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The Ubiquitin-Conjugating Enzyme UBCH7 Acts as a Coactivator for Steroid Hormone Receptors

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We investigated the role of the ubiquitin-conjugating enzyme UBCH7 in nuclear receptor transactivation. Using transient transfection assays, we demonstrated that UBCH7 modulates the transcriptional activity of progesterone receptor (PR) and glucocorticoid, androgen, and retinoic acid receptors in a hormone-dependent manner and that the ubiquitin conjugation activity of UBCH7 is required for its ability to potentiate transactivation by steroid hormone receptors (SHR). However, UBCH7 showed no significant effect on the transactivation functions of p53 and VP-16 activation domain. Depletion of endogenous UBCH7 protein by small interfering RNAs suggests that UBCH7 is required for the proper function of SHR. Furthermore, a chromatin immunoprecipitation assay demonstrated the hormone-dependent recruitment of UBCH7 onto estrogen receptor- and PR-responsive promoters. Additionally, we show that UBCH7 and E6-associated protein (E6-AP) synergistically enhance PR transactivation. We also demonstrate that UBCH7 interacts with steroid receptor coactivator 1 (SRC-1) and that UBCH7 coactivation function is dependent on SRC-1. Taken together, our results reveal the possible role of UBCH7 in steroid receptor transactivation and provide insights into the mechanism of action of UBCH7 in receptor function.

Steroids, retinoids, thyroid hormones, and vitamin D control various biological processes, including growth, development, and homeostasis, via their cognate nuclear receptors, which are comprised of a superfamily of structurally related intracellular ligand-activated transcription factors (2, 57). In the absence of hormones, these receptors are transcriptionally inactive and often found in a large complex consisting of heat shock proteins (hsp90, hsp70, and hsp56) and other chaperone proteins. When bound to hormone, these receptors undergo a conformational change, dissociation from heat shock proteins, receptor dimerization, phosphorylation, DNA binding to the enhancer elements of target genes, interaction with coactivators, and subsequent recruitment of general transcription factors to form a preinitiation complex followed by induction of target gene transcription (4, 36).

In recent years we have witnessed rapid progress in our understanding of the cellular factors that are recruited by activated nuclear hormone receptors. Most of these cellular factors act as either coactivators or corepressors for nuclear receptors (15, 19, 28, 36). Coactivators are molecules that interact with receptors in the presence of hormones and stimulate receptor-mediated transcription of target genes. The most thoroughly studied coactivators include the following members of the p160 family of coactivators: SRC-1 (steroid receptor coactivator 1), SRC-2 (transcription intermediary factor 2 [TIF-2]/glucocorticoid receptor [GR] interacting protein 1 [GRIP-1]), SRC-3 (p300/CREB-binding protein [CBP] interacting protein [p/CIP]/activator of thyroid and retinoid acid receptors [ACTR]/amplified in breast cancer 1 [AIB-1], retinoid acid receptor coactivator 3 [RAC-3]/thyroid receptor activator molecule 1 [TRAM-1]), and the CBP/p300 family (7, 35, 36). Among the coactivators that have been cloned and characterized in detail are nuclear receptor cointegrator (NRC)/activating signal cointegrator 2 (ASC-2)/peroxisome proliferator-activated receptor (PPAR gamma)-interacting protein (PRIP)/thyroid hormone receptor-binding protein (TRBP)/nuclear receptor-activating protein 250 (RAP250)/amplified in breast cancer 3 (AIB-3), PPAR gamma coactivator (PGC), androgen receptor (AR)-associated protein 70 (ARA70), p300/CREB-binding protein-associated factor (P/CAF), TR-associated protein (TRAP) complex, vitamin D receptor-interacting protein (DRIP) complex, E6-associated protein (E6-AP), coactivator-associated arginine methyltransferase (CARM-1), and steroid receptor RNA activator (SRA) (5, 13, 23, 26, 27, 39, 47).

Recently, it has been demonstrated that various enzymatic activities are associated with coactivators that contribute to their ability to enhance receptor-mediated transcription. Acetyltransferase activity was the first enzymatic function found to be associated with SRC-1, RAC-3/ACTR/AIB-1, and CBP/p300, and this activity has been associated with the coactivators' ability to promote transcriptional activation (6, 42, 54). Recently, more coactivators associated with enzymatic function have been found, such as CARM-1, protein arginine methyltransferase (PRMT) (5), ATPase-containing multiprotein, switch/sucrose nonfermentation (SW1/SNF) complex (38, 49), and the ubiquitin-protein ligase E6-AP (21). It has been suggested that these catalytic activities promote transcriptional activation via chromatin remodeling or covalent modification

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of members of the transcriptional machinery, coactivators, and the receptor (8, 49).

It has been suggested that the ubiquitin-proteasome pathway plays an important regulatory role in nuclear receptor function, adding a new dimension to the field of nuclear hormone receptors (10, 32, 33, 39). Besides ubiquitin-protein ligases, such as E6-AP; RSP5 (reverses Spt phenotypes) and its human homologue, RPF-1 (receptor potentiation factor 1) (23, 39); UBA3, a ubiquitin-activating enzyme of the NEDD8 pathway (12, 16); the GAL4 suppressor SUG1 (suppressor of Gal4D lesions 1) (14, 33); and UBC9 (46), the human homologue of *Saccharomyces cerevisiae* E2 ubiquitin-conjugating enzyme of the sumoylation pathway, are some of the components of the ubiquitin-proteasome pathway and ubiquitin-like pathways that have been shown to modulate the properties of steroid receptors. It is possible that these components modulate steroid receptor-dependent gene transcription by degrading and/or modifying factors that influence gene transcription.

Ubiquitin-conjugating enzymes are important components of the ubiquitin-proteasome pathway, facilitating the transfer of activated ubiquitin from ubiquitin-activating enzyme to target proteins with the help of ubiquitin-protein ligases (18, 24, 45, 58). The ubiquitin-conjugating enzyme UBCH7 (E2-F1) is an 18-kDa protein (41), and it is involved in the E6-AP-mediated degradation of p53 (9, 50). Furthermore, it has been suggested that UBCH7 acts as a ubiquitin-conjugating enzyme for E6-AP and also promotes E6-AP degradation (20, 40, 50, 60).

In this study, we describe a role for UBCH7 in steroid hormone receptor function. We found that UBCH7 modulates the hormone-dependent transcriptional activity of various steroid and nuclear hormone receptors. Furthermore, we demonstrate that UBCH7 is recruited to progesterone receptor (PR)- and estrogen receptor (ER)-responsive promoters in a hormone-dependent manner. Additionally, we found that depletion of endogenous UBCH7 protein with small interfering RNA (siRNA) significantly reduced the transactivation of PR. Our data also suggest that coexpression of UBCH7 and E6-AP enhances PR transactivation synergistically. Furthermore, we have shown that UBCH7 is physically associated with SRC-1, and data from the SRC-1 knockout (KO) cell line indicate that SRC-1 is required for UBCH7 to modulate steroid hormone receptor function. Together, our results demonstrate the role of UBCH7 as a coactivator in modulating nuclear receptor function.

MATERIALS AND METHODS

Plasmid construction. The mammalian expression plasmids for PR-B (pCR3.1.PR-B), GR (pCR3.1.GR), AR (pCR3.1.AR), retinoic acid receptor (RAR; RSV.RAR), and GAL-VP16 have been described previously (1, 39, 44, 56). The progesterone/glucocorticoid/androgen-responsive reporter (PRE.TATA.LUC), retinoic acid-responsive reporter, p53-responsive reporter, and 17mer-LUC reporter plasmids also have been described previously (11, 31, 39). The expression vectors for p53, SRC-1, pGEM.E6-AP (3003), pRShPRB, pRShPRA/B, pRShPRΔE, and pPRE3-E4 have been published previously (11, 30, 43, 59).

To construct the mammalian expression vector pBKRSV-UBCH7, a BamHI-EcoRI fragment containing UBCH7 was amplified by PCR with the primer pair 5′-GCGGATCCCCGCGGCCAGCAGGAGGCTGAT-3′ and 5′-CCGGAATTCTTAACAAAAA-3′ using pET-UBCH7 as a template and was subcloned into the corresponding sites of plasmid pBKRSV. The ubiquitin conjugation-defective mutant UBCH7 (C-S) was generated by PCR with the following primer pair: 5′-GAAGATCTATCACCCAAACATCGACGAAAAGGGGCAGGTCAGTCTGCCAGTA-3′ and 5′-CCGGAATTCTTAGTCCACAGGTCGCTTTTCCCCATATTTCTTTGTAAACTC-3′. The PCR product was digested with EcoRI-BglII and cloned into the corresponding sites of plasmid pBKRSV.UBCH7. For glutathione-*S*-transferase (GST) expression vector, GST-UBCH7 was constructed by subcloning the BamHI-EcoRI fragment from pBKRSV.UBCH7 into the pGEX4T-1 plasmid. In order to generate siRNA against UBCH7, the siRNA target finder program from Ambion, Inc. was used. The GAPDH (glyceraldehyde-3-phosphate dehydrogenase) (control) siRNA was purchased from Ambion, Inc. The oligonucleotides used were as follows: for GAPDH siRNA, 5′-GGATATTGTTGCCATCATT-3′; for UBCH7 siRNA 1, 5′-AATTCAGAGCCAGCAATGCCT-3′; for siRNA 2, 5′-AAATGTGGGATGAAAAACTTC-3′; and for siRNA 3, 5′-GGACCGTAAAAAATTCTGT-3′. All constructs were verified by DNA sequencing.

In vitro interaction assay. In vitro expression of radiolabeled SRC-1, SRC-2, and SRC-3 was performed by in vitro transcription and translation (TNT) from rabbit reticulocyte extracts in the presence of [^{35}S]methionine according to the conditions recommended by the manufacturer (Promega). GST-UBCH7 and GST-UBCH5B were expressed in *Escherichia coli* DH-5α cells and purified on glutathione-Sepharose beads. The purified and glutathione-bound UBCH7 and UBCH5B were incubated with in vitro-translated SRC-1, SRC-2, or SRC-3 in NETN buffer (50 mM NaCl, 1 mM EDTA, 20 mM Tris [pH 8.0], 0.1% Nonidet P-40) overnight at 4°C. After washing four times with NETN buffer, UBCH7 and UBCH5B bound SRC proteins were eluted and separated on sodium dodecyl sulfate (SDS)–7.5% polyacrylamide gels and detected by autoradiography.

Coimmunoprecipitation. Twenty-four hours after growth, cells were washed in TEN buffer (40 mM Tris-HCl [pH 7.5], 1 mM EDTA, 150 mM NaCl) and lysed in ice-cold radioimmunoprecipitation assay (RIPA) buffer containing salt (400 mM NaCl, 1× phosphate-buffered saline, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 10 mg of phenylmethylsulfonyl fluoride/ml [10 μl/ml], 30 μl of aprotinin/ml, 100 nM sodium orthovanadate [10 μl/ml]) by pipetting up and down. Thereafter, cell lysates were placed on ice for 30 min. In order to bring the NaCl concentration of the cell lysates to 150 mM, 150 μl of NaCl-free RIPA buffer (1× phosphate-buffered saline, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 10 mg of phenylmethylsulfonyl fluoride/ml [10 μl/ml], 30 μl of aprotinin/ml, 100 nM sodium orthovanadate [10 μl/ml]) was added to the lysates. After centrifugation (21,000 × *g*) at 4°C, the lysates were incubated with 20 μl of protein A-Sepharose and rocked at 4°C for 30 min. After centrifugation, the supernatants were transferred to fresh tubes, and the lysates were mixed with either serum or specific antibody (anti-UBCH7 and anti-UBCH5) at 4°C for 2 h on a rocker. Afterward, 20 μl of protein A-Sepharose beads was added, and the lysates were incubated for an additional hour at 4°C on a rocker. Finally, after extensive washing with NaCl-free RIPA buffer, the immunoprecipitates were subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and analyzed by Western blotting using either anti-SRC-1, anti-UBCH7, or anti-UBCH5 antibody.

In vitro transcription. Cell-free transcription assays using a chromatin template were performed as described previously (30). To assemble DNA onto chromatin, plasmid DNAs pPRE3-E4 and E4 control template lacking PR binding sites were incubated with Sf190 extracts (derived from *Drosophila melanogaster* embryos) and core histones. Purified receptor protein (PR-B), progesterone, wild-type and ubiquitin-conjugating defective mutant UBCH7 (Boston Biochemicals), and HeLa extract were added to a preassembled chromatin assembly, and the reaction was allowed to continue for 30 min at 27°C. The samples were then subjected to in vitro transcription analysis as described previously (30).

Transient transfection. HeLa cells were maintained in Dulbecco modified Eagle medium containing 10% fetal bovine serum. Cells (3×10^5) were plated 24 h before transfection in six-well plates containing 5% dextran-coated, charcoal-stripped serum. Cells were transfected with the indicated amount of DNA using FuGene 6 transfection reagent (Roche Diagnostics). After 4 h, cells were treated with the indicated hormones, and they were harvested 24 h later. Luciferase assays were performed using the Promega luciferase assay system.

SRC-1 and E6-AP KO fibroblasts were plated at a density of 3×10^5 cells in six-well plates in Dulbecco modified Eagle medium containing 10% fetal bovine serum, β-mercaptoethanol, and antibiotics. The next day, medium with stripped serum was added, and the cells were incubated overnight at 37°C. The cells were transfected with PR and progesterone receptor element (PRE) expression vectors as well as with appropriate coactivator expression vectors. After 4 h, the cells were treated with progesterone (10^{-7} M) or a vehicle and were incubated at 37°C overnight. The following day, the cells were harvested, and luciferase assays were performed using Promega's luciferase kit.

ChIP. The MCF-7 cells (ATCC HTB22) or T47D/CAT0 cells were used in chromatin immunoprecipitation (ChIP) analyses following a modified procedure based on previously described protocols (29, 51). The DNA was purified using the QIAquick PCR purification kit (QIAGEN, Valencia, Calif.) and eluted in 50 μl of H_2O. Total input samples were eluted in 100 μl of H_2O and diluted 1:10

before PCR analysis. Each PCR mixture contained 6 μl of immunoprecipitate or input, a 0.5 μM concentration of each primer, 0.4 mM deoxynucleoside triphosphate mixture, 1× Titanium *Taq* PCR buffer (Clontech, Palo Alto, Calif.), and 1× Titanium *Taq* DNA polymerase (Clontech) in a total volume of 25 μl. The primers for the PS2 promoter were as follows: forward, 5′-GGCCATCTCTCA CTATGAATCACTTCTGC-3′, and reverse, 5′-GGCAGGCTCTGTTTGCTTA AAGAGCG-3′. The primers for the mouse mammary tumor virus (MMTV) promoter were as follows: forward, 5′-TATGGTTACAAACTGTTCTTAAAA CGAGGATG-3′, and reverse, 5′-GCAAGTTTACTCAAAAACAGCACTCTT T-3′. PCR was performed for 29 cycles with 1 min of denaturing at 94°C, annealing at 62°C, and extension at 68°C.

RESULTS

UBCH7 modulates the transcriptional activities of various nuclear hormone receptors. To determine if UBCH7 is involved in receptor-dependent activation of target gene expression, transient transfection assays were carried out in HeLa cells. HeLa cells were cotransfected with mammalian expression plasmids for PR, GR, AR, and RAR along with reporter plasmids containing the cognate hormone response element with or without an expression vector for UBCH7. It was observed that UBCH7 had minimal effects on the transactivation functions of various receptors in the absence of hormone. However, UBCH7 significantly enhanced (~4.5- to 6.5-fold) the hormone-dependent transcriptional activity of PR, GR, AR, ER (not shown), and RAR (Fig. 1A). These data suggest that UBCH7 modulates the ligand-dependent transcriptional activities of various nuclear receptors. Since HeLa cells are derived from a papillomavirus type 18-positive cervical carcinoma patient and thus express the viral E6 protein that can functionally interact with UBCH7, it was necessary to rule out the possibility that the viral E6 protein influences the coactivation function of UBCH7. As shown in Fig. 1B, UBCH7 was able to stimulate the hormone-dependent transcriptional activity of PR in the E6-negative T47D cells, suggesting that the coactivation observed in HeLa cells is not dependent on the E6 protein. These data are consistent with previously published data which suggest that the coactivation function of E6-AP is not dependent on the viral E6 protein (39). In view of the fact that UBCH7 is a component of the ubiquitin-proteasome pathway, we also examined the expression levels of PR in both the absence and the presence of exogenously expressed UBCH7. As shown in Fig. 1C, UBCH7 has no significant effect on the expression levels of PR. The expression levels of PR were identical in both the absence and the presence of UBCH7, suggesting that the increased reporter activity observed in transcription assays in the presence of UBCH7 reflects a true increase in the specific transcriptional activity of the PR.

In order to determine whether UBCH7 specifically modulates the transcriptional activities of steroid hormone receptors, we also examined the effects of expression of UBCH7 on the transcriptional activities of other transcriptional factors, such as p53 and the VP-16 activation domain. As shown in Fig. 1D, UBCH7 had no significant effect on the transactivation function of either of these transactivators. The transcriptional activities of p53 and the VP-16 activation domain are identical in both the absence and the presence of exogenously expressed UBCH7. Our observations suggest that UBCH7 preferentially modulates the hormone-dependent transcriptional activity of nuclear hormone receptors.

A number of ubiquitin-conjugating enzymes have been identified. In order to determine if only UBCH7 is involved in receptor-dependent activation of target gene expression, transient transfection assays were carried out. Cells were cotransfected with mammalian expression plasmid for the PR along with reporter plasmid containing a progesterone response element along with either control plasmid or expression plasmid for other ubiquitin-conjugating enzymes, such as UBCH5B, UBCH8, and UBC12. UBCH5B, UBCH8, and UBC12 were unable to stimulate the hormone-dependent transcriptional activity of PR, whereas under similar conditions, UBCH7 was able to stimulate the transcriptional activity of PR (Fig. 1E), suggesting that only UBCH7 is involved in the steroid hormone receptor activation pathway.

Depletion of endogenous UBCH7 levels reduces transcriptional activity of PR. In order to confirm that UBCH7 is indeed required for PR activation, we depleted endogenous expression of UBCH7 in HeLa cells by siRNA directed against UBCH7. HeLa cells were transiently transfected with different UBCH7 siRNAs (siRNA 1, 2, or 3) and GAPDH control siRNA, along with PR and PR-responsive reporter plasmids. It was observed that expression of the different siRNAs directed against UBCH7 resulted in reduced levels of UBCH7 expression, whereas control siRNA had no effect on the expression of UBCH7 (Fig. 2B). Furthermore, we observed that depletion of endogenous UBCH7 by siRNAs 1 and 2 resulted in a 70% reduction of PR transcriptional activity (Fig. 2A). Like siRNAs 1 and 2, siRNA 3 was also able to reduce the transcriptional activity of PR (data not shown). Similarly, the UBCH7-specific siRNA also reduced the expression levels of UBCH7 in E6-negative T47D cells. Furthermore, the transcriptional activity of the endogenous PR in T47D cells was also reduced (data not shown). These data confirm that UBCH7 is required for the proper functioning of this steroid hormone receptor.

UBCH7 reverses transcriptional interference between ER and PR. It has been reported that the transcriptional activity of one receptor can be squelched by the overexpression of another receptor, indicating that both receptors compete for limited pools of common factors (3, 37). To examine if UBCH7 can relieve ER-induced squelching of the transcriptional activity of PR, we used a transient transfection assay. We observed that PR-mediated transcriptional activity was reduced by 82% upon coexpression of estradiol-bound ER (Fig. 3, compare lanes 2 and 3). Addition of UBCH7 reversed this squelching by as much as 9.6-fold (Fig. 3, compare lanes 2 and 8) in a dose-dependent manner. In control cells that do not express ER, UBCH7 enhanced the PR-mediated transcriptional activity from eight- to ninefold (Fig. 3, compare lanes 2 and 9). Thus, coexpression of UBCH7 can reverse the interference between ER and PR, suggesting that UBCH7 is one of the limiting factors that is necessary for efficient PR and ER transcriptional activities.

Effect of UBCH7 on the transcriptional activity of different regions of PR. Since UBCH7 modulates the transcriptional activity of PR in a hormone-dependent manner, we investigated whether UBCH7 has any effect on the transactivation function of the AF-1 and AF-2 regions of PR. HeLa cells were cotransfected with expression plasmids for the AF-1 region of PR, the AF-2 region of PR, or the full length of PR along with the progesterone response reporter gene with or without UBCH7 expression plasmid. The results demonstrate that in

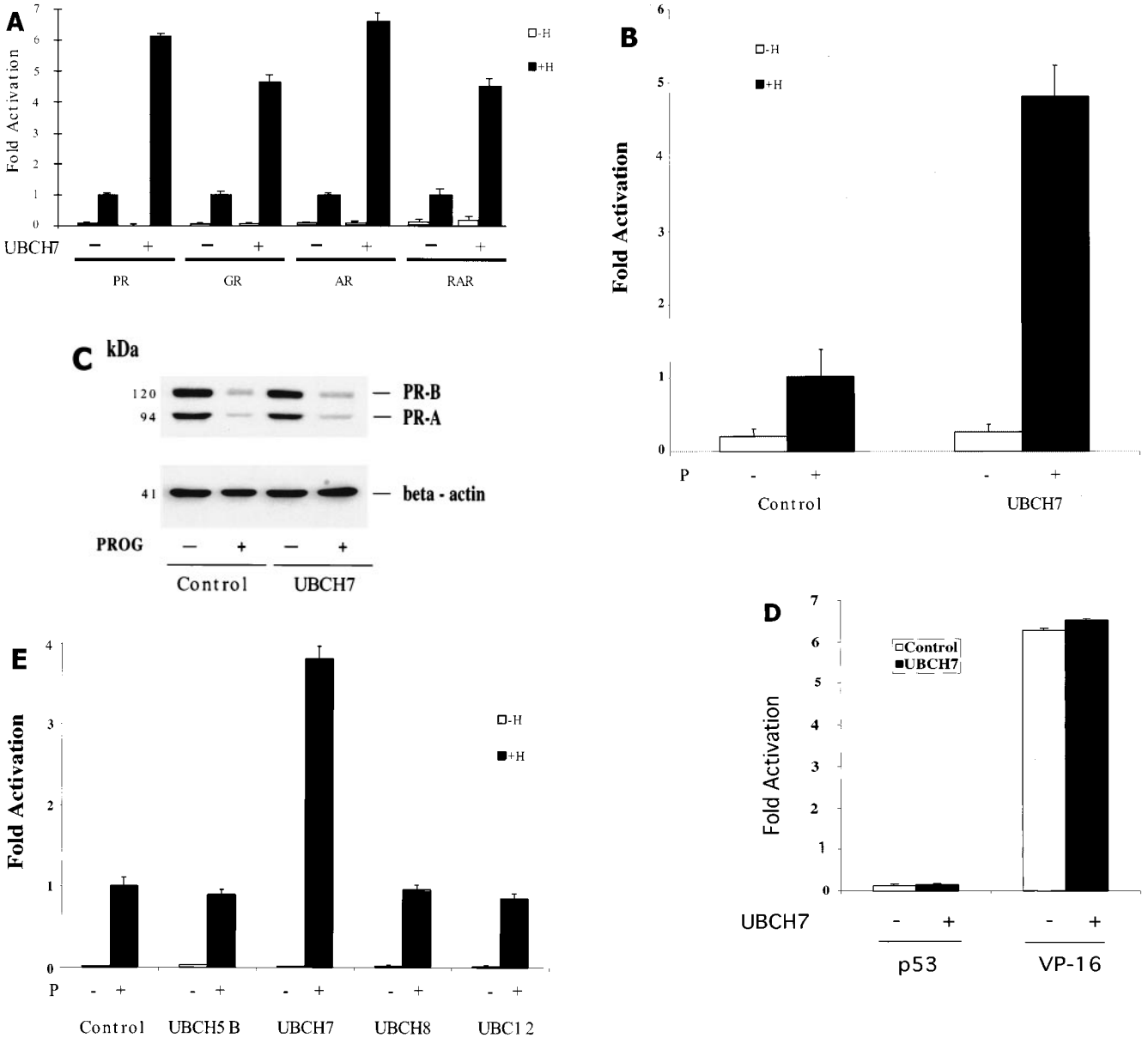

FIG. 1. (A) UBCH7 modulates the hormone-dependent transcriptional activity of various nuclear hormone receptors. HeLa cells were transiently transfected with receptor expression plasmid for PR-B, GR, AR, RAR, and their cognate hormone response elements in the presence or absence of UBCH7. Later, the cells were treated with hormones (H) as follows: PR, progesterone (10^{-7} M); AR, R1881 (2.5×10^{-10} M); GR, dexamethasone (10^{-7} M); and RAR, retinoic acid (10^{-7} M), respectively. Cells were harvested after 24 h and assayed for luciferase activity. Bars indicate means and standard deviations of three different determinations. The data are presented as *n*-fold activation. The activity of receptor in the presence of hormone and the absence of UBCH7 was defined as onefold, and the other bars were scaled accordingly. (B) UBCH7 modulates the hormone-dependent transcriptional activity of endogenous PR in T47D cells. T47D cells were transiently transfected with a reporter plasmid that contained the progesterone response element in the presence and absence of UBCH7. Cells were treated with progesterone (P) (10^{-7} M), and after 24 h cells were harvested and assayed for luciferase activity. The data are presented as *n*-fold activation. The activity of receptor in the presence of hormone (H) and in the absence of UBCH7 was defined as onefold, and the other bars were scaled accordingly. (C) Overexpression of UBCH7 in T47D cells had no significant effect on the expression levels of PRs. T47D cells were transfected with either control vector (pBKRSV) or UBCH7 vector (pBKRSV-UBCH7). Cells were treated with progesterone (PROG) (10^{-7} M), and after 48 h cells were harvested. The expression levels of PR protein were assessed by Western blot analysis using PR-specific antibodies. β-Actin expression was used as a loading control. (D) Coexpression of UBCH7 had no significant effect on the transcriptional activity of nonnuclear hormone receptor transcription factors p53 and VP-16 activation domain. HeLa cells were transiently transfected with expression plasmid and either p53 or VP-16 activation domain along with their respective reporter plasmids, p21 promoter-LUC and 17mer-LUC, in the presence or absence of UBCH7. Data are expressed as means and standard deviations of *n*-fold activation results from three different transfections. (E) Other ubiquitin-conjugating enzymes are not involved in the steroid receptor transactivation pathway. Cells were transiently transfected with PR-B expression and progesterone-responsive reporter plasmids in the presence of either control vector, UBCH5B expression plasmid, UBCH7 expression plasmid, UBCH8 expression plasmid, or UBC12 expression plasmid. Cells were treated with progesterone (P) (10^{-7} M), and after 24 h cells were harvested and assayed for luciferase activity. The data are presented as *n*-fold activation. The activity of receptor in the presence of hormone (H) and in the absence of ubiquitin conjugation enzyme was defined as onefold, and the other bars were scaled accordingly.

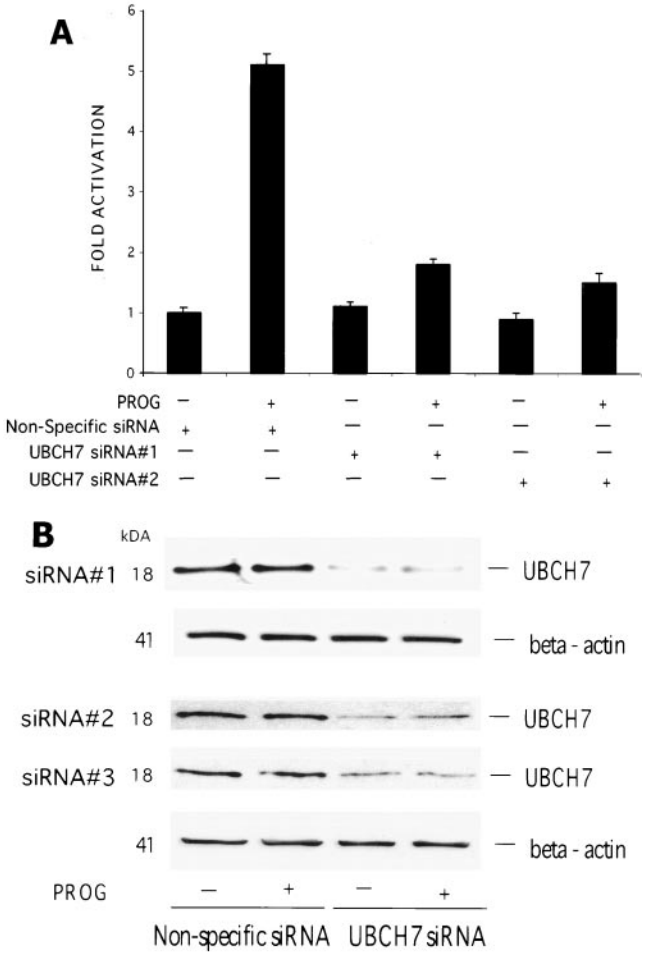

FIG. 2. (A) Depletion of endogenous UBCH7 levels reduces transcriptional activity of PR. HeLa cells were transiently transfected with PR and PRE.TATA.LUC expression plasmid and its response element in the presence or absence of different UBCH7 siRNAs. Transfection was also done using GAPDH siRNA as a control. Four hours after transfection, cells were treated with either progesterone (PROG) (10^{-7} M) or a vehicle (ethanol). Twenty-four hours after transfection, cells were harvested, and luciferase activity was assayed. The data are presented as *n*-fold activation. (B) Expression analysis of UBCH7 protein after siRNA treatment. Cell extracts were separated on a 4 to 20% gradient gel and transferred onto nitrocellulose paper, and protein levels were assessed by Western blotting using UBCH7 specific antibodies. Equal loading of samples was confirmed using β-actin-specific antibodies.

the absence of the AF-1 region of PR, UBCH7 had only a minimal effect on the expression of the reporter gene. However, addition of hormone significantly enhanced the activity of the AF-2 region of PR. Expression of UBCH7 further enhanced the transcriptional activity of the AF-2 region of PR by only 1.5-fold over that in the absence of UBCH7 expression (Fig. 4). It has been shown that the AF-1 region of PR is a hormone-independent activation domain and that addition of hormone has no effect on its activity. Our data also suggest that addition of hormone has no significant effect on the transcriptional activity of the AF-1 region of PR (Fig. 4). Like the AF-2 region of PR, the addition of UBCH7 increases the transcriptional activity of the AF-1 region of PR by only 1.5-fold (Fig. 4)

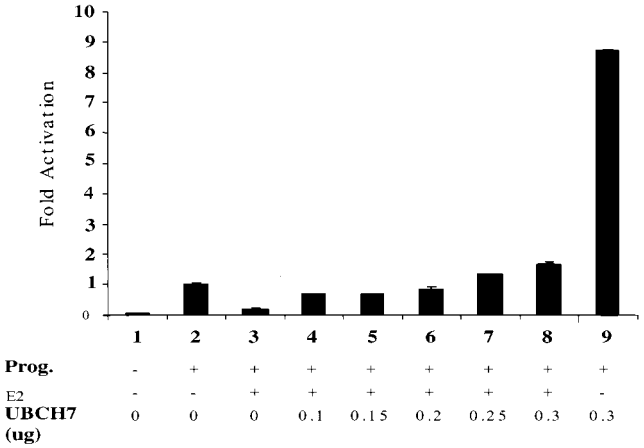

FIG. 3. UBCH7 reverses the transcriptional interference between PR and ER. HeLa cells were transfected with 0.2 μg of PR expression plasmid, 0.3 μg of ER expression plasmid, 1.0 μg of PRE.TATA.LUC, and increasing concentrations (0, 0.1, 0.15, 0.2, 0.25, and 0.3 μg) of UBCH7. Cells were then treated with progesterone (Prog.) alone or progesterone and estradiol together (each at 10^{-8} M). The last bar corresponds to control cells transfected with UBCH7 and PR expression plasmids only. Data are expressed as means and standard deviations of *n*-fold activation results from three independent transfections. The activity in the presence of hormone and absence of UBCH7 was defined as onefold, and the other bars were scaled accordingly.

compared to the activity in the absence of UBCH7. In contrast, UBCH7 was able to enhance the transcriptional activity of full-length PR by 11-fold. These data suggest that UBCH7 has only a minimal effect on the transcriptional activities of the

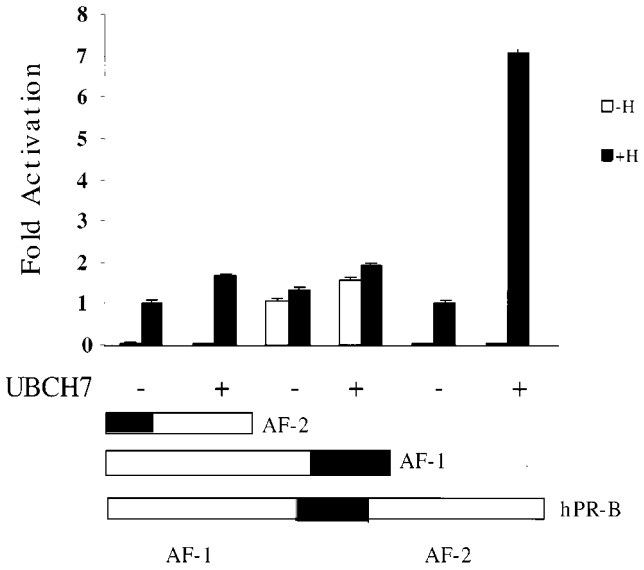

FIG. 4. UBCH7 had minimal effects on the transcriptional activity of AF-1 and AF-2 regions of hPR-B; however, it significantly enhanced the transcriptional activity of the full-length PR-B. HeLa cells were transiently transfected with either RSV.hPR-B (full-length PR-B), RSV.hPRΔA/B (AF-2), or RSV.hPRΔE (AF-1) expression plasmid along with progesterone-responsive reporter (PRE.TATA.LUC) plasmid. After 4 h, cells were treated with or without progesterone (10^{-7} M). Twenty-four hours later, cells were harvested and assayed for luciferase activity. Data are expressed as means and standard deviations of *n*-fold activation results for three different experiments. H, hormone.

isolated AF-1 and AF-2 regions of PR. However, UBCH7 synergistically enhanced the transcriptional activity of the AF-1 and AF-2 regions of PR in the context of the full-length receptor.

The ubiquitin conjugation enzymatic activity of UBCH7 is required for the ability of UBCH7 to activate PR transcriptional activity. Since UBCH7 is an E2 ubiquitin-conjugating enzyme, it is pertinent to understand if its ability to modulate the transcriptional activity of PR is dependent on its ability to form a thioester bond with ubiquitin at its conserved cysteine residue (C87). It has been shown that mutation of the conserved cysteine residue into either alanine or serine abolishes the ubiquitin conjugation activity of the UBCH7 enzyme. We tested the ability of a C-to-S mutant of UBCH7 to modulate PR transcriptional activity by both transient transfection and in vitro transcription assays. As shown in Fig. 5A, wild-type UBCH7 was able to enhance the transcriptional activity of PR in a hormone-dependent manner in a transient transfection assay. In contrast, the mutant (C87S) UBCH7 was unable to activate the transcriptional activity of PR. To confirm that the loss of coactivation function of mutant UBCH7 is not due to the loss of expression of the mutant UBCH7, we also analyzed the expression of UBCH7 by Western blot analysis. Our data suggest that the wild-type and mutant UBCH7 are expressed at equal levels (Fig. 5B). Our results indicated that the ubiquitin conjugation activity of UBCH7 is required for the ability of UBCH7 to enhance nuclear hormone receptor activities.

UBCH7 potentiates PR transactivation on chromatin templates. To further confirm that UBCH7 modulates the transcriptional activity of PR in a ligand-dependent manner and that the ubiquitin conjugation activity of UBCH7 is required for the coactivation function, we employed a cell-free in vitro transcription system. Naked plasmid DNA containing either a progesterone response element or control template that contains no progesterone response element was assembled into chromatin using a *Drosophila* embryo extract. The PR, progesterone, and UBCH7 were added after the chromatin assembly was completed. We found that addition of progesterone resulted in an activation of transcription on preassembled chromatin templates by PR. Addition of wild-type UBCH7 purified from *E. coli* further enhanced the hormone-dependent transcriptional activity of PR, whereas UBCH7 containing the C87S mutation had a minimal effect on the transcription activity of PR (Fig. 5C). However, no significant transcription was observed from the control template that lacks the progesterone response element (Fig. 5C). Our in vitro results confirmed that UBCH7 potentiates PR-mediated transactivation and that the ubiquitin conjugation activity of UBCH7 is required for its coactivator function.

In vivo recruitment of UBCH7 onto the ER- and PR-responsive promoters. In order to better understand the coactivation function of UBCH7, we employed a ChIP assay to examine the recruitment of UBCH7 to ER- and PR-responsive promoters in vivo. Formaldehyde cross-linked chromatin complexes were immunoprecipitated with the appropriate antibodies from MCF-7 and T47D/CAT0 cells in the presence or absence of estrogen and progesterone. The precipitated genomic DNA associated with ER, PRs, and UBCH7 were amplified by a PCR using primers specific for the PS2 and MMTV promoters. ChIP analyses demonstrated the recruitment of UBCH7 to ER- and PR-responsive promoters in vivo in the presence of estrogen or progesterone (Fig. 6A and B). These results are consistent with previously published findings which suggest that coactivators are recruited to the target promoters by receptors in

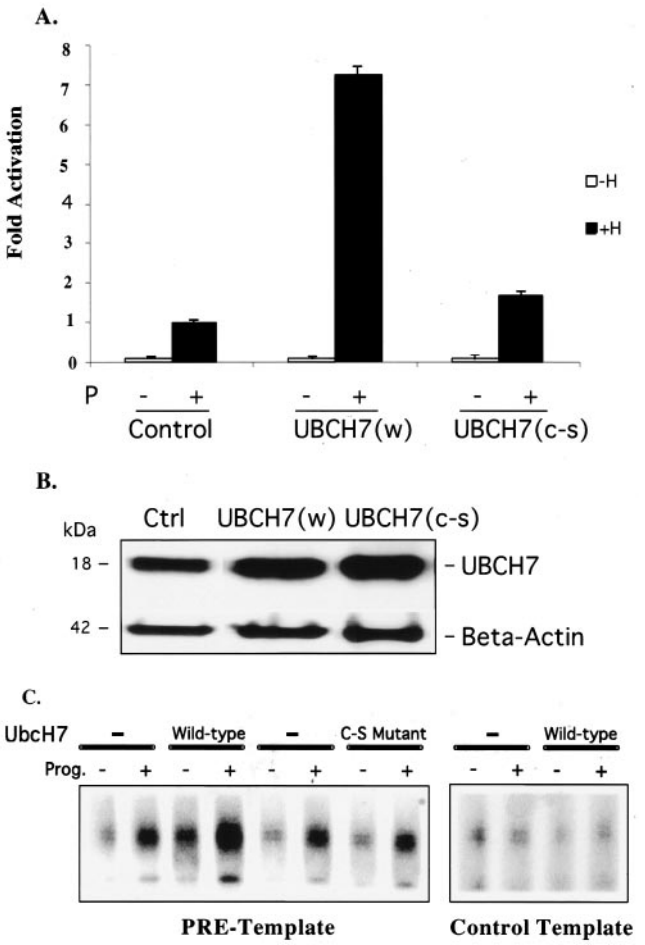

FIG. 5. The ubiquitin-conjugating activity of UBCH7 is required for its coactivation function. (A) The ubiquitin conjugation-defective mutant UBCH7 (c-s) had no significant effect on the transcriptional activity of PR in the transient transfection assay. HeLa cells were transfected with PR-B expression and reporter plasmids in the presence or absence of either wild-type (w) or mutant UBCH7. Cells were treated with progesterone (P) (10^{-7} M), and luciferase activity was measured. The data are presented as *n*-fold activation. The activity of PR-B in the presence of hormone (H) and absence of UBCH7 was defined as onefold, and the other bars were scaled accordingly. (B) Wild-type and ubiquitin conjugation-defective mutant UBCH7 expressed at equal levels. Protein levels were analyzed by Western blotting using anti-UBCH7 antibody. The control lane (Ctrl) represents cells that were transfected with an empty vector. β-Actin expression was used as a loading control. (C) UBCH7 potentiates PR transcriptional activity in a cell-free transcription system on a chromatin template. Briefly, an initial reaction was carried out with *Drosophila* embryo extract, and core histones were assembled on a control template that lacks a PR binding site and a template that contains PR binding site PRE3-E4, purified PR-B, progesterone (Prog.) (10^{-7} M), no progesterone (−), or purified UBCH7 (wild-type or ubiquitin conjugation-defective mutant [C-S]). HeLa extract was added to a preassembled chromatin assembly, and the reaction was allowed to continue for 30 min at 27°C. The samples were then subjected to in vitro transcription analysis.

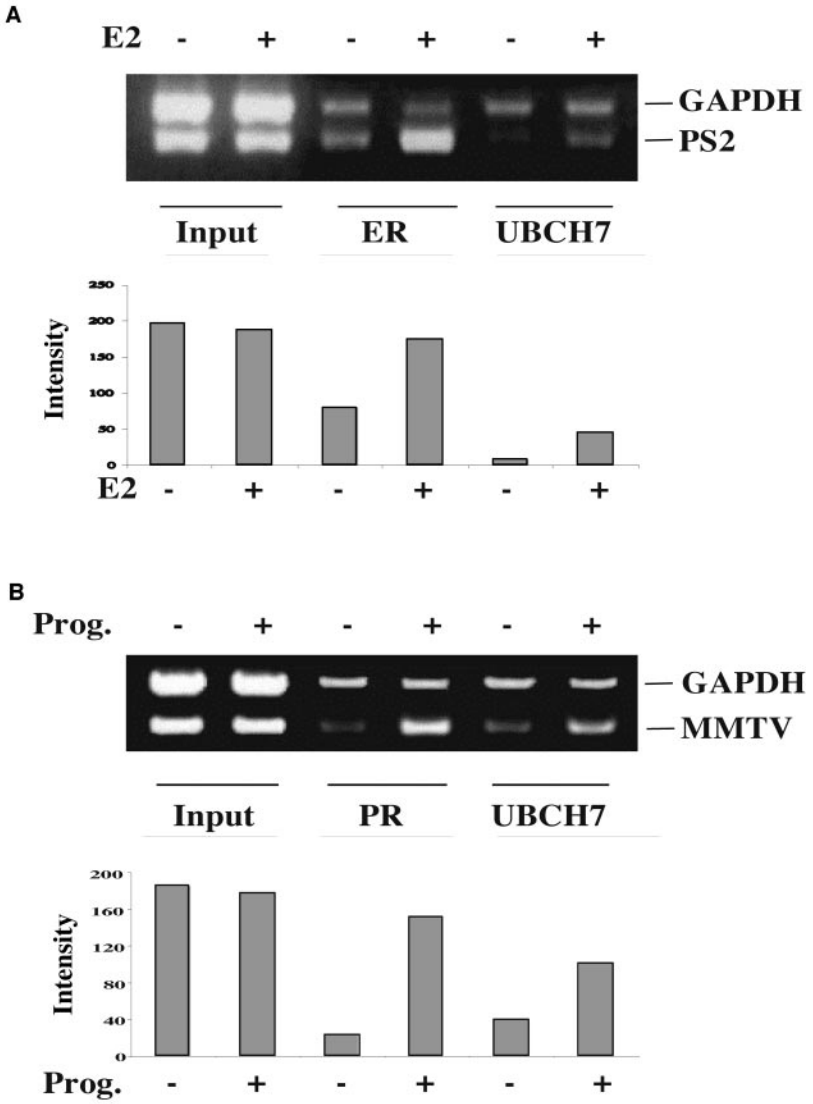

FIG. 6. UBCH7 is recruited onto ER- and PR-responsive promoters. ChIP was performed using MCF-7 and T47D/CAT0 cells in the presence or absence of estrogen or progesterone (Prog.) hormones. (A) Primers specific for the pS2 promoter were used to amplify the genomic DNA associated with ER and UBCH7 in MCF-7 cells. (B) Primers specific for the MMTV promoter were used to amplify the genomic DNA associated with PR and UBCH7 in T47D/CAT0 cells. In these experiments, 0.1% input DNA was used in the control lanes (Input). Primers specific for the GAPDH coding region were used as a loading control. The data were quantified by using the NIH image scan program version 1.62, and data are plotted as intensity versus hormone treatment.

a hormone-dependent manner and demonstrate that UBCH7 is physically present on the promoter of these target genes.

UBCH7 and E6-AP synergistically enhance receptor activity. Since the E3 enzyme E6-AP has been reported to interact with steroid hormone receptors and UBCH7, we wished to further explore the functional interaction between E6-AP and UBCH7. HeLa cells were transiently transfected with wild-type E6-AP and UBCH7 expression plasmids. UBCH7 and E6-AP alone significantly enhance the activity of PR (Fig. 7A). However, when present together, UBCH7 and wild-type E6-AP synergistically enhanced the transactivation function of PR (Fig. 7A). E6-AP is an E3 ubiquitin-protein ligase enzyme, and a previous study has shown that the ligase activity of E6-AP is not required for its coactivation function (39). However, it is still possible that the synergy between E6-AP and UBCH7 may require the ligase activity of E6-AP. In order to test whether the ligase activity of E6-AP is required for its synergy with UBCH7, HeLa cells were transiently transfected with the ligase-defective mutant E6-AP and UBCH7 expression plasmids. As shown in Fig. 7A, like wild-type E6-AP, the ligase-defective mutant E6-AP and UBCH7 were also able to synergistically enhance the transactivation function of PR. These data suggest that the ligase activity of E6-AP is not required for its ability to synergize with UBCH7. Together, these findings suggest that E6-AP and UBCH7 functionally interact with each other.

Since E6-AP has been reported to interact with UBCH7, we wished to examine the ability of UBCH7 to function as a

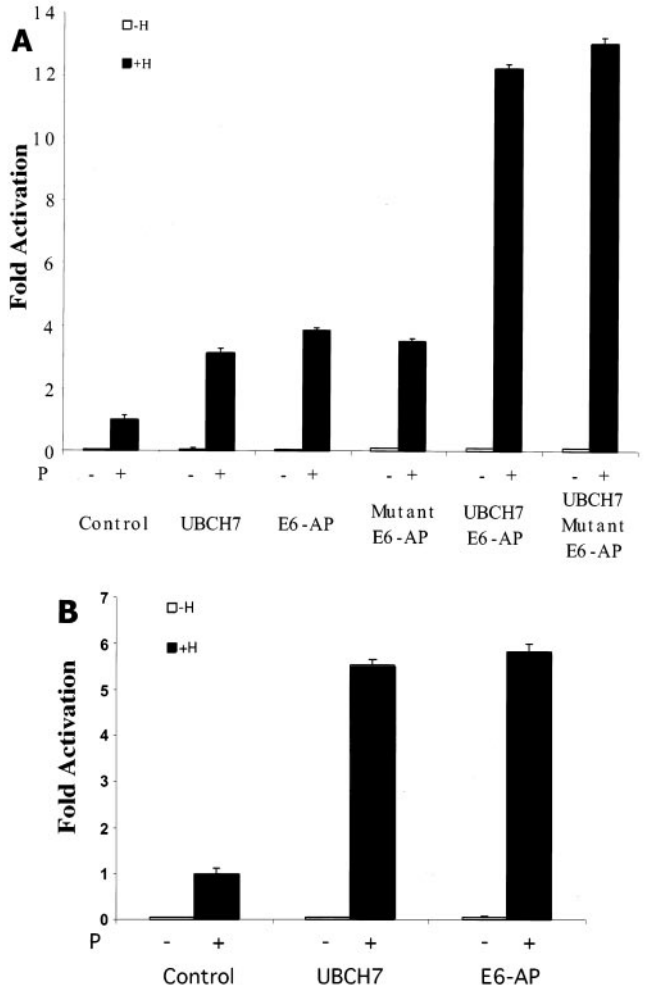

FIG. 7. (A) UBCH7 and E6-AP synergistically enhance PR transactivation. Cells were transiently transfected with PR-B expression plasmid and progesterone-responsive reporter plasmid (PRE.TATA.LUC) in the absence (Control) or presence of UBCH7, wild-type E6-AP, ligase-defective mutant E6-AP (Mutant E6-AP), UBCH7 plus wild-type E6-AP, or UBCH7 plus mutant E6-AP. Cells were treated with or without progesterone (P) (10^{-7} M). Data are expressed as means and standard deviations of *n*-fold activation results for three different transfections. The activation in the presence of hormone (H) using an empty vector was taken as onefold. (B) E6-AP is not required for UBCH7 to potentiate transcriptional activity of PR in vivo. E6-AP KO fibroblasts were transiently transfected with full-length PR (PR-B) expression plasmid along with the progesterone-responsive reporter plasmid (PRE.TATA.LUC). Cells were also transfected with either E6-AP or UBCH7 expression plasmid vector. After 4 h, cells were treated with progesterone (P) (10^{-7}M) or a vehicle. Twenty-four hours later, cells were harvested and assayed for luciferase activity. Data are expressed as *n*-fold activation in the presence of progesterone. The activation in the presence of hormone (H) using an empty vector was taken as onefold. Data expressed are means and standard deviations of results from three different experiments.

coactivator in the absence of E6-AP. In order to test the ability of UBCH7 to function as a coactivator in the absence of E6-AP protein, we utilized the E6-AP KO cells that were derived from E6-AP KO animals. Transient transfection assays indicate that UBCH7 was able to activate PR activity in the E6-AP KO cell line (Fig. 7B). These data suggest that UBCH7 coactivation function is not dependent on E6-AP expression.

UBCH7 interacts with SRC-1. In order to determine whether UBCH7 also interacts with other coactivators, we also examined the ability of UBCH7 to interact with members of the p160 family of coactivators. [^{35}S]methionine-labeled SRC-1, SRC-2, and SRC-3 proteins were synthesized in vitro. Control (GST), GST-UBCH7, and GST-UBCH5B proteins were expressed and purified from *E. coli*. The in vitro-translated SRC-1, SRC-2, and SRC-3 proteins and GST-fused UBCH7/UBCH5B protein, along with GST control protein, were incubated together with glutathione-Sepharose beads and analyzed by SDS-PAGE and autoradiography. Figure 8A shows that UBCH7 was able to interact with SRC-1. However, UBCH7 was unable to interact with either SRC-2 or SRC-3 (Fig. 8A). In order to determine whether SRC-1 specifically interacts with UBCH7, we also examined the ability of UBCH5B that has no significant effect on the transactivation function of receptor to interact with SRC-1. The UBCH5B failed to interact with SRC-1, whereas under similar conditions UBCH7 was able to interact with SRC-1 (Fig. 8A). These data suggest that UBCH7 specifically interacts with SRC-1 protein. To further confirm that UBCH7 indeed interacts with SRC-1, we also examined the in vivo interaction of UBCH7 with SRC-1 by coimmunoprecipitation analysis. Cell lysates were immunoprecipitated with either serum or specific antibodies, such as anti-UBCH7 and anti-UBCH5B, followed by Western blotting with an anti-SRC-1, anti-UBCH7, or anti-UBCH5 specific antibody (Fig. 8B). The results presented in Fig. 8B demonstrate that SRC-1 was coimmunoprecipitated with UBCH7. In contrast, control serum and UBCH5B antibody failed to coimmunoprecipitate SRC-1, suggesting that in vivo UBCH7 also interacts with SRC-1. These data are consistent with our GST pull-down data, which suggest that SRC-1 specifically interacts with UBCH7. Taken together, these findings suggest that UBCH7 and SRC-1 interact with each other both in vitro and in vivo.

In view of the fact that UBCH7 is a component of the ubiquitin-proteasome pathway and that, furthermore, it interacts with SRC-1, we also examined the expression levels of SRC-1 in both the absence and the presence of exogenously expressed UBCH7. The expression levels of SRC-1 are identical in both the absence and the presence of UBCH7 (Fig. 8C). These data are consistent with previously published results, which suggest that the ubiquitin conjugation enzymes UBC2 and UBC3 degrade SRC-1, whereas UBCH7 has no significant effect on the degradation of SRC-1 (60).

Coactivation of PR by UBCH7 requires SRC-1. Since UBCH7 specifically interacts with SRC-1 protein, we tested the ability of UBCH7 to function as a coactivator in the absence of SRC-1. In order to test the ability of UBCH7 to function as a coactivator in the absence of SRC-1 protein, we utilized the SRC-1 KO cells that were derived from SRC-1 KO animals. Transient transfection assays indicate that UBCH7 was unable to activate PR activity in the SRC-1 KO cell line. However, in the presence of exogenously added SRC-1, the coactivation function of UBCH7 was restored (Fig. 8D). These data suggest that SRC-1 expression is essential for the coactivation function of UBCH7 (Fig. 8D).

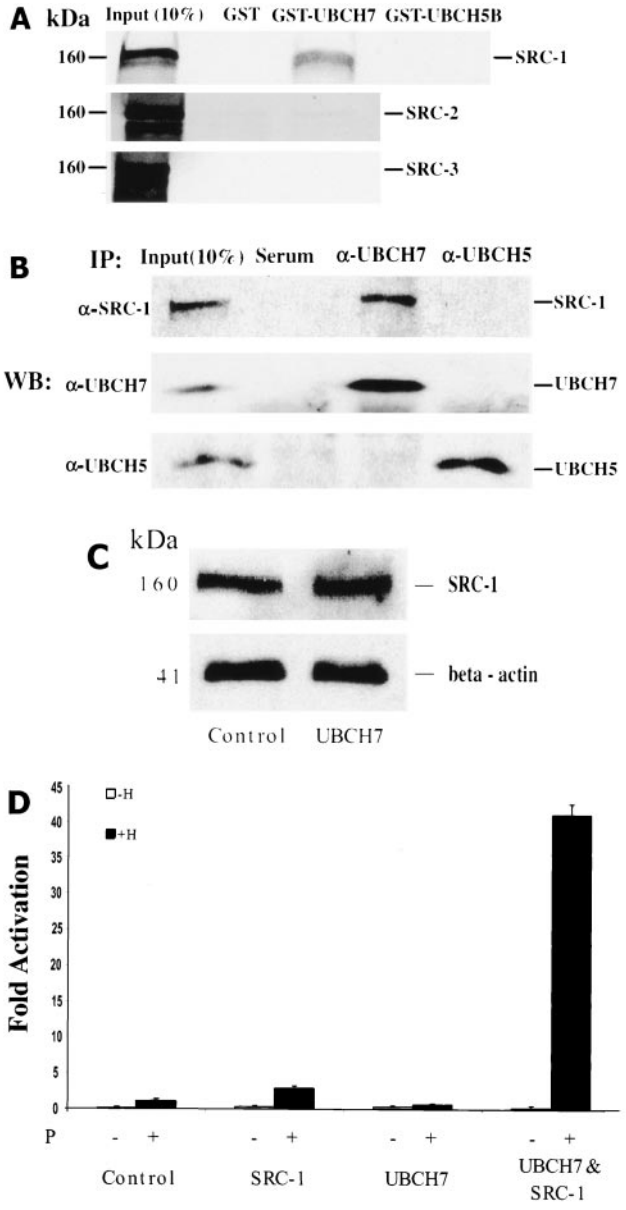

FIG. 8. (A) UBCH7 interacts with SRC-1 protein. Interaction of UBCH7 with SRC-1, SRC-2, and SRC-3 was determined in a GST pull-down assay. SRC-1, SRC-2, and SRC-3 were labeled by in vitro translation and incubated overnight at 4°C with *E. coli*-expressed GST alone (control), GST-UBCH7 bound to beads, or GST-UBCH5B in NETN buffer (50 mM NaCl, 1 mM EDTA, 20 mM Tris [pH 8.0], 0.1% NP-40). Bound proteins were analyzed by 7.5% SDS-PAGE and autoradiographed. (B) In vivo UBCH7 interacts with SRC-1 protein. After cells were grown for 24 h, the cell lysates were prepared in RIPA buffer. Then lysates were immunoprecipitated (IP) with either control serum, UBCH7-specific antibody (α-UBCH7), or UBCH5 specific antibody (α-UBCH5), and precipitated proteins were immunoblotted with either anti-SRC-1 antibody (top panel, WB: α-SRC-1), anti-UBCH7 antibody (middle panel, WB: α-UBCH7), or anti-UBCH5 antibody (bottom panel, WB: α-UBCH5). (C) Overexpression of UBCH7 has no effect on the expression levels of SRC-1 protein. HeLa cells were transfected with either control or UBCH7 expression plasmid. Cells were harvested 48 h after transfection, and SRC-1 levels were assessed by Western blotting using SRC-1 specific antibodies. Equal loading of samples was confirmed using β-actin-specific antibodies. (D) SRC-1 is required for UBCH7 to potentiate transcriptional activity of PR in vivo. SRC-1 KO fibroblasts were transiently transfected with full-length PR (PR-B) expression plasmid along with the progesterone-responsive reporter plasmid (PRE.TATA.LUC). Cells were also transfected with either SRC-1 or UBCH7 expression plasmid vectors or both. After 4 h, cells were treated with progesterone (P) (10^{-7} M) or a vehicle. Twenty-four hours later, cells were harvested and assayed for luciferase activity. Data are expressed as *n*-fold activation in the presence of progesterone. The activation in the presence of hormone (H) using an empty vector was taken as onefold. Data expressed are means and standard deviations of results from three different experiments.

DISCUSSION

UBCH7, an E2 ubiquitin-conjugating enzyme, was isolated as an E6-AP interacting protein (25, 41). UBCH7 acts as an E2 ubiquitin-conjugating enzyme for E6-AP. In this report, we describe the involvement of UBCH7 in steroid hormone and nuclear receptor transactivation pathways. We demonstrated that UBCH7 preferentially modulates the ligand-dependent transcriptional activities of various nuclear hormone receptors. These results are consistent with previously published reports indicating that coactivators can modulate the hormone-dependent transcriptional activity of different nuclear receptors and most coactivators exhibit little detectable receptor specificity (44). It has been suggested that most coactivators interact with receptors via the LXXLL motifs contained within the coactivators (17, 34). Unlike these coactivators, UBCH7 does not contain LXXLL motifs, and it fails to directly interact with receptors. However, previously published reports and our data from GST pull-down and coimmunoprecipitation experiments demonstrate that UBCH7 directly interacts with E6-AP and SRC-1, which contain LXXLL motifs and interact with receptors in a hormone-dependent manner. Our data also suggest that E6-AP expression is not required for UBCH7 coactivation function. Based on these findings, we suggest that UBCH7 likely modulates receptor function through its interaction with SRC-1. Hence, it is possible that UBCH7 interacts with SRC-1 and forms a protein complex which interacts with receptors via SRC-1 and thereby modulates the hormone-dependent transcriptional activity of the target gene. This possibility is supported by our findings from experiments with SRC-1 KO cells, wherein we observed that UBCH7 is unable to coactivate steroid receptor functions unless SRC-1 protein is coexpressed.

The existence of modulatory proteins in the nuclear hormone receptor transactivation pathway is supported by the finding that the transcriptional activity of one receptor can be squelched by the overexpression of another receptor, indicating that both receptors compete for a limited pool of common factors (3, 37, 52). This observation led us to determine whether UBCH7 is one of these limiting factors and whether its overexpression can inhibit this squelching phenomenon. Our results indicate that overexpression of UBCH7 in HeLa cells reverses the squelching effect of ER on PR transactivation in a dose-dependent manner. These results are consistent with previously published studies indicating that a genuine coactivator should be able to reverse squelching between two receptors (37).

There are two distinct activation function domains in nuclear hormone receptors, the AF-1 and the AF-2. The activity of the amino-terminal AF-1 domain is not regulated by hor-

mone, and this domain is constitutively active, whereas the activity of the carboxy-terminal AF-2 domain is regulated by hormone (53). Our data demonstrated that UBCH7 had a marginal effect on the transcriptional activities of AF-1 and AF-2 receptor domains when these domains were analyzed individually. However, UBCH7 synergistically enhanced the transcriptional activity of the AF-1 and AF-2 regions in the context of the full-length receptor. These results indicate that UBCH7 requires both activation domains of the receptor to properly modulate the transcription activity of the receptor. It is possible that UBCH7 exerts its effect on both transactivation domains of the receptors via the SRC-1 protein that is known to interact with both transactivation domains of the receptors as well as UBCH7 (43).

The ubiquitin-proteasome pathway involves three classes of enzymes—an E1 ubiquitin-activating enzyme, E2 ubiquitin-conjugating enzymes, and E3 ubiquitin-protein ligases. It is known that ubiquitin-conjugating enzymes form a thioester bond with ubiquitin through a conserved cysteine residue (C87) contained within these proteins and that mutation of this residue abolishes its ability to bind to and transfer ubiquitin. Our in vitro and in vivo experiments both show that the coactivation function of UBCH7 is dependent on its enzymatic activity, suggesting that the ubiquitin conjugation activity of UBCH7 is required for steroid hormone receptor action. These data build upon a common feature of coactivators, which is that the enzymatic activities of coactivators such as SRC-1, p300/CBP, RAC3/ACTR/AIB1, and CARM-1 contribute to the receptor's ability to stimulate transcription (6, 22, 42, 54). Previously published studies have shown that the ubiquitin-protein ligase activities of the E3 ligases E6-AP and RSP5 are not required for their coactivation function (39). In contrast, here we report that the ubiquitin conjugation activity of UBCH7 is essential for its ability to modulate receptor function, suggesting that an intact ubiquitin-proteasome pathway is requisite for proper functioning of the receptor.

Our ChIP analyses demonstrate the recruitment of UBCH7 onto ER- and PR-responsive promoters, indicating that UBCH7 physically associates with the pS2 and MMTV promoters. These results are consistent with previously published findings, which suggest that coactivator proteins, such as SRC family members, are recruited by receptors to target promoters in a hormone-dependent manner (51). Our data are also in agreement with previously published studies demonstrating that the E3 ubiquitin-protein ligase E6-AP and the components of the regulatory subunit of the proteasome are recruited to the hormone-responsive promoter of the pS2 gene (48). Since UBCH7 fails to physically associate with receptors, it is likely that UBCH7 is recruited to the target promoters in a hormone-dependent manner by its association with E6-AP and/or SRC-1.

Identification of the ubiquitin-conjugating enzyme UBCH7 as a modulator of nuclear hormone receptors implicates the ubiquitin-proteasome pathway as an integral part of eukaryotic gene transcription. Consistent with this hypothesis, it has been shown that an intact ubiquitin-proteasome pathway is required for proper execution of receptor function (10, 55). This hypothesis is further strengthened by the fact that the ubiquitin conjugation activity of UBCH7 is required for its coactivation function and, furthermore, that the ubiquitin-proteasome pathway enzymes are recruited to the promoter of hormone-responsive target genes. It is possible that in order to activate transcription, a hormone-bound receptor recruits ubiquitin-proteasome pathway enzymes, such as E6-AP and UBCH7, to the promoter of target genes. These enzymes then modulate transcription by disrupting the preinitiation complex, allowing subsequent steps in transcription to proceed. It is also plausible that UBCH7 is involved in complex remodeling and turnover of the nuclear receptor-transcription complex via the ubiquitin-proteasome pathway for transcription initiation, elongation, and RNA processing. The enzymatic activity of UBCH7 appears to be involved in exchange of coactivator complexes. This scenario is supported by a recently published report showing that UBCH7 selectively promotes the degradation of TIF-2 and E6-AP (60); therefore, UBCH7-dependent protein degradation is essential to maintain efficient transcription of the target promoter gene.

In conclusion, the results presented in this study demonstrate that UBCH7, an E2 ubiquitin-conjugating enzyme of the ubiquitin-proteasome pathway, acts as an enzymatic coactivator and modulates the transcriptional activities of steroid hormone receptors, substantiating the hypothesis that the ubiquitin-proteasome pathway is intimately involved in nuclear hormone receptor gene transactivation.

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