<table>
<thead>
<tr>
<th>UNCLASSIFIED</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>AD NUMBER</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>ADB251613</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>NEW LIMITATION CHANGE</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>TO</td>
</tr>
<tr>
<td>Approved for public release, distribution unlimited</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>FROM</td>
</tr>
<tr>
<td>Distribution authorized to U.S. Gov’t. agencies only; Proprietary Info.; Jun 99. Other requests shall be referred to U.S. Army Medical Research and Materiel Command, 504 Scott St, Fort Detrick, MD 21702-5012.</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>AUTHORITY</td>
</tr>
<tr>
<td>USAMRMC ltr, 23 Aug 2001</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>THIS PAGE IS UNCLASSIFIED</td>
</tr>
</tbody>
</table>
Award Number DAMD17-97-1-7186

TITLE: Roles of ER, SRC-1, and CBP Phosphorylation in Estrogen Receptor-Regulated Gene Expression

PRINCIPAL INVESTIGATOR: David M. Lonard, Ph.D.

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

REPORT DATE: June 1999

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

Distribution authorized to U.S. Government agencies only
(proprietary information, Jun 99). Other requests for this
document shall be referred to U.S. Army Medical Research
and Materiel Command, 504 Scott Street, Fort Detrick,
Maryland 21702-5012.

The views, opinions and/or findings contained in this report are
those of the author(s) and should not be construed as an official
Department of the Army position, policy or decision unless so
designated by other documentation.
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 (ER) agonist. Tamoxifen resistance may also occur through cellular alterations in the balance of coactivators and corepressors. In this report, the contribution of phosphorylation and proteasome-mediated ER degradation to its transactivation function is explored. An inhibitor of the proteasome, MG132, interferes with ER function 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. Deletion of the N-terminal AB domain of ER results in a highly unstable truncated ER indicating that the N-terminus of the receptor may contribute to ER stability. These results expand upon the findings which may indicate a clinical use for proteasome inhibitors in blocking tamoxifen antagonist/agonist switching in women receiving long-term tamoxifen therapy.
Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the U.S. Army.

Where copyrighted material is quoted, permission has been obtained to use such material.

Where material from documents designated for limited distribution is quoted, permission has been obtained to use the material.

Citations of commercial organizations and trade names in this report do not constitute an official Department of Army endorsement or approval of the products or services of these organizations.

In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and use of Laboratory Animals of the Institute of Laboratory Resources, national Research Council (NIH Publication No. 86-23, Revised 1985).

For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.
### TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>I. Front Cover</td>
<td>1</td>
</tr>
<tr>
<td>II. Report Documentation page</td>
<td>2</td>
</tr>
<tr>
<td>III. Foreword</td>
<td>3</td>
</tr>
<tr>
<td>IV. Table of Contents</td>
<td>4</td>
</tr>
<tr>
<td>V. Introduction</td>
<td>5</td>
</tr>
<tr>
<td>VI. Body</td>
<td>6</td>
</tr>
</tbody>
</table>
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 of estrogen-dependent breast cancer. The purpose of this research is to elucidate the mechanism through which tamoxifen antagonist/agonist switching occurs. Evidence suggests that stimulation of the cAMP/protein kinase A and growth factor/MAP kinase pathways may contribute to this switching phenomenon, prompting further investigation of how phosphorylation of the estrogen receptor (ER), coactivators and corepressors might contribute to the receptors response to 4-hydroxyltamoxifen (4HT) in cell culture. Based upon results which were described in last year’s report 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 in tamoxifen antagonist/agonist switching has been examined in more detail. New data presented here expands upon data implicating the ubiquitin-proteasome pathway in the qualitative response to tamoxifen and the role that ligand-mediated ER phosphorylation plays in conjunction with receptor degradation.
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. This 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 and is also preferentially degraded 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 for steroid receptor coactivation and the response to estradiol and 4HT. Data presented in last year's report suggests that the ubiquitin-proteasome protein degradation pathway has an impact both for coactivation by E6-AP and SRC-1 and for how ER responds to E2 and 4HT.

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 this year’s report expands 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. In spite of the fact that ER is stabilized in MG132-treated cells and is thus present at higher 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.

Last year, it was reported 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 stimulated 2-fold and 8-fold respectively 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 and indicated that 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 potentiate 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, indicating its potential value for abrogating tamoxifen agonist activity in women receiving long-term tamoxifen therapy.

Results presented in this 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 must be degraded in order to release the RNA polymerase from the promoter for the subsequent RNA elongation step to occur. Additional experiments need to be performed to address whether ER mutants which can not bind DNA and mutants which are transcriptionally defective but are still able to bind DNA and ligand are also degraded like the wild-type receptor to explore whether ER degradation depends upon ligand-binding, DNA-binding or transactivation.
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
- MG132-mediated disruption of ER transactivation is correlated with stabilization of the corepressor, N-CoR, suggesting that the proteasome promotes transcription through the degradation of transcriptional repressors
- 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

REPORTABLE OUTCOMES

Published manuscripts:


Abstracts:

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. TSAL,1 MING-JER TSAL,1 AND BERT W. O'MALLEY1*

Department of Cell Biology1 and Department of Molecular and Human Genetics,2 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-protocosome 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 hormone, 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), pCIP (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/CREB, 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 papillomavirus 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 specificity of the ubiquitin-proteasome degradation system. Protein ubiquitination also involves two other classes of enzymes, namely, E1 ubiquitin-activating enzymes and E2 ubiquitin-conjugating 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 hept (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 carboxyl-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 (26, 30, 42). AS is a neurological 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 majority of AS patients examined. In this report, we also show that the ubiquitin ligase activity of E6-AP is not required for the coactivation function of E6-AP. Furthermore, our data indicate that the catalytic function located within the first 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 functions, as both a coactivator and a ubiquitin-protein ligase.

**MATERIALS AND METHODS**

**Plasmid construction.** The bait plasmid 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 (Invitrogen), lactose inducible protein (LacI) and E2F, Sp1, E1A, and E1B responsive reporters (33) have been described previously. To construct the glucocorticoid receptor (GR) expression vector, the pSG5 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), pGREGRE, E1B.LUC and pERE1B.LUC were constructed by inserting PstI/Smal fragments of pPREGRE1B.LUC and pERE1B.LUC into the Smal site of pGL3-basic (Promega). To construct mammalian expression plasmids for wild-type E6-AP (aa 1 to 851), 76-kDa E6-AP (aa 170 to 851), and C833S (change of cysteine 833 to serine) mutant E6-AP (aa 1 to 851), the BamHI-HindIII fragment ligated into the corresponding sites of pGEM E6-AP (Stratagene). The C-terminal fragment of E6-AP (aa 1 to 851), the truncated mutant E6-AP (aa 1 to 649), and the 58-kDa (aa 1 to 854) form of E6-AP, found the transactivation functions of members of the nuclear receptor superfamily, were screened on a yeast two-hybrid plasmid, HindIII-digested (and filled) pGEM E6-AP (100 kDa) was redigested with BamHI. The resulting fragment was cloned into the corresponding sites of the mammalian expression vector pEGEM3. To reconstitute the mutation in the mammalian expression plasmid pEGEM3, the E6-AP cysteine 833 to serine mutant form was used.

**RESULTS**

**Isolation and characterization of E6-AP as a PR-interacting protein.** To identify novel proteins which selectively modulate the transcriptional functions of members of the nuclear receptor 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 12 colonies which strongly interacted 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 carboxy-terminal aa 680 to 851 of the E6-AP (see Fig. 2A).

Full-length E6-AP interacts with the liganded 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 in a progesterone-dependent manner. In the absence of ligand or in the presence of the antihormone compound RU486, we observed no signifi-
coactivator 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.

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.

**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 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 sodium dodecyl sulfate-polyacrylamide gel electrophoresis on a 7.5% gel followed by Western blot analysis using anti-PR-B expression plasmid. The entire coding sequence of PR-A was fused in frame with the yeast ligase activity; --, negative for ubiquitin-protein ligase activity.

<table>
<thead>
<tr>
<th>Form of E6-AP</th>
<th>Coactivationa</th>
<th>Ubiquitin-protein ligase activityb</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type (aa 1–851)</td>
<td>+ + + +</td>
<td>+</td>
</tr>
<tr>
<td>Mutant</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 1–834c</td>
<td>+ + + +</td>
<td>–</td>
</tr>
<tr>
<td>aa 1–714</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>R417Xc</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>aa 1–240</td>
<td>Not tested</td>
<td>–</td>
</tr>
<tr>
<td>C833S</td>
<td>+ + + +</td>
<td>+</td>
</tr>
<tr>
<td>I804K</td>
<td>+ + + +</td>
<td>+</td>
</tr>
<tr>
<td>P782Δ</td>
<td>+ + + +</td>
<td>+</td>
</tr>
<tr>
<td>1–885Asnop</td>
<td>+ + + +</td>
<td>Not tested</td>
</tr>
</tbody>
</table>

a HeLa cells were transfected with 0.1 µg of PR-B expression plasmid, 1 µg of pRc/RSV, E1bLuc, and 0.25 µg of expression plasmid for wild-type E6-AP or 0.25 µg of each of the mutant indicated 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 + + + +.

b 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.

**Mutant form of E6-AP cloned from an AS patient.**

**FIG. 1.** (A) Interaction of PR with wild-type E6-AP in a yeast two-hybrid assay. The entire coding sequence of PR-A was fused in frame with the yeast GAL4 DBD, and the resulting GAL-DBD-PR-A construct was coexpressed with either control vector or the GAL4-AD-E6-AP construct (GAL4 activation domain fused in frame with wild-type E6-AP) along with a reporter plasmid in yeast strain B1789. The transformants were propagated, and β-galactosidase activities from three independent colonies were determined. The yeast cells were treated with either vehicle alone (–H), 10^{-8} M progesterone (–P), or 10^{-7} M RU486 (+RU). Each bar depicts the average of three assays. (B) In vitro interaction of E6-AP with PR. Baculovirus-expressed purified PR was incubated with a purified GST–E6-AP fusion protein or with GST alone (control) bound to glutathione-Sepharose beads either in the absence or in the presence of 10^{-7} M progesterone. E6-AP-bound PR was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on a 7.5% gel followed by Western blot analysis using antibodies which specifically recognize PR.
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 coactivation 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, transferable 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...
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 6 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 1804K, in which isoleucine 804 was mutated to lysine; F782A, an internal in-frame deletion of phenylalanine 782; and 1-885 Astop, 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, separated 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 E6-AP. 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 1804K, in which isoleucine 804 was mutated to lysine; F782A, an internal in-frame deletion of phenylalanine 782; and 1-885 Astop, 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, separated in the central nervous system phenotype of AS (Table 1).
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 F782Δ, were unable to ubiquitinate a protein (HHR23A) implicated as a target of E6-AP ubiquitin-protein ligase activity in an in vitro ubiquitin assay (27, 49), the results suggest that loss of ubiquitin-protein ligase activity contributes to the AS phenotype in these patients. 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 carboxy 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 this 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/CIP (ACTR/RAC3/AB1/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/RPF1) has been implicated as a coactivator that regulates steroid hormone receptors. This receptor with the hect domain with 37% identity to that of E6-AP (24), UREB1, is a DNA-binding protein which also contains a hect domain (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 hect domain alone is not sufficient for coactivation.

The results presented here for mutant E6-AP proteins identified in AS patients suggest that the coactivation 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-protein ligase 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 receptor activation of transcription, a mechanism is required to dissociate 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/RPF1 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 coactivating and enzymatic activities.

ACKNOWLEDGMENTS

We thank Andrew Denies and Sam Cho for technical support. We also thank Peter Howley and Sushant Kumar for the wild-type E6-AP, N-terminally truncated E6-AP, C833S mutant E6-AP, HH23A, and ubiquitin reagents; Arthur Beaudet for AS mutant E6-AP cDNAs; and Austin Cooney and Neil McKenna for critical reading of the manuscript.

This work was supported by a grant from the NIH to B.W.O.

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

Contributed by Bert W. O’Malley, December 22, 1998

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 followed by 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 carboxy 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 ER 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 (25). 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 vitro 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 pGLO3 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-rat 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. and UbcH5B and UbcH7 (UBCs) and reactions were terminated by boiling samples in the presence with either vehicle (DMSO) or proteasome inhibitor (1 μM MG132) both in the absence (−) and presence of 10^{-9} M estradiol (E2).

**Protein Degradation and Ubiquitination 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 lacks 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 radiolabeled bands was 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 *vivo*, UBA and UBC enzymes promoted the degradation of ER protein compared with a control that lacks 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
degradation is analogous to that of tramtrack and vitamin D receptor degradation, which are also degraded into slightly 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 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. 3A). However, the proteasome inhibitors MG132 or lactacystin significantly inhibited the UBA/UBC-mediated degradation of ER. These data are consistent with our intact cell data (Fig. 1), which indicate that the ER protein is degraded through the proteasome pathway, and that inhibitors of this pathway inhibit ER degradation. The in vitro inhibition of ER protein degradation by MG132 is less effective than inhibition by lactacystin. The weaker effect of MG132 may be caused by the fact that MG132 binds to the proteasome in a reversible manner, and that ubiquitinated ER effectively competes for binding to the proteasome because of a higher affinity for the proteasome. In contrast, lactacystin binds to the proteasome in an irreversible manner.

Because the UBA/UBCs promote ER degradation, and proteasome inhibitors decrease the degradation of ER protein both in vivo and in vitro, we asked whether the UBA/UBCs, MG132, and lactacystin-treated reactions promote the accumulation of higher molecular weight forms of ER. Because ubiquitin is conjugated to multiple lysine residues of target proteins and forms polyubiquitin chains, ubiquitin-tagged proteins can be seen as a ladder of higher molecular weight species on SDS/PAGE gels (11, 23, 31). As shown in Fig. 3B, the Western blot analysis of ER protein reveals that the control reaction without proteasome inhibitors (vehicle) exhibited UBA- and UBCs-dependent degradation of ER. Addition of MG132 and lactacystin to the reaction decreased ER degradation. Furthermore, a ladder of higher molecular weight species of ER is visible only in the reactions treated with UBA/UBCs regardless of the presence of proteasome inhibitors compared with that of the −UBA/UBCs reaction. Similarly, higher molecular weight species of ER can be seen in Fig. 3A after exposing the gel ∼10 times longer than the one shown in Fig. 3B (data not shown). It is possible that the ER degradation pattern seen in Fig. 3B is slightly different from that of Fig. 3A because of increased sensitivity in the Western blot, which preferentially amplifies the signal of some minor ER species. The high molecular weight species of ER presumably represent the ubiquitinated form of ER since the −UBA/UBCs reaction did not exhibit the higher molecular weight species ER protein (Fig. 3B). These data are similar to the previously published report that indicates that ubiquitinated

![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 (UbeH5B 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.](image)

![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 (UbeH5B 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 (UbeH5B 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.](image)
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. 4A, 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. 4A). 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 in which the involvement of the ubiquitin–proteasome pathway in the cellular regulation of transcription factors and coactivators was reported (4, 33). In this manuscript, we present data that suggest that the ubiquitin–proteasome pathway is not 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 to 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](image-url)
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.

We thank Peter Howley and Sushant Kumar for the UbcH7 expression plasmids and Allan Weissman for A. thaliana UBA1 and UbcH5B expression plasmids. The H222 antibody was the generous gift of Abbot Laboratories. We also thank Neil McKenna for critical reading of the manuscript. This work was partially supported by an award (Flaming/Davenport) to Z.N. and by National Institutes of Health grants to C.L.S. and B.W.O.


The Ubiquitin-Protein Ligase E6-AP, Mutated in Angelman Syndrome, is a Steroid Receptor Coactivator

David M. Lonard, Zafar Nawaz, Carolyn L. Smith, Sophia Y. Tsai, Ming Jer Tsai and Bert W. O'Malley. Dept. of Cell Biology, Baylor College of Medicine, Houston, TX 77030

Through a yeast-two hybrid screen using the progesterone receptor (PR) ligand-binding domain as bait, we identified E6-AP as a protein that interacted with PR in a progesterone-dependent manner. E6-AP also interacted with PR in a ligand-dependent manner in GST-pull-down and mammalian two-hybrid assays. Transient transfection of E6-AP into HeLa cells revealed that E6-AP coactivates the hormone-dependent transcriptional activity of PR, estrogen receptor (ER) and androgen receptor. Also, coexpression of E6-AP reverses the ability of ER to squelch activation of PR. Although E6-AP was previously identified as a ubiquitin-protein ligase (E3), cotransfection of a mutant form of E6-AP which is unable to act as a ubiquitin-protein ligase could still coactivate steroid receptors. Furthermore, the proteasome inhibitor, brefeldin A, did not affect the ability of E6-AP to coactivate PR indicating that ubiquitin-protein ligase activity is unnecessary for E6-AP to act as a coactivator. E6-AP is mutated in Angelman Syndrome (AS), a genetic neurological disorder characterized by severe mental retardation, absence of speech, and inappropriate laughter. In order to correlate the E6-AP coactivator function with the AS disease, we created an E6-AP mutation similar to that found in some AS patients and found that it is defective as a steroid receptor coactivator, suggesting that the coactivation function of E6-AP may be involved in the AS phenotype. E6-AP thus represents another coactivator involved in a genetic disease along with CBP which causes Rubinstein-Taybi syndrome when mutated. Supported by a NIH grant to B.W.O.
Involvement of ubiquitin-proteasome mediated protein degradation in steroid receptor function. David M. Lonard, Zafar Nawaz, Carolyn L. Smith and Bert W. O'Malley. Dept. of Cell Biology, Baylor College of Medicine, Houston, TX 77030

Through a yeast-two hybrid screen using the progesterone receptor (PR) ligand-binding domain as bait, we identified the ubiquitin-protein ligase, E6-Associated protein (E6-AP), as a protein that interacts with PR in a ligand-dependent manner. Transient transfection experiments revealed that E6-AP is a nuclear hormone receptor coactivator. E6-AP was previously characterized as a ubiquitin-protein ligase which associates with p53 and promotes its degradation in the presence of the viral E6 protein encoded by the high risk human papillomavirus types 16 and 18. A number of other receptor-interacting proteins have also been implicated in ubiquitin-proteasome mediated protein degradation, suggesting that protein degradation plays an important mechanistic role in nuclear hormone receptor-mediated transcription. Ubiquitin-proteasome mediated degradation of a number of transcription factors in addition to p53, such as c-Jun, STAT5a and tramtrack have also been reported, implicating ubiquitin-proteasome function in the repression of transcription. Conversely, another report which showed that N-CoR is degraded in a proteasome-dependent manner suggests that protein degradation may also stimulate transcription by down-regulating transcriptional repressors. The identification of E6-AP as a coactivator and the existence of another ubiquitin-protein ligase, receptor potentiating factor-1 (RPF-1), that also stimulates receptor-mediated transcription supports the possibility that protein degradation is also involved in the stimulation of transcription.

We investigated the role of proteasome-mediated protein degradation on nuclear hormone receptor transcriptional activity and determined that proteasome function is necessary for nuclear hormone receptor mediated gene expression. Post-translational modification of proteins through acetylation, deacetylation and phosphorylation have been shown to play important roles in modulating transcription by nuclear hormone receptors. The identification of coactivators and other receptor-interacting proteins which are involved in targeted protein degradation represents another potential form of post-translational processing in nuclear hormone receptor mediated gene expression.
MEMORANDUM FOR Administrator, Defense Technical Information Center (DTIC-OCA), 8725 John J. Kingman Road, Fort Belvoir, VA 22060-6218

SUBJECT: Request Change in Distribution Statement

1. The U.S. Army Medical Research and Materiel Command has reexamined the need for the limitation assigned to the technical reports listed at enclosure. Request the limited distribution statement for these reports be changed to "Approved for public release; distribution unlimited." These reports should be released to the National Technical Information Service.

2. Point of contact for this request is Ms. Judy Pawlus at DSN 343-7322 or by e-mail at judy.pawlus@det.amedd.army.mil.

FOR THE COMMANDER:

[Signature]

PHYLIS M. RINEHART
Deputy Chief of Staff for Information Management
<table>
<thead>
<tr>
<th>ADB241560</th>
<th>ADB253628</th>
<th>ADB249654</th>
<th>ADB263448</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADB251657</td>
<td>ADB257757</td>
<td>ADB264967</td>
<td>ADB245021</td>
</tr>
<tr>
<td>ADB263525</td>
<td>ADB264736</td>
<td>ADB247697</td>
<td>ADB264544</td>
</tr>
<tr>
<td>ADB222448</td>
<td>ADB255427</td>
<td>ADB263453</td>
<td>ADB254454</td>
</tr>
<tr>
<td>ADB234468</td>
<td>ADB264757</td>
<td>ADB243646</td>
<td></td>
</tr>
<tr>
<td>ADB249596</td>
<td>ADB232924</td>
<td>ADB263428</td>
<td></td>
</tr>
<tr>
<td>ADB263270</td>
<td>ADB232927</td>
<td>ADB240500</td>
<td></td>
</tr>
<tr>
<td>ADB231841</td>
<td>ADB245382</td>
<td>ADB253090</td>
<td></td>
</tr>
<tr>
<td>ADB239007</td>
<td>ADB258158</td>
<td>ADB265236</td>
<td></td>
</tr>
<tr>
<td>ADB263737</td>
<td>ADB264506</td>
<td>ADB264610</td>
<td></td>
</tr>
<tr>
<td>ADB239263</td>
<td>ADB243027</td>
<td>ADB251613</td>
<td></td>
</tr>
<tr>
<td>ADB251995</td>
<td>ADB233334</td>
<td>ADB237451</td>
<td></td>
</tr>
<tr>
<td>ADB233106</td>
<td>ADB242926</td>
<td>ADB249671</td>
<td></td>
</tr>
<tr>
<td>ADB262619</td>
<td>ADB262637</td>
<td>ADB262475</td>
<td></td>
</tr>
<tr>
<td>ADB233111</td>
<td>ADB251649</td>
<td>ADB264579</td>
<td></td>
</tr>
<tr>
<td>ADB240497</td>
<td>ADB264549</td>
<td>ADB244768</td>
<td></td>
</tr>
<tr>
<td>ADB257618</td>
<td>ADB248354</td>
<td>ADB258553</td>
<td></td>
</tr>
<tr>
<td>ADB240496</td>
<td>ADB258768</td>
<td>ADB244278</td>
<td></td>
</tr>
<tr>
<td>ADB233747</td>
<td>ADB247842</td>
<td>ADB257305</td>
<td></td>
</tr>
<tr>
<td>ADB240160</td>
<td>ADB264611</td>
<td>ADB245442</td>
<td></td>
</tr>
<tr>
<td>ADB258646</td>
<td>ADB244931</td>
<td>ADB256780</td>
<td></td>
</tr>
<tr>
<td>ADB264626</td>
<td>ADB263444</td>
<td>ADB264797</td>
<td></td>
</tr>
</tbody>
</table>