We have recently identified the hADA3 protein, the human homologue of yeast transcriptional coactivator yADA3, as a novel HPV16 E6 target. Using ectopic expression approaches, we further demonstrated that hADA3 directly binds to the 9-cis retinoic acid receptors α and β, and functions as a coactivator for retinoid receptor-mediated transcriptional activation. Here, we examined the role of endogenous hADA3 as a coactivator for estrogen receptor (ER), an important member of the nuclear hormone receptor superfamily. We show that ADA3 directly interacts with ERα and ERβ. Using the chromatin immunoprecipitation assay, we also show that hADA3 is a component of the activator complexes bound to the native ER response element within the promoter of the estrogen-responsive gene pS2. Furthermore, using an ER response element-luciferase reporter, we show that overexpression of ADA3 enhances the ERα- and ERβ-mediated sequence-specific transactivation. Reverse transcription-PCR analysis showed an ADA3-mediated increase in estrogen-induced expression of the endogenous pS2 gene. More importantly, using RNA interference against hADA3, we demonstrate that inhibition of endogenous hADA3 inhibited ER-mediated transcription and the estrogen-induced increase in the expression of pS2, cathepsin D, and progesterone receptor, three widely known ER-responsive genes. The HPV E6 protein, by targeting hADA3 for degradation, inhibited the ERα-mediated transactivation and the protein expression of ER target genes. Thus, our results demonstrate that ADA3 directly binds to human estrogen receptor and enhances the transcription of ER-responsive genes, suggesting a broader role of mammalian hADA3 as a coactivator of nuclear hormone receptors and the potential role of these pathways in HPV oncogenesis.

The nuclear hormone receptors (NRs) are a family of structurally related, ligand-induced transcriptional activators that function as critical regulators of diverse physiological processes, including growth, differentiation, development, and reproduction (1, 2). Various NRs control the transcription of distinct subsets of genes in the chromatin environment of the nucleus (3, 4). In general, NRs share a conserved structural and functional organization, with DNA-binding, ligand-binding, and transcriptional activation domains (3, 4). Depending on their DNA binding and dimerization properties, NRs are subdivided into two classes: class I NRs function as homodimers and include the steroid hormone receptors, such as the estrogen (ER), androgen, and progesterone (PR) receptors, and class II NRs function as heterodimers with the 9-cis retinoic acid receptor (RXR) and include the receptors for retinoic acid (RAR), vitamin D, thyroid hormone, and peroxisome proliferators (1, 2). Binding of cognate ligands induces a conformational change in the ligand-binding domain of NRs, which influences their function with respect to subcellular localization, dimerization, coactivator binding, and transcriptional activity (5).

Recent studies have shown that a wide range of NR cofactors perform distinct functions at target promoters, including chromatin modification and remodeling and recruitment of the RNA polymerase II holoenzyme (6). A number of cofactors have been shown to bind the ligand-binding domains directly via short amphipathic α-helices containing the LXXLL sequence (7, 8). Structural studies have demonstrated that a hydrophobic channel (AF2) is exposed on the surface of the ligand-binding domain because of ligand binding, promoting dimerization or interaction with coactivators (4–9). Two groups of NR coactivators have been widely studied: 1) the p300 and the highly related cAMP response element-binding protein-binding protein (CBP); and 2) the mammalian mediator complexes (TRAP, DRIP, ARC, CRSP, SMCC, etc.) (6, 10–12). The p300 and CBP proteins are recruited to ligand-activated, DNA-bound NRs by the steroid receptor coactivators (SRC-1, -2, and -3) family of bridging factors (6, 11). The steroid receptor coactivator proteins have receptor interaction domains containing LXXLL motifs that contact the ligand-binding domains (13). The p300 and CBP proteins are histone acetyltransferases.
MCF-7 and T-47D, breast cancer cell lines, were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. These cell lines were obtained from the American Type Culture Collection.

Genetic studies in yeast have demonstrated yet another co-activator complex, the ADA (alteration/deficiency in activation) complex. The core components of this complex include the adapter proteins ADA3 and ADA2, and a histone acetyltransferase GCN5 (general control non-repressed 5) (16). It is noteworthy that hADA3 exists as a component of a yeast ADA-like complex that includes hADA2 and hGCN5, indicating that the functional roles of the ADA complex are evolutionarily conserved (17). Studies of mammalian RXR and glucocorticoid receptor expressed in the yeast have shown a requirement for the components of yeast ADA complex, including the γADA3 gene product, for transcriptional activation (17, 18). However, a functional role of hADA3 in NR transactivation in mammalian cells was not known until our recent work (19).

Using the yeast two-hybrid interaction system, we recently identified the hADA3 protein as a novel human papilloma virus type 16 (HPV16) E6-binding protein (20). We have demonstrated that the hADA3 protein binds to the high-risk (cancer-associated) but not to the low-risk HPV E6 proteins and to the immortalization-competent but not the immortalization-defective HPV16 E6 mutants, implying a role for E6-hADA3 interaction in oncogenic transformation. In view of the strong yeast genetic data for a potential role of hADA3 as a NR coactivator, isolation of hADA3 as an E6-binding protein raised the possibility that E6 may inactivate the mammalian nuclear hormone receptor function by targeting hADA3. Indeed, by using ectopic gene expression approaches, we demonstrated that hADA3 associates with and functions as a coactivator for human RXRa-mediated transactivation and that E6 inhibited this coactivator function by targeting hADA3 for degradation (19). We were surprised to find that a recent study reported that mouse ADA3, which shows >90% amino acid sequence similarity with hADA3, did not interact with human ER (21), suggesting the possibility that hADA3 may selectively regulate some NRs. Given the importance of this question, we have systematically examined the interactions of human as well as mouse ADA3 proteins with ER. Contrary to findings reported by Benecke et al. (21), we demonstrate that both human and mouse ADA3 directly interact with ERα and enhance the ERα-mediated transactivation of ER-responsive proteins. It is noteworthy that RNAi-mediated reduction in the level of the endogenous hADA3 protein led to an inhibition of the expression of ER-responsive genes. Thus, hADA3 protein functions as a critical component of a mammalian coactivator for the estrogen receptor, supporting the likelihood that ADA complex plays a wider role as a coactivator for NRs.

**EXPERIMENTAL PROCEDURES**

**Plasmid Constructs—**Construction of the vectors encoding hADA3 (pCR3.1-ADA3), GST-hADA3, histidine-tagged hADA3 and FLAG-tagged E6 (pCr3.1-16 E6) has been described previously (20). A mouse ADA3 cDNA expression construct (in pcMV-SPORT6 vector) was obtained from the American Type Culture Collection (expressed sequence tag clone no. 5349223). An EcoRI-NheI PCR product of mADA3 (sense primer, TGGAAATCTTGAGCTGAGACTGTC; antisense primer, ATGGCGCGCTACCTCGAGTACGCTAC) was subcloned into pGEX-4T1 for GST-mADA3 expression and in pEF-6B (Invitrogen) to express histidine-tagged mADA3. A FLAG-ERα expression construct in V19 T3 was obtained from Dr. Richard Karas (New England Medical Center, Boston, MA). The Vit-ERE-Luc reporter was generated by cloning three copies of an ERE, corresponding to that present in the ER-responsive vitellogenin gene, in pLUC vector (Strategene). The pEF-16E6-MYC construct, encoding the Myc-tagged E6, was kindly provided by Dr. M. Ishibashi (Aichi Cancer Center, Japan).

**Cells and Media—**Saos-2, a p53-negative osteosarcoma cell line, and MCF-7 and T-47D, breast cancer cell lines, were grown in α-minimal essential medium (Invitrogen) supplemented with 10% fetal calf serum (Hyclone, Logan, UT). Human embryonic kidney cell line 293T was grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. These cell lines were obtained from the American Type Culture Collection.

**Antibodies—**Generation of hADA3 antibodies has been described previously (19, 20). Rabbit polyclonal antibody against progesterone receptor, c-Myc, p300, and goat polyclonal antibody against cathepsin D were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

**In Vitro Binding Assays—**The indicated [35S]-labeled proteins (ERα and hADA3 in Fig. 1, A, B, and D; and hADA3 or mADA3 in Fig. 1C, ERα and its mutants in Fig. 1E, and ERβ in Fig. 1F) were generated using a rabbit reticulocyte lysate-based coupled in vitro transcription-translation system (TnT Rabbit reticulocyte lysate system; Promega, Madison, WI) in the presence of [35S]cysteine. Aliquots of [35S]-labeled proteins were incubated with 1 µg of GST, GST-hADA3 (Fig. 1, A and E, or mutants in Fig. 1D), GST-mADA3 (Fig. 1A), or GST-ER (Fig. 1C), covalently bound to glutathione-Sepharose beads in 300 µl of lysis buffer (100 mM Tris, pH 8.0, 100 mM NaCl, and 0.5% Nonidet P40) for 1 h in the presence or absence of estradiol (1 µM) or in the presence of tamoxifen (1 µM) (Fig. 1A) at 4 °C. The beads were washed with lysis buffer, and bound proteins were resolved by SDS-PAGE and visualized by fluorography. The expression of GST fusion proteins was determined using the Coomassie blue staining.

**In Vitro Association of hADA3 or mADA3 and ERα—**5 × 10⁵ 293T cells were cultured in phenol red free α-minimal essential medium for 48 h. The cells were then co-transfected with 5 µg of FLAG-ERα and 8 µg of His-tagged hADA3 (Fig. 2A) or mADA3 (Fig. 2B) using the calcium phosphate co-precipitation method. After 12 h of transfection, the cells were treated with 100 nM of estradiol or solvent in fresh medium. Twenty-four hours later, the cell lysates were prepared in the binding buffer (phosphate, pH 8.0, 100 mM NaCl, and 0.5% Nonidet P40) and 800-µg aliquots of protein lysates were incubated with His-Bind resin (Novagen, Madison, WI) for 5 min at 4 °C. The beads were washed five times with the binding buffer, and the bound proteins were eluted by boiling in the sample buffer followed by SDS-PAGE and immunoblotting with anti-FLAG antibody to detect FLAG-ERα. The membranes were probed with anti-His antibody (Santa Cruz Biotechnology) to assess the expression of ADA3.

**Chromatin Immunoprecipitation (ChIP) Assay to Detect Native pS2 Promoter-bound ADA3 and ERα—**The ChIP assay was performed as described previously (22). In brief, 5 × 10⁶ MCF-7 cells per 100-mm diameter dish were treated with formaldehyde to cross-link the chromatin-associated proteins to DNA. The cells were trypsinized and re-suspended in lysis buffer, and nuclei were isolated and subjected to sonication to obtain chromatin-containing supernatants. Equal aliquots of chromatin supernatants were subjected to overnight IP with anti-FLAG antibody to detect FLAG-ERα. The membranes were probed with anti-His antibody (Santa Cruz Biotechnology) to assess the expression of ADA3.

**Transient Transfection and Luciferase Assay—**5 × 10⁵ Saos-2 per 100-mm (Fig. 4A) or 1 × 10⁵ MCF-7 cells (Fig. 4, B and C) per 35-mm diameter dish were cultured in phenol red-free α-minimal essential medium for 48 h. The cells were then transfected with vit-ERE luciferase reporter and various expression plasmids indicated in figure legends, using the Fugene reagent (Roche, IN). Each dish also received 20 µg of SV40-Benilla reniformis luciferase reporter (pRL-SV40) to normalize the differences in transfection. The total amount of transfected DNA was kept constant by adding the vector DNA. After 12 h of transfection, the cells were either mock-treated or treated with 10 pM (for MCF-7 cells) or 1 nM (for Saos-2 cells) of E2 for an additional 24 h. The cells were lysed, and the lysates were assayed for luciferase activity using a dual-luciferase reporter (Promega) system.

**RNA Extraction and RT-PCR for pS2 Expression—**MCF-7 cells stably expressing either vector or pCR3.1-hADA3 (5 × 10⁵ per 100-mm dish) were cultured in phenol red-free medium for 3 days followed by either mock-treatment or treatment with 0.01 or 1 nM E2 for an additional 24 h. The total RNA was then extracted using the TRizol reagent (Invitrogen). Equal aliquots of RNA were used for first-strand cDNA synthesis, and 2 µl of cDNA were used to amplify the pS2 sequence (forward primer, 5′-GCCTTTGGACAGAGAGGA-3′; reverse primer, 5′-TCCCTGGACAGTGTTCA-3′), followed by visualization using ethidium bromide-stained 1% agarose gels. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (forward primer, 5′-ACATGCCGT-CAGACACATGG-3′; reverse primer, 5′-GTAGTGAGTCAAT-GAAGG-3′) was used as an internal control for the PCR.
were maintained in estradiol deprived medium for 3 days followed by transfection of either control or hADA3 specific RNAi oligonucleotide (sense, 5'-GGUGACAGAGAUUCUGUTdTdT-3' and antisense 3'-dT-dTCCACUGUCUGCAAGGACU-5') using Lipofectamine 2000 (Invitrogen, CA) according to the manufacturer's protocol. Forty hours after transfection, cells were treated with 10, 50, or 100 pM concentration of 17β estradiol (E2) for 24 h, total RNA was extracted and RT-PCR was performed (as above) with 1 μg of total RNA using pS2 (primers as above), GAPDH (as above), Cathepsin-D (forward primer, 5'-TACATGATCCCTGCTGAGAAGGT-3', reverse primer, 5'-GGGACAGCTTGTA-
**RESULTS**

Both hADA3 and mADA3 Interact Directly with ERα and ERβ—Genetic analyses have shown a requirement for yeast ADA3 for transcriptional activation by human RXR and other NRs when these were expressed in yeast (17, 18). Furthermore, we have recently shown that hADA3 binds to and functions as a coactivator for RXRα (19). Surprisingly, the mouse homologue of ADA3, which has a 99% homology with hADA3, was reported not to bind to human ER (21), suggesting a selective coactivator function of hADA3. These findings prompted us to closely examine the interaction of mouse and human ADA3 with ER.

As a first step, we examined the binding of GST-hADA3 or GST-mADA3 to in vitro translated, 35S-labeled ERs, using procedures described previously (19–21). As expected, hADA2 (used as a positive control) showed clear binding to hADA3 (Fig. 1B), whereas GST alone showed no binding (Fig. 1, A, lane 2, and B, lane 2). It is noteworthy that both GST-hADA3 and GST-mADA3 bound to ERα, and this binding was observed in the presence as well the absence of the E2 ligand (lanes 3, 4, 6, and 7). In addition, incubation of the reaction mixture with tamoxfen, an antiestrogen, did not affect the hADA3 or mADA3 binding to ERα (lanes 5 and 8). These results clearly demonstrate that hADA3 and mADA3 can bind directly to ER in a ligand-independent manner, similar to our previous results with hADA3-RXRα binding (19). Because Benecke et al. (21) used a reverse binding assay, with in vitro-translated mADA3 and GST-ER, we also performed further binding assay using a similar strategy. Although the overall binding was lower in this assay (which may account for the inability of Benecke et al. to observe binding), both hADA3 and mADA3 could clearly bind to ERs in a ligand-independent manner (Fig. 1C, lanes 3, 4, 7, and 8). Further specificity of ER binding to hADA3 was demonstrated by its binding to the N-terminal region of hADA3 (1–215) but not to the C-terminal half (216–542) (Fig. 1D) and by domain analysis of ERα (AF2 and DBD but not AF1 bind to hADA3; Fig. 1E). It is noteworthy that, similar to binding to ERα, hADA3 also binds to ERβ (Fig. 1F). Taken together, these results demonstrate a clear binding of

---

**A.**

<table>
<thead>
<tr>
<th></th>
<th>-E2</th>
<th>+E2</th>
</tr>
</thead>
<tbody>
<tr>
<td>FLAG-ERα</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>His-hADA3</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>I.B.: anti-FLAG</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>I.B.: anti-His</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

**B.**

<table>
<thead>
<tr>
<th></th>
<th>-E2</th>
<th>+E2</th>
</tr>
</thead>
<tbody>
<tr>
<td>FLAG-ERα</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>His-mADA3</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>I.B.: anti-FLAG</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>I.B.: anti-His</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

**FIG. 2. In vivo interaction of hADA3 and mADA3 with human estrogen receptor (ERα).** A total of 5 × 10⁵ 293T cells were transfected with 8.0 μg of His-hADA3 (A) or mADA3 (B) or 5.0 μg of FLAG-ERα alone or in combination. After 12 h, cells were treated with 100 nA estradiol or same amount of ethanol vehicle. At 24 h after treatment, the cells were lysed in binding buffer, and 800-μg aliquots of lysates were allowed to bind with His-Bind resin. The bead-bound proteins were subjected to SDS-PAGE, followed by anti-FLAG immunoblotting to detect transfected FLAG-ERα (top, lanes 4–6 and 10–12). Lanes 1–3 and 7–9 represent direct immunoblotting of cell lysates from transfectants.
ER to mouse and human ADA3 and show that the ER-binding domain resides in the N-terminal portion of the protein.

**ER**

**ER**

**hADA3 and mADA3 transactivates ERE-luciferase activity.** A, 5 × 10^5 Saos2 cells per 100-mm dish were transfected with 850 ng of vit-ERE-luciferase reporter and 20 ng of Eα plasmids; B, 1 × 10^5 MCF-7 cells per 35-mm dish were transfected with 100 ng of vit-ERE-luc plasmid, together with increasing amounts of hADA3 using the FuGENE reagent. After 24 h, MCF-7 cells were either mock-treated or treated with 10 px of 17β-estradiol (E2) and Saos2 cells were either mock-treated or treated with 1 nx of 17β-estradiol for 24 h before lysis. Equal amounts of cell lysates were used to measure luciferase activity and the results were calculated as -fold activation over vector. 20 ng of SV40-*R. reniformis* luciferase reporter (pRL-SV40) was concurrently transfected and used to normalize differences in transfection. Expression levels of hADA3 and ERα were shown by Western blotting for lysates with E2 treatment. C, 1 × 10^5 Saos2 cells were plated in 6-well plates, transfected with either 10 ng each of Eα or 100 ng of ERβ with SV40-R. reniformis luciferase reporter (pRL-SV40), and 100 ng of vit-ERE-luc plasmid together with increasing amounts of hADA3 as above. Cells were either mock-treated or treated with 1 nx 17β-estradiol (E2), and luciferase activity was measured as above. Data indicates mean ± S.D. of triplicates from a representative experiment of three independent experiments.

**ER**

**ER**
For this purpose, we transfected His-tagged hADA3 (Fig. 2A) or mADA3 (Fig. 2B) together with FLAG-tagged ERα in 293T cells, affinity purified hADA3 on His-Bind resin, and assessed the presence of any associated FLAG-ERα using anti-FLAG immunoblotting. A clear association of hADA3 as well as mADA3 with ERα was seen in the absence as well as the presence of E2 (Fig. 2, A and B, top, lanes 6 and 12). Anti-His immunoblotting showed His-hADA3 expression in the appropriate lanes (Fig. 2, A and B, bottom). Likewise, immunoblotting of the cell lysates showed the expected expression of the transfected proteins. Taken together, our results demonstrate a ligand-independent association of hADA3 or mADA3 with ERα in a human cell system.

hADA3 Is Present in Activator Complexes Bound to the ERE of Native pS2 Promoter—A crucial aspect of the coactivator function involves their ability to assemble into complexes with transcriptional activators bound to specific promoters in the context of the native chromatin (27, 28). To directly assess whether hADA3 is assembled into transcriptional activator complexes bound to a native estrogen response element in its native chromatin configuration upon ligand stimulation. As expected, PCR products were amplified in anti-ERα as well as anti-p300 ChIPs, primarily in chromatin preparations of E2-treated cells (lanes 4 and 10). These results are consistent with a ligand-dependent binding of ER to the pS2 promoter, as previously demonstrated by gel-shift analyses of estrogen responsive promoters (29). Lack of amplification of promoter sequences of GAPDH, which is not an ER target, demonstrated the specificity of results. Overall, these results demonstrate that hADA3 becomes a part of the activator complexes bound to an estrogen response element in its native chromatin configuration upon ERs ligand stimulation.

hADA3 and mADA3 Enhance the ER-mediated Transactivation of Reporters Linked to an ERE—Given the ability of hADA3 and mADA3 to interact with ERα, and the previously defined role of ADA3 as a component of the ADA coactivator complex, we examined whether hADA3 and mADA3 function as coactivators of transcription mediated by ERα. For this purpose, we transiently transfected the ER-positive breast cancer cell line MCF-7 (Fig. 4B) and the ER-negative osteosarcoma cell line Saos2 (Fig. 4A) with hADA3 or mADA3 (together with ER in the case of Saos2 cells) and the Vit-ERE-luciferase reporter. A low level of Vit-ERE-luciferase activity was detected in mock-treated cells, whereas E2 treatment led to a 2- or 8-fold increase in the activity in MCF-7 and Saos2 cells, respectively, under the experimental conditions used. It is noteworthy that transfection of hADA3 or mADA3 (in the

![Fig. 5. HPV E6 abrogates hADA3 coactivator function for ER.](http://www.jbc.org/)
presence of cotransfected ERα in Saos-2 led to a substantial hADA3 or mADA3 dose-dependent increase in ligand-induced luciferase reporter activity in both cell lines. Western blots of cell lysates used in luciferase assays showed the expected expression of hADA3 and ER proteins (Fig. 4, A and B; only E2-treated lysates are shown). Comparison of ERα and ERβ for coactivator function of hADA3 in Saos-2 cells showed that both ERα and ERβ enhanced hADA3-mediated transactivation of ERE-luciferase activity; however, ERα was more efficient (Fig. 4C). These results demonstrate that hADA3 and mADA3 function as coactivators for ER-mediated transcriptional activation in mammalian cells.

**HPV16 E6 Inhibits the Coactivator Function of hADA3**—Given the ability of HPV16 E6 to interact with and induce the degradation of hADA3 (20), we examined the effect of co-expressing E6 on hADA3-induced increase in ERα-mediated transactivation of Vif-ERE-luciferase reporter. For this purpose, both MCF-7 and Saos-2 cells were transfected with hADA3 together with either wild-type E6 or a hADA3-non-binding E6 mutant Δ9–13 (20). Although hADA3 expectedly enhanced the E2-induced Vif-ERE-luciferase reporter activity in both cells (Fig. 5, A and B), neither E6 nor its mutant by themselves had any effect on the ERα-dependent reporter activity (Fig. 5, A, lanes 14 and 16, and B, lanes 8 and 9). It is significant that co-transfection of wild-type E6 dose-dependently inhibited the hADA3-induced increase in Vif-ERE-luciferase reporter activity (Fig. 5, A, lanes 6, 8, and 10, and B, lanes 4–6); in contrast, the hADA3 non-binding E6 mutant Δ9–13 had no effect (Figs. 4B, lane 7, and 5A, lane 12). As anticipated (20), the expression of wild-type E6 but not its ADA3-non-binding mutant was accompanied by a reduction in hADA3 levels (Fig. 5, A and B). Immunoblotting of cell lysates confirmed the expected expression of hADA3 and ER proteins (Fig. 5, A and B, lower, shown only for ER-treated cell lysates). These results clearly demonstrate that HPV16 E6 abrogates the coactivator function of hADA3 toward ERα in human cells.

**hADA3 Enhances the Expression of Endogenous ERα Target Gene pS2**—Given the above results (that hADA3 can function as a coactivator for ER-mediated transactivation), we wished to assess whether ADA3 enhances the expression of known ER target genes. For this purpose, we treated the E2-deprived MCF-7 cells stably expressing either the vector or hADA3 with 0.01 or 1 nM of E2 for 24 h. Total RNA was extracted and...
similar amount of RNA from each sample was used to synthesize the cDNA, followed by a RT-PCR reaction using the primers specific for pS2. As expected, an E2 dose-dependent increase in pS2 expression was seen in vector-transfected cells. It is noteworthy that the transfection of hADA3 further enhanced the ER-mediated increase in pS2 gene expression (Fig. 6A). As expected, no change in the level of the control GAPDH message was observed. Fig. 6B shows the quantification of the data presented in Fig. 6A using NIH Image software.

Knock-down of the Endogenous hADA3 Expression by RNAi

Knock-down of the Endogenous hADA3 in MCF-7 cells reduces the ER-target genes—To further confirm the role of hADA3 as a coactivator for ERα-mediated transactivation, we established MCF-7 cells stably expressing a hADA3 or a control (scrambled) small hairpin RNA (shRNA). Western blotting (Fig. 7A) shows a substantial, albeit partial, decrease in the expression levels of endogenous ADA3 protein. It is noteworthy that RNAi-dependent decrease in the levels of endogenous hADA3 resulted in a substantial decrease in the expression of estrogen target genes (Fig. 7B). These results show a role of endogenous hADA3 in E2-mediated transactivation.

Knock-down of the Endogenous hADA3 Expression by RNAi Oligotransfection Reduces the E2-dependent Up-regulation of Expression of the ER Target Genes—To further confirm the role of endogenous hADA3 in ER function, we examined the mRNA expression of ER target genes (pS2, cathepsin D, and PR) in MCF-7 cells upon knockdown of hADA3 using ADA3 RNAi transfection. For this purpose, we transfected ADA3 oligonucleotide RNAi in MCF-7 cells and analyzed the estrogen-dependent increase in mRNA expression of ER target genes using semiquantitative RT-PCR. A dramatic decrease in the mRNA expression of ER target genes was observed upon transfection of ADA3 RNAi (Fig. 8A–E).

Next, we examined the effect of ADA3 RNAi on the protein expression of ER targets, cathepsin D, and PR. A significant inhibition of E2-dependent increase in the levels of Cathepsin D protein expression was observed in cells expressing hADA3 RNAi compared with control RNAi-expressing MCF-7 cells (Fig. 9A). Furthermore, inhibition of E2-induced progesterone receptor was observed in ADA3 RNAi-expressing cells compared with control RNAi-expressing T47D cells (Fig. 9B). Taken together, these results clearly show a role of endogenous hADA3 in E2-mediated transactivation of several ER-target genes.

HPV16 E6 Inhibits the Estrogen-dependent Increase in the Expression of ER Target Genes—Given the ability of HPV16 E6 to interact with hADA3, and ADA3 to function as a coactivator for ERα-mediated transactivation, we examined the effect of E6 on the expression of estrogen target proteins. It is noteworthy that a significant decrease in estrogen-dependent increase in the levels of PR and c-Myc (another target of ER) proteins were observed in MCF-7 cells expressing E6 compared with vector-expressing MCF-7 cells (Fig. 10A). The effect of E6 on p53 is...
shown in Fig. 10B. These results demonstrate that E6 by targeting a coactivator of ER can perturb ER function, thus providing a link between HPVs and ER function.

**DISCUSSION**

NRs represent crucial ligand-inducible switches that control a variety of physiological processes (1, 2). In recent years, it has become clear that a complex interplay of corepressor and coactivator protein complexes plays an essential role in ensuring the optimal transcriptional activation function of NRs (7, 8). Furthermore, a number of studies have begun to emphasize the importance of selective recruitment of distinct classes of coactivators as a potential determinant of selective gene expression through different NRs (30, 31).

Previous genetic studies of mammalian NRs expressed in the yeast have indicated a coactivator role of the evolutionarily...
our recent results that hADA3 functions as a coactivator for retinoic acid receptors (30, 31). Thus, mammalian ADA3, as a component of ADA complexes, is likely to play an important role in gene expression (17, 18), a possibility directly supported by our recent demonstration of a coactivator role for hADA3, a component of the mammalian ADA complex, in RXR function (19). Given that these studies relied solely on hADA3 overexpression, and given the existence of a recent study reporting the inability of mouse ADA3 to interact with estrogen receptor (21), we undertook a systematic examination of the mammalian ADA3 interaction with ER and the functional role of this interaction in the context of ectopic as well as endogenous ADA3. The findings of this report establish that both the murine and human ADA3 proteins interact with estrogen receptor and that endogenous hADA3 plays an important role in estrogen-induced target gene expression.

The results presented here have significant implications for the potential role of ADA3-containing coactivator complexes in physiological pathways and in oncogenesis. Although yeast ADA3 as a component of ADA and other coactivator complexes has been clearly demonstrated to be important, little was known about the mammalian ADA3 function until recently. Therefore, our present findings, together with our recent results that hADA3 functions as a coactivator for retinoic acid receptors in mammalian cells, strongly support the likelihood suggested by the yeast studies that ADA3 containing complexes may participate as coactivators for multiple nuclear hormone receptors. Thus, mammalian ADA3, as a component of ADA complexes, is likely to play an important role in gene expression relevant to a number of important physiological processes regulated by transcriptional activators of the NR superfamily.

Our results demonstrating the interaction of human as well as the murine ADA3 with ER are in sharp contrast to a recent study by Benecke et al. (21) in which the investigators failed to detect a direct interaction of murine ADA3 to ER. The inability of these investigators to detect the ER-mADA3 interaction is probably the result of technical problems (21). When we measured the ER-ADA3 binding using the method that these investigators used, a considerably lower level of association was noted (Fig. 1C); however, a more robust binding was observed using the reverse analysis with GST-ADA3 and in vitro-translated ER proteins (Fig. 1, A and E). Furthermore, the reported study used considerably less in vitro-translated mADA3 product for binding assays compared with yADA3, which may have further reduced the detection of binding (21).

Whereas some coactivators, such as p300 and CBP, have intrinsic HAT activity and thus can directly participate in chromatin remodeling, other proteins can function as coactivators by recruiting HATs as part of a larger complex. Because ADA3 does not have a HAT domain, it is important to determine which HAT proteins mediate the ADA3 function as a coactivator. The presence of hGCN5 as well as a hGCN5-related gene product P/CAF in hADA3-containing complexes (32), direct interaction of P/CAF with p300/CBP (33), and the interaction of hADA3 itself with p300 (26) suggest that hADA3 may form multiple, distinct coactivator complexes, as also is the case in yeast (34). Thus, it is likely that additional transcriptional activators will emerge as targets of ADA3 coactivator function.

Our results suggest that ADA3 may play a role in oncogenesis. The direct interaction with HPV E6 oncoproteins, and the ability of E6 proteins to abrogate the hADA3 coactivator function for NR receptors support this suggestion. Furthermore, we and others have recently shown the hADA3 protein interacts with p53 and functions as a coactivator of p53-mediated transactivation (20, 26). The p53 coactivator function of hADA3 was also abrogated by HPV E6 (20). The role of presently known ADA3 targets (p53, retinoid receptors, and estrogen receptors) (19, 20, 26; current study) in cell growth and differentiation is well established, and these pathways are frequently affected during oncogenesis. Thus, if E6 indeed targets the various hADA3-containing complexes and influences the transcriptional pathways in which these complexes play a role, this could represent a significant mechanism for the role of E6 in HPV-mediated oncogenesis. The link between HPV and estrogen has long been suspected (35). Although, the mechanism is unknown, it has been shown that 16 α-hydroxylation of estrogens increases the transcription of high-risk papillomaviruses, which is in line with the epidemiologic data showing an increased cervical cancer risk among HPV-infected versus HPV-negative long-term contraceptive users (35–38). Although not functionally demonstrated, a estrogen responsive element has been reported to be present in the promoter region of HPV (39). It has also been reported that HPV E6 and E7 oncoproteins can directly interact with ER and may thus regulate its function (40). Our data that E6 can target a coactivator of ER provides a new molecular insight into the mechanism by which HPV can perturb ER function. It is noteworthy that E6 oncoprotein also associates with p300, which serves as a coactivator for a number of transactivators, including nuclear hormone receptors (41–43). Although the effect of HPV E6 binding to p300 on nuclear receptor-mediated transcription has not been examined, E6 is known to abrogate the coactivator function of p300 toward p53 (20). Considering the important function of ER in physiology and in tumorigenesis (44–46), it will be of significant interest to determine whether expression of hADA3 is mutated or altered in human carcinomas, including cervical cancer, and whether HPV oncogenesis alters ER-regulated gene transcription. Our findings support the emerging concept that viral oncoproteins, such as HPV E6, have attained the ability to perturb the function of multiple transcriptional coactivators apparently by multiple mechanisms. Further biochemical studies of the mammalian ADA complex components and their analyses in human cancers should help assess whether these proteins indeed play a role in cancer.

In conclusion, we have shown here that hADA3 and mADA3...
directly interact with ER, that ADA3 functions as a coactivator for ERα and ERβ-mediated transactivation and enhances the expression of ER-target genes, and that HPV E6 can abrogate ADA3 coactivator function toward ER, thereby inhibiting the expression of ER targets such as PR and c-Myc. Our results also raise the possibility that HPV E6 may target estrogen-dependent transcriptional pathways as a component of HPV-induced oncogenesis.

Acknowledgments—We thank Drs. Pierre Chambon and Richard Karas for PS2 and ER expression constructs and M. Ishibashi for Myc-target E6 construct.

REFERENCES

Human ADA3 Binds to Estrogen Receptor (ER) and Functions As a Coactivator for ER-mediated Transactivation
Gaoyuan Meng, Yongtong Zhao, Alo Nag, Musheng Zeng, Goberdhan Dimri, Qingshen Gao, David E. Wazer, Rakesh Kumar, Hamid Band and Vimla Band

doi: 10.1074/jbc.M404482200 originally published online October 20, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M404482200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 46 references, 20 of which can be accessed free at http://www.jbc.org/content/279/52/54230.full.html#ref-list-1