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ARTICLES

Tip60 Modulates PLAGL2-Mediated Transactivation by Acetylation

Jinying Ning, Gang Zheng, and Yu-Chung Yang*

Department of Pharmacology and Cancer Center, School of Medicine, Case Western Reserve University, Cleveland, Ohio 44106

Abstract Pleiomorphic adenoma gene (PLAG) family proteins are oncogenes involved in various malignancies including lipoblastomas, hepatoblastomas, and acute myeloid leukemia. Overexpression of PLAGL2 induces cell transformation and proliferation, but little is known about how its activities are regulated. We previously showed that transcriptional activity of PLAGL2 is negatively regulated by sumoylation. Here we report that Tip60 modulates PLAGL2 functions through acetylation. Tip60 associates with PLAGL2 through its zinc finger domain and acetylates PLAGL2. Wild-type but not the histone acetyltransferase (HAT)-minus mutant form of Tip60 enhances PLAGL2-mediated transactivation. In addition, coexpression of Tip60 and PLAGL2 completely abolishes the sumoylation of PLAGL2. Both Tip60 and DN-Ubc9 increase transactivation activity of wild-type but not the sumoylation deficient form of PLAGL2 (K250, 269, 356R), indicating that Tip60 acetylates PLAGL2 and abolishes the sumoylation of PLAGL2 possibly through modification of the same lysine residues (K250, 269, 356) within PLAGL2. Tip60 effects vary between different PLAGL2 target gene promoters, suggesting that Tip60 is a novel promoter-specific coactivator of PLAGL2. This is the first demonstration that Tip60 can function as a sumoylation inhibitor in part through its intrinsic acetyltransferase activity to regulate specific gene expression. J. Cell. Biochem. 103: 730–739, 2008.

Key words: PLAGL2; Tip60; acetylation; sumoylation; transcriptional control

Pleiomorphic adenoma gene–like (PLAGL)2 is a member of the PLAG gene family of transcription factors, which are characterized by the presence of seven C2H2 zinc fingers as DNA-binding domains at the N-terminus [Kas et al., 1998]. To date, three members (PLAG1, PLAGL1, and PLAGL2) of this superfamily in human and mouse have been identified with high sequence homology [Pendeville et al., 2006]. Dysregulated PLAG1 expression, which results from chromosomal translocation, is crucial for the formation of pleiomorphic adenomas of the salivary gland [Kas et al., 1997] and lipoblastomas [Astrom et al., 2000; Hibbard et al., 2000; Gisselsson et al., 2001]. Both PLAG1 and PLAGL2 play important roles in the pathogenesis of acute myeloid leukemia in cooperation with Cbfb-MYH11 [Castilla et al., 2004; Landrette et al., 2005]. Conversely, PLAGL1 appears to function as a tumor suppressor protein since it induces apoptosis and cell cycle arrest [Spengler et al., 1997] and is mutated in some breast and pituitary tumors [Bilanges et al., 1999; Pagotto et al., 2000]. PLAG1 and PLAGL2 have similar DNA-binding sequences and their binding affinities to the IGFII promoter are indistinguishable. Overexpression of both PLAG1 and PLAGL2 induces transformation in NIH3T3 cells [Voz et al., 2000; Hensen et al., 2002]. Despite these similarities, the basal-level transcriptional activity of PLAGL2 when fused to the Gal4 DNA-binding domain is much lower than that of PLAG1, suggesting these two proteins might be regulated differently. A recent study showed that PLAGL2, which is highly expressed in adult lung tissue, is a novel transcription factor of surfactant protein (SP)-C promoter, suggesting that PLAGL2 may play a role in lung tissue maturation [Yang et al., 2005]. However, little is known about the mechanisms

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*Correspondence to: Yu-Chung Yang, Department of Pharmacology, School of Medicine, Case Western Reserve University, 2109 Adelbert Road, W353, Cleveland, OH 44106-4965. E-mail: yxy36@cwru.edu

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in modulating the PLAGL2 transactivation functions.

Tip60 was originally identified as a cellular HIV-Tat interacting protein and has been shown to augment Tat-dependent transcription [Kamine et al., 1996]. Subsequently, Tip60 was shown to interact with c-Myc, NFkappaB, as well as androgen receptor (AR) [Baek et al., 2002; Gaughan et al., 2002; Patel et al., 2004; Kim et al., 2005]. As a histone acetyltransferase (HAT), apart from histones, cellular Tip60 can directly acetylate its interacting partners, such as AR, c-Myc, and upstream-binding transcription factor (UBF) [Sapountzi et al., 2006]. However, the functions of Tip60 vary in different gene contexts and cell types. For example, it can either be a coactivator to enhance AR transactivation to initiate transcription of KAI1, one of the NF-kappaB target genes, or be a corepressor to inhibit Stat3 transactivation [Li et al., 2001; Gaughan et al., 2002; Xiao et al., 2003; Kim et al., 2005]. Since p300, a well-established acetyltransferase, is involved in the regulation of PLAG proteins and associates with Tip60 endogenously [Col et al., 2005; Zheng and Yang, 2005], and both Tip60 and p300 are involved in c-Myc regulation [Patel et al., 2004; Faiola et al., 2005], our present study is designed to examine whether Tip60 is also a regulator of PLAGL2.

MATERIALS AND METHODS

Reagents and Antibodies

Monoclonal anti-Flag (M2) antibody was purchased from Sigma. Monoclonal anti-HA antibody was purchased from BAbco (Richmond, CA). Anti-c-Myc rabbit antibody (A-14) and anti-GFP (FL) antibody were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-acetylated-lysine was from Cell Signaling (Beverly, MA). HEK-293 cells were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum.

Plasmid Construction

Wild-type, HAT minus, and different truncation mutants of Flag-tagged Tip60 and Myc-tagged Tip60 were described previously [Xiao et al., 2003]. pcDNA3-HA-Tip60 construct was a gift of Dr. Didier Trouche (Institut d'Exploration Fonctionnelle des Genomes, Toulouse, France) [Legube et al., 2002]. pcDNA3-DNUbc9, Flag-tagged SUMO1, PM-PLAGL2, GFP-PLAGL2, and PM-PLAGL2(RRR) constructs were described previously [Zheng and Yang, 2005]. The IGF-II promoter plasmid [(-1229/+140) of IGF-II promoter 3 in pSLA3 luciferase vector], which contains at least one functional PLAG1-binding site (GGGGCCCCGGGGGGGG) [Voz et al., 2000], was a generous gift of Dr. Elly Holthuizen (Universiteit, Utrecht, The Netherlands). Human surfactant protein (SP)-C-luciferase reporter construct SP-C-luc was provided by Dr. Yih-Sheng Yang through cloning the SP-C promoter fragment from −320 to +1, which contains one PLAGL2-binding site (GGGGCTCTCACAGGGG), into the pGL3 luciferase vector (The University of Texas Southwestern Medical Center at Dallas) [Yang et al., 2005].

Transient Transfection, Immunoprecipitation, and Western Blot Analysis

HEK-293 cells were transfected by the calcium phosphate precipitation method with various plasmid combinations as indicated. Forty-eight hours later, cells were washed with phosphate-buffered saline (PBS), and 1 ml of ice-cold lysis buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 1 mM EGTA, 2 mM Na_3VO_4, 15 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride) was added. Cells were lysed for 30 min at 4°C with occasional vortexing. The lysates were collected into 1.5-ml tubes and cleared of nuclei by centrifugation for 10 min at 14,000 rpm. The supernatants (whole cell extracts) were incubated with different antibodies for 16 h at 4°C, and protein A-agarose beads were added in the last 2 h. The beads were washed five times in TNEN buffer (20 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40, 2 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride, 1 mM NaF). Bound proteins were extracted with SDS–PAGE sample buffer, and analyzed on SDS–PAGE followed by Western blot analysis with the ECL detection system. For sumoylation assays, 36 h after transfection, cells were lysed in a denaturing buffer (2% SDS, 10 mM Tris-HCl pH 8.0, 150 mM NaCl), and analyzed by SDS–PAGE and Western blotting.

Luciferase Assay

Cells were plated in 12-well plates and grown overnight before transfection. The total amount of DNA transfected was adjusted with pcDNA3. Luciferase assay was performed according to

the manufacturer's instructions (Promega). *Renilla* luciferase internal control plasmid was cotransfected with the plasmids as indicated. The relative luciferase activity was normalized based on *Renilla* luciferase activity. For Gal4 fusion-driven luciferase reporter assays, 0.1 μg/well of reporter (pG5-luc) was cotransfected with 0.1 μg of Gal4 fusion expression plasmid in 12-well plates. For IGF-II-luciferase and SP-C-luciferase reporter assays, 0.05 μg/well of reporter plasmid was cotransfected with effector plasmids of indicated amounts in 12-well plates.

RESULTS

Tip60 Interacts With PLAGL2 and Increases its Transactivation Through Acetylation

Our previous study showed that both sumoylation and acetylation are involved in the regulation of PLAG1 and PLAGL2 [Zheng and Yang, 2005]. To test the possibility that PLAGL2 is regulated by Tip60, which is a transcription cofactor with intrinsic acetyltransferase activity, the interaction between PLAGL2 and Tip60 was examined. Myc-Tip60 and Flag-PLAGL2 were ectopically expressed in HEK293 cells. Cell extracts were immunoprecipitated and immunoblotted with antibodies to the tags. In cells expressing both proteins, antibodies to Myc coprecipitated Flag-FLAGL2, and antibodies to Flag coprecipitated Myc-Tip60 (Fig. 1A). Coprecipitation did not occur in cells expressing Flag-PLAGL2 or Myc-Tip60 alone and thus was not the result of antibody crossreactivity. To substantiate the interaction data, the subcellular distribution of Tip60 and PLAGL2 in HeLa cells was examined by immunofluorescence microscopy. Both Tip60 and PLAGL2 are nuclear proteins, and they colocalize in the nucleus (data not shown). As shown in Figure 1B, when fused to the DNA-binding domain of Gal4, PLAGL2 had a low transcriptional activity on the Gal4 reporter gene, which is consistent with our previous report that PLAGL2 is a weak transactivator [Zheng and Yang, 2005]. However, cotransfection of increasing amounts of Tip60 plasmid progressively increased Gal4 activation by PLAGL2, thus suggesting that Tip60 is a PLAGL2 coactivator.

Since Tip60 is a well-established acetyltransferase involved in modulation of c-Myc and other nuclear receptors by acetylation [Gaughan et al., 2002; Patel et al., 2004], to explore the underlying mechanism by which Tip60 increases PLAGL2 transactivation, we examined whether PLAGL2 can be acetylated by Tip60. The proteins were expressed by cotransfection of HEK293 cells. After transfection, cells were lysed and PLAGL2 proteins were immunoprecipitated and subjected to Western blotting with antibodies that specifically recognize acetylated lysines. This analysis revealed that lysine residues in PLAGL2 were specifically acetylated in the presence of Tip60 (Fig. 1C). To further confirm the increase in transactivation of PLAGL2 is dependent on its acetylation, PLAGL2-Gal4 plasmid was cotransfected with either wild-type or HAT-mutant of Tip60 plasmid in HEK293 cells [Ikura et al., 2000]. Luciferase assay shows that although wild-type Tip60 significantly enhanced transactivation of PLAGL2, the HAT-mutant only had modest effect (Fig. 1D), suggesting that modulation of PLAGL2 by Tip60 depends on its HAT activity.

Tip60 Inhibits Sumoylation of PLAGL2 and Modulates its Transactivation

Since our previous study showed sumoylation of PLAGL2 significantly inhibits its transactivation and the three lysine residues (K250, K269, K356) play an important role in the inhibition of PLAGL2-mediated transactivation, we speculated that modulation of PLAGL2 by Tip60 may also be mediated by these lysine residues [Zheng and Yang, 2005]. To test our hypothesis, we first tested if overexpression of Tip60 inhibits sumoylation of PLAGL2. Tip60 and PLAGL2 plasmids were cotransfected in HEK293 cells with or without SUMO1 (*S*mall *U*biquitin-related *Mo*difier; also known as PIC1, UBL1, Sentrin, GMP1, and Smt3). DN-Ubc9, a dominant negative form of Ubc9 (SUMO E2 enzyme) plasmid, was also cotransfected with PLAGL2 as a positive control. The results showed sumoylated bands of PLAGL2 with or without SUMO1 expression, but overexpression of Tip60 or DN-Ubc9 completely abrogated the sumoylation of PLAGL2 (Fig. 2A). This indicates that Tip60 can inhibit PLAGL2 sumoylation. We next examined the effect of sumoylation on PLAGL2's transactivation activity. Gal4-luciferase reporter and Gal4-PLAGL2 plasmid were cotransfected with or without Tip60 or DN-Ubc9 plasmid in HEK293 cells and the luciferase assay was performed 24 h later. As expected, both Tip60 and DN-Ubc9 enhanced

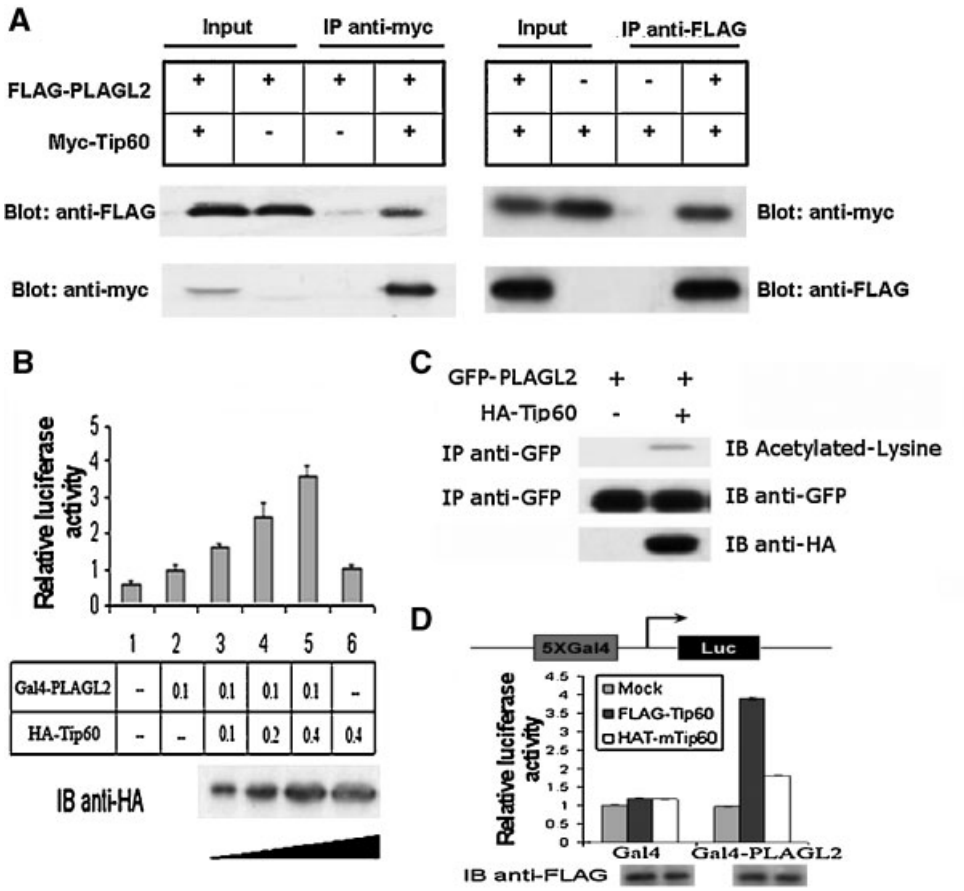

Fig. 1. Tip60 interacts with PLAGL2 and increases PLAGL2-mediated transactivation through acetylation. **A**: Tip60 interacts with PLAGL2. HEK293 cells were transfected with Flag-PLAGL2 (4μg) and Myc-Tip60 (4μg) plasmids. Cell extracts were immunoprecipitated with anti-Myc or anti-Flag antibody. Immunoprecipitates were immunoblotted with anti-Flag or anti-Myc antibody as indicated. **B**: Tip60 increases Gal4-PLAGL2 transactivity. HEK293 cells in 12-well plates were transfected with 0.1 μg of pG5-luc reporter, 0.1 μg of Gal4-PLAGL2, pRL-null (Renilla, 0.005 μg), and different amounts of pcDNA3-HA-Tip60 either alone or in combination as indicated. Activity of the firefly luciferase was normalized to that of the Renilla luciferase and expressed as relative luciferase unit. Total cell lysates were blotted with anti-HA antibody to detect the expression of Tip60 (**bottom panel**). **C**: PLAGL2 can be acetylated by Tip60. Lysates from HEK293 cells (in 60 mm plates) transfected with 2 μg of HA-tagged Tip60 and 2 μg of GFP-PLAGL2 plasmid either alone or in combination were immunoprecipitated with anti-GFP antibody. Immunoprecipitates or whole cell lysates were analyzed by immunoblot with indicated antibodies. **D**: Wild-type but not mutant form of Tip60 enhances transactivation of PLAGL2. HEK293 cells in 12-well plates were transfected with 0.1 μg of pG5-luc reporter, pRL-null (Renilla, 0.005 μg), 0.1 μg of PM-Gal4 (negative control), Gal4-PLAGL2, and Flag-tagged-Tip60 or Flag-tagged HAT-mTip60 plasmid as indicated. Luciferase activity was assayed and normalized as in Figure 1B. Total cell lysates were blotted with anti-Flag antibody to detect the expression of Tip60 (bottom panel). All the data in this figure are representatives of at least three independent experiments.

PLAGL2 transactivation (Fig. 2B), indicating that blocking sumoylation of PLAGL2 increases its transactivation activity. We previously showed that the mutant form of PLAGL2 (K250R, K269R, K356R) has higher transactivation activity than that of wild-type, because these mutations abolished the sumoylation of the three lysine residues that significantly inhibits transactivation function of PLAGL2. If Tip60 also functions through inhibition of sumoylation of the same lysine residues, we expected that the transactivation of mutant PLAGL2 (K250R, K269R, K356R) will no longer be affected by Tip60. We tested this hypothesis with Gal4-luciferase reporter assay, and the results showed that transactivation of the wild-type but not the triple-mutant form of PLAGL2 was significantly increased when cotransfected with Tip60 plasmid (Fig. 2C). To exclude the possibility that Tip60 non-specifically affects histone acetylation or protein sumoylation, we also examined the effect of Tip60 on the

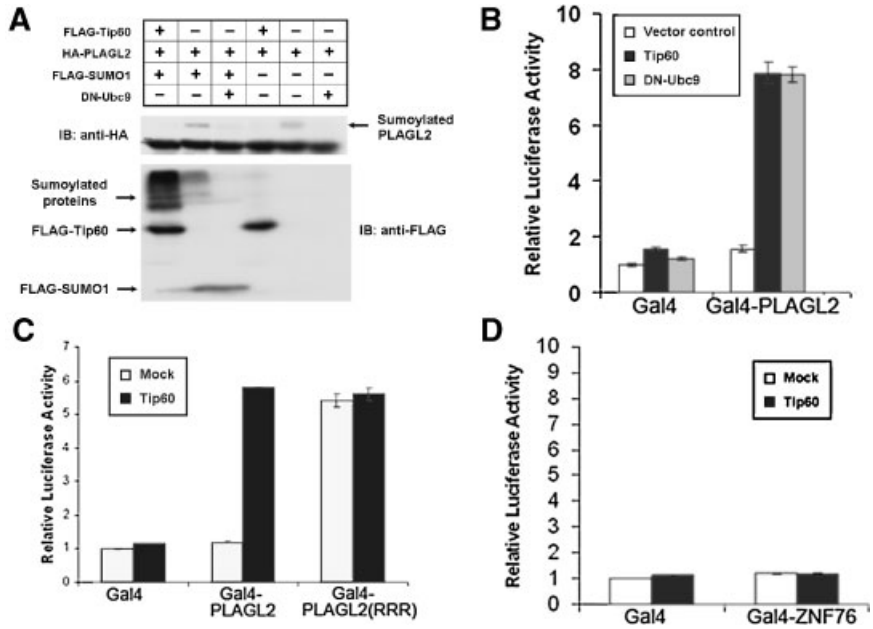

Fig. 2. Tip60 abolishes sumoylation of PLAGL2 and modulates its transactivation. **A**: Tip60 abolishes sumoylation of PLAGL2. HEK293 cells in 6-well plates were transfected with 0.5 μg of pcDNA-HA-PLAGL2 in the presence or absence of 0.3 μg of Flag-SUMO-1 plasmid, 0.5 μg of Flag-Tip60, and 0.5 μg of DNUbc9 plasmid as indicated. Thirty-six hours after transfection, cells were lysed with denaturing buffer and analyzed with indicated antibodies. **B**: Both Tip60 and DNUbc9 increase transactivation of PLAGL2. HEK293 cells in 12-well plates were transfected with 0.1 μg of pG5-luc reporter, pRL-null (Renilla, 0.005 μg), 0.1 μg of PM-Gal4 (negative control), and Gal4-PLAGL2 plasmids plus 0.3 μg of Flag-Tip60 or DNUbc9 plasmid as indicated. Luciferase activity was assayed and normalized as in Figure 1B. **C**: Tip60 increases transactivation of Gal4-PLAGL2 but not the sumoylation mutant. HEK293 cells in 12-well plates were transfected with 0.005 μg pRL-null (Renilla), 0.1 μg of pG5-luc reporter, PM-Gal4 (negative control), PM-PLAGL2, Gal4-PLAGL2 (RRR), and 0.3 μg of Flag-Tip60 as indicated. Luciferase activity was assayed and normalized as in Figure 1B. **D**: Tip60 has no effect on PM-Gal4-ZNF76. HEK293 cells in 12-well plates were transfected with 0.1 μg of pG5-luc reporter, 0.005 μg pRL-null (Renilla), 0.1 μg of PM-Gal4 (negative control), 0.1 μg of Gal4-ZNF76, and 0.3 μg of Flag-Tip60 as indicated. Luciferase activity was assayed and normalized as in Figure 1B. All the data in this figure are representatives of at least three independent experiments.

transactivation activity of ZNF76, a transcriptional factor which can also be acetylated and sumoylated in cells [Zheng and Yang, 2004; Zheng and Yang, 2006] (Fig. 2D). Cotransfection of Tip60 plasmid did not increase ZNF76 transactivation activity, indicating that Tip60 modulation of PLAGL2's transactivation ability is target-specific.

To define the regions in Tip60 required for interacting with PLAGL2, various Flag-tagged Tip60 mutant plasmids described previously [Xiao et al., 2003] were expressed in HEK293 cells together with HA-tagged PLAGL2 plasmid. Anti-HA immunoprecipitates and the total cell lysates were analyzed by SDS–PAGE. Western blot was performed with anti-Flag antibody, stripped, and re-immunoblotted with anti-HA antibody. As shown in Figure 3A, in contrast to full-length Tip60, mutant Tip60 C3 (amino acids 1–255) did not interact with PLAGL2. Since Tip60 C3 expression was comparable with that of wild-type Tip60 and other mutants in total cell lysates, we conclude that the region between amino acid 261 and 366 of Tip60 containing a typical zinc finger motif is required for its interaction with PLAGL2. However, we cannot exclude the possibility that sequences outside the zinc finger domain may also be required for PLAGL2 binding.

To further examine the role of the zinc finger domain of Tip60 in the inhibition of PLAGL2 sumoylation, PLAGL2 plasmid was cotransfected with either wild-type Tip60 or Tip60 C3 plasmid in HEK293 cells and DN-Ubc9 was included as a positive control. In contrast to wild-type Tip60 or DN-Ubc9, Tip60 C3 did not abolish sumoylation of PLAGL2 (Fig. 3B) despite its expression level was higher than that of wild-type Tip60. Furthermore, Tip60 C3 also did not affect transactivation of PLAGL2 (Fig. 3C). These results further support the hypothesis that Tip60 increases PLAGL2 transactivation by inhibiting its sumoylation. Tip60 specifically blocks PLAGL2 sumoylation

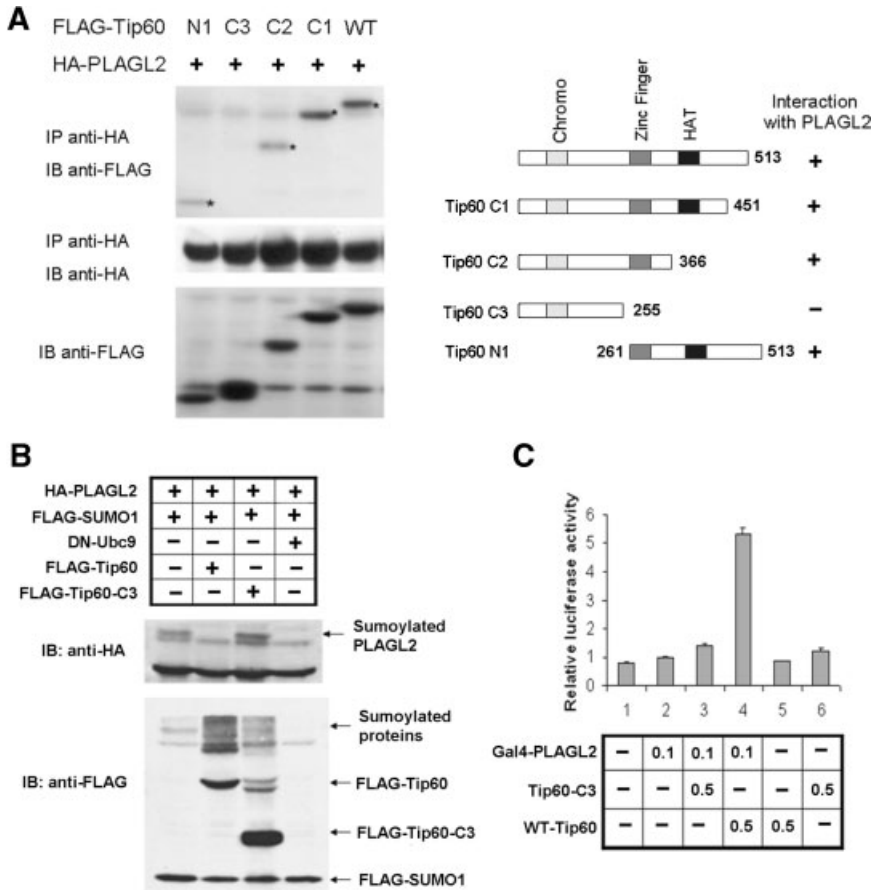

Fig. 3. Zinc Finger domain of Tip60 is necessary for the inhibition of PLAGL2 sumoylation. **A**: Zinc Finger domain of Tip60 mediates its interaction with PLAGL2. HEK293 cells were transfected with HA-PLAGL2 plasmid (5 μg) and various deletion mutants of Flag-Tip60 (5 μg of each). Cell extracts were immunoprecipitated with anti-HA antibody. Immunoprecipitates were immunoblotted with anti-Flag antibody as indicated. **B**: The mutant form of Tip60 which cannot interact with PLAGL2 does not inhibit sumoylation of PLAGL2. HEK293 cells in 6-well plates were transfected with 0.5 μg of pcDNA-HA-PLAGL2 plasmid and 0.3 μg of Flag-SUMO-1 plasmid in the presence or absence of 0.5 μg each of Flag-Tip60, Flag-Tip60-C3, and DNUbc9 plasmids as indicated. Thirty-six hours after transfection, cells were lysed with denaturing buffer and analyzed with indicated antibodies. **C**: The mutant form of Tip60 does not affect transactivation of PLAGL2. HEK293 cells in 12-well plates were transfected with 0.1 μg of pG5-luc reporter, 0.1 μg of Gal4-PLAGL2 plasmid, 0.005 μg pRL-null (Renilla), and 0.5 μg of pcDNA3-HA-Tip60, and 0.5 μg of Tip60-C3 plasmid either alone or in combination as indicated. Luciferase activity was assayed and normalized as in Figure 1B. All the data in this figure are representatives of at least three independent experiments.

through its direct interaction with PLAGL2 because other sumoylated proteins were not affected by Tip60 (Figs. 2D and 3B) and Tip60 C3 (Figs. 2A and 3B).

Tip60 Modulates PLAGL2–Dependent Transcriptional Activity

Since Tip60 enhances transactivation of PLAGL2, we analyzed the impact of Tip60 on the expression of PLAGL2-dependent target genes, such as SP-C and IGFII [Hensen et al., 2002; Yang et al., 2005] (Fig. 4). Both promoters contain PLAGL2-binding sites, a core sequence (GRGGC) and a G-cluster (RGGK) [Voz et al., 2000]. The promoter of the IGFII gene was activated 1.5-fold on average by PLAGL2 in 293 cells. In contrast, Tip60 had no effect on the IGFII promoter, which is consistent with previous findings that Tip60 does not have transactivation potential on its own [Hass and Yankner, 2005]. When both PLAGL2 and Tip60 plasmids were cotransfected, reporter gene expression increased to threefold (Fig. 4A). Thus, Tip60 was capable of modulating the transcriptional activity of PLAGL2 on the IGFII promoter. On the contrary, Tip60 behaved differently on SP-C promoter, which is also a

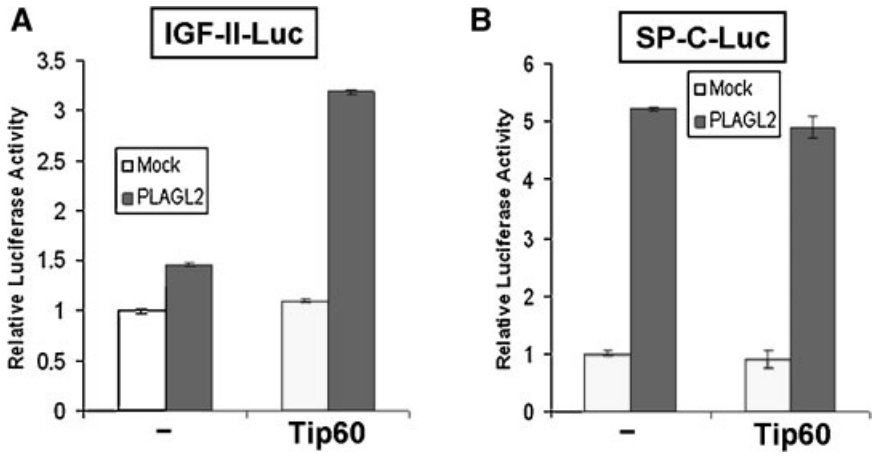

Fig. 4. Tip60 modulates PLAGL2-dependent transcriptional activity. **A**: Tip60 enhances PLAGL2 transcriptional activity on IGF-II promoter. A luciferase reporter under the control of the IGF-II promoter was transfected either alone or in combination with 0.05 μg of PLAGL2 plasmid, 0.1 μg of Tip60 plasmid, or a combination of both as indicated. Luciferase activity was assayed and normalized as in Figure 1B. **B**: Tip60 does not affect PLAGL2 transcriptional activity on SP-C promoter. A luciferase reporter under the control of the SP-C promoter was transfected either alone or in combination with 0.05 μg of PLAGL2 plasmid, 0.1 μg of Tip60 plasmid, or a combination of both as indicated. Luciferase activity was assayed and normalized as in Figure 1B. All the data in this figure are representatives of at least three independent experiments.

target gene for PLAGL2. Tip60 was unable to increase PLAGL2-dependent activation of the SP-C promoter (Fig. 4B). The fact that PLAGL2 by itself activated the SP-C promoter reporter by fivefold may suggest that sumoylation of PLAGL2 does not inhibit its transactivation on the SP-C promoter and Tip60 has no effect on PLAGL2 transactivation on this promoter. The effect of Tip60 on the transcriptional activity of PLAGL2 therefore appears to depend on the promoter and may be context-dependent.

DISCUSSION

In our current study, we report that Tip60 interacts with PLAGL2 and inhibits its sumoylation, possibly through acetylation of the same lysine residues, and acts as a coactivator of PLAGL2 specifically on the IGFII promoter.

Though well established for its HAT activity [Sterner and Berger, 2000], Tip60 can also affect transcription factors, for example, c-Myc and AR, through directly acetylating these substrates [Gaughan et al., 2002; Patel et al., 2004]. Our results showed that wild-type Tip60 (Fig. 1C) but not the HAT-minus mutant acetylates PLAGL2, and only the wild-type Tip60 significantly enhances transactivation of PLAGL2, suggesting that the effect of Tip60 on PLAGL2 also depends on its acetyltransferase activity (Fig. 1D). Interestingly, coexpression of Tip60 and PLAGL2 completely abolished the sumoylation of PLAGL2. As a result, the increased transactivation activity of PLAGL2 can be explained by either acetylation or lack of sumoylation of PLAGL2. However, the mutant form of PLAGL2 (K250R, K269R, K356R) incapable of either being acetylated or sumoylated has higher activity than that of the wild-type PLAGL2 (Fig. 2C), suggesting that sumoylation is the key mechanism in the regulation of PLAGL2 transactivation. Cotransfection of a dominant-negative form of Ubc9 inhibited global sumoylation, and increased PLAGL2 transactivation activity (Fig. 2A and B). Interestingly, Tip60 did not inhibit global sumoylation, instead there was an apparent increase of protein sumoylation in the presence of overexpressed Tip60 (Figs. 2A and 3B). This phenomenon is worthy of further investigation, and it is unlikely that sumoylated Tip60 contributed to the increase of sumoylated proteins. When we coexpressed Tip60 with PIAS1 (SUMO E3 enzyme) and SUMO1, we could not detect any sumoylated Tip60 (data not shown). Nevertheless, the inhibition of sumoylation of PLAGL2 by Tip60 is not a general phenomenon and suggests that such modulation is target specific. When ZNF76, another transcription factor also capable of being sumoylated and acetylated, was examined in a similar fashion, Tip60 had no effect on its transactivation

(Fig. 2D). Another evidence for specific modulation of PLAGL2 activity by Tip60 comes from their specific interaction. Using coimmunoprecipitation, we demonstrated that Tip60 specifically interacted with PLAGL2 (Fig. 1A), and the interaction domain was mapped to the zinc finger domain of Tip60 with minor contribution from the HAT domain (Fig. 3A). We concluded that the specific interaction between Tip60 and PLAGL2 is required for acetylation of PLAGL2 by Tip60. This modification in turn prevents sumoylation of PLAGL2, possibly because sumoylation and acetylation occur at the same lysine residues. Whether the same lysines in PLAGL2 are sumoylated and acetylated needs to be further pursued. Post-translational modifications such as phosphorylation [O'Shea et al., 2002], acetylation [Freiman and Tjian, 2003], methylation [Mowen et al., 2001], ubiquitination [Freiman and Tjian, 2003], and sumoylation [Freiman and Tjian, 2003] are important mechanisms to regulate activities of different transcription factors. Sumoylation, the process of conjugating SUMO, a 101- amino acid polypeptide, to target proteins is highly similar to that of ubiquitination [Kim et al., 2002], but the consequences of sumoylation are markedly distinct. In most cases described to date, sumoylation of transcriptional regulators correlates with inhibition of transcription although this is not due to degradation of the modified transcriptional factors [Gill, 2005]. In contrast, acetylation can activate the modified transcriptional factors, for example, in the case of p53 and Stat3 [Gu and Roeder, 1997; Barlev et al., 2001; Yuan et al., 2005]. Since modification sites of acetylation, ubiquitination, and sumoylation can all occur on lysine residues, transcription factors can potentially undergo a cascade of modifications that modulate their functions. For example, the major site of Sp3 sumoylation is identical to the major site of acetylation, and both of these modifications are involved in modulating Sp3 activation [Braun et al., 2001]. Sumoylation of a lysine residue in IκBα can block its ubiquitination at this site thereby protecting IκBα from degradation [Desterro et al., 1998]. We demonstrate that Tip60 modulates PLAGL2 by blockage of its sumoylation. This function of Tip60 may be a general theme in its regulation of target proteins. Other factors that are regulated by Tip60, for example, AR and LEF1 [Kioussi et al., 2002], can also be modulated by sumoylation [Poukka et al., 2000; Sachdev et al., 2001]. It will be interesting to investigate whether Tip60 also blocks their sumoylation and alters their activities.

We mapped the interaction between Tip60 and PLAGL2 to the zinc finger domain of Tip60. This domain has also been documented to play essential roles in mediating interactions between Tip60 and other proteins. For example, zinc finger region is critical for translocation ETS leukaemia gene (TEL) binding to Tip60 [Nordentoft and Jorgensen, 2003]. We previously reported that Tip60 acts as a corepressor of Stat3 by recruiting HDAC7 through its zinc finger domain [Xiao et al., 2003]. PLAGL2 and other proteins all interact with the common Tip60 region and, as a consequence, PLAGL2 may compete with others for Tip60 binding. Our study shows that Tip60 specifically affects the transcriptional activity of PLAGL2 on the IGFII promoter but not on the SP-C promoter, suggesting that the effects of Tip60 on PLAGL2 may be context-dependent. One possible explanation is that PLAGL2-occupied SP-C promoter is not sensitive to sumoylation since PLAGL2 has higher activity on the SP-C promoter than the IGFII promoter (Fig. 4). Another possibility is that the promoter context dictates the interaction between PLAGL2 and Tip60, especially if PLAGL2 competes with other proteins for Tip60 binding. It is also possible that some other transcription factors bring endogenous HAT activity to PLAGL2 on the SP-C promoter and therefore its activity cannot be further boosted by ectopic expression of Tip60.

Overexpression of PLAGL2 can induce transformation of NIH3T3 cells. However, the underlying mechanisms are not clear. IGFII could be one of the major targets of PLAGL2 to mediate its transformation activity [Voz et al., 2000; Hensen et al., 2002]. Our present study shows that Tip60 acts as a coactivator of PLAGL2 on IGFII promoter, suggesting that Tip60 may be involved in tumorigenesis promoted by PLAGL2. The involvement of Tip60 in AR regulation during prostate cancer development has been well documented [Brady et al., 1999; Gaughan et al., 2002; Halkidou et al., 2003]. Tip60 also enhances c-Myc-transforming activity mediated by human T-cell lymphotropic virus type 1 (HTLV-1) pX splice-variant p30II and may contribute to adult T-cell leukemogenesis [Awasthi et al., 2005]. One recent report showed that Tip60 may be

involved in polyamine-mediated tumor promotion [Hobbs et al., 2006]. Whether Tip60 modulates these transforming events through similar mechanisms described in the current study requires further investigation.

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