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13. ABSTRACT (Maximum 200 words) 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 activity which have been associated with the conversion of the tamoxifen metabolite, 4-hydroxytamoxifen (4HT), into an estrogen receptor agonist. Tamoxifen resistance may also occur through cellular alterations in the balance of steroid receptor coactivators and corepressors. This report describes a recently identified coactivator named E6-associated protein (E6-AP) which is a novel dual function protein that also subserves an independent function in the ubiquitin-proteasome protein degradation pathway. E6-AP was able to coactivate the estrogen receptor (ER) independent of its role in the ubiquitin-proteasome protein degradation system. However, a proteasome inhibitor, MG132, did block coactivation by both E6-AP and steroid receptor coactivator-1, suggesting that ubiquitin-proteasome mediated protein degradation is required for coactivator function. In HepG2 cells where ER normally responds to 4HT as an agonist, MG132 was able to eliminate the agonist response of ER to 4HT, possibly by affecting corepressor turnover. These novel findings may indicate a role for proteasome inhibitors in enforcing the antagonistic state of tamoxifen in women receiving long term tamoxifen therapy.				
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TABLE OF CONTENTS

I. Front Cover.....	1
II. Report Documentation page.....	2
III. Foreword.....	3
IV. Table of Contents.....	4
V. Introduction.....	5
VI. Body.....	8
VII. Conclusions.....	26
VIII. References.....	28

INTRODUCTION

About two-thirds of breast cancer patients possess cancers that are estrogen-dependent and respond well to the antiestrogen, tamoxifen, at least initially (1, 2, 3). 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. Possible mechanisms for this resistance may involve increases in cAMP and protein kinase A (PKA) activity (4, 5), alterations in the balance of expression of steroid receptor coactivators (6) and corepressor proteins (7) or metabolism of tamoxifen into inactive forms (8). Application of pharmacological agents which agonize the cAMP intracellular signal transduction system can activate the estrogen receptor (ER) in the absence of estradiol, concomitant with phosphorylation of the receptor (9). In addition, agents which stimulate cAMP also alter the cellular response to the functionally relevant metabolite of tamoxifen, 4-hydroxytamoxifen (4HT), changing it from an antagonist to an agonist. One possible mechanism for tamoxifen resistance is that the cAMP pathway becomes hyperactive in tamoxifen-resistant tumors leading to an altered response to tamoxifen. It is also possible that steroid receptor coactivator overexpression plays a role in the altered response of breast cancers to tamoxifen. It has recently been shown that AIB1/RAC3 (6, 10), a member of the SRC family of coactivators, is overexpressed in breast cancer biopsies.

Steroid receptor coactivator-1 (SRC-1) (11), CREB-binding protein (CBP) (12) and a new coactivator recently identified in our laboratory, named E6-associated protein (E6-AP) are part of a growing family of proteins that are required to confer full transcriptional activity to many steroid receptor superfamily members, including ER. Coactivators interact with nuclear hormone receptors in a ligand-dependent manner where they serve as bridging factors between the receptor and components of the basal transcription machinery, resulting in increased

transcription of hormone-regulated genes. Coactivators such SRC, CBP and RAC3/AIB1 have also been shown to possess histone acetyltransferase (HAT) activity (13, 14, 15) which may be responsible for stimulating gene expression by loosening the chromatin structure surrounding the promoter of hormone-responsive genes. E6-AP also possesses an enzymatic function, although it is unique among coactivators in that it contains ubiquitin-ligase activity which is part of the ubiquitin-proteasome protein degradation pathway (see below), unlike the other coactivators mentioned above. Corepressors such as N-Cor and SMRT (16, 17) also play an important role in modulating transcription by nuclear hormone receptors. N-Cor and SMRT have been shown to bind to nuclear hormone receptors in the absence of their cognate ligand, imparting a repressive effect on transcription. It has recently been shown that the progesterone receptor (PR) can also associate with N-Cor in the presence of its antagonist, RU486 (18). The same group also provide evidence that ER also associates with a corepressor in the presence of 4HT by showing that when ER was cotransfected into the same culture well as PR and treated with 4HT, it could convert RU486 into an agonist of PR, presumably by competing for a limiting corepressor (18).

The purpose of this research is to explore intracellular alterations that may contribute to the conversion of tamoxifen into an ER agonist and to determine what role coactivator proteins may exert on the ability of ER to activate transcriptional activity in response to estradiol or tamoxifen. Elucidation of the mechanisms through which tamoxifen changes from an antagonist to an agonist in breast tissues would be of valuable clinical significance for controlling breast cancer in women receiving long term tamoxifen therapy. The potential for E6-AP to mediate its coactivator function through the ubiquitin-proteasome protein degradation pathway and for E6-AP to play a part in tamoxifen antagonist/agonist switching may provide an important new

avenue for designing treatments that retain the initially antagonistic state of tamoxifen. New data presented in this progress report implicates the ubiquitin-proteasome protein degradation pathway in the qualitative response of ER to tamoxifen.

BODY

Experimental Methods:

Cell transfections. Cell transfections were carried out in HeLa and HepG2 cell lines. HeLa cells 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 six well plates at a density of 3×10^5 cells per well with phenol red-free DMEM containing 5% dextran-coated-charcoal stripped serum to remove any traces of estrogen in the medium. Transfections were carried out with Lipofectamine (Life Technologies) according to the manufacturer's recommendations. An estrogen responsive reporter construct, pERE-E1b-LUC, was constructed for this project which contains a portion of the vitellogenin A2 promoter (positions -331 to -87) fused to the adenovirus E1b 'TATA' box linked to a firefly luciferase gene in the pGL3-Basic vector (Promega). Each well of HeLa cells was transfected with 750 ng of pERE-E1b-LUC, 5 ng of an expression vector for ER (pCR3.1-hER), 50 ng of a β -galactosidase expression vector to control for transfection efficiency (pCR3.1- β GAL) and expression vectors for coactivators or their parent plasmids to equalize the amount of DNA between wells.

HepG2 cells were maintained and plated similar to HeLa cells except at a density of 8×10^5 cells/well. HepG2 cells were transfected with Lipofectin (Life Technologies) according to the manufacturer's recommendations. In HepG2 cells, 1000 ng of pC3-LUC, an estrogen responsive target gene construct consisting of the complement C3 promoter (positions -1807 to +58) linked to the firefly luciferase gene (19, 20) was transfected along with 50 ng of an

expression vector for wildtype ER (pCMV₃hER) or mutant ER (pCMV₃hER S104A S106A S118A) and 150 ng of pCR3.1- β GAL to control for transfection efficiency.

For both cell types, six hours after addition of Lipofectamine or Lipofectin and DNA, the medium was replaced with DMEM with 5% fetal bovine serum. One day after transfection, cells were treated with estradiol (10^{-9} M for HeLa, 10^{-8} M for HepG2), 4HT (10^{-7}), or ethanol vehicle. A specific inhibitor of the proteasome, MG132 (1 μ M) (21), was added at the same time as hormones. Twenty hour hours after hormone treatment, cells were assayed for luciferase activity using the Luciferase Assay System (Promega), according to the manufacturer's instructions.

Mutagenesis. Mutagenesis was performed using the Gene Editor mutagenesis system (Promega). The construct to be mutagenized was denatured and incubated with a primer containing the desired mutation and another primer which anneals within the ampicillin resistance gene and modifies it so that it subsequently confers resistance to both ampicillin and a proprietary antibiotic supplied by the manufacturer. T4 DNA polymerase was used to synthesize the complementary strand which was then ligated with T4 DNA ligase. The resulting double-stranded plasmid products were transformed into BMH71-18 *mutS*⁻ bacterial cells which are unable to repair DNA mismatches. After twenty-four hours, the plasmid DNA from these bacteria were extracted and used to transform the JM109 *E. coli* strain. The mutagenized plasmid was subsequently identified by restriction analysis for a restriction site incorporated into the plasmid by the mutagenic primer and sequenced to verify its authenticity.

Western analysis. HeLa cells were transfected under the same conditions used for transient transfection assays (see above). Cells were extracted in an ER extraction buffer containing 50 mM TRIS pH 8.0, 5 mM EDTA, 1% NP40, 0.2% Sarkosyl, 0.4 M NaCl, 100 μ M sodium vanadate, 10 mM sodium molybdate and 20 mM sodium fluoride. Cell pellets were resuspended in ER extraction buffer by vortexing and incubated 15 minutes at 4°C. After centrifugation for 15 minutes at 15,000 rpm at 4°C, equal amounts of protein (40 μ g) were loaded onto 7.5% SDS-PAGE gels and run at 38 mA for ~1 hour and transferred to nitrocellulose. Blots were blocked for 1 hour in a 5% dried milk, TBST solution (5% fat-free dried milk, 50 mM TRIS pH 7.5, 150 mM NaCl, 0.05% Tween-20). ER blots were probed with a rat anti-human ER antibody (H222), then with a rabbit anti-rat bridging antibody and then with an anti-rabbit-HRP conjugated antibody. PR Western blots were probed with a mouse anti-human PR antibody, then with a rabbit anti-mouse bridging antibody and then with an anti-rabbit-HRP conjugated antibody. ER and PR signals were detected using an ECL detection kit (Amersham) according to the manufacturer's recommendations. The H222 antibody recognizes ER as a single band migrating with an apparent molecular weight of 66 kDa (22).

Results and Discussion

A new steroid receptor coactivator recently cloned in our laboratory named E6-AP may play an important role in modulating the response of ER to estrogens and tamoxifen. E6-AP was identified in a yeast two-hybrid screen for corepressor proteins which specifically interact with the RU486 bound progesterone receptor (PR) which yielded a 21 kDa clone with 100% sequence identity to the C-terminal portion of E6-AP. However, subsequent analysis of the full-length E6-AP revealed that it interacted with PR in a progesterone dependent manner and not in the

presence of RU486. This discrepancy may be due to the fact that the PR-ligand binding domain was used in the yeast two hybrid screen instead of the full length receptor. Also, RU486 possesses stronger agonist activity in yeast than in mammalian cells which would cause coactivators to interact with PR instead of corepressors in the yeast two-hybrid screen. Despite the change in specificity of the original yeast two-hybrid clone versus the full-length E6-AP, it was still possible that E6-AP could still be involved in binding to the antagonist bound ER. Analysis of E6-AP expression in mouse mammary gland tumors derived from a mouse carcinogen-induced mammary gland tumor model indicated that it is overexpressed in 12 out of 13 tumors examined and not in hyperplastic tissue from the same animals (Lakshmi Sivaraman and Bert O'Malley, personal communication). The role of E6-AP in coactivation of ER and in tamoxifen resistance was subsequently explored in greater detail.

The ability of E6-AP to coactivate ER in the absence and presence of hormone and in the presence of 4HT was assessed (Fig. 1). E6-AP was able to coactivate ER in the presence of estradiol but did not convert 4HT into an agonist of ER. E6-AP was also able to coactivate PR, glucocorticoid receptor, androgen receptor, thyroid hormone receptor and retinoic acid receptor (Fig. 2). E6-AP was also able to stimulate Sp1 transactivation, albeit to a lesser extent and was unable to stimulate transactivation by CREB or E2F (data not shown). This indicates that E6-AP is a general coactivator of nuclear hormone receptors like SRC-1 (11).

During the course of transient transfection analysis of E6-AP, it was noticed that expression of E6-AP stimulated β -galactosidase expression from the cotransfected control vector, pCR3.1- β GAL, which uses the cytomegalovirus (CMV) promoter to drive β -galactosidase expression. Further analysis of E6-AP and other coactivators revealed that they were also able to

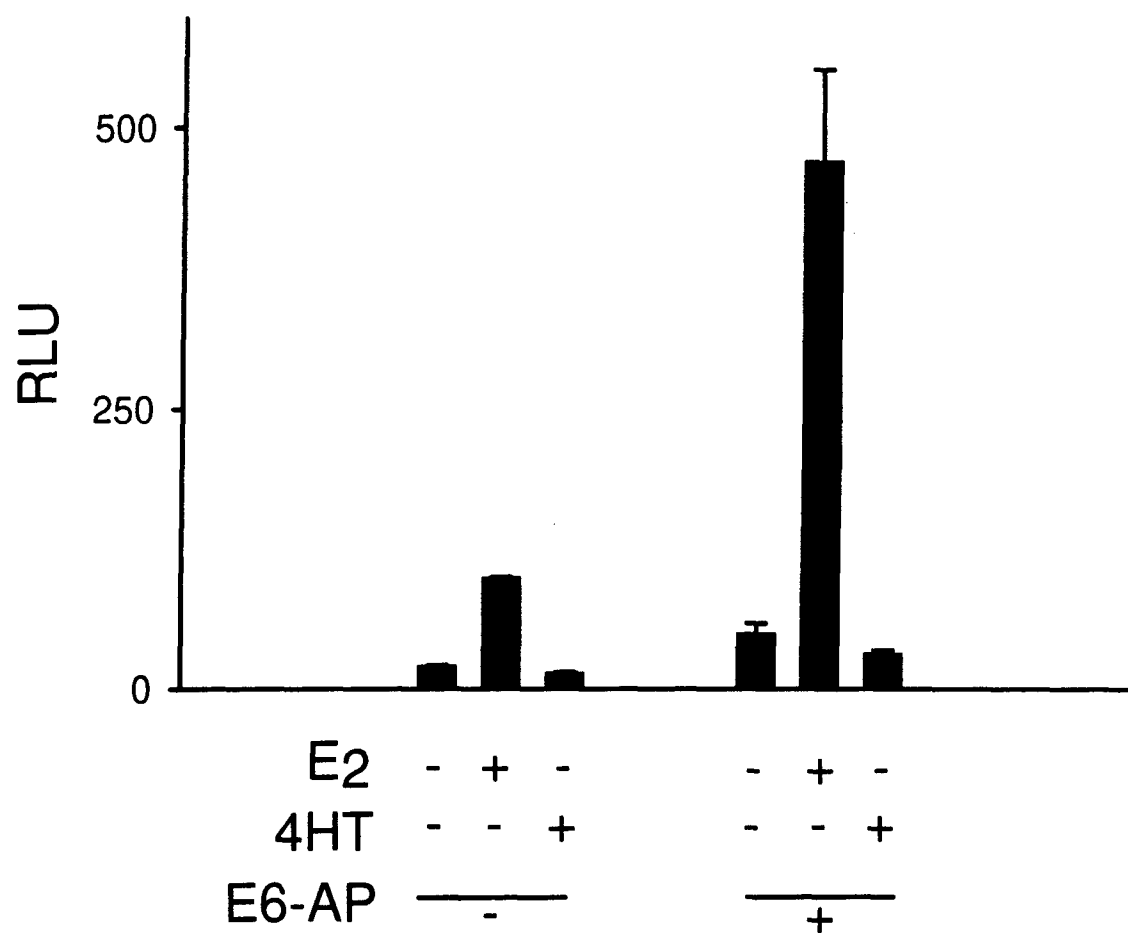


Fig. 1. E6-AP coactivates the transcriptional activity of ER. HeLa cells were transiently transfected with an ER expression construct and pERE-E1b-Luc reporter construct, in the absence or presence of E6-AP expression plasmid. In all transfections, a β -galactosidase expression vector was also cotransfected to monitor transfection efficiency. Cells were treated with vehicle (-), 10^{-9} M E_2 (E_2) or 10^{-7} 4HT (4HT). Results are expressed as relative light units (RLU) adjusted for β -galactosidase activity. $n = 8$, bars indicate s.e.m.

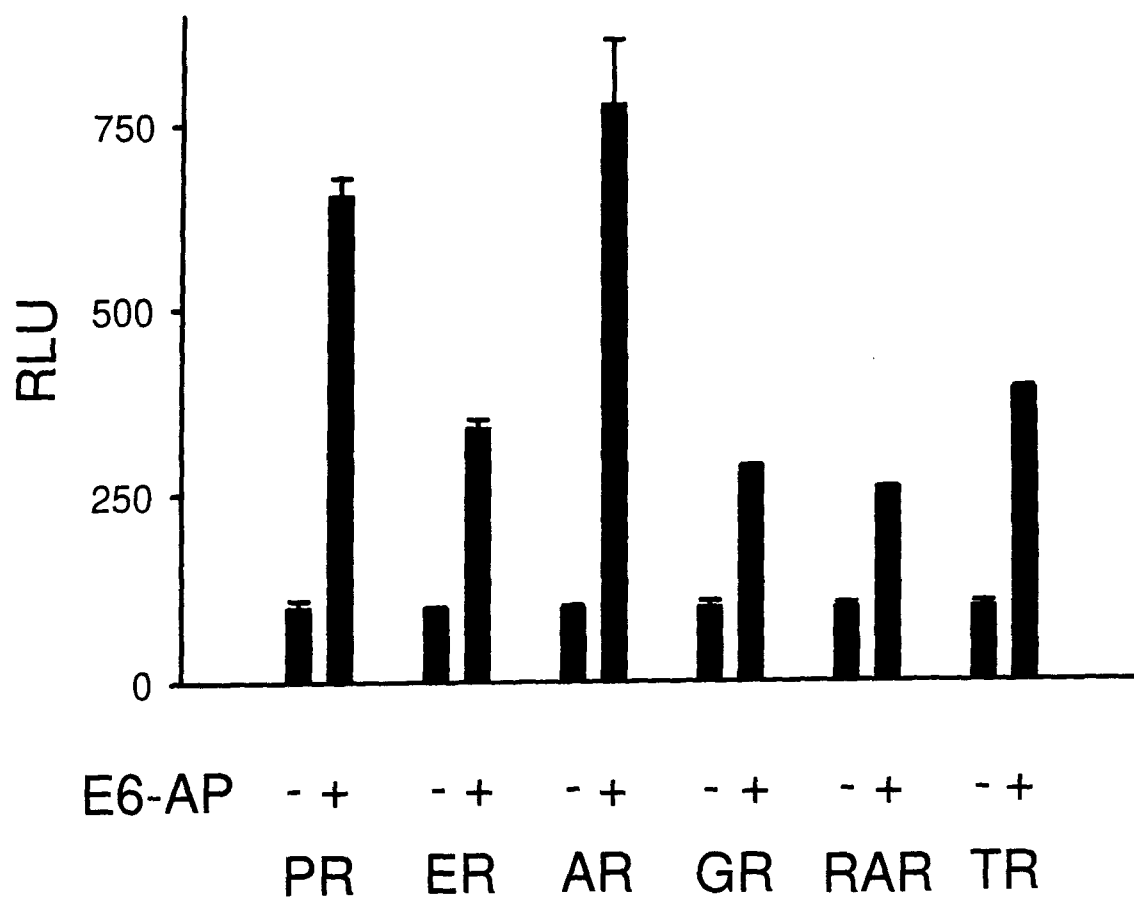


Fig. 2. E6-AP coactivates the hormone-dependent transcriptional activity of nuclear hormone receptors. HeLa cells were transfected with receptor expression vectors for PR, ER, androgen receptor (AR), glucocorticoid receptor α (GR), retinoic acid receptor α (RAR) and thyroid receptor β (TR) and their cognate hormone responsive reporter plasmids, and subsequently treated with appropriate hormones; PR, progesterone (10^{-7}); ER, E_2 (10^{-9} M); AR, R1881 (2.5×10^{-10} M); GR, dexamethasone, (10^{-7} M); RAR, all-*trans* retinoic acid (0.5×10^{-6} M) and TR, triiodothyronine (10^{-7} M).

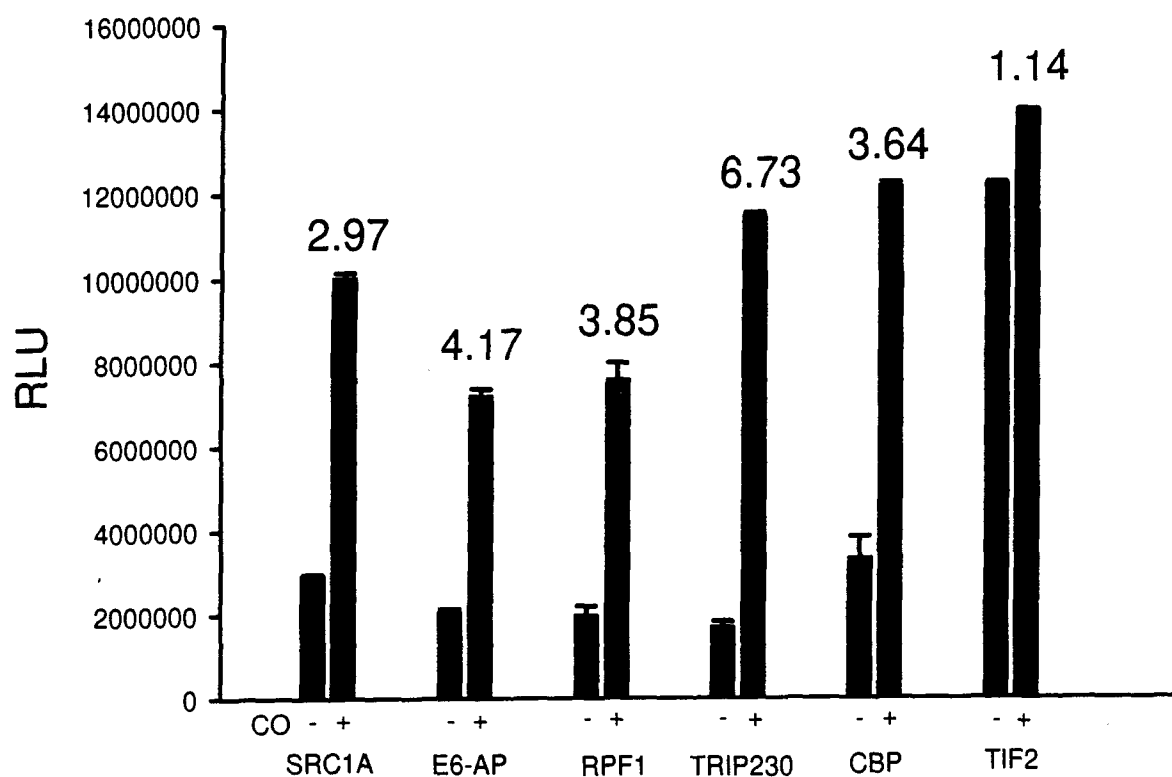


Fig. 3. Coactivators stimulate the CMV promoter, used to drive expression of many steroid receptors in transient transfection assays. 10 ng of a cytomegalovirus (CMV) promoter expression vector, driving expression of the firefly luciferase gene (pCR3.1-LUC) was transfected with 500 ng of carrier DNA and expression vectors for the indicated coactivators (+) or their corresponding parent plasmids (-) to equalize the amount of DNA between assays. Numbers above each set of bars indicate the fold-stimulation of each coactivator on luciferase expression. RLU have been normalized to protein levels. For the indicated coactivators, SRC1A (250 ng pCR3.1-SRC1A), E6-AP (250 ng pCR3.1-E6-AP), RPF1 (250 ng pBK-CMV-RPF1), TRIP230 (250 ng pBK-CMV-TRIP230), CBP (500 ng pRc-RSV-HA-mCBP), TIF2 (500 ng pSG5-TIF2) were used.

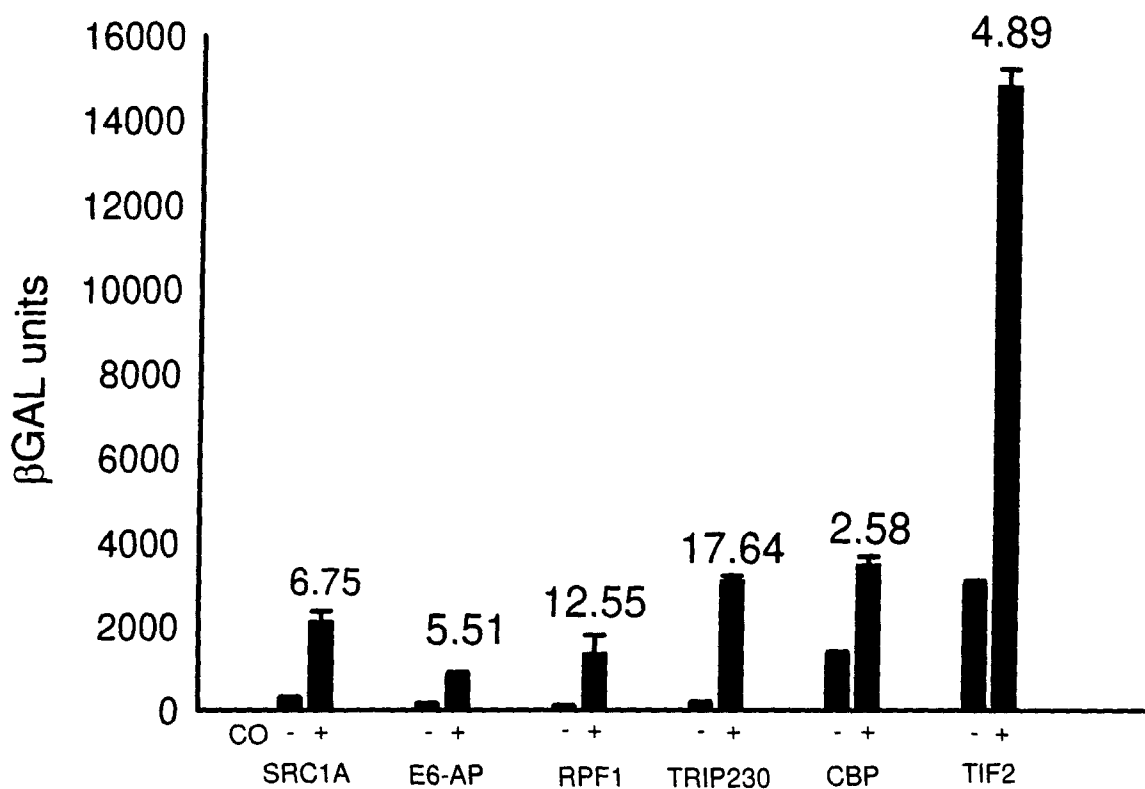


Fig. 4. Coactivators stimulate the RSV promoter, used to drive expression of many steroid receptors in transient transfection assays. 500 ng of a Rous sarcoma virus (RSV) promoter expression vector, driving expression of the β -galactosidase gene (pRSV- β GAL) was transfected with indicated coactivators (+) or their corresponding parent plasmids (-) to equalize the amount of DNA between assays. Numbers above each set of bars indicate the fold-stimulation of each coactivator on β -galactosidase expression. β -galactosidase units have been normalized to protein levels. Coactivators were cotransfected as in fig. 3.

stimulate expression from an expression vector containing a cytomegalovirus (CMV) promoter fused to the firefly luciferase gene (pCR3.1-LUC) (Fig. 3). The effect was not confined to CMV promoters, as an expression vector containing the Rous sarcoma virus (RSV) promoter fused to the β -galactosidase gene (pRSV- β GAL) was also stimulated by coactivator overexpression (Fig. 4). Coactivators were also similarly able to stimulate SV40 promoter-driven gene expression (data not shown). Because the ER expression vectors used in our transient transfection assays also utilized the CMV promoter to drive expression of ER, it was important to determine if ER expression would also be affected. Western analysis of ER expressed under the same condition as the transient transfection assays for coactivator activity reveal that E6-AP and SRC-1 were both able to stimulate ER protein levels (Fig. 5). Western analysis of PR protein levels from transient transfection assays with E6-AP provided the same results (Fig. 5). When cell extracts corresponding to equal amounts of β -galactosidase activity were loaded however, the protein levels of PR were equal. This indicates that the internal β -galactosidase vector can be used to control for hormone receptor levels in transient transfection assays. Transient transfection assays which are normalized for protein levels recovered from each culture well do not adequately control for increases in reporter gene expression which will occur due to increased hormone receptor expression in cells cotransfected with coactivators. All the ER coactivator data presented in this report is standardized to β -galactosidase activity because of this important observation.

E6-AP was previously identified as a 100 kDa cellular protein which interacts with the human papillomavirus type 16 and 18 E6 protein and p53. The E6 and E6-AP complex promotes the degradation of p53 via the ubiquitin-proteasome degradation pathway (23, 24). E6-AP is a

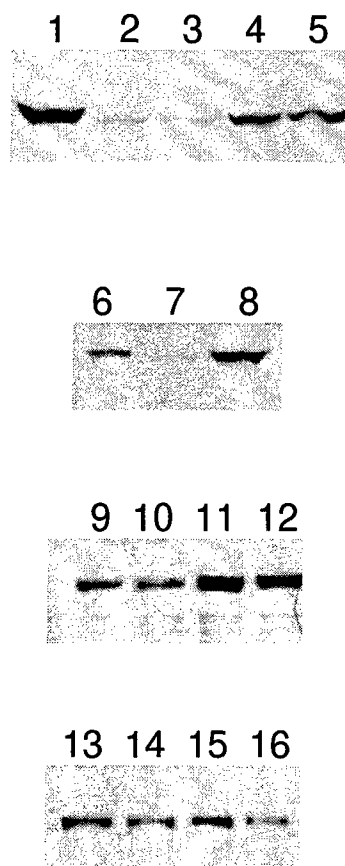


Fig. 5. E6-AP and SRC-1 increase steroid receptor protein levels in transient transfection assays as revealed by Western analysis (see Materials and Methods). ER Western blots (1-8); 1, ER positive control; 2, no expression vector for E6-AP or estradiol; 3, no expression vector for E6-AP with estradiol (10^{-9} M); 4, expression vector for E6-AP without estradiol; 5, expression vector for E6-AP with estradiol (10^{-9} M); 6, ER positive control; 7, no expression vector for SRC-1 with estradiol; 8, SRC-1 expression vector with estradiol (10^{-9} M). PR Western blots (9-16); 9, no expression vector for E6-AP or progesterone; 10, no expression vector for E6-AP with progesterone; 11, E6-AP expression vector without progesterone; 12, E6-AP expression vector with progesterone. Lanes 13-16 are the same as 9-12, except equal β -galactosidase activity was loaded in each lane.

member of a class of functionally related ubiquitin-protein ligases (E3's) which accept ubiquitin from an ubiquitin-conjugating enzyme (E2) and transfer it to the protein targeted for degradation. The carboxyl-terminal 350 amino acids (aa) of E6-AP contain a 'hect' (homologous to the E6-AP carboxy terminus) domain (25) which is conserved among many E3 ubiquitin protein-ligases characterized so far. The extreme carboxyl-terminal 100 aa contains the catalytic region of E6-AP which transfers ubiquitin to the protein targeted for degradation (25, 26). The identification of a coactivator with ubiquitin-protein ligase activity opens the possibility that the ubiquitin-proteasome pathway is involved in stimulating transcription. Because E6-AP is involved in the ubiquitin-proteasome protein degradation pathway, we wanted to determine if E6-AP's ability to coactivate ER is related to the ubiquitination activity of E6-AP. A mutant form of E6-AP (C833S) in which the cysteine at residue 833 is replaced with a serine that is unable to accept ubiquitin from an E2 and transfer ubiquitin to the terminal target protein (26) was assessed for its ability to coactivate ER in HeLa cells (Fig. 6). Coactivation was essentially equal to that seen with the wild-type E6-AP, indicating that the E3 ubiquitin-protein ligase activity of E6-AP is unrelated to its coactivation function. However, through a separate approach (see below), ubiquitin-proteasome function was demonstrated to be essential for E6-AP or SRC-1 to function as a coactivator. Treatment of HeLa or HepG2 cells with the proteasome inhibitor, MG132, a cell-permeable leupeptin analog, was able to block the coactivator function of E6-AP and SRC-1. (Figs. 7 and 8). This suggests that coactivators impart their effect through the ubiquitin-proteasome protein degradation pathway in addition to their roles as bridging factors or histone acetyltransferases. The mechanism through which this occurs will require further investigation. It is also possible that corepressors or other proteins which repress transcription accumulate

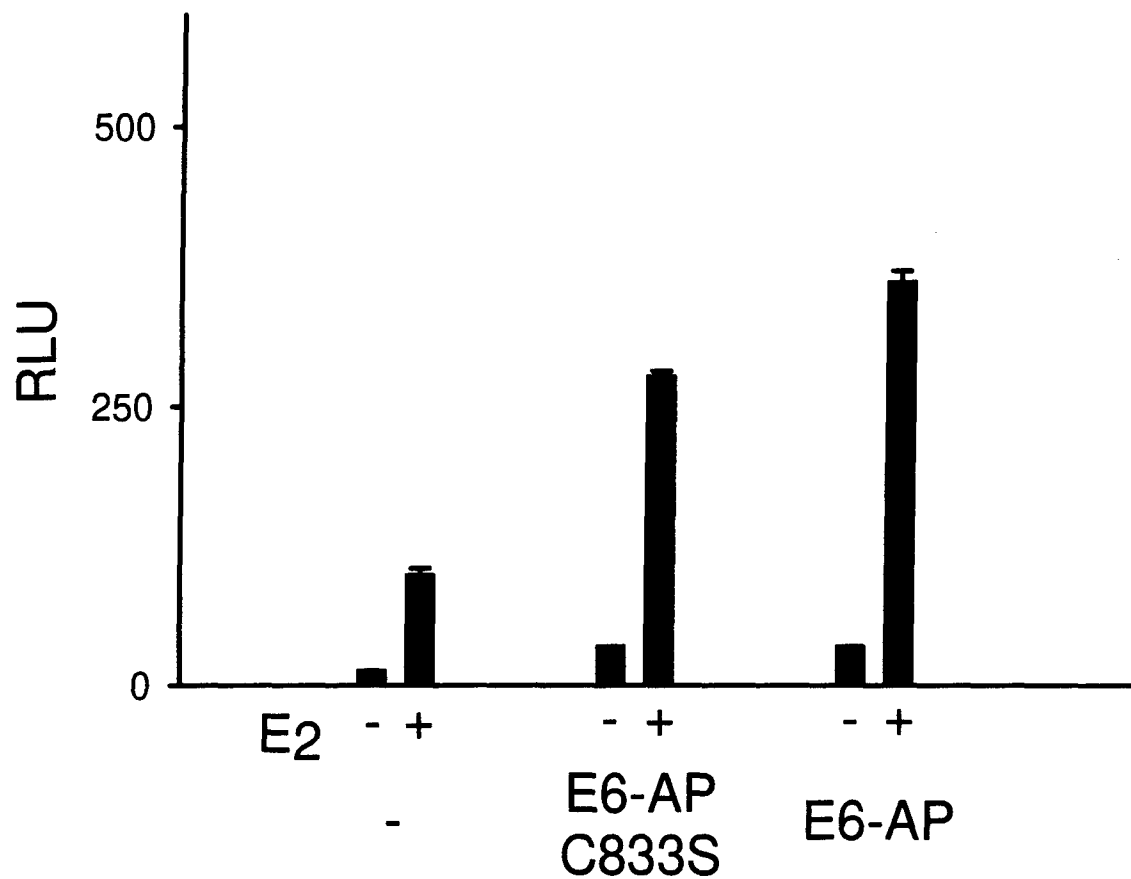


Fig. 6. Coactivation of ER by C833S E6-AP, a mutant that is defective for E3 ubiquitin-protein ligase activity. HeLa cells were transfected as above, along with an expression vector for the C833S E6-AP mutant or the wild-type E6-AP, or the parent plasmid for either E6-AP expression vector to equalize the amount of DNA transfected into each well. $n = 6$, error bars indicate s.e.m.

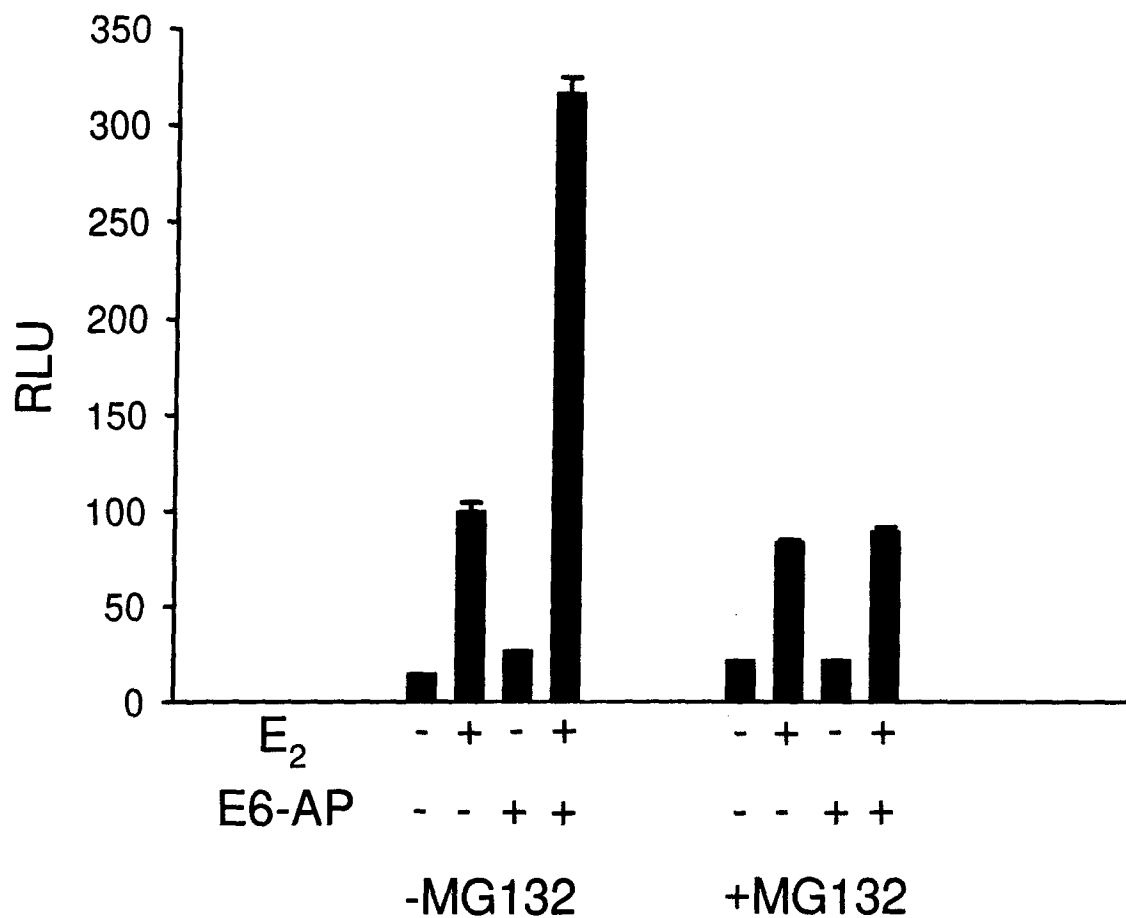


Fig. 7. The proteasome inhibitor, MG132, blocks E6-AP coactivation. HeLa cells were transfected as above with 250 ng E6-AP expression vector. At the time of E_2 addition (10^{-9} M), 1 μ M MG132 was added to the indicated wells.

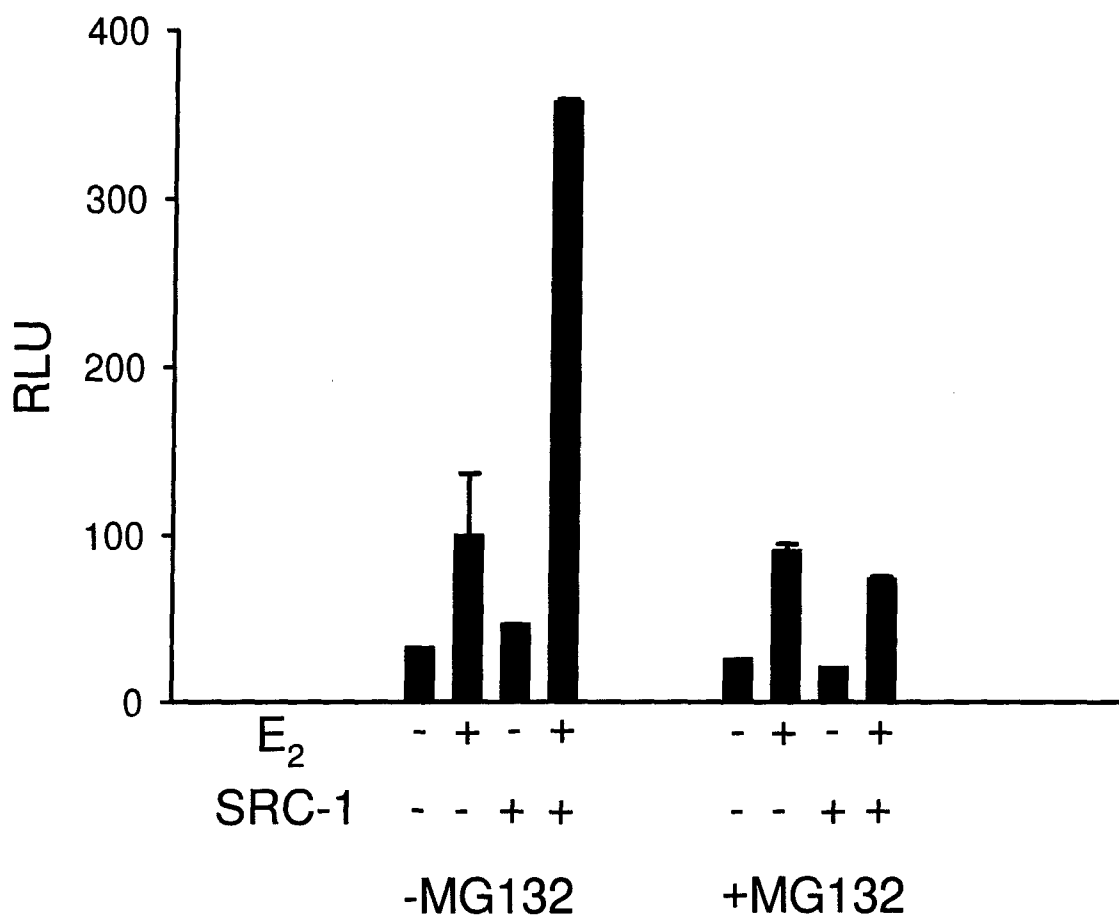


Fig. 8. The proteasome inhibitor, MG132, blocks SRC-1 coactivation. HeLa cells were transfected as above with 250 ng SRC-1 expression vector. At the time of E₂ addition (10⁻⁹ M), 1 μ M MG132 was added to the indicated wells.

when the ubiquitin-proteasome protein degradation pathway is inhibited, interfering with coactivator function.

Because inhibition of the ubiquitin-proteasome protein degradation pathway was able to block exogenous coactivator function, it was important to determine what effect this impairment of coactivator function would have on the response of ER to 4HT. The HepG2 cell line was chosen to explore what role coactivators had in 4HT agonist activity because 4HT functions as a relatively good agonist of ER in this cell line (19, 20). ER was transfected into this cell line along with a target gene construct containing the complement C3 promoter linked to luciferase (pC3-LUC) which provides a strong ER-dependent agonist response to 4HT in this cell line. Treatment of these transfected cells with 4HT resulted in the expected agonist response. However, in the presence of MG132, 4HT possessed no agonist activity (Fig. 9). It is possible that a coactivator is responsible for converting 4HT into an agonist in HepG2 cells which can be inhibited by MG132 or MG132 might block the turn-over of a corepressor that is essential for coactivators to function and for 4HT to function as an agonist. Further studies will be required to test these possibilities. This novel finding indicates that proteasome inhibitors such as MG132 might be a potential solution for restoring 4HT to its originally antagonistic state in breast cancer patients who have been taking tamoxifen for a number of years.

After establishing that the ubiquitin-proteasome pathway is involved in the response of ER to 4HT, I have begun to examine whether there is any connection between phosphorylation of ER, the ubiquitin-proteasome pathway and the effects on 4HT response observed. In HepG2 cells, the AF-1 region of ER has been shown to be very active in stimulating ER-dependent stimulation of transcription and that 4HT functions by activating this portion of the receptor. Phosphoserine residues in the AF-1 region of ER have also been implicated in the ligand-

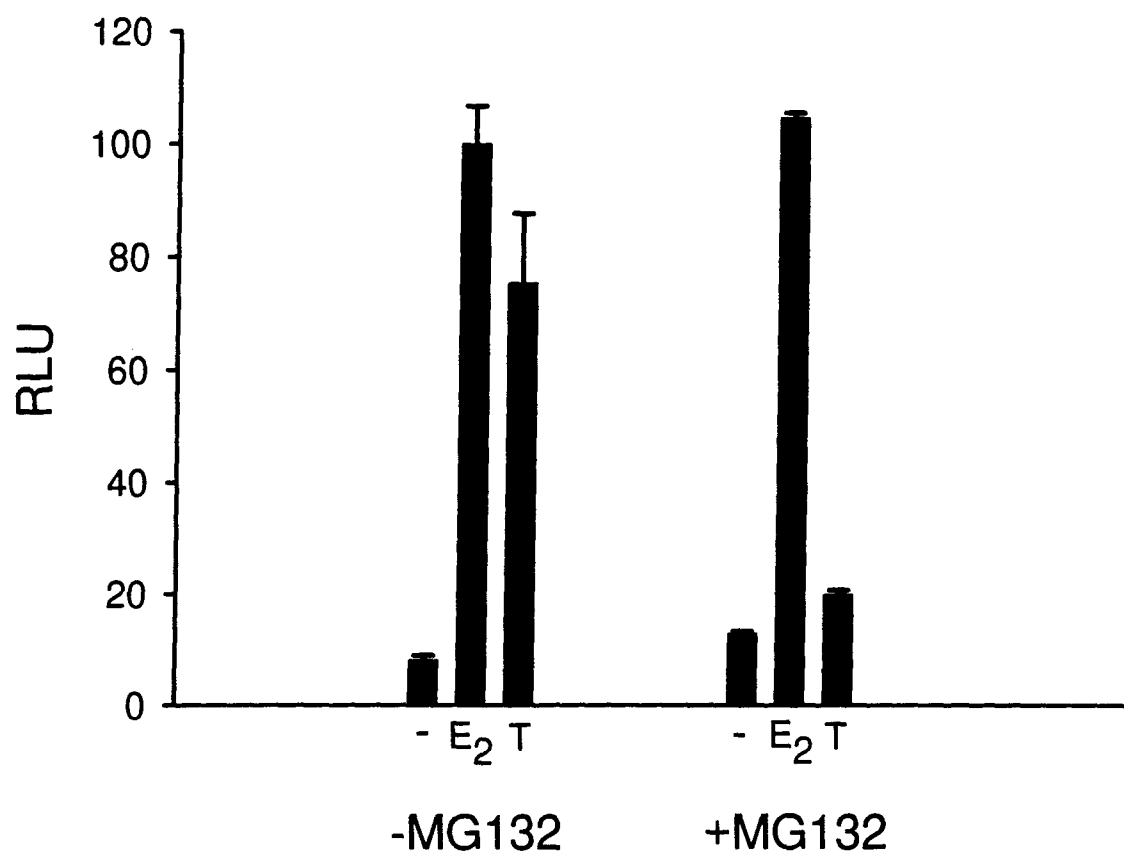


Fig. 9. MG132 converts 4HT from an agonist to an antagonist in HepG2 cells. HepG2 cells were transfected with 1000 ng pC3-LUC and 50 ng pCMV_hER along with 150 ng pCR3.1- β GAL to control for transfection efficiency. At the time of E₂ addition (10^{-8} M), 1 μ M MG132 was added to the indicated wells.

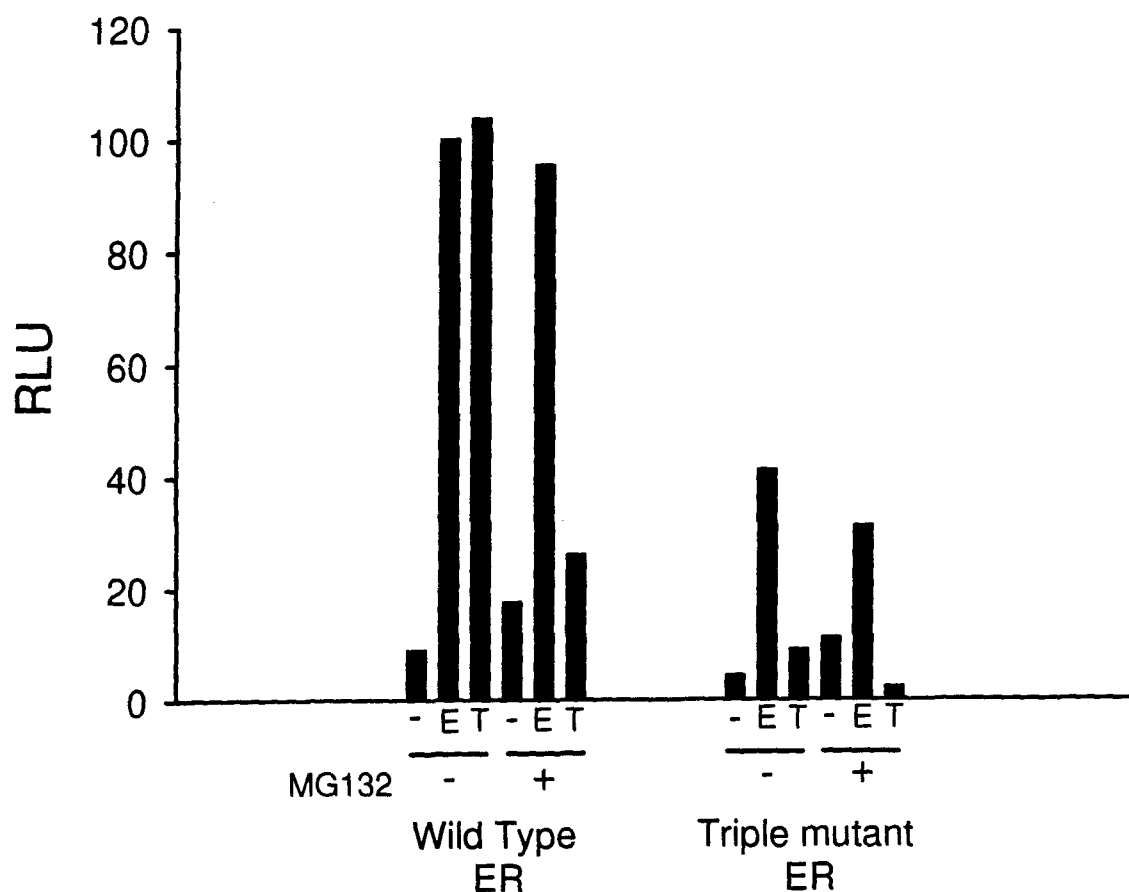


Fig. 10. ER with serines mutated to alanines at positions 104, 106 and 118 (triple mutant ER) possesses no 4HT agonist activity in HepG2 cells. Cells were transfected as above, except 50 ng of a mutant ER expression vector (pCMV_hER S104A S106A S118A) was cotransfected instead of the wildtype ER expression vector. Cells were treated with vehicle (-), 10^{-8} M E_2 (E) or 10^{-7} 4HT (T).

independent activation of ER by cAMP or MAP kinase (27, 28, 29). A mutant form of ER which contains alanines which can not be phosphorylated in place of serines at amino acid positions 104, 106 and 118 was tested for its ability to stimulate ER-dependent gene expression in the presence of estrogen or 4HT (Fig. 10). The triple-mutant ER was unresponsive to 4HT suggesting that phosphorylation of the AF-1 region of ER is essential for 4HT to function as an agonist. In the presence of MG132, 4HT suppressed the transcriptional activity of the triple-mutant ER even further.

Future directions of this research will be to substantiate the involvement of the ubiquitin-proteasome pathway in tamoxifen agonist/antagonist switching and ER function through approaches independent of MG132. Using anti-ubiquitin antibodies, the differential ubiquitination of ER, coactivators or corepressors in the presence or absence of estradiol or 4HT can be explored and could provide corroborative evidence to support an important role for the ubiquitin-proteasome protein degradation pathway in ER function. Additionally, it will be informative to assess the effect of MG132 on coactivator and corepressor turnover. Western analysis of E6-AP, SRC-1, CBP, N-Cor and SMRT levels in the absence or presence of MG132 may indicate that turnover of one or more of these proteins is responsible for the effects seen on coactivation and 4HT responsiveness demonstrated in this report.

CONCLUSIONS

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. The recent cloning and characterization of a new steroid receptor coactivator in our laboratory, E6-AP, suggested that ubiquitination and protein degradation might represent a novel mechanism for steroid receptor coactivation and the response of ER to estradiol and 4HT. Although the ubiquitin-protein ligase activity of E6-AP does not appear to contribute to its ability to coactivate ER, other data in this report suggests that the ubiquitin-proteasome protein degradation pathway is important both for coactivation and how ER responds to 4HT. Investigations which could further substantiate the ubiquitin-proteasome protein degradation pathway in coactivator function and the ER response to 4HT will be needed to bolster these findings. It will be important to determine how E6-AP and estradiol influence the ubiquitination of ER and other coactivators such as SRC-1 and CBP. Additionally, it will be interesting to determine if coactivators and estradiol influence the association of ER and other transcription regulators with components of the ubiquitin-proteasome degradation pathway. Ultimately, the ubiquitin-proteasome pathway may be linked to phosphorylation of ER and coactivators. Kim and Maniatis (30) have shown that phosphorylation of the STAT1 transcription factor is coupled to its ability to stimulate transcription and its subsequent degradation by the ubiquitin-proteasome protein degradation pathway, allowing for more precise regulation of IL-2 mediated gene expression. On the other hand, phosphorylation of c-Jun by MAP kinase has been shown to reduce the degree to which c-Jun is ubiquitinated and degraded allowing the MAP kinase pathway to stimulate c-Jun mediated transcription (31). It is conceivable that phosphorylation of coactivators, ER, or other transcription regulators may also both render them transcriptionally active and target them for

degradation or make them more resistant to degradation to provide an additional means to regulate ER mediated gene expression.

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