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Trihydrophobin 1 Attenuates Androgen Signal Transduction Through Promoting Androgen Receptor Degradation

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ABSTRACT

The androgen-signaling pathway plays critical roles in normal prostate development, benign prostatic hyperplasia, established prostate cancer, and in prostate carcinogenesis. In this study, we report that trihydrophobin 1 (TH1) is a potent negative regulator to attenuate the androgen signal-transduction cascade through promoting androgen receptor (AR) degradation. TH1 interacts with AR both in vitro and in vivo, decreases the stability of AR, and promotes AR ubiquitination in a ligand-independent manner. TH1 also associates with AR at the active androgen-responsive prostate-specific antigen (PSA) promoter in the nucleus of LNCaP cells. Decrease of endogenous AR protein by TH1 interferes with androgen-induced luciferase reporter expression and reduces endogenous PSA expression. Taken together, these results indicate that TH1 is a novel regulator to control the duration and magnitude of androgen signal transduction and might be directly involved in androgen-related developmental, physiological, and pathological processes. J. Cell. Biochem. 109: 1013–1024, 2010.

KEY WORDS: TH1; ANDROGEN RECEPTOR; INTERACTION; UBIQUITINATION; DEGRADATION

Sex steroid hormones, in particular testosterone (T) and dihydrotestosterone (DHT), play a major role in normal prostate development, benign prostatic hyperplasia (BPH), and established prostate cancer [Montie and Pienta, 1994; Ross et al., 1998]. The action of T and DHT in the prostate is mediated by the androgen receptor (AR), a member of the nuclear receptor superfamily of transcription factors [El Sheikh et al., 2003]. Upon T or DHT binding, the AR dissociates from chaperone proteins, homodimerizes, translocates into the nucleus, and turns on the expression of its target genes by binding to the androgen-responsive elements [Quigley et al., 1995]. AR consists of an N-terminal transactivation domain (NTD), a DNA-binding domain (DBD), a hinge region that contains the bipartite nuclear translocation signal which is involved in AR nuclear translocation, and a ligand-binding domain (LBD), which is responsible for ligand binding and transactivation [Jenster et al., 1991; MacLean et al., 1997].

Since androgen–AR signaling plays important roles in cell progresses, it is conceivable that this signal needs to be tightly controlled to maintain normal cell function. One way to control this signal is via regulation of AR protein synthesis or turnover. Although control of AR synthesis has been studied extensively, relatively little is known about how AR is targeted for degradation. AR, like other members of the steroid hormone receptor family, requires dimerization to execute its function [Mangelsdorf et al., 1995]. The androgen-dependent intramolecular interaction between the NH2-terminal domain and the COOH-terminal domain of AR decreases a dissociation rate of the bound androgen and increases AR protein half-life [Wong et al., 1993]. Accumulating evidence indicates that AR is targeted for degradation through ubiquitin–proteasome pathways. There is a highly conserved PEST sequence, which is thought to target proteins for ubiquitination and degradation, located in the hinge region of AR throughout many

Yanzhong Yang and Weiying Zou contributed equally to this work.

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species [Sheflin et al., 2000]. Moreover, several ubiquitin ligases (E3s) were reported to mediated AR ubiquitination and degradation [Kang et al., 1999; Poukka et al., 2000; Yeh et al., 2000]. Phosphorylation-dependent AR ubiquitination and degradation by the PI3K/Akt pathway require Mdm2 E3 ligase [Lin et al., 2002]. Results from E6-AP-knockout animals indicate that the expression level of AR is increased in E6-AP-null prostate glands, compared with that of normal control animals, suggesting that E6-AP modulates the protein levels of AR in prostate glands [Gao et al., 2005]. A recent study demonstrates that COOH terminus of the *H*sp70-*i*nteracting *p*rotein (CHIP) can interact with AR and function as a negative regulator of AR transcriptional activity by promoting AR ubiquitination and degradation [He et al., 2004].

The human *trihydrophobin 1* (*th1*) gene, which is located in chromosome 20q13, was originally identified and characterized by the D.T. Bonthron group in 2000 during the positional cloning of *mei-41* [Hari et al., 1995; Bonthron et al., 2000]. Though highly conserved and ubiquitously expressed, the function of human TH1 is not well understood. Our previous studies have demonstrated that TH1 could specifically interact with A-Raf kinase and negatively regulate its kinase activity [Yin et al., 2002; Liu et al., 2004]. TH1 can be ubiquitinated by E3 ubiquitin ligase E6-AP and degraded by proteasome [Yang et al., 2007]. Recently, an independent study indicated that TH1 is identical to NELF-D, an integral subunit of the human negative transcription elongation factor (NELF) complex [Yamaguchi et al., 1999; Narita et al., 2003]. Under normal growth conditions, NELF and DSIF cause RNA Pol II to pause in the promoter proximal region of the *hsp70* gene in *Drosophila*, and heat shock induction results in dissociation of NELF from the elongation complex [Wu et al., 2003]. Nevertheless, the in vivo physiological roles of TH1 in human cells are poorly understood.

In this report we demonstrate that TH1 interacts with AR and decreases AR protein levels through promoting AR protein ubiquitination. TH1 also associates with AR at the active androgen-responsive PSA promoter in LNCaP cells. As judged by the decreased reporter gene activity and PSA expression in LNCaP cells, TH1 interferes with androgen-signal transduction. These results may suggest a novel role for TH1 in the regulation of androgen signaling pathway.

MATERIALS AND METHODS

PLASMID CONSTRUCTION AND REAGENTS

TH1 expression plasmids were constructed as described previously [Liu et al., 2004; Yang et al., 2007]. The plasmids pSG5-AR, MMTV-LUC, ARE_{2X}-TATA-LUC and PSA-LUC were kindly provided by Dr. Roland Schüle (Universitäts-Frauenklinik und Zentrum für Klinische Forschung, Klinikum der Universität Freiburg, Freiburg, Germany). The pACT, pBind, 17mer-luc were kindly provided by Dr. Nancy Weigel (Baylor College of Medicine, Houston, TX). The pGALD-H (GALD-H), pVPAR1-660 (VP-ABC), and 5×GAL4Luc3 reporter were from Dr. Elizabeth M. Wilson (University of North Carolina, Chapel Hill, NC). AR NTD (1–556 amino acids), DBD (559–670 amino acids), LBD (712–919 amino acids) were PCR amplified and cloned into pGEX-4T-1 vectors. The following sequences were chosen to construct the TH1 shRNA expression vectors: 5′-GATCCGCAGAATTGAGCACACTTTATCGAAATAAAG-TGTGCTCAATTCTGCTTTTTTGGAAA-3′ and 3′-GCGTCTTAACTC-GTGTGAAATAGCTTTATTTCACACGAGTTAAGACGAAAAAACC-TTTTCGA-5′, the oligonucleotides were inserted into the *Bgl*II and *Hin*dIII sites in pU6 vector (Ambion). The identities of the clones were verified by sequencing.

Cells and C-57 black mice were obtained from the Institute of Cell Biology, Academic Sinica. TH1 antiserum was raised against the full-length GST–TH1 protein purified from *E. coli* [Yang et al., 2007]. AR(C-19), AR(N-20), anti-HA tag antibody, HRP-conjugated goat antirabbit, and HRP-conjugated goat antimouse IgG secondary antibodies were purchased from Santa Cruz Biotechnology. The mouse anti-Myc antibody was purchased from Invitrogen. [^{35}S]methionine, glutathione–Sepharose beads were purchased from Amersham Biosciences. β-Glycerolphosphate disodium and DHT was purchased from Fluka. Human KLK3/PSA Immunoassay kit was bought from R&D Systems.

CELL CULTURE AND TRANSIENT TRANSFECTION

COS-1 cells were maintained in Dulbecco's modified Eagle's medium (Sigma, Inc.), supplemented with 10% fetal bovine serum. LNCaP cells were maintained in F12 medium supplemented with 10% fetal bovine serum. All media contained 100 U of penicillin, 50 μg of streptomycin per ml, and 2 mM L-glutamine. Approximately 3×10^6 cells were plated on 100 mm dishes 24 h before transfection. Eight micrograms of total plasmids and 30 μl of Lipofectamine™ reagent were used for transfection according to the manufacturer's instructions.

IMMUNOPRECIPITATION

Approximately 16 h after transfection, cells were cultured in the medium containing 5% charcoal stripped serum with EtOH or 10 nM DHT. After another 24 h, cells were washed with ice-cold PBS and solubilized with 1 ml of CoIP buffer (50 mM Tris–HCl (pH7.5), 150 mM NaCl, 0.1% Nonidet P-40). Detergent insoluble materials were removed by centrifugation. Whole cell lysates were incubated with 2 μg relevant antibody at 4°C for 2 h. Pre-equilibrated protein G-agarose beads were added and collected by centrifugation after incubation overnight and then gently washed three times with the lysis buffer. The bound proteins were eluted and analyzed by Western blots. For the coimmunoprecipitation from mouse tissues, the testes and the prostates of 8-week-old male C-57 black mice were extracted in CoIP buffer. One milligram of total tissue lysates were subjected to coimmunoprecipitation as described above.

GST PULL-DOWN ASSAY

GST–TH1, GST, pcDNA3-AR, and TH1 constructs were used to generate [^{35}S]methionine-labeled proteins individually with the TNT® Coupled Reticulocyte Lysate System (Promega) according to the manufacturer's instructions. GST–AR–NTD, GST–AR–DBD, and GST–AR–LBD were expressed in *E. coli*, and preimmobilized on glutathione–Sepharose 4B beads. A GST pull-down assay was performed as described previously [Liu et al., 2004]. In brief, the labeled proteins were addressed immediately by constant mixing with 25 μl of glutathione–Sepharose beads in the binding buffer (20 mM Tris, pH 7.5, 50 mM NaCl, 10% glycerol, 10 mM NaF, 1%

Nonidet P-40, 1 mM NaVO4, 1 mM phenylmethylsulfonyl fluoride) with or without 10 nM DHT for 2 h at 4°C. Then the beads were washed three times with the binding buffer and the bound proteins were eluted in protein sample buffer and subjected to 10% SDS–PAGE analysis. The gel was then dried and autoradiographed.

REAL-TIME RT-PCR ASSAY

Total RNA was isolated from LNCaP by using TRIzol reagent (Invitrogen) according to manufacturer's instructions. The real-time PCR assay was conducted following the manufacturer's procedures (Applied Biosystems). Essentially, total RNA was isolated and reverse transcribed by using the random primer method (ImPrompII from Promega). Real-time PCR was conducted by using the fluorescent dye SYBR Green and an ABI PRISM 7700 Sequence Detection System (Applied Biosystems). The following primers were used: β-actin, 5′-CAGCACTGTGTTGGCGTACAGGTC-3′, and 3′-GACTAACGGTTACCACTACTGGAC-5′; TH1, 5′-TATACCTCTG-ACTTCGTGCAACT-3′, and 3′-GGACACTGCCTCAAAATCGAGT-5′; AR, 5′-AAGGCTATGAATGTCAGCCCA-3′, and 3′-CGGAACGAGA-GATCGGAGTTAC-5′.

SUBCELLULAR FRACTIONATION

COS-1 cells were transfected with indicated plasmids. Approximately 16 h after transfection, cells were cultured in the medium containing 5% charcoal stripped serum with EtOH or 10 nM DHT. After another 24 h, cell monolayers were harvested with ice-cold phosphate-buffered saline and pelleted. Cold buffer A (10 mM HEPES–KOH, pH 7.9, at 4°C, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol, and 0.2 mM phenylmethylsulfonyl fluoride) equal to five times the packed cell volume was used to resuspend the cells. After swelling on ice for 10 min, plasma membranes were disrupted by vortexing for 10 s. The nuclei were pelleted and the supernatants containing the cytoplasmic fraction of proteins were recovered. The remaining pellets were resuspended in two times the pellet volume of cold buffer C (20 mM HEPES–KOH, pH 7.9, 25% glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM dithiothreitol, and 0.2 mM phenylmethylsulfonyl fluoride) and incubated on ice for 60 min. Samples were then centrifuged and the supernatants containing the nuclear fraction were recovered.

IN VIVO UBIQUITINATION ASSAY

COS-1 cells were transfected with indicated plasmids. After 48 h, cells were treated with 50 μM MG132 for 5 h prior to harvest. Lysates were prepared by trypsinizing cells and washed three times with ice-cold PBS. Cell pellets were resuspended in modified RIPA buffer (150 mM NaCl, 1% NP-40, 0.5% deoxycholate, 0.1% SDS, 50 mM Tris–HCl, pH 7.5) with 1% SDS to disrupt protein–protein interaction and boiled for 10 min, then diluted in ten volumes of RIPA buffer. The lysates were sonicated on ice and clarified by centrifugation followed by preclearing with protein G agarose for 45 min at 4°C. The lysates were quantitated, and 1 mg of total cell lysates was subsequently divided for individual immunoprecipitation with anti-AR antibody or anti-HA antibody. Each 30 μg of cell lysates together with the immunoprecipitated proteins was analyzed by Western blots.

CHROMATIN IMMUNOPRECIPITATION

LNCaP cells were grown in F12 medium supplemented with 5% charcoal–dextran-stripped fetal bovine serum. After 3 days of cultivation, cells were treated with appropriate ligands, and cross-linked with 1% formaldehyde at 37°C for 10 min. Cells then were rinsed twice with ice-cold PBS, collected, and centrifuged for 5 min. The pellets were then resuspended in 0.3 ml of lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris–HCl, pH 8.1) and sonicated three times at 15 s each at a submaximal input (Fisher Sonic Dismembrator, Model 300) followed by centrifugation for 10 min. Supernatants were collected and diluted in dilution buffer (1% Triton X-100, 2 mM EDTA, 150 mM NaCl, and 20 mM Tris–HCl, pH 8.1) followed by preclearing for 2 h at 4°C with 2 μg sheared salmon sperm DNA and 20 μl of a 50% slurry of protein G-Sepharose. Immunoprecipitation was performed for 6 h or overnight at 4°C with specific antibodies. Samples were processed as described. For amplifying PSA gene fragments, 36 PCR cycles were used. Each cycle consisted of a 45-s denaturation at 95°C, a 45-s annealing at 60°C, and a 45-s elongation at 72°C. The primer sequences were as follows: ARE I (PSA (−170/+19)): 5′-AGAACAGCAAGTGCTAGCTC-3′ and 3′-GTCGGGGTTCGAATGGTGGA-5′; ARE III (PSA (−4288 to −3922)), 5′-GGGGTTTGTGCCACTGGTGAG-3′ and 3′-TTGGTACCTCTTAA-CGGAGGG-5′; Exon 4 (PSA (+3909 to +4067)), 5′-GTGTGTGG-ACCTCCATGTTATT-3′ and 3′-GAACTCCCCTTTCCACTCACC-5′; GAPDH, 5′-TCCTCCTGTTTCATCCAAGC-3′ and 3′-TTTCATCCC-GGGCCGATGA-5′. LNCaP cells transfected with Myc-TH1 or pcDNA3.1-Myc vector were subjected as described above. Each sample was run in triplicate.

DUAL LUCIFERASE REPORTER ASSAY

LNCaP (3×10^4 cells per well in 24-well plates) was incubated in 5% charcoal-stripped FBS supplemented F12 or DMEM for 24 h prior to transfection. For reporter gene assays, cells were cotransfected with 200 ng each of MMTV-LUC, ARE2×-TATA-LUC, PSA-LUCreporter vectors; 2 ng of control Renilla luciferase plasmid (pRL), indicated amounts of TH1 expression plasmids using Lipofectamine™ according to the manufacturer's instructions. The total plasmids were balanced up to 600 ng with pcDNA3.1-Myc empty vector. At 16 h after transfection, the culture medium was replaced with F12 or DMEM containing 5% charcoal-stripped FBS supplemented with EtOH or 10 nM DHT. After another 24 h, cells were lysed using Passive Lysis Buffer (Promega) according to the manufacturer's specifications and assayed immediately for reporter and control gene activities with the Dual-Luciferase Reporter Gene Assay (Promega) using a Lumat LB 9507 luminometer (EG&G Berthold, Bad Wildbad, Germany).

ELISA DETECTION OF LNCaP CELL CULTURE SUPERNATES

LNCaP cells were transfected with TH1 or pcDNA3.1-Myc and TH1 shRNA or pSilence 2.1 constructs. At 16 h after transfection, the culture medium was replaced with F12 containing 5% charcoal-stripped FBS and supplemented with EtOH or 10 nM DHT. After another 24 h, LNCaP cells were transfected and treated with or without DHT. Secreted PSA in cell culture supernates were detected using Human KLK3/PSA Immunoassay kit according to the manufacturer's instructions.

RESULTS

EXPRESSION OF TH1 IN MOUSE TISSUES AND MULTIPLE CELL LINES

TH1 is highly conserved and ubiquitously expressed, but its function is little known. To examine the expression level of TH1 protein in the tissues and cell lines, rabbit antiserum for TH1 was raised against the full-length GST–TH1 protein purified from *E. coli* and was observed to react with protein with predicted molecular weight of TH1 [Liu et al., 2004; Yang et al., 2007]. Next, Western blot analysis was performed to detect the expression level of TH1 in mouse tissues. As shown in Figure 1A, TH1 with predicted molecular mass of 65.8 kDa was expressed at variable levels in various mouse tissues. High level of TH1 protein was seen in prostate and heart, while moderate to low expression levels were detected in other tissues. Further, the expression level of endogenous TH1 in several cell lines was tested. TH1 is ubiquitously expressed with various levels in all cell lines analyzed (Fig. 1B), which is consistent with previous reports [Hari et al., 1995; Bonthron et al., 2000]. We noticed that this antiserum gave two closely spaced species. To clarify the specific band(s) for TH1, TH1 shRNA constructs were transfected into T47D cells with high expression level of TH1. The effect of knockdown was evaluated by RT-PCR analysis, suggesting that TH1 mRNA was decreased about 70% (Fig. 1C, right panel). Further, Western blot analysis with this antiserum showed that there was a reduction about 90% for the amount of lower band (Fig. 1C, left panel).

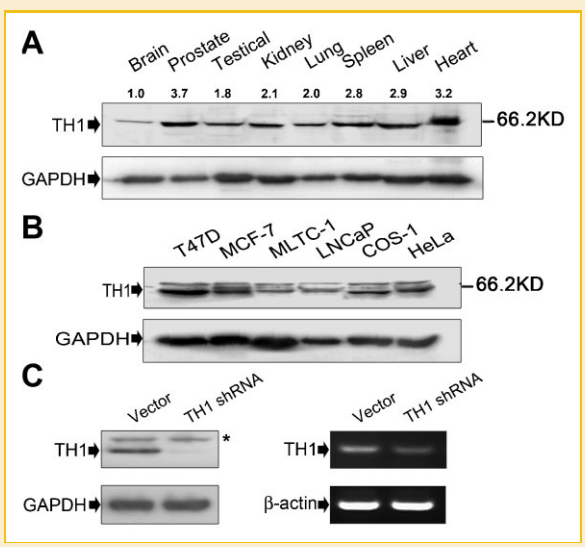

Fig. 1. Expression of TH1 in mouse tissues and multiple cell lines. A: Western blot detection of extracts from normal mouse tissues shows the expression level of TH1 protein. About 30 μg protein from each tissue extracts was subjected to 10% SDS–PAGE analysis. B: Expression of TH1 protein in multiple cell lines. Extracts from two breast cancer cell lines T47D and MCF-7, mouse Leydig tumor cell line MLTC-1, prostate cancer cell line LNCaP, African green monkey kidney COS-1, and cervical cancer cell line HeLa were subjected to Western blot analysis. C: Immunoblots (left) and RT-PCR (right) analysis of endogenous TH1, GAPDH proteins or TH1, β-actin mRNA in the control and TH1 knockdown of T47D cells. These experiments were performed at least three times.

However, the amount of upper band was not obviously influenced, which implied that the upper band may be a nonspecific detection.

TH1 INTERACTS WITH AR IN VIVO AND IN VITRO

In light of the high level of TH1 expression in the prostate of mouse tissues, we speculated that TH1 may have some novel function in human prostate. To examine whether TH1 associates with AR in mammalian cells, we performed coimmunoprecipitation experiments. TH1 protein was detected in the anti-AR immunoprecipitates from COS-1 cells cotransfected with TH1 and AR, but not with TH1 and empty vector (Fig. 2A). Furthermore, this interaction is not dependent on DHT. This interaction was confirmed in a reciprocal coimmunoprecipitation assay (Fig. 2B). To determine that a physical interaction occurs between endogenous AR and TH1 in vivo, we performed coimmunoprecipitation experiments using extracts from LNCaP cells, mouse prostate, and testicle. Immunoprecipitates of TH1 were detected with a mouse monoclonal antibody against AR. As shown in Figure 2C, AR protein could be precipitated in LNCaP cells by TH1 antiserum but not by normal rabbit serum. TH1 antiserum, but not normal rabbit serum, could immunoprecipitate AR from mouse prostate and testicle extracts (Fig. 2D). These results indicate that TH1 is associated with AR in vivo.

To determine if TH1 could interact with AR directly, a GST pull-down assay was carried out. As seen in Figure 3A, a strong retention of AR protein was obtained only in the sample containing GST–TH1. Furthermore, addition of DHT in this assay did not obviously alter the binding of AR with GST–TH1. To further map the interaction domains within AR, several AR fragment constructs were generated for testing their ability to interact with TH1 by GST pull-down assays (Fig. 3B). As shown in Figure 3C, TH1 could interact with N-terminal domain (NTD) and LBD of AR in a ligand-independent manner. Taken together, these results suggest that the AR and TH1 form a complex in cells.

TH1 DOWN-REGULATES AR PROTEIN LEVEL BUT NOT mRNA LEVEL

TH1 can interact with both NTD and LBD domain of AR, and association between the N- and C-terminal domains of the receptor stabilizes the AR homodimer. To test whether TH1 regulates AR protein turnover, Western blot analysis was performed. Overexpression of TH1 obviously decreased both cytoplasmic and nuclear AR protein level in the presence or absence of DHT treatment in COS-1 cells (Fig. 4A). To determine whether endogenous TH1 also influence the AR protein level, we detected the AR protein level in TH1 knockdown LNCaP cells treated with or without DHT. Knockdown of TH1 increased AR protein level in LNCaP cells (Fig. 4B), which is consistent with exogenous transfection assays. Real-time PCR analysis of the native AR gene expression shows that neither overexpression of TH1 nor knockdown of TH1 affects AR mRNA level (Fig. 4C,D). These results demonstrate that TH1 regulates AR protein turnover.

It is possible that TH1 may influence the stabilization of AR by blocking AR N/C interaction. To further investigate the mechanism, we analyzed the effect of TH1 overexpression on AR intermolecular interaction between the NH2 and the COOH domains. With mammalian two-hybrid assay, we showed that TH1 suppressed DHT-promoted AR N/C interaction in a dose-dependent manner

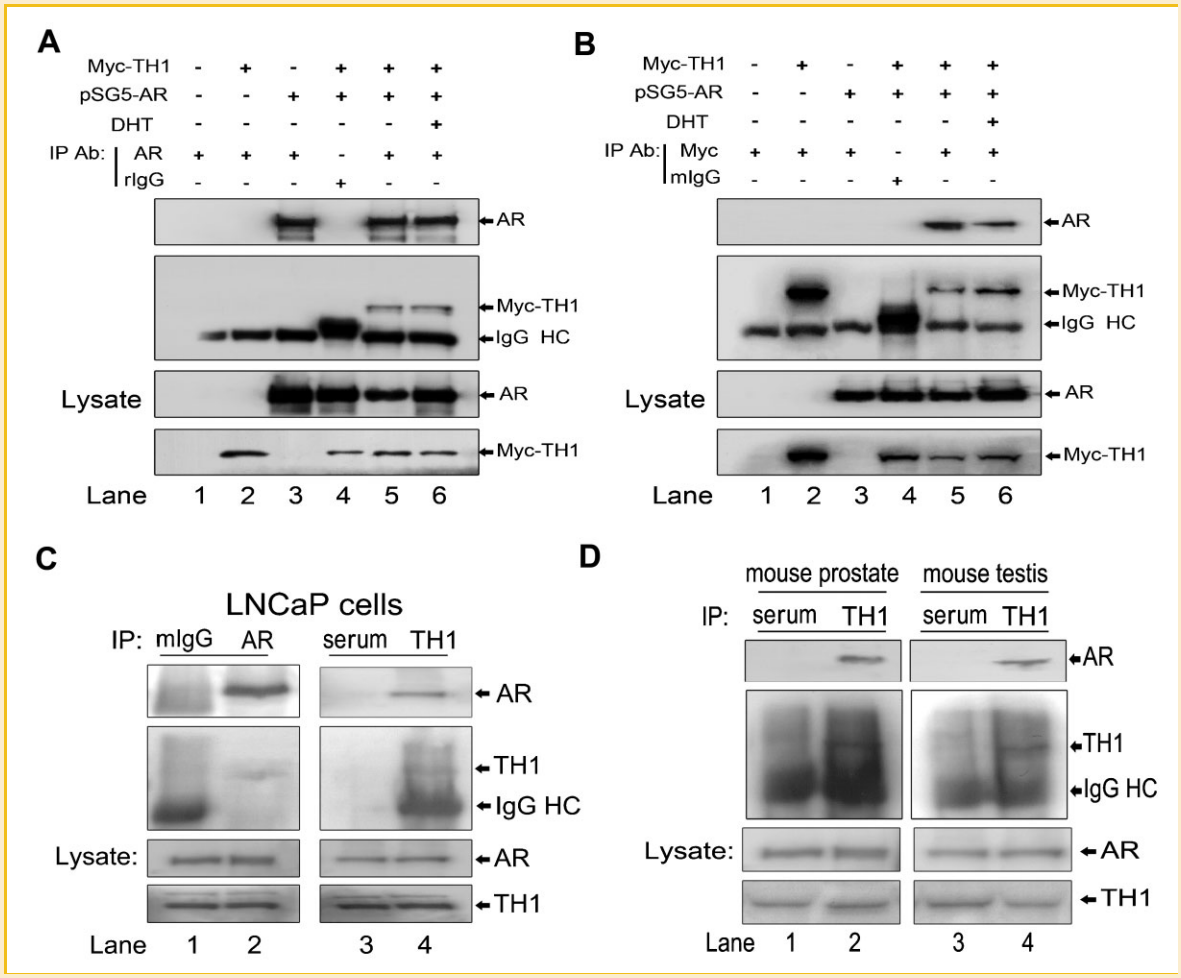

Fig. 2. TH1 interacts with AR in vivo. A: COS-1 cells were transiently transfected with the indicated combinations of pSG5-AR and pcDNA3.1-Myc-TH1 expression constructs, respectively. After 24 h of transfection, cells were treated with EtOH or 10 nM DHT. Another 24 h later, cells were harvested and the lysates were immunoprecipitated (IP), followed by Western blot analysis with antibodies as indicated (top panels). The bottom panels show the expression levels of the AR and TH1 from the transfected cell lysates. B: Similar experiment was performed except that anti-Myc IPs were immunoblotted with anti-AR antibody (N-20). C: Cell lysates from LNCaP cells treated with 10 nM DHT for 24 h were subjected to immunoprecipitation experiments, followed by Western blot analysis with the indicated antibodies. The bottom panel shows the expression levels of the AR and TH1 from the cell lysates used for coimmunoprecipitation experiments. D: Association of AR with TH1 in normal mouse prostate and testis. Totally 1 mg each of lysates from C-57 black mouse testis and prostate was incubated with TH1 antiserum. The immunoprecipitates and lysates were blotted with AR antibody (C-19) and TH1 antiserum, respectively. These experiments were performed at least three times. Ab, antibody; rIgG, normal rabbit IgG; mIgG, normal mouse IgG; serum, normal rabbit serum; IgG HC, IgG heavy chain. These experiments were performed at least three times.

without the alteration of AR ABC and AR D–H protein levels (Fig. S1).

TH1 DECREASES AR STABILITY AND ENHANCES AR UBIQUITINATION

Since TH1 reduced AR protein level but not mRNA level, we next determined whether TH1 affected AR protein half-life. As shown in Figure 5A (data plotted from three independent experiments), the half-life of AR in LNCaP cells is longer than 10 h in the presence of 10 nm DHT. In cells transfected with Myc-tagged TH1, AR protein turnover was faster (approximate 6.5 h), compared with controls. To directly examine whether the change of AR protein level was due to ubiquitination, HA-tagged ubiquitin and AR were transfected in COS-1 cells with or without TH1, and cells were mock-treated or treated with MG132. Lysis buffer containing 1% SDS was used, which is supposed to disrupt also the protein–protein interactions, with no effect on post-translational modification like ubiquitination. Overexpression of TH1 led to an accumulation of the ubiquitinated AR (AR-Ub_n) protein (Fig. 5B, upper panel, compared lane 2 with 3; Fig. 5C, upper panel, compared lane 2 with 3) and a degradation of the full-length AR (Fig. 5B,C, lower). However, in the presence of MG132, TH1 overexpression led to a modest decrease in AR protein level (Fig. 5B, right lower). Taken together, these results indicate that TH1 enhances the degradation and ubiquitination of AR.

TH1 IS RECRUITED ONTO THE PSA PROMOTER AND ENHANCER IN NUCLEUS

TH1 interacts with AR in a DHT-independent manner, and TH1 also increases AR turnover with or without DHT treatment. To examine

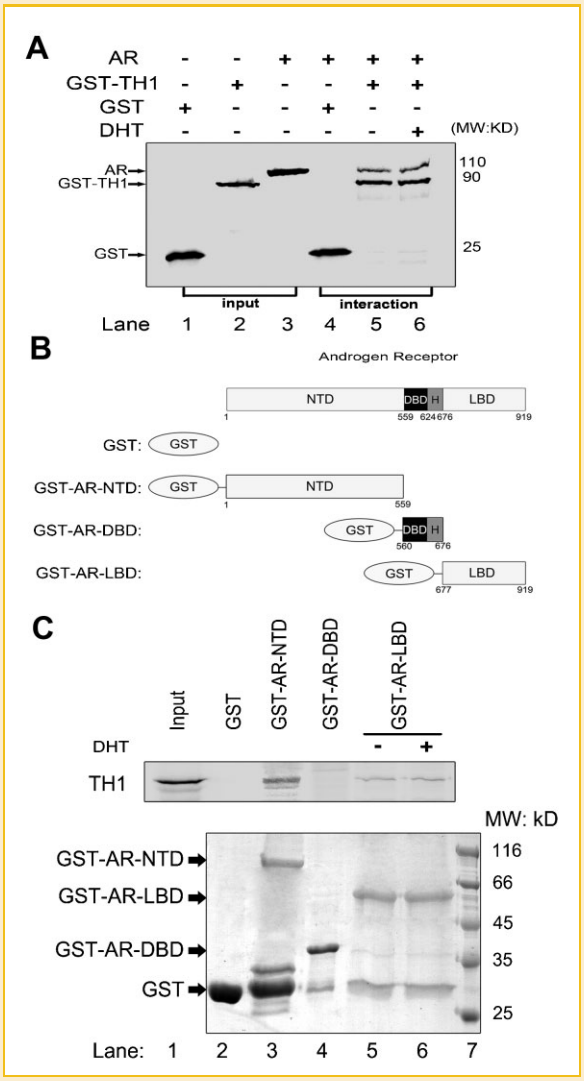

Fig. 3. TH1 interacts with AR in vitro. A: In vitro interaction of GST–TH1 or GST with AR. GST–TH1, GST and AR was in vitro translated using TNT coupled reticulocyte lysate system, labeled with [^{35}S]methionine, and then used for binding reaction with or without 10 nM DHT. After 2 h of incubation, the beads were washed and eluted with SDS sample buffer. Twenty-five percent of GST eluate and others were run with 20% input on an SDS–PAGE, and detected by phosphorimaging. B: Schematic presentation of constructs used in the interaction assays. NTD, N-terminal domain; DBD, DNA-binding domain; H, hinge region; LBD, ligand-binding domain. C: GST–AR–NTD, GST–AR–DBD, GST–AR–LBD, and GST proteins were bacterially expressed and purified as a GST-fusion protein. TH1 was in vitro translated using TNT-coupled reticulocyte lysate system, labeled with [^{35}S]methionine, and incubated with 2 μg of preimmobilized GST–AR–NTD, GST–AR–DBD, GST–AR–LBD, or GST control treated with or without DHT. The beads were washed and eluted with SDS sample buffer. The eluates were run with 20% input on an SDS–PAGE and detected by phosphorimaging. The gel was stained with Coomassie Brilliant Blue R-250, dried, and photographed (lower panel). These experiments were performed at least three times.

whether TH1 is recruited onto the PSA promoter/enhancer and involved in the transactivation of AR target gene, LNCaP cells treated with or without DHT were subjected to chromatin immunoprecipitation (ChIP). Genomic DNA corresponding to the androgen response elements ARE I and ARE III, located in the promoter and enhancer of the prostate-specific antigen (PSA) gene, respectively, were immunoprecipitated in a ligand-dependent manner with anti-AR antibodies (Fig. 6A). In the presence of ligand, TH1 was associated with chromatin on the PSA promoter (ARE I) and enhancer (ARE III), together with AR (Fig. 6B). Additionally, DNA from neither exon 4 of the PSA gene nor the promoter of the GAPDH genes was not enriched, suggesting that TH1 was specifically recruited onto PSA promoter and enhancer. These studies demonstrate that TH1 associates with AR at the active androgen-responsive PSA promoter and enhancer. To gain more insight into the influence of TH1 on AR promoter/enhancer interaction in vivo, we compared the recruitment of AR on PSA enhancer and promoter regions. The loading of AR onto both PSA enhancer and promoter were significantly decreased in Myc-TH1 transfected cells than parental LNCaP cells (Fig. 6C). It is possible that the interaction between TH1 and active AR in nucleus more effectively regulates the function of AR.

TH1 ATTENUATES ANDROGEN-DEPENDENT TRANSCRIPTIONAL ACTIVATION BY AR

The questions arise about whether the repression of the endogenous AR protein by TH1 interferes with the androgen signal transduction cascade and modifies AR target gene expression. We analyzed the effect of TH1 overexpression on three AR target gene reporters: mouse mammary tumor virus (MMTV)-LUC reporter, PSA promoter-LUC reporter, and two tandem copies of androgen-responsive element (ARE_{2X}-TATA)-LUC reporter. LNCaP cells were transiently transfected with a constant amount of reporter expression plasmids and increasing amount of Myc-tagged TH1 plasmid was balanced with the corresponding vector control. As shown in Figure 7A, TH1 suppressed, in a dose-dependent manner, the activation of androgen signal transduction. To study the effect of endogenous TH1 on androgen-signal transduction, LNCaP cells were transiently transfected with shRNAs directed against TH1. As shown in Figure 7B, knockdown of TH1 supported a higher level of ligand-dependent transcription from MMTV promoter compared with the control cells. Endogenous PSA protein expression can be induced by the DHT treatment in LNCaP cells and serum PSA is proportional to tumor volume and correlates positively with the clinical stage of the disease. We determined, using an ELISA assay, the levels of secreted PSA in the cell culture media. As shown in Figure 7C, overexpression of TH1 inhibited the secretion of PSA to some extent. Knockdown of TH1 relatively increased amount of PSA into the medium in LNCaP cells (Fig. 7D). Taken together, these results clearly indicate that TH1 acts as a special regulator to attenuate the androgen-signal transduction by AR.

DISCUSSION

In this report, we report that TH1 has potent inhibitory effects on the androgen signal-transduction cascade through promoting AR degradation. First, we show that TH1 interacts with AR in vitro and in vivo by GST pull-down and co-IP approaches. Second, TH1 decreases the stability of AR and enhances AR ubiquitination. Third,

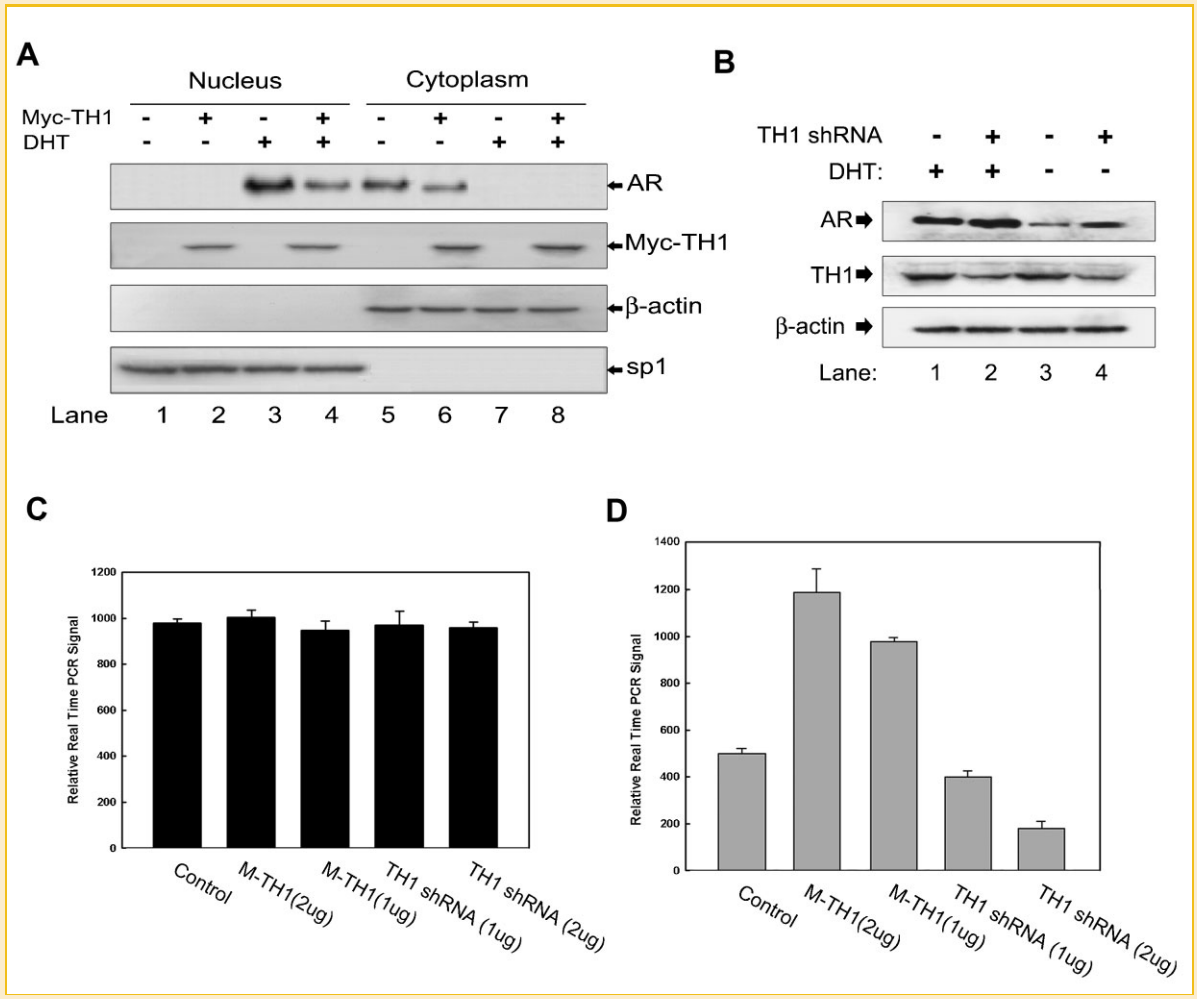

Fig. 4. TH1 down-regulates AR protein level but not mRNA level. A: COS-1 cells were transfected with 2 μg of pSG5-AR in combination with 4 μg of pcDNA3.1-Myc-TH1 or pcDNA3.1-Myc vector. About 24 h after transfection, cells were treated with 10 nM DHT or EtOH for another 24 h. Western blots analysis was performed to detect AR, Myc-TH1, sp1, and β-actin. B: LNCaP cells were transfected with control shRNA or TH1 shRNA, respectively, and then treated as described above. Western blot analysis was performed to detect the AR, TH1, and β-actin protein expressions. C: LNCaP cells were transfected with different doses of pcDNA3.1-Myc-TH1 or TH1 shRNA (as shown), and then treated with 10 nM DHT for 24 h. Cellular RNA was isolated and 1 μg of total RNA was subjected to real-time PCR for detection of AR transcript levels. D: Real-time PCR determines the TH1 transcript levels in the control, TH1 overexpression, and knock-down LNCaP cells. Results are averages of triplicates that are normalized against transcript levels of β-actin. Each experiment was performed at least three times.

TH1, associated with AR, is recruited to the endogenous AR responsive PSA gene promoters in a ligand-stimulated manner in LNCaP cells. Last but not least, we show that TH1 represses androgen-signal transduction and endogenous AR-responsive genes, PSA, from expression in LNCaP cells by both ectopic expression and shRNA knockdown approaches.

The human *trihydrophobin 1* (*th1*) gene, the homolog of Drosophila *th1*, was originally identified during the positional cloning of *mei-41* [Hari et al., 1995]. It lies adjacent to *mei-41* and was characterized by Bonthron et al. [2000]. According to their studies, the *TH1* gene was located in chromosome 20q13 and highly conserved from *Drosophila* to human by sequence comparison. Northern blots demonstrated that *TH1* was widely expressed in multiple tissues. The human TH1 protein has been predicted to have a molecular mass of 65.8 kDa and found at high level in cardiac and skeletal muscle, kidney, adrenal, and thyroid.

Though highly conserved and ubiquitously expressed, the function of TH1 is not well understood. Since our previous work has identified TH1 as a negative regulator of A-Raf kinase [Yin et al., 2002; Liu et al., 2004], which is one of MKKs in mammalian MAPK signaling pathway. Although MEKK1 stimulates the transcriptional activity of the AR in the presence or absence of ligand [Abreu-Martin et al., 1999], repression of A-Raf kinase may not account for the mechanism how TH1 suppress AR transactivation. These may include the following: (i) TH1 directly interacts with AR in in vitro recombinant system; (ii) TH1 interacts with both in vitro purified NTD and LBD of AR; (iii) in association with AR, TH1 is recruited to the PSA gene promoter in the stimulation of ligand. Taken together these findings suggest that TH1 may exert its regulatory effect through other pathways than suppressing A-Raf kinase activity.

Motif scan suggests that TH1 contains one LXXLL motif, a signature motif of transcriptional cofactors [McInerney et al., 1998].

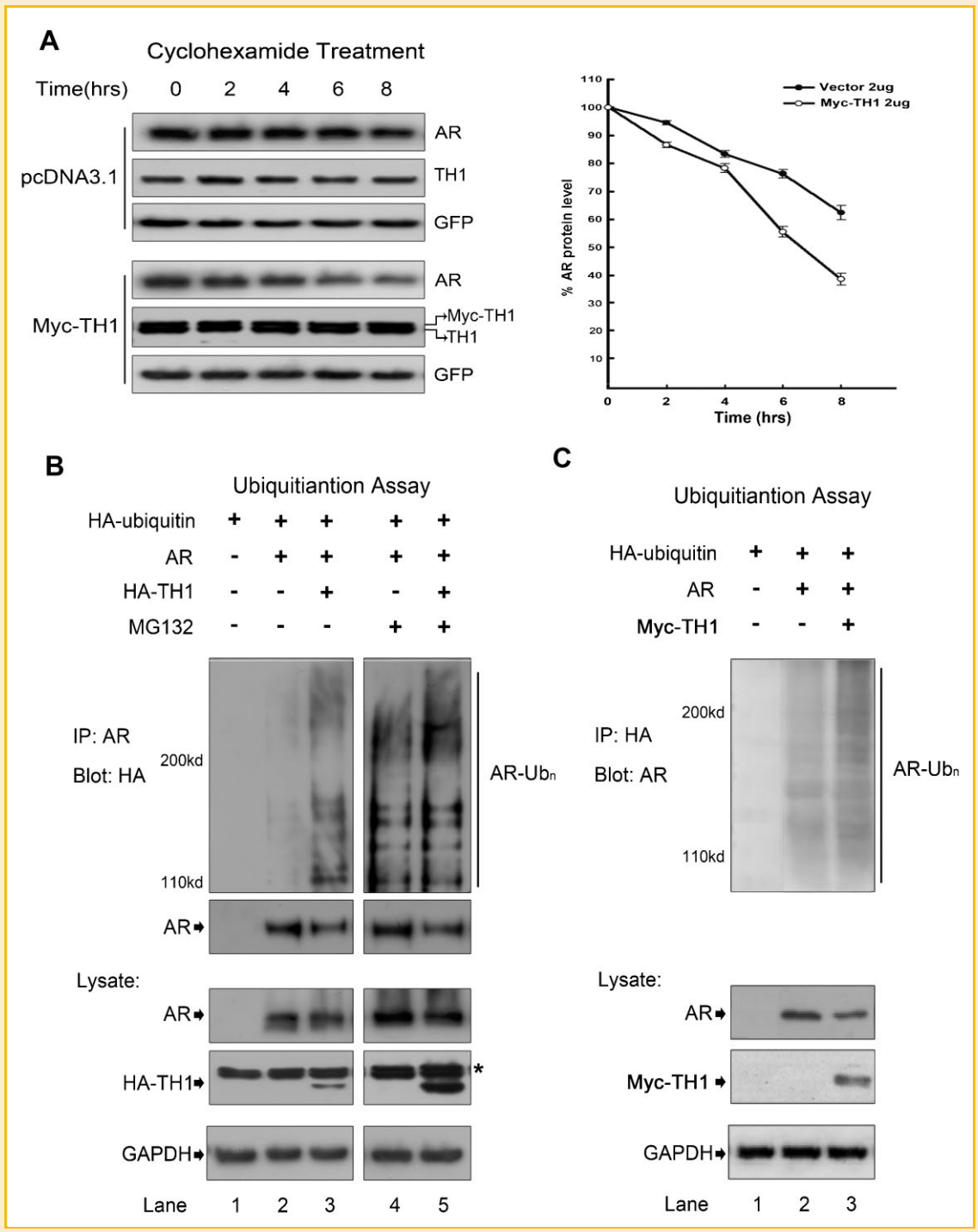

Fig. 5. TH1 decreases the stability of AR and enhances AR ubiquitination. A: LNCaP cells were transfected with 2 μg of pcDNA3.1-Myc-TH1 or pcDNA3.1-Myc vector, together with 0.5 μg of GFP constructs to control the transfection efficiency. After 24 h, cells were treated with 10 nM DHT for another 24 h, then cells were treated with 50 μg/ml CHX for the times indicated, and 30 μg of the lysates was run on a 10% polyacrylamide gel and blot-probed with AR, TH1, and GFP antibodies. AR expression was quantified by densitometric analysis. Expression is represented as the percentage remaining relative to time zero. Half-life values were calculated using lines of best fit. B,C: Overexpression of TH1 induces ubiquitination of AR in vivo. COS-1 cells were transfected with pSG5-AR, pcDNA3-HA-TH1 or pcDNA3.1-Myc-TH1, and pcDNA3-HA-ubiquitin constructs as indicated. After 48 h, cells were treated with or without 50 μM of MG132 for 5 h. The cell lysates were prepared using modified RIPA buffer as described in the Materials and Methods Section. The complex immunoprecipitated by anti-AR antibody was separated and blotted with anti-HA antibody (B), and the complex immunoprecipitated by anti-HA antibody was blotted with anti-AR antibody (C). The smear indicates the ubiquitinated AR (upper section). The lower panel shows the expression levels of the AR and HA-TH1 or Myc-TH1 from the cell lysates used for immunoprecipitation experiments. GAPDH protein level was shown as a loading control. These experiments were performed at least three times.

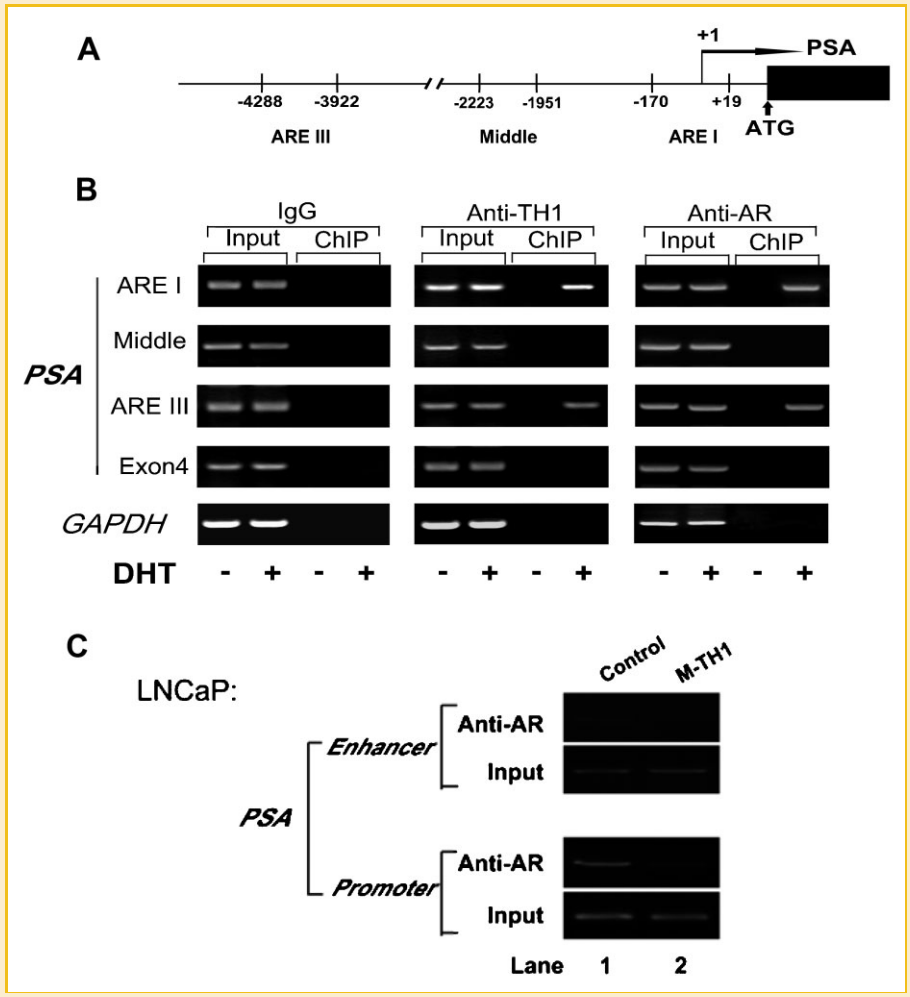

Fig. 6. TH1 is recruited to the enhancer and the promoter of the PSA gene in response to DHT treatment in nucleus. A: Schematic representation of the PSA promoter region analyzed by the ChIP assay. The localization of the PCR primers and androgen-response elements (ARE) is shown. B: ChIP assays of TH1 and AR occupancy on the PSA gene regulatory region. LNCaP cells were treated with or without 10 nM DHT for 1 h. ChIP was performed with the anti-AR antibody, TH1 antiserum, or normal rabbit IgG. The precipitated chromatin was amplified by PCR using primers flanking the promoter region (ARE I), the middle region (middle), the enhancer region (ARE III), exon 4 of the PSA gene, or the promoter of the GAPDH genes, followed by agarose gel electrophoresis and ethidium bromide staining as described in the Materials and Methods Section. Input, DNA prior to immunoprecipitation. C: LNCaP parental and Myc-TH1 transfected LNCaP cells were treated with 10 nM DHT for 1 h, and ChIP assays were performed with anti-AR antibody as described in (B). These experiments were performed at least three times.

The LXXLL motif is located at the N-terminus of TH1 (aa119–123), which implies that TH1 possesses a receptor interaction region in its N-terminus. The interaction of TH1 with NTD and LBD of AR leads to the interference of AR intramolecular interaction, decrease of AR stability, and enhance of AR ubiquitination. With mammalian two-hybrid assay, we showed that TH1 suppressed DHT-promoted AR N/C interaction in a dose-dependent manner (Fig. S1). AR N/C interaction is unique in nuclear receptors and may play important roles in the full function of AR via the following mechanisms: receptor dimerization, stabilization of a ligand in the LBD ligand binding pocket, and retardation of AR degradation [Wong et al., 1993; Zhou et al., 1995]. The relationship between AR N/C interaction and AR transactivation was equivocal and whether the influence of AR N/C interaction can totally translate into AR transactivation and/or in vivo cell growth remains unclear [Hsu et al., 2005]. Coactivator ARA70N not only has a strong functional domain that can enhance AR transactivation but also contains one FXXLF motif, which can compete with the AR N-terminal FXXLF motif to bind the AR C-terminal AF-2 domain. This competition may result in the blockage of AR N/C interaction [Yeh and Chang, 1996]. Corepressor ARA67 can interact with both N- and C-terminus of the AR, which may then be able to hold AR N–C together to enhance AR N/C interaction [Zhang et al., 2004]. ARA70 and ARA54 have no effect on AR N/C interaction, while both of them can enhance AR transactivation [Yeh and Chang, 1996; Kang et al., 1999]. The effects of both Rad9 and SRC-1 on AR N/C interaction correlate with their coregulator functions, with Rad9 suppressing and SRC-1 enhancing AR transactivation [Onate et al., 1995; Wang et al., 2004]. N-terminal domain of AR has a specific role in stabilizing the receptor by slowing the rate of ligand dissociation and AR degradation. Interactions of TH1 with both N- and C-terminus of the AR may abolish AR N/C interaction and hence decrease the stability of AR.

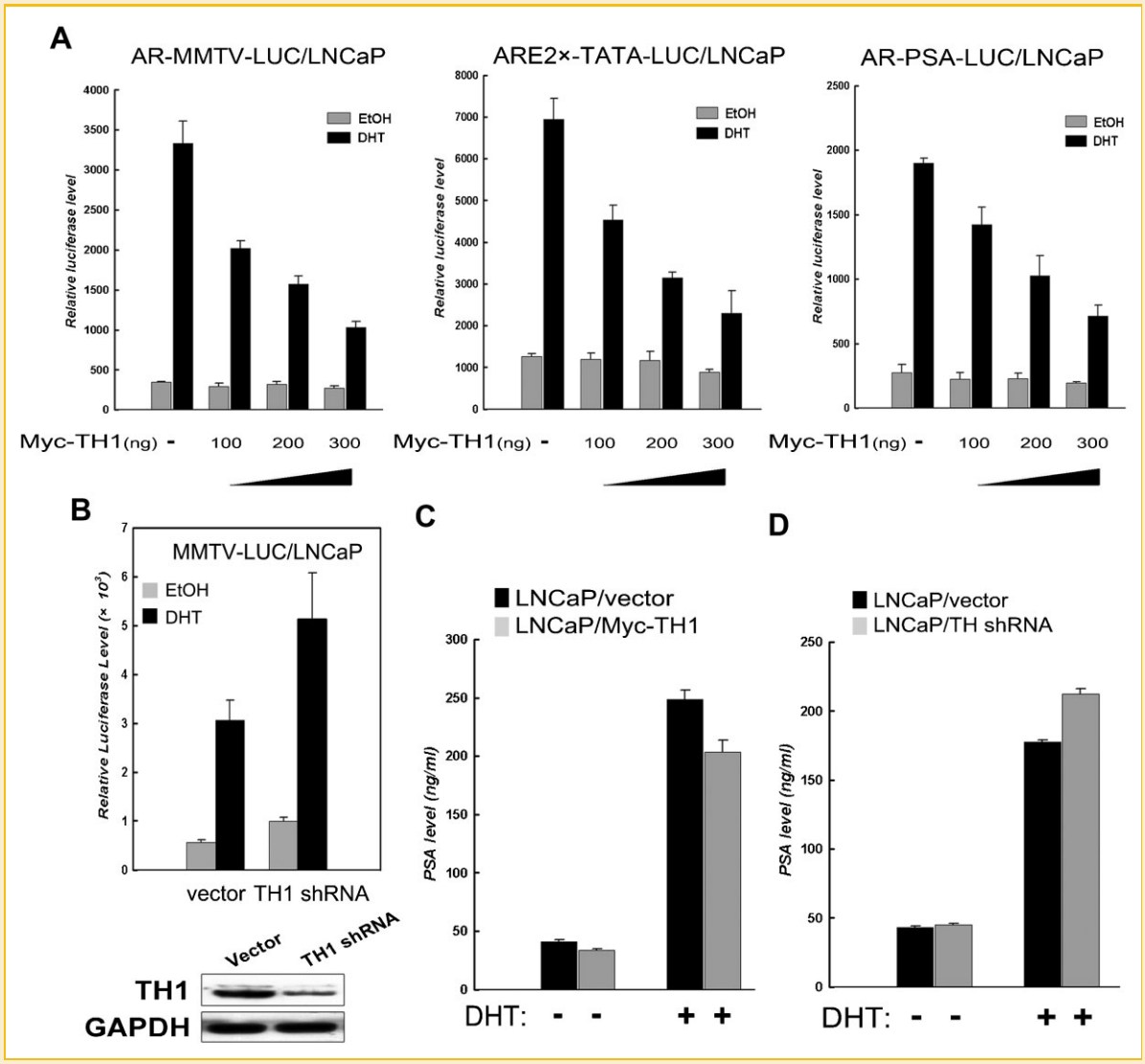

Fig. 7. TH1 attenuates androgen-dependent transcriptional activation by AR. A: LNCaP cells were transfected with different doses of pcDNA3.1-Myc-TH1 and/or pcDNA3.1-Myc vector together with 200 ng each of the reporter constructs and 2 ng of the internal control constructs. The total plasmids were balanced up to 600 ng with pcDNA3.1-Myc empty vector. About 16 h after transfection, cells were treated with EtOH or 10 nM DHT for 20–24 h. Luciferase activities were measured as described in the Materials and Methods Section. B: LNCaP cells were transfected with MMTV-Luc and control shRNA or TH1 shRNA, cultured for 40–48 h, followed by 10 nM DHT or EtOH treatment for 24 h, and assayed for luciferase activity. pRL was used as the internal control. Immunoblots with TH1 antiserum indicating the specific knockdown effect of the TH1 shRNA on the endogenous TH1 protein level (lower). C: LNCaP cells, which were grown in 35 mm plates, were transfected with 2 μg of pcDNA3.1-Myc-TH1 or pcDNA3.1-Myc vector constructs, respectively, cultured for 24 h, followed by EtOH or 10 nM DHT treatment for another 24 h. Each of 50 μl from 2 ml of cell culture medium was subjected to ELISA analysis to detect the secreted PSA using Human KLK3/PSA immunoassay kit according to the manufacturer's instructions. EtOH, ethanol. D: LNCaP cells were transfected with control shRNA or TH1 shRNA, respectively. Cells were treated, and the secreted PSA was detected by ELISA assay as described above. Results shown in (A) and (B) are the means ± standard deviations for three independent experiments. EtOH, ethanol.

The AR is critical for the normal growth and differentiation of the prostate and other urogenital structures. Deregulation of AR signaling may be a causal factor in prostate cancer development [Jaworski, 2006]. The AR, similar to many NRs, is a target for several post-translational modifications that govern numerous facets of receptor function [Gaughan et al., 2005]. Among them, ubiquitylation has been demonstrated to signal receptor destruction providing an absolute mechanism of AR inactivation [Lin et al., 2002]. Previous studies have identified E6-AP as an E3 ubiquitin ligase to catalyze AR ubiquitination [Gao et al., 2005]. Another well-studied example is probably the E3 RING finger ligase MDM2, whose ubiquitylating activity is linked to other post-translational modifications of AR, namely phosphorylation by Akt and deacetylation by histone deacetylase 1 [Lin et al., 2002; Gaughan et al., 2005]. The E3 U box ligase CHIP also increases the levels of ubiquitylated AR and promotes AR degradation [Cardozo et al., 2003; He et al., 2004].

Here, we report that TH1 promotes ubiquitination and degradation of AR, which may represent a novel mechanism of attenuating hormone-responsive gene expression. TH1, discriminated by its

protein structure character, belongs to neither *H*omologous to the *E*6-AP *C*-*T*erminus (HECT) domain E3s [Scheffner et al., 1993], nor *R*eally *I*nteresting *N*ew *G*ene (RING)-finger domain E3s. Furthermore, E6-AP catalyzes TH1 ubiquitination and degradation through ubiquitin–proteasome pathway [Yang et al., 2007]. Then, how TH1 promotes AR ubiquitination? One possible explanation is that TH1 may function as a regulator to hold AR and E3 ligase together, and promote receptor ubiquitination. Further work is necessary to test this hypothesis.

Some studies indicate that 26S proteasome involves in AR degradation in COS-1 cells [Lin et al., 2002], while others suggested the opposite. Treatment with proteasome inhibitor MG-132 for 24 h decreases AR protein level in LNCaP cells [Lin et al., 2002]. Moreover, treatment with PS-341, another proteasome inhibitor, not only decreases basal AR protein levels but also fails to prevent the reduction in AR protein levels elicited by ICI in LNCaP cells [Bhattacharyya et al., 2006]. Interestingly, hyperthermia, which inhibits 26S proteasome activity, dramatically decreases AR protein levels in human prostate cancer cells [Pajonk et al., 2005]. And recently, it is shown that it is calpain, but not 26S proteasome, that is involved in AR breakdown in prostate cancer cells [Libertini et al., 2007; Yang et al., 2008]. Therefore, there is an intricate process for AR degradation.

Current prostate cancer therapies are based on androgen deprivation. Although the tumors will be regressed to extend, they usually relapse as a result of the outgrowth of androgen-independent cells. The so-called androgen-independent cells may no longer require androgen for growth, in reality they may still be responsive to androgen. This is because AR may be activated by many cell signaling pathways, namely, MAPK [Abreu-Martin et al., 1999; Yeh et al., 1999], PI3K/Akt [Wen et al., 2000], PKC [Darne et al., 1998], or PKA [Nazareth and Weigel, 1996]; mutated AR may be activated by antagonists [Veldscholte et al., 1990a]; due to amplification, AR can be activated by small doses of remaining androgens [Koivisto et al., 1996]; and mutations in AR may alter its specificity toward adrenal androgens or even toward nonandrogenic steroids such as progesterone and estradiol [Veldscholte et al., 1990b; Culig et al., 1993]. Superior therapeutic interventions are therefore needed which could take advantage of the fact that prostatic tumors are androgen-independent but remain AR-dependent. So targeting AR degradation is an effective promising approach. Suppression of androgen–AR signaling through promoting receptor ubiquitination and degradation by TH1 provides a novel clue to develop novel therapies for the treatment of androgen-independent prostate cancer.

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