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Ubiquitin-Dependent Proteolysis of Trihydrophobin 1 (TH1) by the Human Papilloma Virus E6-Associated Protein (E6-AP)

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Abstract Human Papilloma virus E6-associated protein (E6-AP), which is known as an E3 ubiquitin ligase, mediates ubiquitination and subsequent degradation of a series of cellular proteins. In this paper, we identify here trihydrophobin 1 (TH1), an integral subunit of the human negative transcription elongation factor (NELF) complex, as a novel E6-AP interaction protein and a target of E6-AP-mediated degradation. Overexpression of E6-AP results in degradation of TH1 in a dose-dependent manner, whereas knock-down of endogenous E6-AP elevates the TH1 protein level. TH1 protein turnover is substantially faster, compared to controls, in cells that overexpressed E6-AP. Wild-type E6-AP promotes the ubiquitination of TH1, while a catalytically inactive point mutant of E6-AP abolishes its ubiquitination. Furthermore, in vitro ubiquitination assay also demonstrates that TH1 can be ubiquitinated by E6-AP. The degradation is blocked by treatment with proteasome inhibitor MG132. Herein, we provide strong evidence that TH1 is a specific substrate that is targeted for degradation through E6-AP-catalyzed polyubiquitination. J. Cell. Biochem. 101: 167–180, 2007.

Key words: TH1; E6-AP; interaction; ubiquitin; degradation

Ubiquitin-mediated degradation of regulatory proteins plays important roles in the control of numerous processes, including cell-cycle progression, signal transduction, transcriptional regulation, receptor downregulation, and endocytosis [Hershko and Ciechanover, 1998]. Ubiquitination of a target protein is highly specific and involves multi-step enzymatic reactions catalyzed by a cascade of enzymes, including ubiquitin-activating enzyme E1, ubiquitin-conjugating enzyme E2, and ubiquitin ligase E3. After the linkage of ubiquitin to the protein substrate, a polyubiquitin chain is usually formed in which the C-terminus of each ubiquitin unit is linked to a specific lysine residue (most commonly Lys48) of the previous ubiquitin. The polyubiquitinated substrates are rapidly recognized and degraded by the 26S proteasome [Hershko and Ciechanover, 1998; Finley, 2002].

Despite this fairly well characterized enzymatic pathway for ubiquitin conjugation, how proteins are selected for their ultimate demise is only beginning to be clarified. It is likely that the intrinsic E3 ligase activity serves as the rate-limiting step of ubiquitin modification of proteins and plays a central role in determining the specificity of ubiquitylation [Hershko and

Abbreviations used: TH1, trihydrophobin1; E1, ubiquitin-activating enzyme; E2, ubiquitin-conjugating enzymes; E3, ubiquitin protein ligase; E6-AP, E6-associated protein; E6, human papilloma virus E6 oncoprotein; GST, glutathione S-transferase; WGE, wheat germ extracts; HPV, human Papilloma virus; HA, hemagglutinin; IP, immunoprecipitation; PMSF, phenylmethylsulfonyl fluoride; PCR, polymerase chain reaction; CHX, cycloheximide.

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Ciechanover, 1998; Laney and Hochstrasser, 1999]. The E6-associated protein (E6-AP), as its name implies, is a human cellular protein, which interacts with the human Papilloma virus E6 protein [Huang et al., 1999; Be et al., 2001; Cui et al., 2003]. E6-AP belongs to the HECT E3s, characterized by a homology to E6-AP carboxy-terminal domain (HECT), which is one of the three families of E3 ubiquitin ligases based upon their domain structure and substrate recognition [Huibregtse et al., 1995]. The HECT E3 ubiquitin ligases share a common catalytic domain, the HECT domain, with a conserved cysteine residue, through which HECT domain E3s form thioester intermediates with ubiquitin. The N-terminal regions are highly variable and may be involved in substrate recognition [Zheng, 2003]. E6 and E6-AP can form a complex that functions as a p53-specific E3 ubiquitin ligase [Scheffner et al., 1993]. Src family tyrosine kinases Blk and Src [Harris et al., 1999; Oda et al., 1999], nucleotide excision repair factor HHR23A [Kumar et al., 1999], a subunit of replication licensing factor Mcm7 [Kuhne and Banks, 1998], E6TP1 [Gao et al., 2002] and Human Scribble (Vartul) [Nakagawa and Huibregtse, 2000], and one of the two splice variants encoding isoforms with identical N-termini and variant C-termini of NFX1 (NFX1-91) [Gewin et al., 2004] are identified as targets of E6-AP.

The human trihydrophobin 1 (*TH1*) gene, which is located in chromosome 20q13, was originally identified and characterized by the Bonthron et al. [2000] during the positional cloning of mei-41. Previous studies showed that TH1 displayed high levels of expression in cardiac and skeletal muscle, kidney, adrenal, and thyroid [Bonthron et al., 2000]. Though highly conserved and ubiquitously expressed, the human TH1 is not well understood in terms of its function. Recently, it is found to be identical to NELF-C/D, an integral subunit of the human negative transcription elongation factor (NELF) complex [Narita et al., 2003]. NELF is a four-subunit complex (A, B, C/D, and E) that is biochemically purified based upon its role in repressing RNA polymerase II (RNAPII)-dependent transcription elongation in vitro [Yamaguchi et al., 1999]. NELF-A and NELF-E bind to RNAPII and RNA respectively, and both interactions being critical for NELF function in transcriptional pausing in vitro [Yamaguchi et al., 2002]. Although TH1 (NELF-C/D) and NELF-B are both required for the assembly of a functional NELF complex, their biochemical functions in the context of the NELF complex remain to be elucidated.

In this current report, we performed a yeast two-hybrid experiment using TH1 as bait, and identified E6-AP as an interaction partner of TH1. Both in vivo and in vitro assays indicated that TH1 might be a novel E6-AP interaction protein and a target of E6-AP-mediated ubiquitination and subsequent degradation.

MATERIALS AND METHODS

Materials

HeLa and HepG2 cells were obtained from the Institute of Cell Biology, Academic Sinica. MATCHMAKER LexA two-hybrid system and human fetal liver MATCHMAKER LexA cDNA library were products of Clontech (Palo Alto, CA). Protein G-agarose, the mouse monoclonal anti-HA (12CA5), anti-GFP antibodies were purchased from Roche (Mannheim, Germany). The anti-p53, anti-Ubiquitin antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The mouse monoclonal E6-AP antibody was purchased from BD Bioscience (Palo Alto, CA). The rabbit polyclonal E6-AP antibody was purchased from Abcam Ltd (Cambridge, UK). The TH1 antiserum was raised against the GST-TH1 protein purified from Escherichia coli (*E. coli*). The mouse anti-Myc antibody and LipofectAMINETM were purchased from Invitrogen (Carlsbad, CA). TNT-coupled Wheat Germ Extract Systems, and Reticulocyte Lysate Systerms were purchased from Promega (Madison, WI). E1, UbcH7 (E2), and ubiquitin were purchased from Sigma Chemical Co (St. Louis, MO). [35S]methionine, glutathione-Sepharose beads were purchased from Amersham Biosciences (Uppsala, Sweden). Proteasome inhibitor MG132 (carbobenzoxy-L-leucyl-L-leucyl-L-leucinal) was purchased from Calbiochem (La Jolla, CA).

Plasmid Construction

TH1 expression plasmids were constructed as described previously (30). The plasmid pcDNA-HA-ubiquitin was kindly provided by Dr. Hohfeld J. (Bonn,Germany). pGEX-E6-AP, pGEX-E6-AP(C833A) were kindly provided by Dr. Peter M. Howley (Harvard Medical School, Boston, MA). The pcDNA3.1-Myc-E6-AP,

pcDNA3.1-Myc-E6-AP (C833A), E6-AP-deficient mutants F1, F2, F3, and F4 were generated by PCR using pGEX-E6-AP and pGEX-E6-AP (C833A) as templates and the PCR product were cloned in-frame into the *Bam*HI/*Hind*III sites of pcDNA3.1. The E6-AP shRNA targeted sequences as follows: esh1 5′-CTAATAGAA-CGCTACTACCACCAGTTAAC-3′, esh2 5′-AG-AGATTGTTGAAGGCCATCACGTATGCC-3′, and esh3 5′-ACAATGAAGAAGATGATGA-AGAGCCCATC-3′ were kindly provided by Dr. Denise A. Galloway (University of Washington, Seattle). The pEF-Myc-16E6 and pEF-Myc-18E6 were from Dr. Tohru Kiyono (National Cancer Center Research Institute, Tokyo).

Yeast Two-hybrid cDNA Library Screening

MATCHMAKER LexA two-hybrid system was used to perform yeast two-hybrid screening according to the manufacturer's instruction. The full-length TH1 was cloned in-frame into LexA coding sequence and then used as bait to screen by the reporter genes LEU2 and lacZ. The transformed yeast was grown on plates lacking both histidine to select the bait plasmid and tryptophan to select the prey plasmid, respectively. The individual cDNA of interest was purified from the positive clones and retransformed into yeast to confirm its specific interaction with TH1.

Cell Culture and Transient Transfection

Cells were cultured with DMEM supplemented with 10% (v/v) fetal bovine serum −1% (w/v) penicillin, streptomycin in a humidified atmosphere containing 5% CO_2 at 37°C. For transient transfection assays, the cells (8×10^5) were grown on 60-mm tissue culture dishes. The following day, the cells were transfected with relevant plasmids. The transfection was performed with LipofectAMINETM according to the manufacturer's recommendations. After 48 h, the cells were harvested for further analysis.

Production of Recombinant Proteins

GST fusion proteins were obtained by sonicating bacteria induced with IPTG in suspension buffer (20 mM HEPES pH 7.4, 2 mM DTT, 1 mM PMSF, 1 mM $MgCl_2$, 10 μM $ZnCl_2$, 10 μg/ml aprotinin, 10 μg/ml leupeptin). Triton X-100 was added to a concentration of 1% (v/v), and debris was pelleted for 10 min at 14,000*g*. Glutathione-Sepharose (Amersham) was added for 2 h. After washing the beads in suspension buffer, bound proteins were eluted with 50 mM glutathione in 20 mM Tris-HCl pH 8.0, 120 mM NaCl, 0.1% (v/v) Triton X-100.

In Vitro Binding Assay

Rabbit reticulocyte lysates from Promega were used to generate [35S]Methionine-labeled proteins, which were used immediately for binding studies with recombinant bacterial proteins. The binding reactions were performed in protein binding buffer (20 mM Tris, pH 7.5, 50 mM NaCl, 10% glycerol, 10 mM NaF, 1% (v/v) Nonidet P-40, 1 mM $NaVO_4$, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM PMSF) at 4°C for 2 h with constant rotation. The beads were washed, and the bound proteins were eluted in protein sample buffer and subjected to 10% SDS–PAGE analysis. The gel was then dried and autoradiographed.

In Vivo Interaction Assay

Forty-eight hours after transfection, the cells were solubilized with 1 ml of lysis buffer (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.1% (v/v) Nonidet P-40, 5 mM EDTA, 5 mM EGTA, 15 mM $MgCl_2$, 60 mM β-glycerophosphate, 0.1 mM sodium orthovanadate, 0.1 mM NaF, 0.1 Mm benzamide, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM PMSF). Detergent insoluble materials were removed by centrifugation. Whole cell lysates were incubated with 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 using Western blots.

Pulse-Chase Experiments

Cells were transiently transfected with either pcDNA3 mock plasmids or pcDNA3-E6-AP constructs described above. After 48 h, cells were metabolically pulse-labeled with 300 μCi of [35S] methionine/cysteine for 1 h and chased for the indicated time periods. Equal amounts of radiolabeled lysates (based on counts) were immunoprecipitated with TH1 antiserum and analyzed by SDS–PAGE, followed by fluorography.

In Vivo Ubiquitination Assay

Cells were treated with 50 μM MG132 for 5 h prior to harvest. Lysates were prepared by trypsinizing cells and washing with PBS. Cell pellets were resuspended in modified RIPA buffer (150 mM NaCl 1% (v/v), NP-40 0.5% (w/v), deoxycholate 0.1% (w/v), SDS 50 mM Tris-HCl, pH 7.5, 1 mM PMSF, 10 μg/ml aprotinin, 5 mg/ml leupeptin) with 1% (w/v) SDS to disrupt protein–protein interaction and boiled for 10 min, then diluted in 10 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 subsequently divided for individual immunoprecipitation with the appropriate antibody. Immunoprecipitated proteins were analyzed using Western blots.

In Vitro Ubiquitination Assay

TH1 was labeled with [35S] methionine in an in vitro translation reaction with Wheat Germ Extract according to the manufacturer's protocol (Promega). Exponentially growing cells were trypsinized, and lysised by sonicating on ice in 100 μl of 20 mM Tris, pH 8.0, for 10 min. Cell debris was removed and 200 μg of the total protein was mixed with 20 μl of [35S]-labeled TH1, and the volume was adjusted to 120 μl using reaction buffer (20 mM Tris, pH 7.6, 50 mM NaCl, 10 mM $MgCl_2$). Following incubation at 30°C for various periods of time, 30 μl of the ubiquitination mixture was retrieved and the reaction was stopped by adding 3 × SDS sample buffer. For inhibition of the 26 S proteasome, MG132 was added to the ubiquitination mixture to a final concentration of 100 μM. Samples were boiled for 5 min and separated on an 8% SDS–polyacrylamide gel. Following migration, the gel was fixed, dried, and subjected to phosphorimaging.

To identify E6-AP as an E3 ubiquitin ligase participating in TH1 ubiquitination, E1 and Ubc7 (E2) were used in the in vitro ubiquitination assays. Ubiquitination reaction mixtures containing E1 (50 ng), Ubc7 (200 ng), ubiquitin (6 μg), ATP (4 mM), in vitro translated TH1 (5 μl), and immunoprecipitated E6-AP on Sepharose beads were incubated at 30°C for 90 min. Samples were analyzed by SDS–PAGE and subjected to phosphorimaging.

RESULTS

Identification of E6-AP as a New Interaction Partner of TH1

To identify TH1-interacting proteins, we used the full-length *TH1* gene as bait to screen an expression library generated from human fetal liver cDNA. We plated a pool of cells that contained 5×10^7 primary library transformants at a multiplicity of 10 onto selection induction medium. Twenty-five clones were obtained whose LEU_2 and lacZ reporter genes were activated. Sequence analysis of these genes revealed that one of them encoded the N terminal truncate of human E6-AP. The interaction between TH1 and E6-AP in the yeast two-hybrid assay was specific as suggested by the observation that neither the TH1 bait plasmid nor E6-AP target plasmids conferred significant growth to yeast cells when transfected independently (data not shown). Characterization of the other clones is in progress in our laboratory.

Interaction of TH1 With E6-AP In Vitro

To further confirm the interaction between TH1 and E6-AP, a GST pull-down experiment was performed as follows. GST-TH1 protein expressed and purified in bacteria was assayed for its ability to bind radiolabeled E6-AP synthesized in vitro using the Reticulocyte Lysate System. GST-TH1 efficiently interacted with E6-AP (Fig. 1A, lane 2).

The functional domains of E6-AP are schematically shown (Fig. 1B). To investigate the region in E6-AP responsible for TH1 binding, we

Fig. 1. TH1 interacts with E6-AP in vitro. **A**: In vitro interaction between GST-TH1 and E6-AP. E6-AP was synthesized in Rabbit reticulocyte lysates in the presence of [^{35}S] methionine and incubated with glutathione-Sepharose-coupled GST or GST-TH1 proteins expressed in *E. coli*, for 2 h at 4°C. Samples were washed and resolved by SDS–PAGE and visualized by fluorography (**upper panel**). The sane gel was stained with Coomassie Brilliant Blue for the expression of GST-fusion proteins (**lower panel**). Molecular mass markers are indicated on the right. **B**: Mapping of the regions responsible for TH1 binding in E6-AP. Schematic representation of the interaction between TH1 and E6-AP mutants with GST-pull down assay. Regions and residue numbers are indicated. The column on the right summarizes whether constructs did (+) or did not (–) interact. **C**: In vitro interaction between E6-AP mutants and GST-TH1. [^{35}S]methionine-labeled E6-AP (C833A), E6-AP deletion mutants, and nonradiolabeled GST or GST-TH1 were used for interaction analysis. Molecular mass markers are indicated on the **right**, and individual proteins are indicated on the **left**. The interaction of TH1 and A-Raf is shown as a positive control.

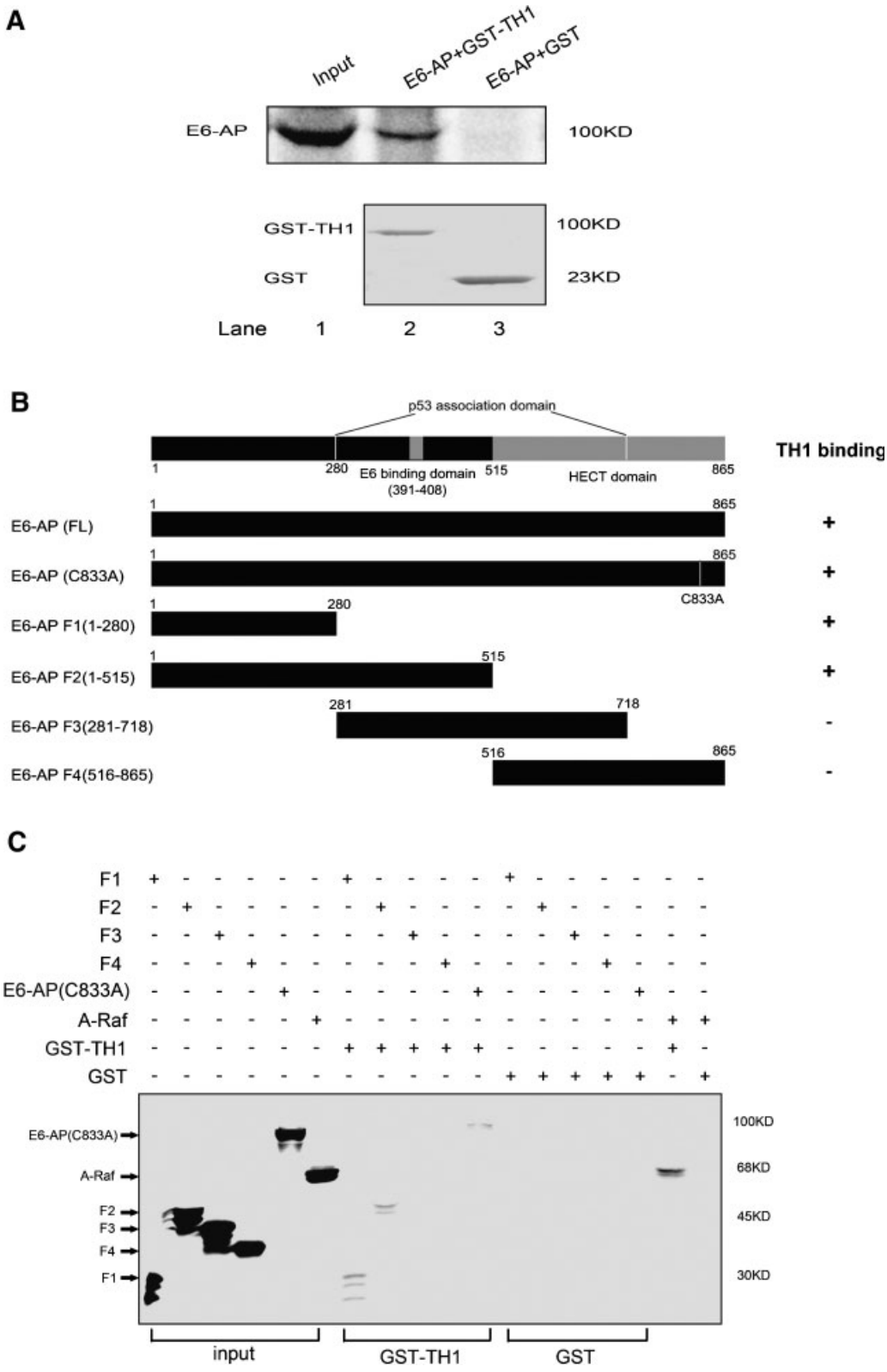
A
Input
E6-AP+GST-TH1
E6-AP+GST
E6-AP
100KD
GST-TH1
GST
100KD
23KD
Lane 1 2 3
B
p53 association domain
TH1 binding
1
280
E6 binding domain
(391-408)
515
HECT domain
865
E6-AP (FL)
E6-AP (C833A)
C833A
E6-AP F1(1-280)
E6-AP F2(1-515)
E6-AP F3(281-718)
E6-AP F4(516-865)
C
F1
F2
F3
F4
E6-AP(C833A)
A-Raf
GST-TH1
GST
100KD
68KD
45KD
30KD
input
GST-TH1
GST

Fig. 1.

constructed a series of E6-AP deletion mutants: F1 (amino acids 1–280), F2 (amino acids 1–515), F3 (amino acids 281–718), F4 (amino acids 516–865), and a catalytically inactive point mutant, E6-AP (C833A), into plasmid pcDNA3.1, and tested for their binding abilities with TH1 in a GST pull-down assay. As shown in Figure 1C, TH1 could bind directly with E6-AP (C833A), F1, and F2, whereas no binding with F3 (p53 association domain) and F4 (HECT domain) were detected. Taken together, the N-terminal 1–280 amino acids of E6-AP are necessary and sufficient for the interaction with TH1.

In Vivo Interaction of TH1 and E6-AP

TH1 antiserum was raised against the full-length GST-TH1 protein purified from *E. coli*. This antiserum was observed to react with protein of size matching those predicted by the human genome (Fig. 2A). To evaluate whether TH1 and E6-AP associate with each other in

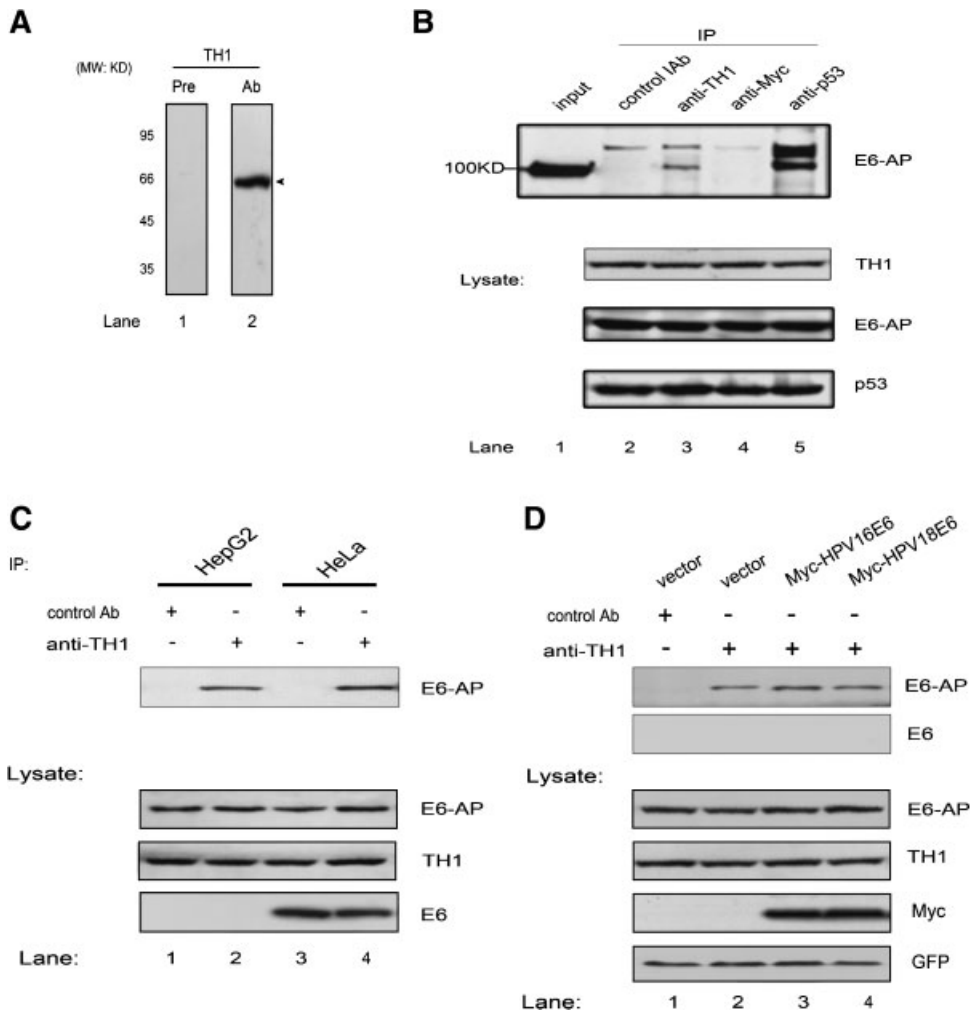

Fig. 2. In vivo interaction of TH1 and E6-AP. **A**: Immunoblot analysis of HeLa cells lysates with preimmune antiserum (**lane 1**) and TH1 antiserum (**lane 2**). The polypeptide marked by an asterisk matches the predicted size of TH1. **B**: Interaction of TH1 and E6-AP in cells. A total of 10^6 HeLa cells were plated onto the 100-mm dishes and 48 h later, the cell lysates were subjected to immunoprecipitation with indicated antibodies, followed by blotting with E6-AP antibody. 5% of whole-cell lysates were directly blotted with indicated antibodies to assess the expression of proteins. **C**: The interaction of TH1 and E6-AP is E6-independent. Both HepG2 and HeLa cells were immunoprecipitated with control antibody and TH1 antiserum respectively, followed by blotting with E6-AP antibody. The expressions of proteins were assessed by Western blot. IP, immunoprecipitation; Ab, antibody. **D**: E6 overexpression does not influence the interaction of TH1 and E6-AP. HeLa cells were transfected with indicated plasmids respectively and the cell lysates were immunoprecipitaed with TH1 antiserum (**lanes 2–4**) and control antibody (lane 1). E6-AP and E6 antibody were used to detect the anti-TH1 immunocomplex. The expressions of individual proteins were assessed by Western blotting.

cells, lysates from HeLa cells were immunoprecipitated with the indicated antibodies, followed by immunoblotting with E6-AP antibody (Fig. 2B, upper panel). E6-AP was reported to interact directly with p53 and subsequently mediate the ubiquitination and degradation of p53 in E6-positive cells (e.g., HeLa cells). The interaction of E6-AP and p53 was shown as a positive control (Fig. 2B, upper panel, lane 5). The monoclonal anti-Myc antibody and normal rabbit IgG were added to exclude any nonspecific interaction. TH1 specifically interacted with E6-AP in cells (Fig. 2B, upper panel, lane 3).

Although E6-AP and E6 oncoprotein together provide the E3-ubiquitin protein ligase activity in the transfer of ubiquitin to p53, a wealth of evidence strongly supports the paramount importance of the E6-independent ubiquitin ligase activity of E6-AP. To investigate whether the interaction of TH1 and E6-AP was E6-dependent, we performed the co-IP experiment in HepG2 cells, which is an E6-deficient cell line. Figure 2C shows, the interaction of TH1 with E6-AP is not dependent on the presence of E6 oncoprotein.

We next determined whether E6 and TH1 bound simultaneously to E6-AP. HeLa cells were transfected with or without plasmids encoding Myc-tagged HPV16E6 (Fig. 2D, lane 3) and HPV-18E6 (lane 4) respectively. The results indicate that E6 was not detectable in the anti-TH1 immunocomplex. Furthermore, overexpression of E6 did not influence the binding of TH1 to E6-AP (Fig. 2D, compared lanes 2, 3, and 4).

E6-AP Downregulates TH1 Protein Levels

The interaction of TH1 with E6-AP raised the possibility that this protein might be a substrate for E6-AP-mediated protein degradation. To test this hypothesis, we first examined the degradation of TH1 induced by E6-AP. As expected, overexpression of E6-AP indeed dose-dependently decreased TH1 protein levels in a manner sensitive to the proteasome inhibitor MG132, implying that E6-AP may directly or indirectly induce the proteasomal degradation of TH1 (Fig. 3A,B). Next we asked whether knock-down of the endogenous E6-AP could elevate the TH1 protein levels. To address this question, we used three shRNAs targeting three different sequences of E6-AP, which showed different knockdown efficiency, to block the expression of E6-AP [Gewin et al., 2004]. Esh1 is the best hairpin for reducing mRNA of E6-AP (70% reduced), whereas esh1 and esh2 show 55% and 40% knock-down efficiency respectively. Intriguingly, knock-down of the endogenous E6-AP elevated the TH1 protein levels (Fig. 3C,D). The effect of E6-AP shRNAs on p53 protein levels were shown as a positive control.

E6-AP Shortens the Half-Life of TH1 Protein

To further investigate the destabilization of TH1 protein by E6-AP, we performed the following two experiments. In Figure 4A, cells were transfected with mock vectors or pCMV-E6-AP, and CHX was used to inhibit the protein synthesis. TH1 protein level was detected and the half-life was measured. Transfected cells were pulse-labeled with [^{35}S] methionine and [^{35}S] cycteine, followed by a chase for various time periods. Anti-TH1 immunoprecipitates of these lysates were subjected to fluorography. As seen in Figure 4C (a representative autoradiogram) and 4D (data plotted from three independent experiments), TH1 protein turnover was substantially faster, compared to controls, in cells cotransfected with E6-AP. Thus by either method, our study suggested that TH1 was a stable protein with a half-life of over 12 h, whereas ectopic expression of E6-AP made TH1 less steady with the half-life of less than 6 h.

TH1 Is Ubiquitinated In Vivo

To directly examine whether the change of TH1 protein level was due to ubiquitination, HeLa cells transfected with HA-tagged ubiquitin, with or without E6-AP were either mock-treated or treated with MG132 for 5 h before cell lysis. Treatment with MG132 led to an accumulation of the ubiquitinated TH1 protein both in the absence and presence of E6-AP (Fig. 5A, upper panel, lanes 2 and 4). MG132 treatment also led to an increase in TH1 protein levels compared with untreated cells both in the presence and absence of E6-AP overexpression (lower panel, compared lane 1 with 3, and 2 with 4). These results implied that TH1 protein was regulated by the Ub-proteasome pathway both basally and when degradation was induced by E6-AP protein. The levels of p53 protein were monitored as a positive control.

In order to ascertain whether E3 ubiquitin ligase activity of E6-AP was dispensable in mediating TH1 ubiquitination, we detected the

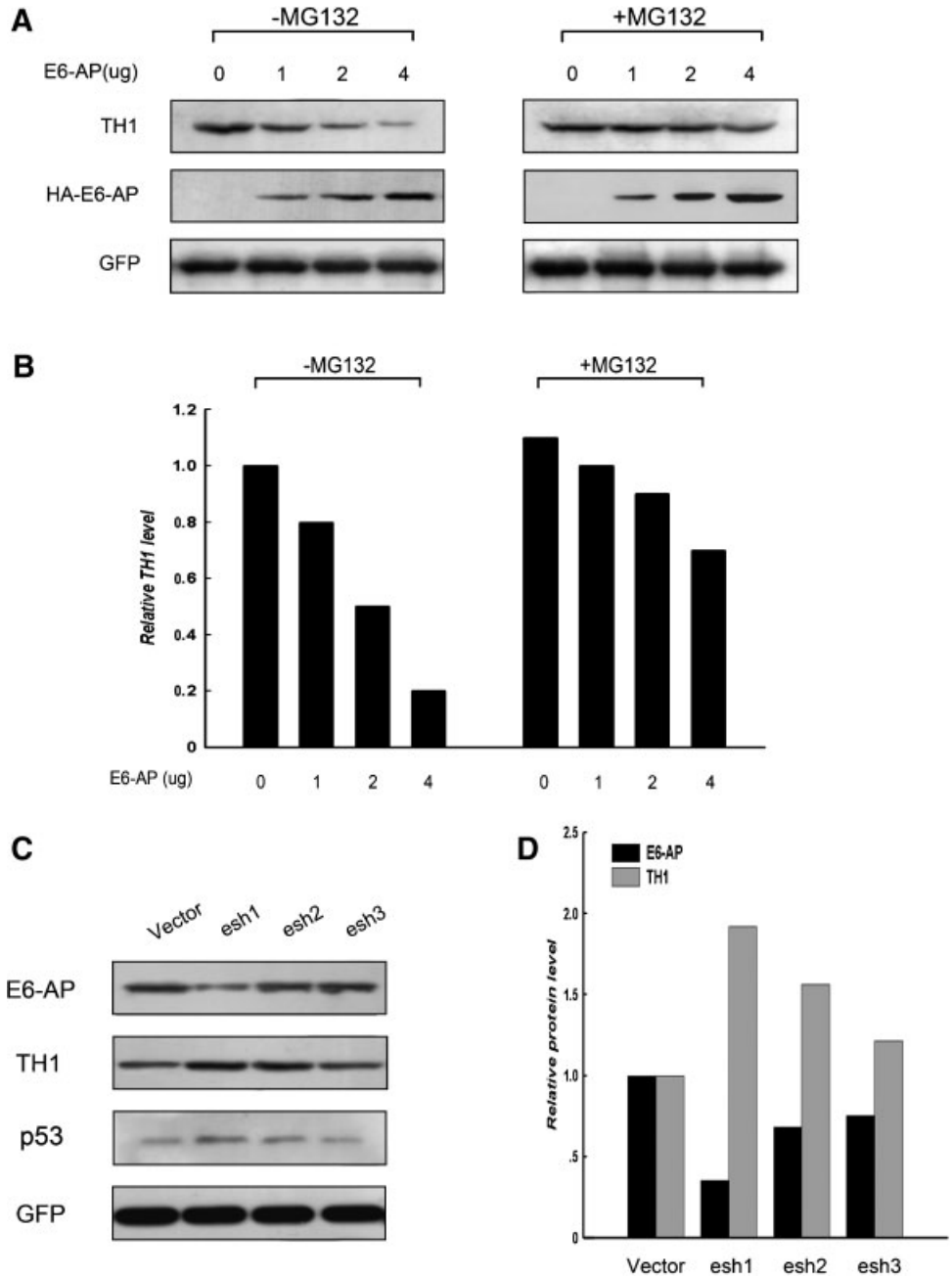

Fig. 3. E6-AP mediates TH1 degradation. **A**: Overexpression of E6-AP results in degradation of TH1 in a dose-dependent manner. HeLa cells were transfected with the plasmids to overexpress HA-tagged E6-AP protein in the presence (**right**) or absence (**left**) of 50 μM MG132. An immunoblot was performed with the TH1 antiserum. The **middle section** indicates the dose-dependent increased expression of E6-AP, and the **bottom section** shows even loading of the samples. **B**: Graphical presentation shows quantitative densities of the relative TH1 abundance in the transfection of different doses of HA-E6-AP after normalization with co-expressed GFP. **C**: Knock-down of E6-AP elevates TH1 protein levels. HeLa cells were transfected with shRNA targeting three different sequences of E6-AP (esh1, esh2, esh3). Levels of E6-AP, TH1, and p53 were analyzed after separation of 30 μg of protein extracts on a SDS–PAGE and immunoblotting using specific antibodies. **D**: Graphical presentation shows quantitative densities of the relative TH1 abundance after normalization with co-expressed GFP.

effect of enzyme function mutant E6-AP on TH1 ubiquitination in vivo. Wild-type and enzyme function mutant E6-AP were introduced into cells and their effects on TH1 ubiquitination and protein levels were detected. In Figure 5B, transient expression of wild type E6-AP resulted in an enhancement of TH1-ubiquitinated forms. In contrast, expression of the E6-AP

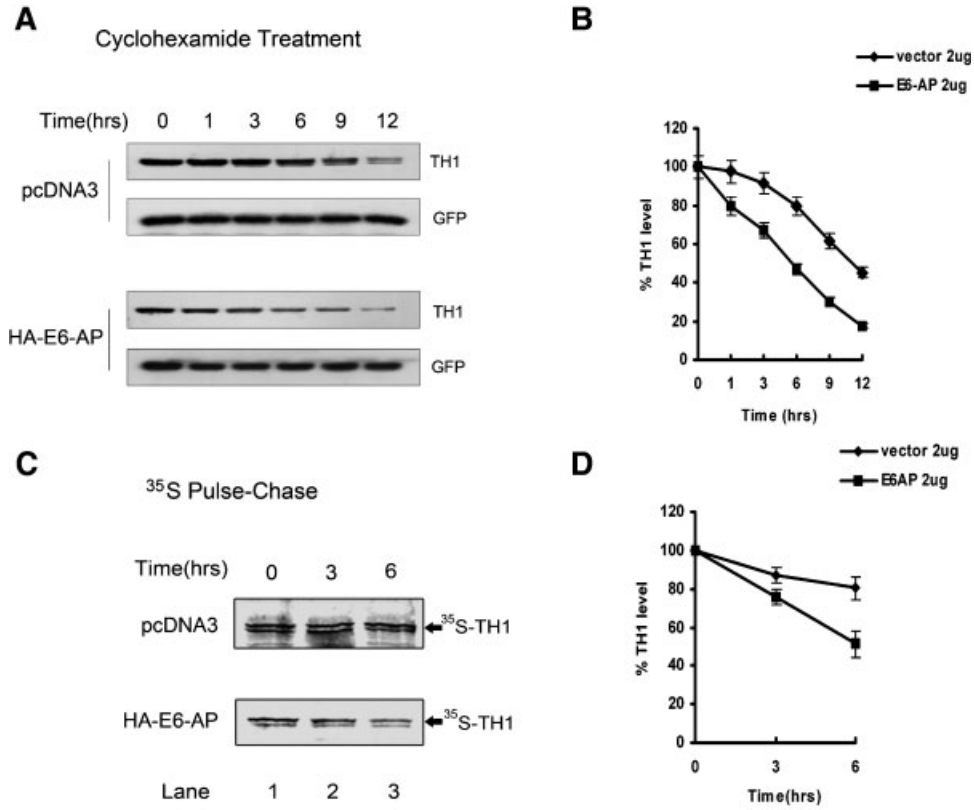

Fig. 4. Overexpression of E6-AP shortens TH1 protein half-life. **A**: TH1 turnover is reduced in E6-AP overexpression HeLa cells versus parental HeLa cells. HeLa cells were transfected with 2 μg of either mock vector or pcDNA-E6-AP. After 48 h, cells were treated with 50 μg/ml CHX for the times indicated, and 20 μg of the lysates was run on a 10% polyacrylamide gel and blot-probed with TH1 antiserum. **B**: TH1 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. **C**: Overexpression of E6-AP decreases the metabolic stability of TH1. HeLa cells transfected with either mock vector or HA-E6-AP constructs were subject to a pulse-chase metabolic labeling with [^{35}S]-methionine plus [^{35}S]-cysteine for 1 h and chased for the indicated time periods. Equal amounts of radiolabeled lysates were immunoprecipitated with TH1 antiserum and resolved by SDS–PAGE, followed by autofluorography. A representative experiment is shown. **D**: Quantitative presentation of the pulse-chase experiments. Three independent experiments were performed. The [^{35}S]-labeled TH1 signals were quantified and valued relative to that at time zero. The data are represented as the average and standard errors.

catalytically inactive mutant E6-AP (C833A), which retains efficient interaction with TH1 (Fig. 1C), behaved in a dominant negative fashion resulting in a decrease in the overall ubiquitination of TH1 and an elevation of TH1 protein level. Taken together, the enhancement of TH1 ubiquitination by wild type E6-AP and the negative effects of the catalytically inactive mutant substantiated that TH1 was a substrate for E6-AP-mediated ubiquitination in vivo.

E6-AP-Mediated Ubiquitination and Degradation of TH1 In Vitro

We have obtained evidence that E6-AP-mediated ubiquitination might be involved in the degradation of TH1 in vivo. We therefore examined the ability of E6-AP to ubiquitinate TH1 in vitro. Protein extractions were performed in the absence of both detergent and reducing agent to preserve ubiquitination activity in the extracts. Incubation of [^{35}S]methionine-labeled TH1 synthesized in WGE in the presence of HeLa cell extracts resulted in the rapid formation of high molecular weight species of [^{35}S]-TH1 (Fig. 6B). We could also detect the degradation of in vitro translated [^{35}S]-TH1 as the time indicated. Furthermore, this degradation could be inhibited by proteasome inhibitor MG132 (Fig. 6A). These results indicate that TH1 is ubiquitinated in the presence of cell extract in vitro (Fig. 6A,B).

To further confirm the involvement of E6-AP in the ubiquitination and degradation of TH1 in vitro, we performed the in vitro recombinant

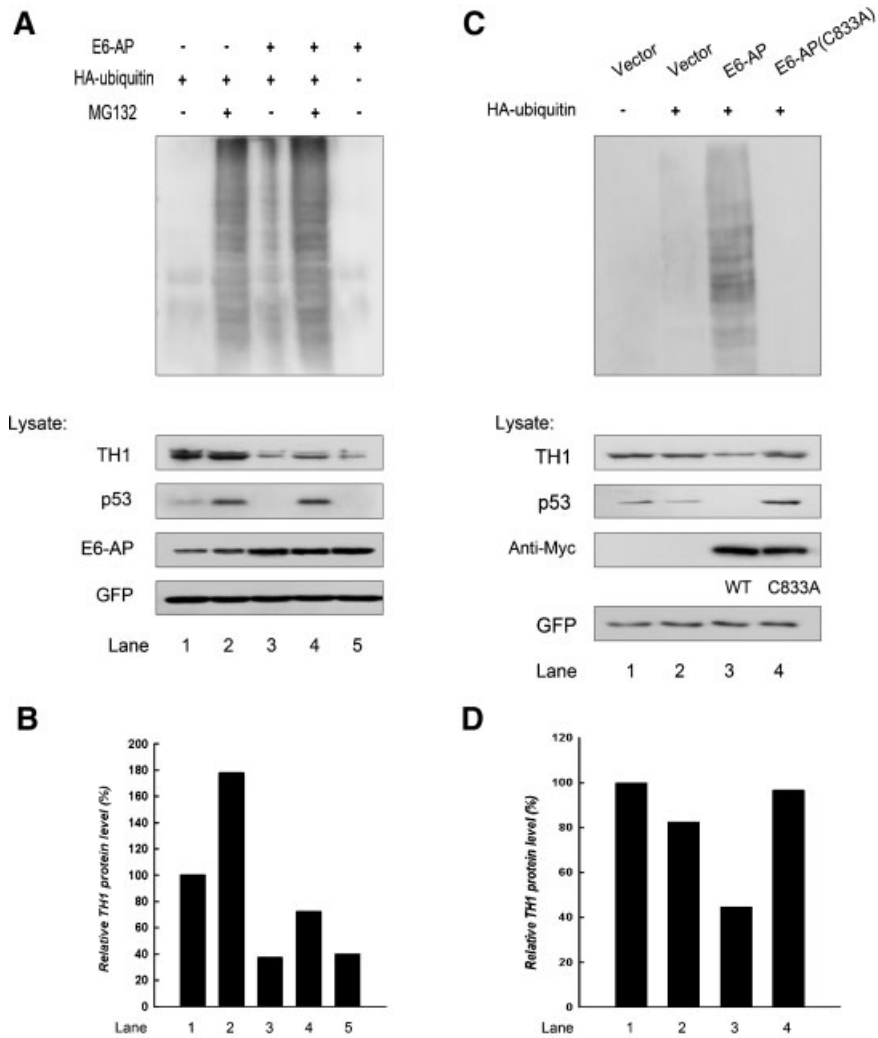

Fig. 5. Ubiquitinylation of TH1 by E6-AP in vivo. **A**: Overexpression of E6-AP induces ubiquitination of TH1 in vivo. HeLa cells were transfected with the plasmids to overexpress HA-ubiquitin and E6-AP proteins as indicated in the presence (+) or absence (−) of 50 μM of MG132 for 5 h before harvest. The cell lysates were precipitated with TH1 antiserum. The complex was separated and blotted with anti-HA antibody. The smear indicates the ubiquitinated TH1 (**upper section**). The expression of E6-AP, p53, and TH1 are demonstrated as indicated in the **middle section**. **B**: Quantitative presentation of the remaining TH1 proteins is shown in the **lower section**. The TH1 signals were quantified and valued relative to that in HA-ubiquitin solo transfected cells without MG132. **C**: E3 ubiquitin ligase activity of E6-AP is dispensable in mediating TH1 ubiquitination. HeLa cells were transfected with the plasmids to overexpress HA-ubiquitin, Myc-E6-AP, and Myc-E6-AP (C833A) proteins as indicated. The ubiquitination experiment was carried out as described for panel A (upper section). The middle sections indicate the expression of TH1, p53, Myc-E6-AP, and Myc-E6-AP (C833A). **D**: The TH1 signals were quantified and valued to that in mock-transfected cells.

ubiquitination assay using E1, E2 (UbcH7), and ubiquitin, either in the presence of immunoprecipited E6-AP or control anti-rabbit normal IgG. As shown in Figure 5C, incubation of TH1 with E1, E2, and ubiquitin did not result in any ubiquitination. Upon addition of immunoprecipited E6-AP, either from HepG2 cells (lane 7) or from HeLa cells (lanes 5 and 6) with or without overexpressed E6, TH1 was significantly ubiquitinated as seen by the appearance of higher molecular weight bands. It is inferred from these data that E6-AP induced the ubiquitination and consequent degradation of TH1 proteins.

DISSCUSSION

This study identified an intriguing E3 ubiquitin-protein ligase, E6-AP, which specifically interacted with TH1, an integral subunit of

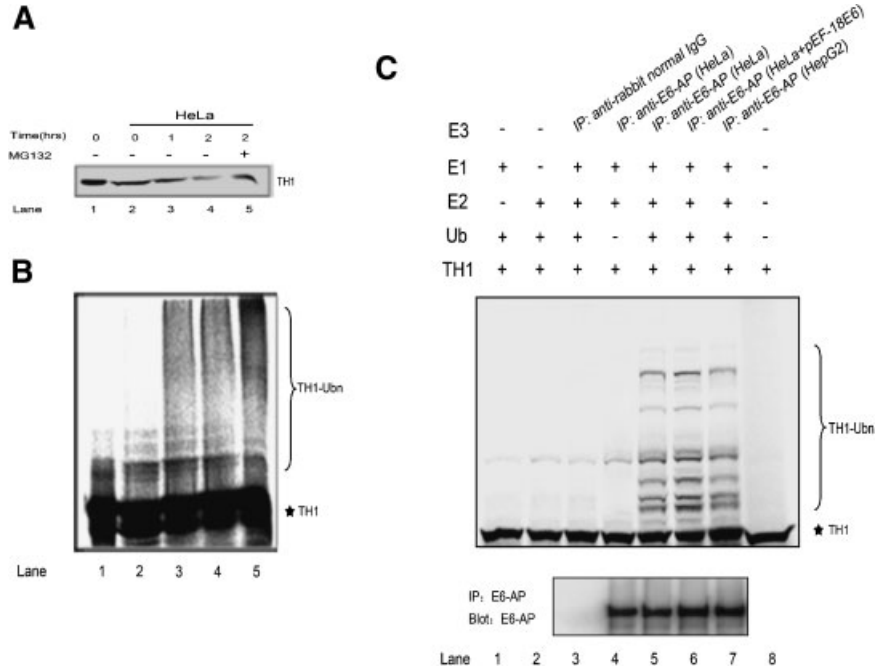

Fig. 6. In vitro ubiquitination of TH1. **A**: In vitro translated ^{35}S-TH1 (20 μl) was incubated in the presence of 200 μg (100 μl) of total protein extract from HeLa cells (**lanes 2–5**) for the indicated periods of time at 30°C. Ubiquitination mix (30 μl) was retrieved and the reaction was stopped by adding 3 × SDS sample buffer. Following electrophoresis, [^{35}S]-TH1 was visualized by autoradiography. MG132 was added to the ubiquitination mix to a final concentration of 100 μM. In vitro translated [^{35}S]-TH1 (5 μl) was loaded as nonreactivation control. **B**: Longer exposures of results shown in (A). **C**: E1 (50 ng), E2 (UbcH7) (100 ng), ubiquitin (4 μg), ATP (4 mM), in vitro translated TH1 using wheat germ extracts and immunoprecipitated E6-AP either from HeLa or HepG2 cells on Sepharose beads were incubated in reaction buffer at 30°C for 90 min. TH1 ubiquitination was analyzed as above. The immunoprecipitation efficiency of E6-AP was monitored in a parallel experiment.

the NELF complex and promoted its ubiquitination both in vitro and in vivo. In a series of recent studies, the protein modifying peptide ubiquitin has been found to mediate an additional layer of RNAPII transcription regulation and to do so by a variety of previously unknown and unanticipated mechanisms. The well-studied function of ubiquitination is its role in regulating the degradation of multiple components of the transcriptional machinery [Conaway et al., 2002]. In a departure from the classic paradigm of ubiquitin-dependent destruction of target proteins, several RNAPII transcription factors have now been shown to be proteolysically processed from inactive precursors to their active forms by ubiquitin-dependent action of the proteasome. The nuclear factors kappa B1 (NF-κB1) p105 and p100 precursors are processed into the active NF-κB p52 and p50 subunits by ubiquitylation and cleavage by the proteasome [Lin and Ghosh, 1996; Lin et al., 1998; Orian et al., 1999, 2000]. In addition to NF-κB, two *Saccharomyces cerevisiae* transcription factors, SPT23 and MGA2, which play key roles in the activation of expression of genes required for synthesis of polyunsaturated fatty acids, have recently been shown to be regulated by ubiquitin-dependent processing by the proteasome [Hoppe et al., 2000]. The prototypical HECT domain family member is the E6-AP, a 100-kDa protein required for ubiquitination and rapid degradation of P53 in the presence of the human papilloma virus oncoprotein [Scheffner et al., 1993; Huang et al., 1999; Be et al., 2001; Cui et al., 2003]. E6-AP is also a steroid hormone receptor coactivator, and ablation of the E6-AP gene in mice is associated with hormone resistance [Nawaz et al., 1999; Smith et al., 2002]. The HECT domain E3 ubiquitin ligase Rsp5, which is also a steroid hormone receptor coactivator, has been shown to ubiquitinate and target RNAPII to the proteasome during DNA damage [Imhof and McDonnell, 1996; Beaudenon et al., 1999].

Transcription elongation by RNAPII is controlled by a number of *trans*-acting factors as well as by *cis*-acting elements [Uptain et al.,

1997; Conaway et al., 2000]. The levels and activities of transcriptional activators and repressors are tightly controlled. The recent discovery of a new class of positive and negative elongation factors, including DRB sensitivity-inducing factor (DSIF), negative elongation factor (NELF), and positive transcription elongation factor b (P-TEFb), have shed new light on the control of RNAPII elongation [Peng et al., 1998; Wada et al., 1998; Yamaguchi et al., 1999, 2001]. Biochemical data indicate that NELF and DSIF might provide a checkpoint during early elongation that ensures proper capping of nascent transcripts [Pei et al., 2003; Mandal et al., 2004]. A subsequent report has characterized the entire NELF complex from Drosophila, and this provides a foundation for further study of this protein complex in Drosophila [Wu et al., 2005]. NELF-A and NELF-E bind to RNAPII and RNA, respectively. NELF-B is found to directly interact with estrogen receptor α (ERα) and is recruited with the rest of the NELF-complex to a number of endogenous ERα-responsive promoters in an estrogen-stimulated fashion. The promoter-bound NELF complex acts to stall RNAPII movement and attenuate ERα-dependent transcription [Aiyar et al., 2004]. As an integral subunit of NELF complex, which act together with DSIF to inhibit transcription elongation in vitro [Wada et al., 2000; Ping and Rana, 2001; Kim et al., 2003] and implicate in causing promoter proximal pausing on the *hsp 70* gene in *Drosophila* [Wu et al., 2003], TH1 is much more like a scaffold which links NELF-A, NELF-B, and NELF-E together to assemble a functional complex.

We obtained substantive evidence that E6-AP functions as an E3 ubiquitin-protein ligase of TH1. The evidence is noted as follows. (1) E6-AP and TH1 form a stable complex both in vitro and in vivo. (2) E6-AP directly catalyzes TH1 ubiquitination. (3) The enzymatic reaction is dependent on ubiquitin thioester bond formation at the active-site cysteine. Therefore, TH1 might be a physiological target for the E6-AP E3 ligase. Identification of E6-AP as an E3 Ub-protein ligase of TH1 provides an important clue to the further understanding of the link between the ubiquitin–proteasome pathway and transcription elongation regulated by RNAPII, since the ubiquitin–proteasome pathway tightly regulates a large number of key transcriptional regulators including p53, c-Fos, c-Jun, and more recently nuclear hormone receptors and their cofactors [Salvat et al., 1999; Nawaz and O'Malley, 2004]. Although previous studies suggested that the coactivator function of E6-AP was independent of its ubiquitin liagase activity, whether the ubiquitin liagase function contributes to the coactivator activity of E6-AP is still not clear.

In summary, we have confirmed in our study that TH1 served as a new HPV-18 E6 independent substrate of ubiquitin ligase E6-AP, and these might provide a novel clue to the relationship between proteolysis and transcription regulation.

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