the receptor-interacting proteins already identified as a component of the ubiquitin-proteasome protein degradation system. The role of phosphorylation as a signal for protein degradation has been established for a number of proteins and data presented here suggests that ER is similarly down-regulated when phosphorylated. Thus, phosphorylation of the receptor should be considered as an additional requirement for ligand-mediated down-regulation of the receptor. The ability of proteasome inhibitor compounds to selectively block the agonist activity of 4HT in HepG2 cells suggests that these compounds may also be useful in a clinical setting to combat resistance to tamoxifen therapy. Consistent with this observation is the finding that proteasome inhibitors can also lead to an increase in the steady-state level of the N-CoR corepressor which has been shown to also block 4HT agonist activity.
REFERENCES


BIBLIOGRAPHY


APPENDICES

1. Attached article: Nawaz et al. (1999a)

2. Attached article: Nawaz et al. (1999b)

3. Attached article: Lonard et al. (2000)
The Angelman Syndrome-Associated Protein, E6-AP, Is a Coactivator for the Nuclear Hormone Receptor Superfamily

ZAFAR NAWAZ,1 DAVID M. LONARD,1 CAROLYN L. SMITH,1 EFRAT LEV-LEHMAN,2 SOPHIA Y. TSAI,1 MING-JER TSAI,1,2 AND BERT W. O'MALLEY1*2

Department of Cell Biology and Department of Molecular and Human Genetics,1 Baylor College of Medicine, Houston, Texas 77030

Received 6 August 1998/Returned for modification 9 September 1998/Accepted 27 October 1998

In this study, we found that the E6-associated protein (E6-AP/UBE3A) directly interacts with and coactivates the transcriptional activity of the human progesterone receptor (PR) in a hormone-dependent manner. E6-AP also coactivates the hormone-dependent transcriptional activities of the other members of the nuclear hormone receptor superfamily. Previously, it was shown that E6-AP serves the role of a ubiquitin-protein ligase (E3) in the presence of the E6 protein from human papillomavirus types 16 and 18. Our data show that the ubiquitin-protein ligase function of E6-AP is dispensable for its ability to coactivate nuclear hormone receptors, showing that E6-AP possesses two separable independent functions, as both a coactivator and a ubiquitin-protein ligase. Disruption of the maternal copy of E6-AP is correlated with Angelman syndrome (AS), a genetic neurological disorder characterized by severe mental retardation, seizures, speech impairment, and other symptoms. However, the exact mechanism by which the defective E6-AP gene causes AS remains unknown. To correlate the E6-AP coactivator function and ubiquitin-protein ligase functions with the AS phenotype, we expressed mutant forms of E6-AP isolated from AS patients and assessed the ability of each of these mutant proteins to coactivate PR or provide ubiquitin-protein ligase activity. This analysis revealed that in the majority of the AS patients examined, the ubiquitin-protein ligase function of E6-AP was defective whereas the coactivator function was intact. This finding suggests that the AS phenotype results from a defect in the ubiquitin-proteosome protein degradation pathway.

Steroids, thyroid hormones, vitamin D, and retinoids regulate diverse biological processes including growth, development, and homeostasis through their cognate nuclear hormone receptors, which make up a superfamily of structurally related intracellular ligand-activated transcription factors (18, 34, 40, 47). Nuclear hormone receptors contain common structural motifs which include a poorly conserved amino-terminal activation function (activation factor 1 [AF-1]) that affects transcription efficiency, a central DNA-binding domain, which mediates receptor binding to specific DNA enhancer sequences and determines target gene specificity, and a carboxy-terminal hormone-binding domain. The latter domain contains AF-2, a region which mediates the hormone-dependent activation function of receptors (40). When bound to hormones, these receptors undergo a conformational change, dissociation from heat shock proteins, receptor dimerization, phosphorylation, DNA binding at an enhancer element of the target gene, interaction with coactivators, and subsequent recruitment of basal transcription factors to form a stable preinitiation complex. These events are followed by either up-regulation or down-regulation of target gene transcription (40).

Nuclear hormone receptor coactivators represent a growing class of proteins which interact with receptors in a ligand-specific manner and serve to enhance their transcriptional activities (33). Prior to their identification, coactivators were predicted to exist based on experiments which showed that different receptors compete for a limiting pool of factors required for optimal transcription. Stimulation of one receptor resulted in transrepression of another receptor, indicating the depletion of a common coactivator pool (6, 10, 31, 39). Among the coactivators cloned to date are steroid receptor coactivator 1 (SRC-1) (33), TIF2 (GRIP1) (17, 51), p/CIP (ACTR/RAC3/AIB1/TRAM-1) (2, 9, 28, 46, 48), and ARA70 (54). Coactivators were originally envisioned to serve a bridging role, linking the receptor to the basal transcription machinery (36, 45). Recently, they were shown to possess enzymatic activities which contribute to their ability to enhance receptor-mediated transcription; SRC-1, p300/CBP, and RAC3/ACTR/AIB1 possess histone acetyltransferase activity (HAT) (2, 9, 28, 32, 41). Ligand-activated receptors are thought to bring these HAT activity-containing coactivators to the chromatin surrounding the receptor, disrupting the local repressive chromatin structure by acetylating histones and possibly other chromatin-associated factors (41). Because of their ability to enhance receptor-mediated gene expression, coactivators are thought to play an important role in regulating the magnitude of the biological response to steroids, vitamin D, and retinoids in different tissues or individuals. The level of coactivator expression may contribute to variations in hormone responsiveness seen in the population, and disruption in coactivator expression could lead to the pathological hyper- or hyposensitivity to steroid hormones. Recently, it was shown that disruption of the SRC-1 locus in mice resulted in an attenuated response to steroid hormones, a finding consistent with this hypothesis (53).

In this report, we describe the cloning and characterization of E6-associated protein (E6-AP) (21), a protein linked to Angelman syndrome (AS) (26, 30, 42), as a progesterone receptor (PR)-interacting protein. E6-AP was previously identified as a protein of 100 kDa, present in both the cytoplasm and the nucleus (14). E6-AP mediates the interaction of human papillomavirus type 16 and 18 E6 proteins with p53, a growth-suppressive and tumor-suppressive protein (14, 22). Initial in
vitro studies suggested that the E6–E6-AP complex specifically interacts with p53 and promotes the degradation of p53 via the ubiquitin-proteasome degradation pathway, but recent in vivo studies show that E6-AP can directly interact with p53 and promote its degradation even in the absence of the papilloma-

virus E6 protein (11, 20, 38). E6-AP is a member of a family of proteins, known as E3 ubiquitin-protein ligases, which have been proposed to play a role in defining the substrate spe-

tric activity of the ubiquitin-proteasome degradation system. PAG6 (or ubiquitin-conjugating enzymes) is composed of two subunits: ubiquitin-conjugating enzymes and ubiquitin-modifying enzymes, which activate ubiquitin moieties and transfer them to target proteins and E3, respectively (19). The carboxyl-terminal 350 amino acids (aa) of E6-AP constitute a heat (homologous to the E6-AP carboxy terminus) domain which is conserved among many E3 ubiquitin-protein ligases and E6-AP-related proteins (19). The extreme carboxy-terminal 100-

aa segment contains the catalytic region of E6-AP, which transfers ubiquitin to the protein targeted for degradation (19). The E6-binding domain consists of an 18-aa region located within the central portion of the E6-AP protein (22).

Recently, it was shown that a genetic disorder, AS, is caused by the absence of a functional maternal copy of the E6-AP gene (16). AS is a neurodevelopmental disorder characterized by severe mental retardation, seizures, speech impairment, and other symptoms (5). However, the exact mechanism by which the defective E6-AP gene causes AS remains unknown. Our analysis of mutant E6-AP proteins from AS patients revealed that the ubiquitin-protein ligase function of E6-AP was defective, whereas the coactivator function was intact, in the major-

ity of AS patients examined. In this report, we also show that the ubiquitin ligase activity of E6-AP is not required for the coactivating function of E6-AP. Furthermore, our data indi-

cate that the catalytic function located within the Nect domain of E6-AP is not necessary for the ability of E6-AP to interact with and coactivate steroid hormone receptor function. These findings suggest that E6-AP possesses two independent func-

tions, as both a coactivator and a ubiquitin-protein ligase.

MATERIALS AND METHODS

Plasmid construction. The plasmid used for the yeast two-hybrid system (pAS1-PRLBD) (33), mammalian expression plasmids for PR-B (1), estrogen receptor (ER) (7), and androgen receptor (AR) (44), E2F reporter plasmid UAS-TATA-LUC (LUC denotes luciferase) (41), and E2F, Sp1, and CREB-

related response elements (CREBRES) have been previously described. To construct the pro-

cortisol receptor (GR) expression vector, the pSTCGR vector was digested with BamHI and then the BamHI fragment containing the GR cDNA was cloned into the corresponding sites of plasmid pCR3.1 (Invitrogen). pPRE/GR:

E1b-LUC and pERE/E1b-LUC were constructed by inserting PstI-SmaI frag-

ments of pPRE/EREb.CAT and pERE/E1b.CAT into the SmaI site of pG321-based (Promega). To construct mammalian expression plasmids for wild-

type E6-AP (aa 1 to 851), 76 kDa E6-AP (aa 170 to 851), and C3358 (change of cysteine 833 to serine) mutant E6-AP (aa 1 to 851), the BamHI/HindIII frag-

ments of pGEM E6-AP (100 kDa), gGEM-6-AP (76 kDa), and pGEM E6-

AP(C3358-S) were cloned into the corresponding sites of plasmid pBK-RSV (Stratagene). The C-terminal fragment of E6-AP (aa 680 to 851), the truncated mutant E6-AP (aa 1 to 849), and the 99-kDa (aa 1 to 845), 86-kDa (aa 1 to 714), 47-kDa (aa 450 to 851), and 24-kDa (aa 1 to 240) forms of E6-AP were amplified by PCR with the following primer pairs: 5'-GCGGATCCATCGGAGGATCCGCGGACAGTGATACCTAAGAAGGA-3' (upper strand) and 5'-GGGCAATGGTCAACGTCTTAAAGGTT4 (lower strand); 5'-GGGCAATGGTCAACGTCTTAAAGGTT4 (lower strand) and 5'-GGGCAATGGTCAACGTCTTAAAGGTT4 (lower strand); and 5'-GGGCAATGGTCAACGTCTTAAAGGTT4 (lower strand) and 5'-GGGCAATGGTCAACGTCTTAAAGGTT4 (lower strand).

RESULTS

Isolation and characterization of E6-AP as a PR-interacting protein. To identify novel proteins which selectively modulate the transactivation functions of members of the nuclear recep-

tor superfamily, we screened a HeLa cDNA library by using the ligand-binding domain of PR as a bait in a yeast two-hybrid screening assay. We isolated 13 colonies which strongly in-

teracted with this domain of PR. These colonies contained cDNAs with identical sequences. A sequence similarity search in the GenBank database revealed that all colonies encoded the carboxyl-terminal aa 680 to 851 of the E6-AP (see Fig. 2A).

Full-length E6-AP interacts with the ligand-bound form of PR both in vivo and in vitro. As shown in Fig. 1A, in a yeast two-hybrid assay, E6-AP interacts with PR-B in a ligand-dependent manner. In the absence of ligand or in the presence of the antihormone compound RU486, we observed no signifi-

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E6-AP COACTIVATES NUCLEAR HORMONE RECEPTOR SUPERFAMILY

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TABLE 1. Coactivation and ubiquitin-protein ligase activities of wild-type and mutant forms of E6-AP

<table>
<thead>
<tr>
<th>Form of E6-AP</th>
<th>Coactivation</th>
<th>Ubiquitin-protein ligase activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type (aa 1–851)</td>
<td>++ ++ ++</td>
<td>+</td>
</tr>
<tr>
<td>Mutant&lt;sup&gt;1&lt;/sup&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>aa 450–851</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>aa 680–851</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>aa 1–845</td>
<td>+ + +</td>
<td>-</td>
</tr>
<tr>
<td>aa 1–834&lt;sup&gt;2&lt;/sup&gt;</td>
<td>+ + +</td>
<td>-</td>
</tr>
<tr>
<td>aa 1–714</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>R417X&lt;sup&gt;3&lt;/sup&gt;</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>aa 1–240</td>
<td>Not tested</td>
<td>-</td>
</tr>
<tr>
<td>C835S</td>
<td>++ ++ ++</td>
<td>-</td>
</tr>
<tr>
<td>I804K&lt;sup&gt;4&lt;/sup&gt;</td>
<td>++ ++</td>
<td>-</td>
</tr>
<tr>
<td>F792A&lt;sup&gt;5&lt;/sup&gt;</td>
<td>++ +</td>
<td>-</td>
</tr>
<tr>
<td>1–855stop&lt;sup&gt;6&lt;/sup&gt;</td>
<td>++ ++</td>
<td>Not tested</td>
</tr>
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<sup>1</sup> HeLa cells were transfected with 0.1 μg of PR-B expression plasmid, 1 μg of pPRE/GRE/E1bLUC, and 0.25 μg of expression plasmid for wild-type E6-AP or 0.25 μg of each of the indicated mutant forms of E6-AP. The cells were incubated with 10–7 M progesterone. Coactivation by each mutant form of E6-AP is presented as relative to coactivation by wild-type E6-AP, scored as + + + + + .

<sup>2</sup> The wild-type and mutant forms of E6-AP were expressed and purified from E. coli as GST fusion proteins, and their ubiquitin-protein ligase activities were measured with HHR23A as a target protein. +, positive for ubiquitin-protein ligase activity; –, negative for ubiquitin-protein ligase activity.

<sup>3</sup> Natural mutant form of E6-AP cloned from an AS patient.

Significant level of interaction relative to the control between E6-AP and PR. To document further that the progesterone-dependent interaction observed in the yeast two-hybrid assay is due to a direct physical association of E6-AP and PR, we purified baculovirus-expressed His-tagged PR on a nickel affinity column and then incubated it with GST–E6-AP which was purified and subsequently bound to glutathione-Sepharose beads in the absence or presence of progesterone. As a control, purified GST was incubated with PR. After extensive washing, E6-AP-bound PR was analyzed by immunoblotting using a PR-specific antibody (Fig. 1B). A significant level of E6-AP interaction with PR was observed in the presence of progesterone but not in its absence.

Regions of E6-AP required for interaction with PR. Since E6-AP interacts with PR in a hormone-dependent manner, we next defined the regions of E6-AP important for interaction with PR. For this purpose, we used an in vivo mammalian two-hybrid interaction assay system (41). In this assay, full-length E6-AP and various deletion fragments of E6-AP were fused to the VP16 activation domain (Fig. 2A), and the ability of each of the VP16–E6-AP hybrid proteins to interact with PR was determined in the absence or presence of progesterone. As shown in Fig. 2B, wild-type E6-AP, N-terminal deletion fragments (aa 170 to 851 and 680 to 851), and C-terminal deletion fragments (aa 1 to 714 and 1 to 449) of E6-AP were able to interact with PR, while the control vector lacking E6-AP cDNA and the N-terminal fragment (aa 1 to 240) of E6-AP did not interact, suggesting that at least two PR interaction sites are located within the E6-AP protein. One site is located within the C-terminal fragment (aa 680 to 851; 21 kDa) of E6-AP, the fragment originally isolated in the yeast two-hybrid screen. The second PR interaction site is located within aa 240 to 449 and overlaps the E6-binding site. Each of these regions of E6-AP interacts with both transcriptional activation factor 1 and transcriptional activation factor 2 of PR.

E6-AP as a coactivator for the nuclear hormone receptor superfamily. To investigate whether E6-AP may play a role in receptor-dependent activation of target gene expression, we performed transient cotransfection assays of HeLa cells. HeLa cells were transfected with expression vectors for PR and a reporter plasmid containing a progesterone response element with or without an expression vector for E6-AP. In the absence of ligand, PR had a minimal effect on reporter gene expression either in the absence or in the presence of E6-AP (Fig. 3A). Addition of the hormone yielded an 8-fold increase in PR activity in the absence of E6-AP; when E6-AP was coexpressed with PR, the activity of PR was further stimulated by ~5-fold, a total of 40-fold over the basal level. In contrast, coexpression of E6-AP with PR had no significant effect on the transcription of the reporter gene when receptor was bound to the antihormone compound RU486 (Fig. 3A). These data are consistent with previously published data which indicate that RU486 induces a distinct conformational change in the receptor molecule that has reduced affinity for coactivators (1, 33, 50, 52).

Since (i) HeLa cells are derived from a papillomavirus type 18-positive cervical carcinoma patient and thus express the E6 protein and (ii) E6-AP was originally cloned as an E6-interacting protein, it was necessary to rule out the possibility that the E6 protein influences the coactivation function of E6-AP. E6-AP was able to stimulate the hormone-dependent transcriptional activity of steroid hormone receptors in the E6-negative HepG2 and SK-N-SH cell lines (data not shown), suggesting that the coactivation observed in HeLa cells is not dependent on the E6 protein.
Our data suggest that E6-AP stimulates the hormone-dependent transcriptional activity of PR by acting as a coactivator. To determine if E6-AP functions as a coactivator for members of the nuclear receptor superfamily, we examined the effect of E6-AP expression on the ligand-dependent transcriptional activities of different nuclear hormone receptors and on several other transcription factors (Fig. 3B and C). E6-AP significantly enhanced the hormone-dependent transcriptional activity of PR, ER, AR, and glucocorticoid receptor (GR). It also enhanced the transcriptional activity of retinoic acid receptor alpha and thyroid hormone receptor (data not shown). E6-AP had minimal or no effect on the transcriptional activity of E2F and CREB. Coexpression of E6-AP had only a moderate effect on the activation function of Sp1 (Fig. 3C). These data suggest that E6-AP preferentially coactivates the hormone-dependent transcriptional activity of nuclear hormone receptors but is not uniquely specific for them as is the case for other coactivators such as SRC-1 (33).

E6-AP relieves squelching between ER and PR. It has been shown that ER and PR share certain coactivators since hormone-bound ER can sequester limited pools of coactivators from PR; a phenomenon known as squelching (10, 31, 39). We examined whether coexpression of E6-AP was able to reverse this squelching phenomenon. The hormone-induced transcriptional activity mediated by PR was reduced by 91% upon coexpression of estradiol-bound ER (Fig. 4A; compare lanes 2 and 3). Addition of E6-AP reversed this squelching by as much as 9.6-fold (Fig. 4A; compare lanes 3 and 6) in a dose-dependent manner. At the highest concentration of E6-AP used in this reverse squelching experiment, PR activity was enhanced only 2.6-fold (compare lanes 2 and 7). However, in control cells which do not express ER, E6-AP enhanced the transcriptional activity of PR from four- to fivefold (compare lanes 2 and 8). These data suggest that E6-AP is a limiting factor which is necessary for efficient PR and ER transactivation. The fold coactivation by E6-AP is lower in this experiment than in that shown in Fig. 3B, due to differences in experimental conditions. In Fig. 4A (lane 7), the coactivating effect of E6-AP on the transcriptional activity of PR was observed in the presence of the ER expression plasmid, whereas in Fig. 3B, only a single receptor was transfected. As expected, no significant reverse squelching was observed (Fig. 4B; compare lanes 3 and 6) with the C-terminal fragment of E6-AP (aa 680 to 851) (Fig. 2A), which weakly interacts with ER (data not shown) and has no activation function (Fig. 5). However, this fragment did not possess dominant negative activity under our experimental conditions. Western blot analysis confirmed that the C-terminal fragment of E6-AP (aa 680 to 851) and full-length E6-AP are equally expressed (data not shown).

E6-AP contains an intrinsic activation domain. To ascertain whether E6-AP possesses an intrinsic, transferrable activation domain, wild-type and deletion fragments of E6-AP were recruited to DNA by linking them to the GAL4 DBD. Wild-type E6-AP (aa 1 to 851) and the N-terminal (aa 170 to 851, 76 kDa) and C-terminal (aa 1 to 714, 86 kDa) deletion fragments stimulated the transcriptional activity of the reporter gene compared to that of the control vector containing only the GAL4 DBD (Fig. 5), while the 21-kDa fragment (aa 680 to 851) did not. This finding suggests that E6-AP itself contains a transcriptional activation domain located between aa 170 and 680.

E6-AP contains two independent, separable functions, coactivation and ubiquitin-ligase activity. Since E6-AP is a ubiquitin-protein ligase, we examined whether the coactivation function of E6-AP is dependent on this enzymatic function. It has been shown that the conserved C833 residue in E6-AP

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FIG. 2. (A) Schematic representation of E6-AP showing positions of the catalytic region (solid box), helix domain, transactivation domain, and PR-binding domain. Wild-type E6-AP is a 106-kDa (aa 1 to 851) protein: 76 kDa (aa 170 to 851) and 47 kDa (aa 450 to 851) represent E6-AP with a deletion at the N terminus. The 21-kDa (aa 680 to 851) form represents the carboxyl terminus of E6-AP identified in the yeast two-hybrid screen: 99 kDa (aa 1 to 845), 98 kDa (aa 1 to 834), 86 kDa (aa 1 to 714), 53 kDa (aa 1 to 449), and 28 kDa (aa 1 to 240) represent E6-AP C-terminal deletion mutants. C833S represents a cysteine 833-to-serine mutant form of E6-AP. The AS disease mutants are represented by 98 kDa (deletion of 17 aa from the C terminus) and 53 kDa (C-terminally truncated E6-AP) (aa 1 to 449); the 1804K form of E6-AP contains lysine at position 804 instead of isoleucine. F782A contains a deletion of phenylalanine at position 782, and the 104-kDa 1-885 stop form of E6-AP is a readthrough mutant. (B) Localization of the PR interaction site in E6-AP. To determine the PR interaction site on E6-AP, full-length E6-AP and various deletion fragments of E6-AP shown in panel A were fused in frame with the VP16 activation domain, and the ability of E6-AP to interact with PR was determined in a mammalian two-hybrid assay. HeLa cells were cotransfected with 0.3 μg of PR expression plasmid and 0.3 μg of pRE/GR/E1b/LUC in the absence (control) or presence of expression plasmid p8BP/VP16-E6-AP (0.9 μg) (aa 1 to 851 [wild-type], 170 to 851, aa 680 to 851, 1 to 714, aa 1 to 449, and aa 1 to 240). The cells were treated with either vehicle only (□) or 10-7 M progesterone (●). Data are presented as relative light units per microgram of protein, and each bar depicts the average of at least three wells.
forms a thioester bond with ubiquitin and is necessary for the transfer of ubiquitin to the protein targeted for ubiquitination. The mutation of C833 to A or S has been shown to eliminate the ubiquitin-protein ligase activity of E6-AP (19). In cotransfection experiments, an E6-AP bearing a C-to-S mutation at this critical site was still able to coactivate PR (Table 1) and ER (data not shown) to nearly the same extent as wild-type E6-AP. Furthermore, the C833S mutant form of E6-AP also can reverse squelch the hormone-dependent transcriptional activity of PR to a similar extent as wild-type E6-AP (data not shown). Our data suggest that the ubiquitin-protein ligase activity of E6-AP is not required for the coactivation function of E6-AP. To further confirm that the ubiquitin-proteasome pathway is not involved in the coactivation function of E6-AP, we analyzed a deletion mutant of E6-AP (aa 1 to 845) which lacks 5 aa at the carboxy terminus and has been shown to be defective for ubiquitin-protein ligase activity (19). Like the C833S mutant, this mutant also retains the ability to coactivate the hormone-dependent transcriptional activity of PR (Table 1), further confirming that the ubiquitin-protein ligase activity of E6-AP is not necessary for E6-AP to function as a coactivator. Our data indicate that E6-AP possesses two independent, separable functions, coactivation and ubiquitin-protein ligase activity.

The AS phenotype results from defects in the ubiquitin-protein ligase activity of E6-AP. Recently, it was shown that a subset of AS patients express mutant forms of the E6-AP, rather than possessing the more common large-scale deletions of the 15q11-q13 region which contains E6-AP (26, 30, 42). To determine if the coactivator function of E6-AP is necessary for development of the AS phenotype, we generated several mutant E6-AP proteins corresponding to those found in these AS patients (Table 1). First, we tested the effect of an E6-AP mutant with a gross deletion in which the C-terminal half of the protein had been deleted due to a nonsense mutation at codon 417 (R417X). The ability of this AS mutant protein to coactivate PR is much less than that of wild-type E6-AP, but it can still interact with PR (Fig. 2B), indicating that a loss of coactivation is due to disruption of the activation domain located at aa 170 to 680. Furthermore, the loss of coactivation by the R417X mutant is not due to the loss of expression of mutant protein, since this mutant was able to interact with PR to the same extent as wild-type E6-AP in the mammalian cells used to assess coactivation (Fig. 2B). The R417X mutant of E6-AP was also unable to coactivate ER and AR (data not shown).

We then tested another mutant form of E6-AP which contains a small deletion in the hecat domain due to a frameshift mutation which results in the truncation of the last 17 aa of the protein (aa 1 to 834) and the replacement of four different amino acids from the new reading frame. This mutant E6-AP was able to coactivate PR to the same extent as wild-type E6-AP (Table 1). Similarly, an artificial mutant which lacks 6 aa at the extreme C terminus of E6-AP (aa 1 to 845) was also able to act as a coactivator of PR activity (Table 1). We tested three other mutations for the ability to coactivate PR transcription: missense mutation I804K, in which isoleucine 804 was mutated to lysine; F782A, an internal in-frame deletion of phenylalanine 782; and 1-885 stop, a readthrough mutation which results in a longer mutant form of E6-AP. All three of these mutant forms of E6-AP were able to coactivate PR activity, suggesting that the coactivator function of E6-AP is not involved in the central nervous system phenotype of AS (Table 1).

To correlate the ubiquitin-protein ligase activity of E6-AP with AS, we tested the ubiquitin-ligase function of wild-type

FIG. 3. (A) E6-AP coactivates the transcriptional activity of PR-B. HeLa cells were transiently transfected with 0.2 μg of pPR-B expression plasmid and 1 μg of pGRE/GRE.E1b.LUC in the absence or presence of E6-AP expression plasmid pBK.RSV.E6-AP (0.250 μg). The cells were treated with either vehicle only (Δ), 10−7 M progesterone (+P), or 10−7 M RU486 (+RU). Each bar depicts the average of at least three wells. Activity in the presence of hormone and in the absence of exogenous coactivator was defined as 100%, and data for the other bars were scaled accordingly. (B) E6-AP coactivates the hormone-dependent transcriptional activity of nuclear hormone receptors. HeLa cells were transfected with receptor expression plasmids for PR, ER, AR, and GR and their cognate hormone-responsive reporter plasmids in the absence and presence of E6-AP (0.350 μg). The cells were treated with appropriate hormones as follows: PR, progesterone (10−7 M); ER, estradiol (10−7 M); AR, R1881 (2.5 × 10−10 M); and GR, dexamethasone (10−5 M). The extent of coactivation by E6-AP on the hormone-dependent transcriptional activities of various receptors ranges from four- to eightfold. The level of coactivation by E6-AP is dependent on both cell type and cell passage number (data not shown). Each bar depicts the average of at least three wells. Activity in the presence of hormone and in the absence of exogenous coactivator was defined as 100%, and data for the other bars were scaled accordingly. (C) Effect of E6-AP expression on transcriptional activities of diverse transcription factors. HeLa cells were transfected with an E2F expression plasmid (0.05 μg) along with 2.5 μg of an E2F-responsive or Sp1-responsive reporter plasmid. To test the effect of E6-AP on the transcriptional activity of the CREB transcription factor, LMTK cells were transfected with a CREB-responsive reporter plasmid (2.5 μg) in the absence or presence of E6-AP (0.25 μg). The CREB transcription factor was activated by treating cells with 10 μM forskolin (Fsk). Each bar depicts the average of at least three wells.
and AS mutant forms of E6-AP. Some AS mutant forms of E6-AP, such as the fragment comprising aa 1 to 834, R417X, and F782A, were unable to ubiquitinate a protein (HHR23A) in an in vitro ubiquitin assay (27, 49); the results suggest that loss of ubiquitin-protein ligase activity contributes to the loss of ubiquitination. However, the AS missense mutant 804K was able to ubiquitinate the target protein HHR23A to an extent comparable to that of wild-type E6-AP (Table 1).

**DISCUSSION**

Nuclear hormone receptors are ligand-induced transcription factors. To activate transcription of target genes, these receptors undergo a complex multistep activation process (18, 34, 40, 47). These steps, though required for receptor function, are not sufficient to achieve optimal receptor function. Recently, it has been shown that coactivator proteins are necessary for maximal gene activation by the receptors (40). Coactivators enhance receptor function by acting as a bridge between DNA-bound receptor and basal transcription factors of the preinitiation complex or by providing HAT activity which disrupts the local repressive chromatin structure, contributing to increased transcriptional activity of the target gene (2, 9, 28, 36, 41, 45).

In this report, we demonstrate that E6-AP protein interacts only with the liganded form of PR, both in vivo and in vitro, and that it coactivates the transcriptional activity of the hormone-bound receptors. However, E6-AP fails to interact with PR in the presence of RU486, consistent with our previously published data indicating that coactivators do not interact efficiently with receptors in the presence of antihormone both in vitro and in vivo (1, 33, 50, 52). Like other cloned coactivators, E6-AP contains LXXLL motifs, which are thought to be important for receptor interaction (15, 16). Two of these motifs are located within the amino terminus of E6-AP whereas the third is located within the carboxyl terminus, which supports our findings that E6-AP possesses receptor-interacting regions in both amino and carboxy termini.

The existence of coactivators in the signal transduction pathway of nuclear hormone receptors is supported by the finding that transcription activity of one receptor can be squelched by the overexpression of another receptor, indicating that both receptors compete for common factors. This observation led us to determine whether E6-AP is one of these limiting factors that can abrogate the squelching phenomenon (6, 10, 31, 39). Our study shows that overexpression of E6-AP in mammalian cells reverses the squelching effect of ER on PR transactivation in dose-dependent manner. These results further support the observation that E6-AP is a genuine coactivator for nuclear hormone receptors.

To date, several coactivators, e.g., SRC-1 (33), TIF2 (GRIP1) (17, 51), and p/CI (ACTR/RAC3/AIB1/TRAM-1) (2, 9, 28, 46, 48), have been cloned. These coactivators contain intrinsic activation domains and enhance the transactivation of the nuclear hormone receptor superfamily. Most of the coactivators exhibit no receptor specificity and are able to coactivate a wide variety of nuclear hormone receptors (33). Like these other
coactivators. E6-AP has an intrinsic activation domain and coactivates all nuclear hormone receptors tested.

E6-AP represents a unique class of coactivators because it exhibits ubiquitin-protein ligase activity. However, this ubiquitin-protein ligase activity is not part of the coactivator function of E6-AP. The data presented in this report indicate that E6-AP possesses two independent, separable functions, coactivation and ubiquitin-protein ligase activity. On the other hand, previously cloned coactivators such as SRC-1, p300/CBP, and RAC3/ACTR/AIB1 possess HAT activity and presumably manifest part of their in vivo coactivation function through this enzymatic activity (2, 9, 28, 32, 41). E6-AP possesses ubiquitin-protein ligase activity, instead of HAT activity, which is not a prerequisite for coactivation. This finding suggests that E6-AP works as a novel dual-function protein, orchestrating both steroid hormone receptor action and ubiquitin-proteasome-mediated degradation of p53. Another coactivator, TRIP230, has also been shown to be involved in cell cycle control by sequestering the hypophosphorylated form of the retinoblastoma protein (8).

Another potential coactivator identified in yeast and mammalian cells (RSP5/hRPF1) has been implicated as a coactivator of steroid hormone receptors that possesses a hepatocidin with 37% identity to that of E6-AP (24). UBE1, a DNA-binding protein which also contains a hepatocidin (32), is amino-terminally truncated (approximately 300 aa) compared to E6-AP and has no effect on the transactivation function of nuclear hormone receptors (data not shown), again suggesting that the hepatocidin domain alone is not sufficient for coactivation.

The results presented here for mutant E6-AP proteins identified in AS patients suggest that the transactivation function of E6-AP is not associated with the phenotypic manifestation of AS. However, our results do suggest that the AS phenotype results from a defect in the ubiquitin-protein ligase activity of E6-AP. Normally, only the maternal copy of E6-AP is expressed in certain regions of the brain, while the paternal copy is silent due to imprinting (37). However, it is still possible that gross or complete deletions of E6-AP (such as the R417X mutant) can result in defective steroid receptor coactivation in these regions of brain or other tissues where E6-AP is expressed in an imprinted manner. A more detailed analysis of the relationships among AS, E6-AP, and other nuclear hormone receptor-regulated processes awaits further investigation. Interestingly, haploinsufficiency of another nuclear hormone receptor coactivator, CREB-binding protein, is associated with Rubinstein-Taybi syndrome, a hereditary disease also characterized by diverse neurological defects (35).

In conclusion, our results demonstrate that E6-AP, a protein genetically linked to a human hereditary disease (AS), is a bona fide coactivator of nuclear hormone receptors. Although ubiquitin-proteasome pathway-mediated degradation of transcription factors recently has been shown to be important for transcriptional regulation (25, 29, 43), our experiments suggest that E6-AP’s ubiquitin-protein ligase activity is not sufficient to mediate the ability of E6-AP to coactivate nuclear hormone receptors. Nevertheless, it is possible that the ubiquitin-mediated degradation pathway(s) contributes to some aspects of nuclear hormone receptor function in vivo. E6-AP may modulate the transcriptional activity of nuclear hormone receptors by promoting the degradation of negative regulators of transcription such as corepressors. Consistent with this hypothesis, it has been shown that one of the nuclear receptor’s corepressors, N-CoR, can be degraded through the proteasome degradation pathway (55). It is also possible that subsequent to receptor activation of transcription, a mechanism is required to disassociate the preinitiation complex to allow reinitiation of transcription and elongation and ultimately to mediate the degradation of either the receptor or general transcription factors to exert tighter control of transcription. Further evidence of a link between the ubiquitin pathway and gene transcription has been suggested by a report that RSP5/hRPF1 ubiquitinates the C-terminal domain of RNA polymerase II (23). Our report represents another example of a group of coactivators for nuclear receptors whose members contain distinct coactivation and enzymatic activities.

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REFERENCES

Proteasome-dependent degradation of the human estrogen receptor

ZAFAR NAWAZ, DAVID M. LONARD, ANDREW P. DENNIS, CAROLYN L. SMITH, AND BERT W. O’MALLEY

Department of Cell Biology, Baylor College of Medicine, One Baylor Plaza, Houston, TX 77030

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ABSTRACT In eukaryotic cells, the ubiquitin–proteasome pathway is the major mechanism for the targeted degradation of proteins with short half-lives. The covalent attachment of ubiquitin to lysine residues of targeted proteins is a signal for the recognition and rapid degradation by the proteasome, a large multi-subunit protease. In this report, we demonstrate that the human estrogen receptor (ER) protein is rapidly degraded in mammalian cells in an estradiol-dependent manner. The treatment of mammalian cells with the proteasome inhibitor MG132 inhibits activity of the proteasome and blocks ER degradation, suggesting that ER protein is turned over through the ubiquitin–proteasome pathway. In addition, we show that in vitro ER degradation depends on ubiquitin-activating E1 enzyme (UBA) and ubiquitin-conjugating E2 enzymes (UBCs), and the proteasome inhibitors MG132 and lactacystin block ER protein degradation in vitro. Furthermore, the UBA/UBCs and proteasome inhibitors promote the accumulation of higher molecular weight forms of ER. The UBA and UBCs, which promote ER degradation in vitro, have no significant effect on human progesterone receptor and human thyroid hormone receptor β proteins.

The ubiquitin–proteasome pathway is the major system in the eukaryotic cell for the selective degradation of short-lived regulatory proteins (1, 2). A common feature of proteasome-mediated protein degradation is the covalent attachment of ubiquitin, a highly conserved 8.6-kDa protein, to lysine residues of proteins targeted for degradation following the formation of polyubiquitin chains attached covalently to the targeted protein. Ubiquitinated proteins are recognized and degraded by the multi-subunit protease complex, the 26S-proteasome (3–6). In addition to the role it plays in protein degradation, ubiquitination may serve regulatory functions such as directing the subcellular localization of proteins (3, 4). The ubiquitin–proteasome pathway also plays an important role in various cellular processes such as cell-cycle regulation, signal transduction, differentiation, antigen processing, and degradation of tumor suppressors (3, 4, 7–11).

Protein ubiquitination involves three classes of enzymes, namely the E1 ubiquitin-activating enzyme (UBA), E2 ubiquitin-conjugating enzymes (UBCs), and E3 ubiquitin–protein ligases. The UBA first activates ubiquitin in an ATP-dependent manner. The activated ubiquitin then forms a thioester bond between the carboxyl-terminal glycine residue of ubiquitin and a cysteine residue of the UBA. Next, ubiquitin is transferred from the E1 to one of the several E2s (UBCs), preserving the high-energy thioester bond (1, 2, 5, 11). In some cases, ubiquitin is transferred directly from the E2 to the target protein through an isopeptide bond between the ε-amino group of lysine residues of the target protein and the carboxyl terminus of ubiquitin. In other instances, the transfer of ubiquitin from UBCs to target proteins proceeds through an E3 ubiquitin–protein ligase intermediate (12, 13). It has been proposed that the biological specificity of the ubiquitin pathway is modulated by the selective combination of UBCs and E3 proteins. To date, more than 30 UBCs and 25 E3 proteins have been identified (7, 14).

Recent studies from our laboratory and others suggest that the ubiquitin-conjugating enzyme, UBC9, and the E3 ubiquitin–protein ligases, E6-associated protein and RNF1/RSP5, interact with members of the nuclear hormone receptor superfamily and modulate their transactivation functions (15–18). Similarly, yeast SUG1, an ATPase subunit of the 26S-proteasome complex, also interacts with and modulates nuclear hormone receptor functions (19–22). These studies suggest a possible regulatory role for the ubiquitin–proteasome pathway in nuclear hormone receptor-mediated gene activation.

The stability of the human estrogen receptor (ER) is modulated by its ligand, estradiol. In the absence of estradiol, the half-life of ER is ~5 days, but only 3–4 hr in the presence of estradiol (23, 24). Because the ES protein has a short half-life in the presence of ligand (24), it is possible that the receptor itself would be a target of the ubiquitin–proteasome degradation pathway. In fact, a previously published study suggests that the ER protein in uterus may be ubiquitinated (23). However, not all members of the steroid hormone receptor superfamily are similarly regulated. For example, the progesterone receptor (PR) and glucocorticoid receptor are reported to have longer half-lives (~20–25 hr) regardless of the presence of ligand (25, 26).

In this report, we now show that ER is degraded in a hormone-dependent manner and the proteasome inhibitor, MG132, promotes the in vivo accumulation of ER and blocks hormone-induced receptor degradation. We demonstrate that ER is degraded in vitro and that this degradation depends on UBA and UBC enzymes of the ubiquitin pathway and the proteasome inhibitors, MG132 and lactacystin, block ER degradation in vitro. Furthermore, the UBA/UBCs, regardless of the presence of proteasome inhibitors, promote the accumulation of higher molecular weight forms of ER. Our data also indicate that the ubiquitin pathway enzymes that facilitate ER degradation are unable to promote the degradation of PR and human thyroid hormone receptor β (TR) under similar experimental conditions and suggest that specific complexes of UBA and UBCs may target different nuclear receptors for degradation.

MATERIALS AND METHODS

Plasmid Constructs. The mammalian expression plasmid for ER (27), the in vitro expression plasmids for ER, PR, and

Abbreviations: ER, human estrogen receptor; UBA, ubiquitin-activating E1 enzyme; UBCs, ubiquitin-conjugating E2 enzymes; PR, human progesterone receptor; TR, human thyroid hormone receptor β; DMSO, dimethyl sulfoxide; NEDD8, neural precursor cell-expressed developmentally down-regulated; TNT, in vitro transcription and translation.

*To whom reprint requests should be addressed. e-mail: berto@bcm.tmc.edu.
TR (27, 28), the bacterial expression plasmids of Arabidopsis thaliana UBA1 (29), and expression plasmids of various UBCs [UbcH5B (30) and UbcH7 (31)] have been described previously. The estrogen-responsive reporter plasmid, pERE.E1b.LUC, was constructed by ligating a PvuII–SmaI fragment of pERE.E1B.CAT into the SmaI site of the pGL3 Basic plasmid (Promega).

**Transfections.** HeLa cells were maintained in DMEM supplemented with 10% fetal bovine serum. Twenty-four hours before transfection, 3 × 10^5 cells per well were plated in six-well Falcon dishes in phenol red-free DMEM containing 5% dextran-coated charcoal-stripped serum. Cells were transfected with 4 ng of ER expression plasmid and 750 ng of the estrogen-responsive reporter plasmid by using Lipofectamine (Life Technologies, Grand Island, NY), according to the manufacturer's recommended guidelines. Cells were washed and fed with phenol-red free DMEM containing 5% charcoal-stripped serum and subsequently treated with 10^-9 M estradiol (E2) and 1 μM proteasome inhibitor. MG132 (Sigma). As a control, cells were treated with dimethyl sulfoxide (DMSO) both in the absence and presence of estradiol. After 24 hr, cells were harvested and cell extracts were prepared for ER protein analysis.

**Analysis of ER Protein Levels.** To analyze the ER protein levels, transfected cells were harvested and lysed in ER extraction buffer [50 mM Tris-HCl (pH 8.0)/5 mM EDTA/1% Nonidet P-40/0.2% Sarkosyl/0.4 M NaCl/100 μM sodium vanadate/10 mM sodium molybdate/20 mM NaF]. Subsequently, 40 μg of protein extracts was loaded and resolved by 7.5% SDS/PAGE, and then transferred to a nitrocellulose membrane. The nitrocellulose membrane was incubated in a blocking buffer [50 mM Tris-HCl (pH 7.5)/150 mM NaCl/0.5% Tween 20/1% dried nonfat milk] for 1 hr at room temperature. Then the membrane was incubated with the H222 antibody, which specifically recognizes the ER protein. After extensive washing, the membrane was first incubated with rabbit anti-rabbit antibody and then with horseradish peroxidase-conjugated goat-anti-rabbit IgG, and ER protein levels were visualized with the ECL+Plus Western blotting detection system (Amersham).

**Bacterial Expression of Ubiquitin Pathway Enzymes.** A. thaliana UBA1 (pET), and ubiquitin-conjugating enzymes (UbcH5B and UbcH7) were expressed in Escherichia coli BL21 (DE3) by using the pET expression system (Novagen) (32). Bacterial cells harboring appropriate expression plasmids were grown overnight in 400 ml cultures at 25°C. The next morning, expression of proteins was induced with 1 mM isopropyl-β-thiogalactoside for 3-4 hr. Subsequently, cells were lysed in sonication buffer [10 mM Tris-HCl (pH 7.9)/10% glycerol/0.5 M NaCl/0.1% Nonidet P-40/5 mM β-mercaptoethanol and protease inhibitors (100 μg/ml phenylmethylsulfonyl fluoride/2 μg/ml leupeptin/2 μg/ml aprotinin/1 μg/ml pepstatin)]. Coomassie blue staining of an aliquot of each lysate separated by 7.5% SDS/PAGE was used to determine the relative amounts of each protein.

**In Vitro Expression of ER, PR, and TR.** In vitro expression of radiolabeled ER, PR, and TR proteins was performed by using in vitro transcription and translation (TNT)-coupled rabbit reticulocyte extracts in the presence of [35S]methionine, according to manufacturer's recommended conditions (Promega).

**Protein Degradation and Ubiquitination Assays.** [35S]-labeled ER was incubated with and without UBA1/E1 (≈5-10 ng) and UBCs/E2s (~100 ng) in reaction mixtures containing 20 mM Tris-HCl (pH 7.5), 50 mM NaCl, 4 mM ATP, 10 mM MgCl2, 0.2 mM DTT, and 4 μg of ubiquitin (Sigma) for 2-3 hr at 25°C. Reactions were terminated by boiling samples in the presence of SDS-loading buffer [100 mM Tris-HCl (pH 8.0)/200 mM DTT/4% SDS/20% glycerol/0.2% bromophenol blue]. The reaction mixtures were resolved by 7.5% SDS/PAGE, and radiolabeled bands were visualized by autoradiography. However, in Fig. 3B the ER protein was analyzed by Western blot analysis using H222 antibody.

**RESULTS AND DISCUSSION.** It has been reported that ER in the uterus is ubiquitinated and exhibits a short half-life in other estrogen target tissues in the presence of estradiol (23, 24). To determine whether down-regulation of the ER protein is mediated by the ubiquitin–proteasome pathway, we performed transient cotransfection assays in the presence or absence of the proteasome inhibitor, MG132. HeLa cells were cotransfected with an expression plasmid for ER and a reporter plasmid containing an estrogen response element and subsequently incubated with either DMSO (vehicle) or MG132 both in the absence and presence of estradiol. The effect of hormone and MG132 on ER protein levels was analyzed by Western blot analysis of cell extracts from these cells. As shown in Fig. 1, the DMSO-treated control cells exhibit lower levels of ER protein compared with that of MG132-treated cells. Addition of estradiol to the control cells reduces the level of ER protein compared with cells that were not treated with hormone (lane 1 vs. lane 2). However, MG132 blocks the estradiol-induced degradation of the ER protein (lane 3 vs. lane 4). The small molecular weight (<66 KDa) bands apparent in the MG132-treated cells likely are the result of nonproteasomal degradation of overexpressed ER. These data are consistent with the previously published report that indicates that estradiol induces down-regulation of the ER protein (23, 24). Our results also suggest that estrogen-dependent down-regulation of ER proceeds through the proteasome.

To further investigate whether hormone-dependent ER down-regulation was through the ubiquitin–proteasome pathway, we performed in vitro protein degradation and ubiquitin assays. [35S]-labeled ER protein was synthesized in vitro by using TNT-coupled rabbit reticulocyte extracts in the presence of radiolabeled methionine. The [35S]-labeled ER protein was then incubated with ATP and ubiquitin either in the absence of UBA/UBCs or in the presence of bacterially expressed UBA and UbcH5B and UbcH7 (UBCs), and reactions were terminated at varying times. In vitro, UBA and UBC enzymes promoted the degradation of ER protein compared with a control that lacked UBA and UBCs (Fig. 2). Furthermore, ER is degraded in a time-dependent manner. As shown in Fig. 2, most of the full-length receptor protein is degraded into a smaller form within 2 hr. This receptor degradation is not complete. The ER undergoes limited proteolysis that results in a slightly smaller form of ER. This restricted pattern of ER
Fig. 2. In vitro ER degradation depends on ubiquitin pathway enzymes. UBA and UBCs. 35S-labeled ER protein was synthesized in vitro with TNT-coupled rabbit reticulocyte extracts. The labeled ER protein was incubated with ATP and ubiquitin either in the absence of UBA/UBCs (for 120 min) or presence of bacterially expressed UBA and UBCs (UbcHB and UbcH7). Reactions were terminated at varying times by adding SDS-loading buffer and analyzed by SDS/PAGE and autoradiography. Arrows indicate the position of intact and degraded ER protein.

degradation is analogous to that of tramtrack and vitamin D receptor degradation, which are also degraded into smaller forms through a proteasome pathway (22, 33). It is likely that more complete degradation of ER does not occur in these in vitro assays because of limiting amounts of proteasome pathway components. Furthermore, addition of hormone in vitro assays did not change the ER degradation pattern (data not shown). These data support the hypothesis that the proteasome pathway is involved in ER protein degradation.

Next, we asked whether inhibitors of the proteasome pathway were able also to reduce the in vitro degradation of ER. A control reaction in which ER was incubated with vehicle exhibited UBA- and UBCs-dependent ER protein degradation.

Fig. 3. The proteasome inhibitors, MG132 and lactacystin, block ER degradation in vitro. (A) 35S-labeled ER protein was synthesized in vitro in the presence of either vehicle only, 33 μM MG132 or 33 μM lactacystin with TNT-coupled rabbit reticulocyte extracts. The labeled ER protein was then incubated with ATP and ubiquitin either in the absence of UBA/UBCs or in the presence of UBA and UBCs (UbcHB and UbcH7). Arrows indicate the position of intact and degraded ER protein. (B) The UBA/UBCs and proteasome inhibitors, MG132 and lactacystin, promote the accumulation of slower migrating forms of ER (shown by a bracket). 35S-labeled ER protein was synthesized in vitro in the presence of either vehicle only, 33 μM MG132 or 33 μM lactacystin with TNT-coupled rabbit reticulocyte extracts. The labeled ER protein was then incubated with ATP and ubiquitin either in the absence of UBA/UBCs or in the presence of UBA and UBCs (UbcHB and UbcH7). Then the ER protein was analyzed by Western blot analysis using H222 antibody that specifically recognizes ER. The control lane contains reticulocyte extract only. Arrows indicate the position of intact, degraded, and slower migrating forms of ER protein.
forms of ER exhibit a ladder of higher molecular weight species (23). Taken together, these data are highly suggestive that ER is degraded through the ubiquitin–proteasome pathway.

To determine whether the ubiquitin–proteasome pathway also promotes the degradation of other members of the nuclear receptor superfamily, we performed in vitro protein degradation and ubiquitin assays on the PR and TR proteins. The 35S-labeled PR and TR proteins were synthesized by TNT-coupled rabbit reticulocyte extracts in the presence of radiolabeled methionine. The 35S-labeled PR and TR proteins were then incubated with ATP and ubiquitin either in the absence of UBA and UBCs or in the presence of bacterially expressed UBA, UbcH5B, and UbcH7. As shown in Fig. 4.4, the addition of ubiquitin pathway enzymes, UBA and UBCs, has no significant effect on PR protein levels. Furthermore, addition of MG132 also exhibited no significant effect on the level of PR protein (Fig. 4.4). Data from our transfection studies also suggest that PR protein levels are not significantly altered by either hormone or protease inhibitors in mammalian cells (data not shown).

Like PR, ubiquitin–proteasome pathway enzymes have no significant effect on the level of TR. The TR is intact both in the absence and presence of ubiquitin pathway enzymes (Fig. 4B). Similarly, MG132 exhibited no significant effect on TR. These data suggest that PR and TR are not the target of the ubiquitin–proteasome pathway in this assay system. Our PR data appear to be in contrast with a previously published study that reports that chicken PR protein is ubiquitinated (34). Several reasons may account for this difference. Our study used human PR protein instead of chicken PR protein, and it is possible that each protein possesses intrinsic differences that may account for this discrepancy. Alternatively, if the human PR protein is degraded by means of the ubiquitin pathway, it may require a different set of UBC enzymes than those used to demonstrate degradation of ER in our in vitro assays.

In eukaryotic cells, several different ubiquitin–ubiquitin-like pathways exist that are mediated by ubiquitin itself and by two other ubiquitin-like proteins, neural precursor cell-expressed developmentally down-regulated (NEDD8) and sentrin (2, 14, 35–37). To date, only the ubiquitin pathway has been clearly implicated in targeted protein degradation. NEDD8 and sentrin are both small (~8 kDa) proteins that share significant homology to ubiquitin and also are covalently attached to target proteins. The role of the NEDD8 and sentrin pathways in protein degradation is not clear and the significance of modification by NEDD8 and sentrin is unknown (2, 14, 35–37). However, yeast RUB1 and SMT3 proteins, which are closely related to ubiquitin and the mammalian ubiquitin-related factor SUMO1, have been shown to be required for survival and appropriate cell-cycle progression in yeast (38).

The importance of the ubiquitin–proteasome pathway in higher eukaryotes has been well established in cell-cycle regulation, signal transduction, and cell differentiation. Recently, the ubiquitin–proteasome pathway has been linked to transcriptional machinery, and it has been demonstrated that the carboxyl-terminal tail of RNA polymerase II itself is a target of the ubiquitin–proteasome pathway (2–4, 7–11, 39). The involvement of the ubiquitin–proteasome pathway in eukaryotic transcription is further strengthened by the observation that UBCs and E3 ubiquitin–protein ligases interact with steroid hormone receptors and several other transcription factors and coactivate their transactivation functions (15–18). Because the coactivation and ubiquitination activities are distinct, this raises the question as to why ubiquitin pathway enzymes are linked to steroid receptor activation.

Eukaryotic cells exhibit rigorous control over gene expression, and one possible mechanism to control gene expression is to modulate the concentrations of transcriptional regulators in the cell by proteasome-mediated protein degradation. This possibility has been reported for the regulation of protein levels of transcription factors such as STAT5α and tramtrack (4, 33). In this manuscript, we present data that suggest that the ubiquitin–proteasome pathway modulates the concentration of ER protein in mammalian cells by promoting its degradation. Considering that the transcriptionally active ER protein is associated with a diverse group of proteins and forms a preinitiation complex, it is possible that subsequent receptor activation of transcription, proteasome-mediated degradation of the receptor may be a mechanism that dissociates the preinitiation complex. It could be necessary to dissociate the preinitiation complex through targeted protein degradation, since the reinforcing interactions of multiple transcription factors may make passive dissociation of ligand and coactivators impossible. Additionally, it is possible that hormone-

![Fig. 4](https://example.com/fig4.png)

**Fig. 4.** PR and TR proteins are not the target of the ubiquitin–proteasome pathway. (A) 35S-labeled PR protein was incubated with ATP and ubiquitin either in the absence of UBA/UBCs or in the presence of UBA and UBCs for 120 min (UbcH5B and UbcH7). In the presence of UBA/UBCs, the reaction mixtures were treated with either vehicle or 33 μM MG132. The position of intact PR is indicated by the arrow. (B) 35S-labeled TR was incubated with ATP and ubiquitin either in the absence of UBA/UBCs or in the presence of UBA and UBCs for 120 min (UbcH5B and UbcH7). The reaction mixtures containing UBA and UBCs were treated with either vehicle or 33 μM MG132. The position of intact TR is indicated by the arrow.
induced ER degradation serves to control physiological responses in estrogen target tissues by down-regulating ER, which ultimately serves to limit the expression of estrogen-responsive genes.

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The 26S Proteasome Is Required for Estrogen Receptor-α and Coactivator Turnover and for Efficient Estrogen Receptor-α Transactivation

David M. Lonard, Zafar Nawaz, Carolyn L. Smith, and Bert W. O’Malley
Department of Molecular and Cellular Biology
Baylor College of Medicine
Houston, Texas 77030

Summary

Estrogen receptor-α (ERα) is downregulated in the presence of its cognate ligand, estradiol (E2), through the ubiquitin proteasome pathway. Here, we show that ubiquitin proteasome function is required for ERs to serve as a transcriptional activator. Deletion of the last 61 amino acids of ERα, including residues that form the ligand-binding domain that impair coactivator binding. In addition, coactivators are also subject to degradation by the 26S proteasome, but their intrinsic transcriptional activity is not affected. These data provide evidence that protein interactions with ERα coactivator binding surfaces are important for ligand-mediated receptor down-regulation and suggest that receptor and coactivator turnover contributes to ERα transcriptional activity.

Introduction

Estradiol (E2) exerts its biological actions through two estrogen receptors, estrogen receptor-α (ERα) and the recently cloned estrogen receptor-β (ERβ), which are members of the nuclear hormone receptor superfamily (Green et al., 1986; Kuiper et al., 1996; Mosseiman et al., 1996). In the presence of E2, ERα dissociates from heat shock proteins, binds to its cognate estrogen response element (ERE) and to coactivator proteins, and subsequently stimulates expression of estrogen-responsive genes (Tsai and O’Malley, 1994; Glass et al., 1997; McKenna et al., 1999).

Over thirty years ago, it was observed that E2 treatment of ovariecotomized rats reduced the E2 binding capacity of uterine tissue (Jensen et al., 1969). Numerous subsequent investigations of the stability of ERs have been reported, establishing a short half-life of 3-5 hr in uterine tissues and in cultured cells through techniques involving dense amino acid or [35S]methionine/cysteine incorporation, or radiolabeled tamoxifen aziridine binding (Eckert et al., 1984; Monsma et al., 1984; Scholl and Lippman, 1984; Miller et al., 1985; Nardulli and Katzenellenbogen, 1986; Pakdel et al., 1993). It also has been reported that ERα is subject to ligand-mediated proteolysis (Horigome et al., 1988) and that ERα is ubiquitinated preferentially in the presence of E2 (Nirmala and Thampan, 1995). Several recent studies have focused on the involvement of the ubiquitin proteasome pathway in ligand-mediated degradation of ERs, which can be blocked with specific inhibitors of proteasome function, such as MG132 and lactacystin (Alarid et al., 1999; El Khassir and Leclercq, 1999; Nawaz et al., 1999b). Ligand-mediated downregulation of the progesterone receptor (PR) (Horwitz and McGuire, 1978; Syvala et al., 1998; Lange et al., 2000) and retinoid X receptor (Nomura et al., 1999) also have been reported. Other receptors such as the vitamin D receptor (Li et al., 1999) and androgen receptor (Krongrad et al., 1991; Yesp et al., 1999) have been shown to be upregulated in the presence of their cognate ligand in most cell contexts, indicating that ligand-mediated downregulation of nuclear hormone receptors is not universally linked to their activation by ligand or necessary for their function in transcription.

The ubiquitin proteasome pathway is responsible for the selective degradation of a number of short-lived transcription factors whose activity must be tightly regulated, such as NF-κB, STAT1, and fos/jun (Ciechanover et al., 1991; Palombella et al., 1994; Kim and Maniatis, 1996). Through a series of enzymes (ubiquitin-activating [UBA] and ubiquitin-conjugating/ubiquitin ligase enzymes), the 76 amino acid ubiquitin protein is covalently linked to proteins targeted for degradation, marking them for recognition by the 26S proteasome, a large multisubunit protease (Coux et al., 1996; Baumeister et al., 1998). Initially, ubiquitin forms a high-energy thioester bond with UBA, then is transferred to any one of a number of ubiquitin-conjugating enzymes and is subsequently attached to the protein that is to be degraded. This may be done in conjunction with ubiquitin ligases that serve as adaptor molecules between ubiquitin-conjugating enzymes and target proteins. Ubiquitin ligases may also play an enzymatic role by receiving ubiquitin from the ubiquitin-conjugating enzyme and performing the final transfer of ubiquitin to the targeted protein.

Interestingly, an emerging group of steroid receptor-interacting proteins have been reported that were previously identified as components of the ubiquitin proteasome degradation system, including SUG1/TRIP1 (Lee et al., 1995; vom Baur et al., 1998), RSP5/RPF1 (Imhof and McDonnell, 1996), E6-AP (Nawaz et al., 1999a), and UBC9 (Gottlicher et al., 1996; Poukka et al., 1999), suggesting that protein degradation is an integral part of nuclear hormone receptor function. RSP5/RPF1 and E6-AP are both ubiquitin ligase proteins, which also have been shown to stimulate nuclear hormone receptor-dependent gene activation, suggesting that targeted protein degradation may be linked to the induction of gene expression by these transcription factors.

Initially, we examined the impact that inhibition of proteasome activity had on ERα-mediated transcription and found that transcription was abrogated in transiently transfected HeLa cells. Inspection of coactivator proteins involved in ERα-mediated transcription revealed that they are also degraded by the ubiquitin proteasome protein degradation system; however, their intrinsic transcriptional activity was not attenuated when
the proteasome was inhibited. To explore events associated with receptor activation for their involvement in receptor downregulation, residues critical for transcriptional activity and association with coactivators were mutated, and the effect of E2 on the resulting receptors’ steady-state levels was examined. These mutants were not downregulated in the presence of E2. Data presented here suggests that coactivator interaction with the liganded receptor is required for its ligand-mediated degradation and that proteasome-mediated protein degradation plays an important role in contributing to ERα-mediated transcription.

Results

Proteasome Function Is Required for ERα-Mediated Transcription

The observation that ligand-mediated ERα downregulation is concomitant with its ability to stimulate gene transcription raises the important question of how degradation of ERα might contribute to its ability to stimulate transcription. To begin to address this, HEK cells transiently transfected with ERα and an estrogen-responsive luciferase reporter construct were treated with E2 or ethanol vehicle and the proteasome inhibitor, MG132, or its DMSO vehicle, and luciferase activity was assayed 24 hr thereafter (Figure 1A). MG132 attenuated luciferase gene expression, suggesting that proteasome-mediated degradation is required for ERα-mediated transcription. Additionally, treatment of cells with another proteasome inhibitor, lactacystin, produced similar results (data not shown). MG132 treatment also interfered with PR and thyroid hormone receptor-mediated transcription, but interestingly, not with human glucocorticoid receptor transcriptional activity (data not shown), indicating that this effect is not limited to ERα but does not affect all nuclear hormone receptors either. This data suggests that proteasome function is required for efficient transcription to ensue through ERα.

The effect of MG132 treatment on the ability of Sp1, E2F, and p53 nonnuclear hormone receptor transcription factors and the viral CMV promoter to mediate transcription was also tested to assess whether proteasome function is needed for the activity of all transcription factors. In contrast to what was observed for nuclear hormone receptors, these other transcription factors and CMV promoter–mediated transcription were shown to be moderately stimulated (Figures 1B and 1C). This indicates that the inhibition of ERα-mediated transcription by MG132 was not due to general cellular toxicity to this proteasome inhibitor or a global block in the ability of the cells to engage in transcription. p53 is a known target of the ubiquitin proteasome system degradation pathway, and the targeted degradation of p53 by the E6 protein of the high-risk human papilloma virus has been implicated as a mechanism for eliminating p53 from the cell and allowing for transformation of infected tissues (Huibregtse et al., 1991). Therefore, it is possible that an elevation of p53 steady-state levels may account for the increase in transcription from a p53-dependent promoter when proteasome function has been disrupted. However, ERα-mediated transcription is attenuated in MG132-treated cells in spite of the fact that ERα steady-state levels rise (Nawaz et al., 1999b; Figure 3),
suggesting that protein degradation plays a positive role in ERα-mediated gene expression.

ERα transcription is mediated through two discrete activation functions, AF-1 and AF-2, located in the N-terminal A/B domain and the C-terminal E domain of the receptor, respectively. To examine whether proteasome function is necessary for AF-1- and AF-2-mediated transcription, expression vectors for fusion proteins of the GAL4 DNA binding domain linked to either the ERα A/B or D/E/F domains along with a GAL4-responsive luciferase reporter were transfected into HeLa cells and treated with E2 and/or MG132 as indicated (Figures 1D and 1E). MG132 treatment was able to attenuate transcription mediated through both the AF-1 and AF-2 domains of the receptor, demonstrating that proteasome function is required for efficient transcription through either activation function. In order to ensure that MG132 effects were not limited to transfected synthetic target genes, the ability of the proteasome inhibitor to affect ERα-mediated transcription of endogenous genes also was assessed. ERα-positive MCF-7 breast cancer cells were treated with E2 and/or MG132 and harvested 18 hr thereafter. MCF-7 cell extracts were analyzed for the level of two estrogen-responsive genes, PR and pS2 (Horwitz and McGuire, 1978; Masiakowski et al., 1982). MG132 was able to attenuate the ability of E2 to stimulate the expression of both PR (Figure 1F) and pS2 (data not shown). This indicates that the proteasome is also required for ERα-mediated transcription through endogenous promoters.

Importantly, the impact of MG132-mediated disruption of the ubiquitin proteasome pathway on ERα-mediated transcription was substantiated in the tss5 cell line (derived from the F3MA mouse mammary carcinoma cell line), which harbors a temperature-sensitive mutation in the UBA enzyme that disrupts protein ubiquitination and leads to the accumulation of cellular proteins and cell cycle arrest (Finley et al., 1984). The tss5 cells were transiently transfected with ERα and an estrogen responsive reporter, then treated with E2 and incubated at permissive (30°C) or restrictive (37°C) temperatures for 24 hr and subsequently assayed for luciferase activity. Incubation at the restrictive temperature attenuated ERα-mediated transcription 5-fold compared to cells incubated at the permissive temperature (Figure 2A). The activity of ERα in the absence of E2 is relatively high in these cells and also was reduced when they were incubated at a restrictive temperature. pS3-mediated gene expression and the transcriptional activity of the viral CMV promoter were also assessed at the two different temperatures and were found to be unaffected by disruption of UBA (Figure 2B). Western analysis of ERα protein levels in the tss5 cell line at the permissive temperature revealed that the receptor is downregulated, while at the restrictive temperature, the ERα steady-state levels were higher in the presence of E2 (Figure 2C), consistent with the ligand-bound receptor being degraded at an enhanced rate by the ubiquitin proteasome protein degradation system. Incubation of tss5 cells at the nonpermissive temperature had no influence on the steady-state level of luciferase produced from a transfected constitutive expression plasmid (Figure 2D), indicating that disruption of the ubiquitin proteasome pathway is not a general effect. Additionally, luciferase and /β-galactosidase enzymatic activities were unaffected by shifting cells to the nonpermissive temperature (data not shown), further indicating specificity of the ubiquitin proteasome protein degradation pathway for ERα.

**Figure 2.** Disruption of Ubiquitin-Activating Enzyme (UBA) Function Abrogates ERα Function

(A) The temperature-sensitive UBA mutant tss5 cell line was transiently transfected with 1500 ng GERE-E1b-LUC and 16 ng of an ERα expression vector (pCR3.1hER). (B) 1900 ng of a pS3-responsive reporter and 100 ng of a pS3P72 carrier DNA along with 10 ng of a CMV-driven luciferase expression vector (pCR3.1-LUC) were transfected.

(C) ERα protein levels were determined by Western analysis with the H222 antibody. All Western analysis data is representative of experiments performed at least three times. Twenty-four hours after transfection, cells were treated with E2 or vehicle (-H) and shifted to a restrictive (37°C) temperature where indicated and harvested 24 hr thereafter for either luciferase assays or Western analysis.

(D) Cells were transfected with 2 μg of pCR3.1-LUC and shifted to a restrictive temperature where indicated 24 hr after transfection. Twenty-four hours thereafter, cell extracts were subjected to Western analysis for luciferase protein levels using an antibody against luciferase.

**Contribution of the C Terminus of ERα to Its Ligand-Mediated Downregulation**

The nuclear hormone receptor LBD encompasses 12 conserved α helices (Moras and Gronemeyer, 1998). Upon ligand binding, the conformation of helix 12 is rearranged in a way that allows it to interact with AF-2 interacting coactivators, thereby contributing to the re-
comparing the difference in protein levels between cells that were treated with or without MG132. As shown previously (Nawaz et al., 1999b), the steady-state level of wild-type ERα is lower in the presence of E2 (see Figure 3C). Treatment of cells with MG132 resulted in an increase in the amount of ERα protein and blocked its ligand-mediated downregulation. The F domain of ERα contains a PEST sequence (residues 555-567), which has been implicated as a motif involved in rapid protein turnover through either the ubiquitin-proteasome or calpain protein degradation pathways. Luciferase assays revealed that ERα 1–554 possesses transcriptional activity similar to the wild-type receptor as reported previously (Pakdel et al., 1993; Figure 3B). Additionally, ligand-mediated downregulation of this form of ERα was observed and could be blocked by treatment with MG132 (Figure 3C), indicating that the F domain and PEST sequence are dispensable for ligand-dependent downregulation of the receptor.

A slightly larger deletion that removes the F domain and helix 12 of the receptor (ERα 1–534) resulted in an altered pattern of protein stability in response to E2 (Figure 3C). This form of the receptor is stabilized in the presence of E2 with or without MG132. Functionally, ERα 1–534 is transcriptionally impaired (Tzukerman et al., 1994; Figure 3B) and lacks amino acid residues, which are required for contact with coactivators (Danielian et al., 1992; Heery et al., 1997; Henttu et al., 1997; Shiu et al., 1998; Mak et al., 1999), suggesting that the receptor’s AF-2 function and/or coactivator binding contributes to its ligand-dependent downregulation. An ERα mutant lacking the entire ligand binding domain (ERα 1–282) was transcriptionally inactive (Tzukerman et al., 1994; data not shown), and Western analysis revealed that E2 did not decrease the steady-state level of this protein (Figure 3C). However, MG132 was able to stabilize this form of the receptor.

Disruption of Coactivator Binding Surfaces Abrogate ERα Ligand-Mediated Downregulation

Expression vectors for other mutant forms of human ERα were constructed that possess point mutations in helix 3 (I358D, K362D), helix 5 (V376D), or helix 12 (L539A) for which mutation of equivalent residues in the mouse ERα (Figure 4A) resulted in a loss or reduction of binding to steroid receptor coactivator-1 (SRC-1) (Mak et al., 1999). The human ERα forms of the coactivator binding mutants mentioned above were transcriptionally impaired in a manner similar to the corresponding mouse ERα mutants (Figure 4B). We also examined an AF-2 mutant form of ERα in which the charged amino acid residues within helix 12 were replaced with alanine (D538A/E542A/D545A) (Tzukerman et al., 1994; Brzozowski et al., 1997). The stability pattern for all of these mutants was similar to that observed for ERα 1–534 (Figure 3C), further suggesting that coactivator binding is important for ligand-mediated degradation of ERα. The mouse forms of the single residue coactivator binding mutants bind to EREs and E2, indicating that a gross structural disruption of the C terminus is not responsible for the observed change in ERα stability (Mak et al., 1999). It is also evident that the altered pattern of stability observed for ERα 1–534, D538A/E542A/D545A, and the
Coactivator-Mediated Estrogen Receptor Degradation

Figure 4. Effect of AF2 Coactivator Binding Mutations on ERα Steady-State Levels
(A) Alignment of human and mouse ERα residues involved in coactivator binding.
(B) 750 ng pERE-E1b-LUC and 4 ng of expression vectors for the ERα (pCre3.1ERα), LS39A, I358D, K362D, V376D, or D538A/E542A/D546A were transfected and assayed for luciferase activity.
(C) Cells were transfected as in (B), but ERα protein levels were determined by Western analysis. Cells were treated with hormone/ MG132 and harvested for luciferase assays and Western analysis with the H222 antibody.

cocactivator binding mutants was unaffected by MG132 treatment, indicating that in the absence of an intact coactivator binding site(s), the receptor is stabilized by E2 in a proteasome-independent manner.

Assessment of DNA Binding in ERα-Mediated Downregulation
Although coactivator binding to the AF-2 of ERα is required for ligand-mediated downregulation, it was unclear whether DNA binding may be a signal for the receptor to be degraded. To test this, C201H/C205H, an expression vector for a DNA binding mutant that possesses mutations to two of the zinc-coordinating cysteines in the first zinc finger, was transfected, and its steady-state levels in the absence or presence of E2 was determined. This form of the receptor is transcriptionally inactive when assayed on an ERE-containing luciferase reporter (Figure 5A) as it cannot bind to an estrogen response element (Kumar and Chambon, 1988). Although it cannot be discounted that disruption of the DNA binding domain results in gross structural changes in the ERα, this mutant receptor was downregulated in the presence of E2 like the wild-type receptor, indicating that DNA binding and an intact DNA binding structure are not required for ligand-mediated downregulation (Figure 5B). Because this mutant form of ERα is also transcriptionally impaired (Figure 5A), coactivator binding itself may be sufficient for ligand-mediated downregulation, and the engagement of the receptor in transcription at an ERE may not be required.

Impact of Disruption of Proteasome Function on Nuclear Receptor Coactivators
Because ligand-mediated downregulation of ERα depends upon intact coactivator binding surfaces, and proteasome function is required for efficient ERα-mediated transcription, we chose to examine whether coactivators are targets of the proteasome and how ubiquitin proteasome protein degradation might be related to the role of coactivators in mediating nuclear hormone receptor-dependent transcription. HeLa cells were transiently transfected with expression vectors for SRC-1A, TIF2, RAC3, or CBP, treated with MG132 or its DMSO
vehicle alone, and harvested 24 hr later. The CMV promoter that was used to drive expression of these coactivators was not significantly affected by the presence of MG132 (see Figure 1C). Western analysis of the steadystate levels of coactivators overexpressed in HeLa cells in the presence of MG132 revealed that SRC-1A, TIF2, RAC3, and CBP were all targets of degradation via the proteasome (Figure 6A). The intrinsic transcriptional activity of each of these coactivators was assessed also in the absence or presence of MG132. GAL4-DNA binding domain–coactivator fusion proteins were expressed in HeLa cells in the presence of a reporter plasmid containing five GAL4 DNA binding response elements (Figure 6B). In contrast to that seen for ERα itself, the ability of the tested coactivators to stimulate transcription in the presence of MG132 was increased. CBP-mediated transcription was stimulated the most by the addition of MG132, consistent with it being stabilized to the largest extent in the above Western analysis. The fact that the intrinsic transcriptional activity of the tested coactivators is not impaired further indicates that the abrogation of ERα-mediated transcription is not due to a general block in transcription or an effect due to the toxicity of MG132.

Discussion

Ligand-Mediated Downregulation of ERα
Regulation of the steady-state level of ERα protein has been reported to occur through transcriptional and translational mechanisms (Saceda et al., 1988; Read et al., 1989), which is indicative of the importance of precisely regulating biological responses to estrogen. In this study, we sought to determine the relationship of ERα's participation in transcription and its C-terminal LBD in ligand-mediated receptor downregulation. We previously have shown that ERα is downregulated in the presence of E2 and that this downregulation proceeds through a proteasome-dependent mechanism (Nawaz et al., 1999b). Here we show that treatment of transiently transfected HeLa cells with the proteasome inhibitor, MG132, impaired ERα-mediated transcription. MG132 also was able to impair PR- and TR-mediated transcription (data not shown), indicating that this effect is not limited to ERα. In contrast, MG132 modestly stimulated transcription mediated via Sp1, E2F, and p53 and the human glucocorticoid receptor, as well as transcription directed by the viral CMV promoter, indicating that proteasome function is not required for transcription to proceed in general, nor for all nuclear receptors to stimulate gene expression. In support of the data suggesting that MG132 was able to interfere with ERα-dependent gene expression, ERα-mediated transcription was attenuated when the ubiquitin proteasome pathway was

Figure 6. Coactivators Are Targets of the Ubiquitin proteasome Protein Degradation System but Still Retain Their Intrinsic Ability to Stimulate Transcription in the Presence of MG132
(A) One microgram of expression vectors for SRC-1A (Flag-tagged), TIF2, RAC3, and CBP was transiently transfected separately into HeLa cells. Twenty-four hours after transfection, cells were treated with MG132 where indicated and harvested 24 hr thereafter. Cell extracts were subjected to Western analysis with the appropriate anti-coactivator-specific antibodies.
(B) The intrinsic transcriptional activity of coactivators was assessed by transfecting 20 ng of expression vectors for GAL4-DNA binding domain–coactivator fusion proteins or the GAL4 DNA binding domain alone (GAL) from the pBIND vector along with 500 ng of pG5-LUC. Twenty-four hours after transfection, cells were treated with MG132 where indicated and harvested 24 hr thereafter for luciferase activity.
disrupted in the temperature-sensitive UBA mutant ts85 cell line. Additionally, p53-mediated gene expression and transcription driven by the viral CMV promoter were unaffected by disruption of UBA function, further indicating that the ubiquitin proteasome pathway is important for the function of selected nuclear hormone receptors. Proteasome function appears to be required for transcription mediated through either the AF-1 or AF-2 domain of ERα as MG132 treatment was able to attenuate transcription from either domain when tethered to the GAL4 DBD. Both activation functions of steroid receptors have been shown to interact with coactivators (Webb et al., 1998; Ma et al., 1999), consistent with the possibility that MG132 is able to attenuate the transcriptional activity of either activation function by interfering with the function of a common coactivator or coactivators.

Role of Coactivator Binding in Ligand-Mediated Downregulation

It was possible to identify components of the receptor LBD and events in ERα-mediated transcription that were involved in ligand-mediated downregulation of the receptor through Western analysis of deletion and point mutations of ERα transiently expressed in HeLa cells. Ligand-mediated downregulation of ERα in HeLa cells is evident in 2 hr (data not shown), similar to observations made in MCF-7 cells and in the uterus in vivo (Jensen et al., 1998; Saceda et al., 1998; Read et al., 1998), indicating that the protein degradation components necessary for this event are present in this cell line. A C-terminal deletion that removes the F domain of the receptor produced a pattern of stability identical to that observed for the wild-type receptor.

Crystal structural analysis of ERα and other nuclear hormone receptors has revealed the presence of 12 conserved helices in their ligand binding domains (Mora and Gromemeyer, 1998). Helix 12, the most C-terminal of these helices, has been identified as the critical core of the AF-2 function of the receptor and plays an important role in coactivator binding to the ligand-bound receptor (Danielian et al., 1992; Henttu et al., 1997; Shlau et al., 1998). Residues in helix 12 of the ligand-activated receptor interact with LXXLL nuclear receptor (NR) box motifs (where L is leucine and X is any amino acid) present in many coactivators such as SRC-1 and GRIP1 (Heery et al., 1997). The corecrystal structure of the ERα LBD-ligand-NR box complex reveals that residues within this part of the receptor as well as within helices 3 and 5 are important for making contact with an NR box motif of GRIP1 (Mak et al., 1999). The altered pattern of stability observed in all the coactivator binding mutants tested suggests that receptor interaction with an NR box–containing protein is required for the ligand-mediated downregulation of ERα. The identity of the putative protein that promotes this ligand-mediated downregulation is unknown, but it is possible that one of the ubiquitin proteasome pathway–related coactivators such as E6-AP, which also contains two NR boxes, TRIP1 or RPF1, may contribute to this effect.

The hormone-dependent increases in steady-state levels of the coactivator binding mutant forms of ERα could not be blocked by treatment with MG132, indicating that a proteasome-independent mechanism of receptor degradation exists for these mutants. The observation that ligand stabilizes ERα mutants with impaired coactivator binding is reminiscent of the previously reported ability of E2 to protect the receptor from protease digestion in vitro (Allen et al., 1992; Beekman et al., 1993; McDonnell et al., 1995). This suggests that the binding of a coactivator or another protein to the wild-type ligand-bound receptor is able to override the allosteric protection that E2 would otherwise have on the receptor. This model is also consistent with the observation that ERα is stabilized in the presence of 4-hydroxytamoxifen, which binds to the ERα LBD but alters its conformation in such a way that it is unable to bind coactivators (Brzozowski et al., 1997).

The question of whether DNA binding and interaction of the receptor with the basal transcription machinery is also involved in promoting ligand-mediated receptor downregulation cannot be addressed adequately with the coactivator binding mutants since they still bind to DNA and their transcriptional activity is already impaired due to defective coactivator binding. However, analysis of a DNA binding domain mutant form of ERα (ERα C201H/C205H) revealed that its stability pattern resembles that of the wild-type receptor, suggesting that DNA binding and productive engagement of the receptor in transcription are not required for ligand-mediated receptor downregulation. Rather, occupation of the ERα coactivator binding sites appears to be the major determinant in promoting ligand-mediated downregulation. In contrast to our results, it has been reported that treatment of cells with the transcriptional inhibitor, actinomycin D (Horwitz and McGuire, 1978; data not shown), is able to prevent ERα downregulation, suggesting that transcription is required for ligand-mediated downregulation of the receptor. However, a similar inhibition of E2-dependent receptor downregulation is also observed upon inhibition of protein synthesis with cycloheximide (El Khissli et al., 1997). Thus, it is possible that the continued synthesis of a protein or proteins required for ligand-mediated downregulation of the receptor may be necessary for appropriate receptor downregulation rather than ERα transcriptional activity itself. Alternatively, the DBD mutant ERα may still be able to engage in transcription through protein–protein interactions with other transcription factors such as Sp1 and AP1, leaving open the possibility that ERα transcriptional activity may still be required for ligand-mediated downregulation to occur (Krishnan et al., 1994; Webb et al., 1995), or the receptor may be targeted to the proteasome by an alternative pathway. Data presented here indicates that ligand-mediated downregulation of ERα likely depends upon interaction with a coactivator or a receptor-interacting protein that binds to the ligand-bound ERα through similar LBD residue contacts. It is unknown whether the responsible molecule directly interacts with the ligand-bound ERα to promote its degradation, or whether another protein that associates indirectly with the receptor as part of a multisubunit complex is responsible for actually targeting the receptor for degradation.

In hindsight, it is feasible to expect that targeted degradation of the receptor contributes to the cessation of
ERα-mediated transcription, rather than simply a fall in the level of estrogen. The binding of multiple coactivators to the ERα-E2 complex would presumably result in cooperative stabilization of the activated receptor in a complex with DNA, ligand, and numerous coactivators to the point where passive diffusion of E2 from the ligand pocket of the receptor is unlikely to be efficient, necessitating other mechanisms, such as degradation of the receptor itself for cessation of transcription. In support of this hypothesis, it has been shown that simple NR box peptides and a receptor-interacting domain of SRC-1 were able to dramatically reduce the rate at which ERα agonist but not antagonist ligands dissociate from the receptor in vitro, indicating that coactivator binding can impair passive dissociation of ligand from the receptor (Gee et al., 1999). Our data suggest that receptor downregulation is linked to its role in transcription as a function of the activated receptor’s ability to bind coactivators.

The observation that ERα transcriptional activity is abrogated in the presence of MG132 leads to the question of where the proteasome impinges upon nuclear hormone receptor-mediated transcription. A number of coactivators tested were stabilized by MG132, suggesting possible routes through which the proteasome could impact receptor-mediated transcription. All the coactivators tested were present at a higher steady state in MG132-treated cells, with CBP being the most stabilized. When linked to a heterologous GAL4 DNA binding domain and overexpressed in HeLa cells, GAL4-DNA binding domain-coactivator fusions of SRC-1A, TIF2, RAC3, or CBP stimulated gene expression from a GAL4-responsive reporter in the presence of MG132. This was surprising considering that cellular ERα was unable to efficiently engage in transcription, and suggests that proteasome inhibition may alter some aspect of ERα-coactivator complex formation or degradation.

Recent observations suggest that the SRC-1-containing coactivator complex and the TRAP/DRIP complex associate with nuclear receptors in a mutually exclusive fashion (Treuter et al., 1999). Proteasome-mediated downregulation of one complex or its components may be necessary for coactivator complex exchange to occur and ultimately for the ligand-bound receptor to execute all the steps necessary for transcription. This report identifies proteasome-mediated protein degradation as a novel enzymatic activity that contributes to nuclear hormone receptor-mediated transcriptional regulation, and adds to the already characterized concert of other enzymatic functions such as acetylation, phosphorylation, and methylation that are involved in the modulation of transcription.

**Experimental Procedures**

**Cell Transfections**

Transient transfections were carried out in HeLa or MCF-7 cells at 37°C, or the UBA (E1) temperature-sensitive cell line ts5 at 30°C (Finley et al., 1984), which were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum. Twenty-four hours prior to transfection, HeLa cells were plated in 6-well plates at a density of 3 x 10⁴ per well; MCF-7 and ts85 cells were plated at 9 x 10⁴ cells per well, in phenol red-free DMEM containing 10% dextran coated charcoal-stripped serum (FBS). Transfections were carried out with Lipofectamine (Life Technolog-
Coactivator-Mediated Estrogen Receptor Degradation

Analysis (Bio-Rad) were resolved by 7.5% SDS-PAGE and subsequently transferred to nitrocellulose membranes. For ERα blots were probed with an ERα-specific antibody (H222) directed against amino acid residues located in the C-terminus followed by a rabbit anti-rat secondary antibody and an anti-rabbit HRP-linked tertiary antibody or with the ER-NT antibody, directed against the first 8 amino acids of the receptor followed by an anti-mouse HRP-linked antibody. PR levels in MCF-7 cells were assessed using a mouse monoclonal antibody (AB52) directed against PR followed by an anti-mouse HRP-linked antibody. Western analysis for luciferase protein levels were performed using a goat polyclonal antibody against the luciferase protein (Promega) followed by an anti-goat HRP-linked antibody. Western analysis of the Flag epitope-tagged SRC-1A was conducted with an anti-Flag M2 antibody (Sigma), and the other coactivators were probed with antibodies specific to each coactivator protein. All blots were visualized by chemiluminescence (ECL Plus, Amersham).

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