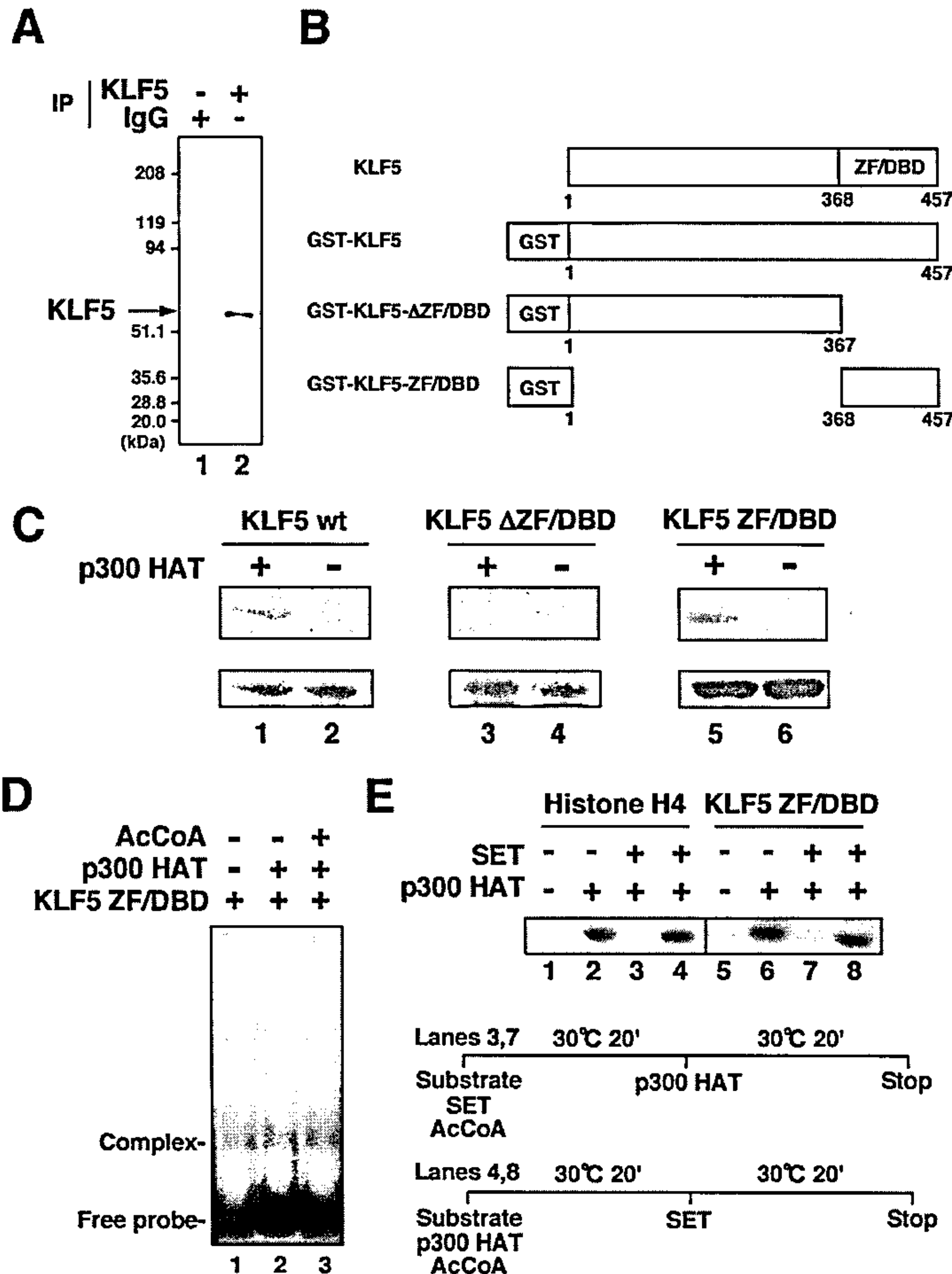

FIG. 4. Acetylation of KLF5 and its regulation by SET. (A) Acetylation of KLF5 in vivo. Cells were treated with trichostatin A and labeled with [^{3}H]acetate followed by immunoprecipitation with KLF5 (lane 2) and control normal IgG antibodies (lane 1). (B) Schematic representation of GST-KLF5 fusion mutant constructs. GST-KLF5 comprises full-length KLF5 fused to GST, GST–KLF5-ΔZF/DBD comprises only the N-terminal regulatory domain fused to GST, and GST–KLF5-ZF/DBD comprises only the C-terminal zinc finger DBD fused to GST. (C) Acetylation of KLF5 mutant constructs in vitro by p300. KLF5 proteins (1.2 μg) were incubated with 50 ng of FLAG-p300 HAT domain protein (amino acids 1195 to 1673) in the presence of [^{14}C]acetyl-CoA. Reaction products were separated by SDS–12% PAGE. The difference between pairs (lanes 1 and 2, 3 and 4, and 5 and 6) is the presence of p300 HAT protein in the reaction mixture for the respective KLF5 mutant proteins. The gel was stained with Coomassie brilliant blue (lower panel) and then analyzed with a BAS 1500 phosphorimager (upper panel). (D) Effects of acetylation on KLF5 DNA-binding activity. Acetylation reactions were performed in the presence (+) of acetyl-CoA (AcCoA) and FLAG-p300 HAT domain (lane 3), in the presence of FLAG-p300 HAT domain (lane 2), and in the absence (−) of acetyl-CoA or FLAG-p300 HAT domain (lane 1). Reaction products were resolved by electrophoresis and analyzed with BAS1500. (E) Effects of SET on KLF5 acetylation (lanes 5 to 8). Histone H4 was used as a control (lanes 1 to 4). A schematic diagram of the protocol for order-of-addition experiments is shown. In lanes 3 and 7, the p300 HAT domain was added following the reaction of SET with the substrate (KLF5 ZF/DBD or histone H4) (prior to acetylation), and in lanes 4 and 8, SET was added following the reaction of p300 HAT with the substrate (after acetylation). Acetylation reactions were done essentially as described above. All experiments were done at least twice with consistent findings.

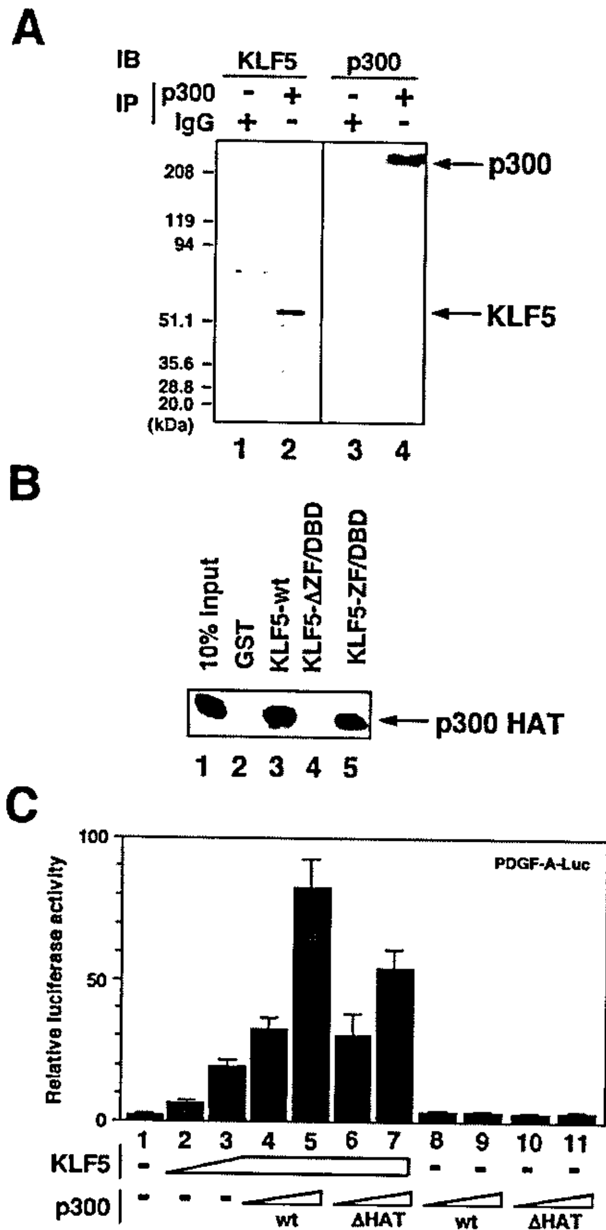

FIG. 5. Interaction and activation of KLF5 by the coactivator and acetylase p300. (A) Interaction of KLF5 and p300 in vivo. p300 was immunoprecipitated from cells followed by immunoblotting against KLF5 (lane 2). Immunoblotting against p300 confirms the immunoprecipitation procedure (lane 4). Normal IgG was used as the control (lane 2). (B) Interaction between KLF5 deletion mutants and p300 HAT domain in vitro. Approximately 2 μg of recombinant GST-KLF5 wild type (wt) (amino acids 1 to 457, lane 3), GST-KLF5-ΔDBD (amino acids 1 to 367, lane 4), and GST-KLF5-DBD (amino acids 368 to 457, lane 5) were used in a GST pull-down assay with 1 μg of the FLAG-p300 HAT domain. The interaction between KLF5 and the p300 HAT domain was analyzed by SDS-10% PAGE of the pull-down reactions and Western blotting with anti-FLAG antibody. The input (lane 1) contains 10% of the FLAG-p300 HAT domain protein. GST protein (lane 2) was used as a control. (C) Effects of p300 on KLF5 transactivation as assessed by reporter cotransfection assay. Cells were transfected with 100 ng of PDGF-A chain luciferase reporter and increasing amounts of KLF5 expression vector plasmids up to 750 ng (lanes 2 to 7). DNA concentrations were maintained constant by addition of the empty vector. Increasing amounts of p300 expression vector plasmids were similarly cotransfected up to 250 ng with 100 ng of PDGF-A–luciferase reporter plasmid in the absence (−) (lanes 8 and 9) or presence of 750 ng of KLF5 expression vector (lanes 4 and 5). Effects of an acetyltransferase region-deleted mutant of p300 (ΔHAT) on KLF5-mediated transcriptional activation were also examined (lanes 6, 7, 10, and 11). All experiments were done at least twice with consistent findings. +, present.

that the effects of SET on KLF5 may at least in part be by inhibition of KLF5 acetylation.

DISCUSSION

Regulatory pathway of coupled interaction and acetylation. We have described a new regulatory pathway of transcriptional activation and inhibition of a DNA-binding transcription factor through the DBD by coupled interaction and modification (e.g., acetylation) as shown through the opposing actions of p300 and SET on the transcription factor KLF5 (Fig. 7F). SET negatively regulates and p300 positively regulates KLF5 actions, and SET further inhibits acetylation of KLF5 by p300. Importantly, our biochemical and cellular data suggest that the effects of KLF5 acetylation are complementary to the effects of SET on KLF5 on the basis of regulation of transcriptional activation and cell growth. Our findings suggest that the actions of SET are therefore likely mediated at least in part by blocking activation as mediated through this chemical modification. The actions of SET on DNA-binding factors have been hitherto unknown, although past studies had addressed its role in adenoviral DNA replication and as a chaperone for histones (15, 25, 26, 34). The present findings therefore implicate a new role for SET in factor-specific regulation of transcription through a mechanism that has previously only been known for histones. As SET has been shown to similarly inhibit transcription of retinoic acid receptor transcription (39), it is tempting to speculate that this action can be generalized to include at least the nuclear receptor family, which contains a zinc finger-type DBD common to KLF5.

The regulation of acetylation is emerging as a new pathway for the control of transcriptional regulation. Catalytic regulation through actions of deacetylases (17, 33) and coupled regulation with other signaling pathways (i.e., regulation of p53 by coupled acetylation and phosphorylation) (38) and by other interactions has received much attention, but noncatalytic regulation by protein interaction with the substrate and/or enzyme is also a notable regulatory mechanism. SET, as a subunit of the INHAT complex (39), likely masks the substrate protein from acetylation by interaction (e.g., competition) and/or further inducing a conformational change, making it inaccessible to the enzyme. It was also previously shown that DNA binding inhibits interaction and acetylation of Sp1 by the acetylase p300 (45). Further, the human immunodeficiency virus-related tat protein has been shown to modulate acetylation, likely by interaction and by conformational changes to the substrate and/or enzyme, thus modulating acetylation activity and its effects on gene expression (6).

Taken together, modulation of acetylation through noncatalytic actions (e.g., blocking or masking interaction) is a pathway which can confer a new regulatory step to transcriptional regula-

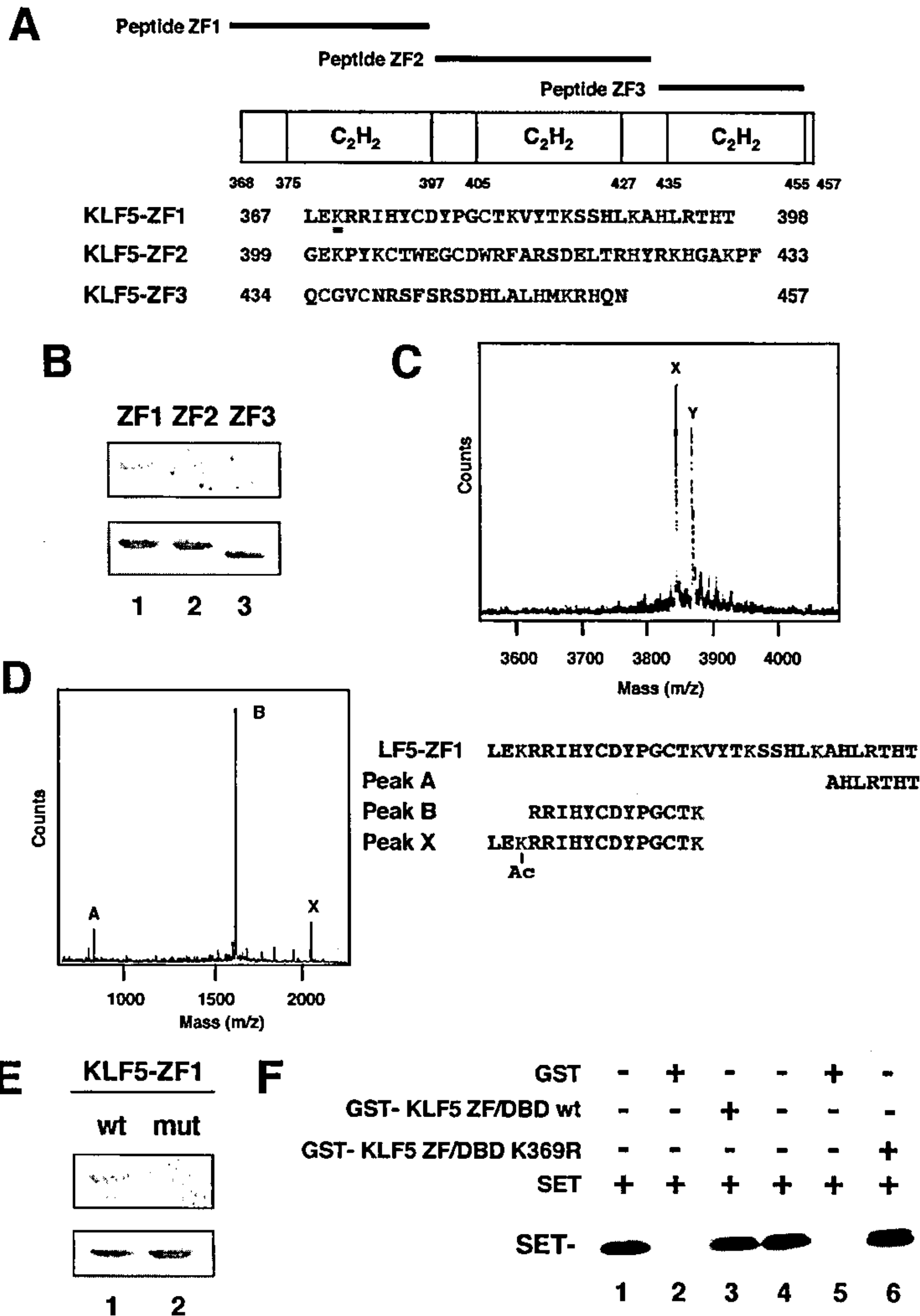

FIG. 6. Mapping of the acetylated region and residue of KLF5. (A) Schematic representation of KLF5 zinc finger peptides. ZF1, ZF2, and ZF3 cover each of the zinc fingers, respectively, from the N terminus. (B) Acetylation of KLF5 zinc finger mutants in vitro. Approximately 1.0 μg of purified GST-KLF5 fusion zinc fingers 1, 2, and 3 were incubated with [^{14}C]acetyl-CoA and recombinant FLAG-p300 HAT domain. Reaction products were separated by SDS–10% PAGE. The gel was stained with Coomassie brilliant blue (lower panel) and then analyzed with a BAS 1500 phosphorimager (upper panel). (C) Mass spectrum quantification of acetylated lysines in peptide KLF5-ZF1. A parallel reaction mixture with unlabeled acetyl-CoA was analyzed by MALDI-TOF (MS). The major peak labeled X corresponds to the expected mass of the unmodified peptide KLF5-ZF1. The major peak labeled Y, larger by 42 atomic mass units, represents monoacetylated peptide. (D) Masses of peptides digested with

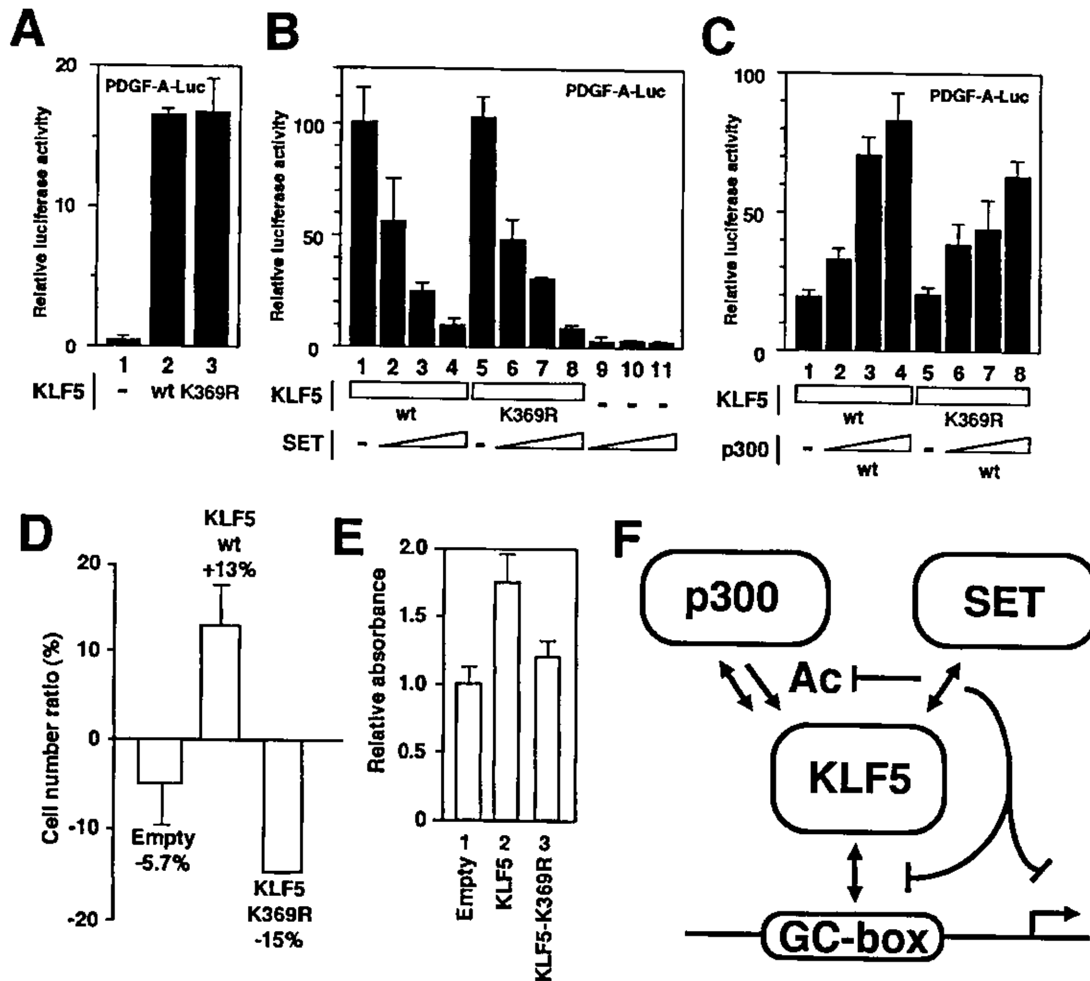

FIG. 7. Effects of the KLF5 K369R point mutant. (A) Effects of KLF5 K369R mutant (lane 3) on PDGF-A chain promoter transcriptional activation compared to that of the KLF5 wild type (wt) (lane 2). Seven hundred fifty nanograms of each expression vector was transfected in the presence of 100 ng of the reporter construct (all lanes). –, absent. (B) Effects of SET on KLF5 wild type and K369R mutant. Up to 250 ng of SET expression vector was transfected in the presence of 750 ng of KLF5 expression vector. (C) Effects of the KLF5 K369R mutant on PDGF-A chain promoter transcriptional activation compared to that of the KLF5 wild type in the presence of p300. Increasing amounts of p300 expression vector up to 250 ng were transfected in the presence of 750 ng of KLF5 (wild type, lanes 1 to 4; K369R mutant, lanes 5 to 8) expression vector and 100 ng of the reporter construct. (D) Effects of KLF5 K369R mutant on cell growth. The KLF5 wild type and K369R mutant were transfected with adenovirus and counted on the sixth day in comparison with nontreated cells. Error bars denote standard errors. (E) Effects of the KLF5 K369R mutant on cell growth were similarly assessed by BrdU assay. Error bars denote standard errors. All experiments were done at least twice with consistent findings. (F) Summary of findings. Note that SET negatively regulates DNA binding, transactivation, and acetylation of KLF5. p300 interacts, transactivates, and acetylates KLF5. We envision that this mechanism of interplay between coupled positive regulation by p300 and negative regulation by SET occurs in an inducible setting (e.g., phorbol ester stimulation).

tion of gene expression. Note, the combined role of noncatalytic acetylation blocking or masking events with those of deacetylases, which act following acetylation, and further regulation by other interacting regulatory proteins (e.g., complex) is still unknown. Future studies aimed at deciphering the complexity of the regulation of the collective transcription reaction as mediated by acetylation will be necessary to understand the precise role of this regulatory pathway.

Lys-C endopeptidase. The peptide sequences that are suggested from measured masses are shown below. Peak X represents the acetylated fragment. (E) Replacement of acetylated lysine by arginine impairs acetylation of GST–KLF5-zinc finger 1. Approximately 1.0 μg of purified GST–KLF5-zinc finger 1 (lane 1, wild type [wt]) and GST–KLF5-mut zinc finger 1 (K369R) (lane 2, mutant [mut]) were incubated with [^{14}C]acetyl-CoA and 50 ng of FLAG-p300 HAT domain protein. Reaction products were separated by SDS–10% PAGE. The gel was stained with Coomassie brilliant blue (lower panel) and then analyzed with a BAS 1500 phosphorimager (upper panel). (F) Binding assay of K369R and wild-type KLF5 with SET. Wild-type and K369R mutant KLF5 fused to GST were immobilized on GST resin followed by a pull-down assay of SET protein. Lanes 1 and 4 are SET input. Lanes 2 and 5 are GST alone. All experiments were done at least twice with consistent findings. +, present; –, absent.

Regulatory pathway through the DBD. Another finding of the present study is that the DBD plays a pivotal role in mediating regulatory interactions. It is well established that the activation and regulatory domain of transcription factors mediates important regulatory interactions (19, 36, 37). In contrast, our findings and those of others suggest that the DBD mediates regulatory interactions particularly for the Sp/KLF family of zinc finger transcription factors. Interaction of the DBD with acetylases has been shown in the past (45), and others have shown interaction with SWI/SNF (12), deacetylase (8), and cell cycle regulatory factor (13, 20) as well as interaction with other zinc finger transcription factors, including Krüppel-like factors (21).

In the present study, we show that there is regulatory interplay by interacting proteins, namely through physical interaction and modification (e.g., acetylation) as mediated by acetylase and its inhibitor (e.g., masking protein) on the DBD. It is tempting to envision that the DBD of at least this subgroup of zinc finger transcription factors mediates a convergence of multiple regulatory pathways contributing to temporospatial regulation of DNA-involved processes ranging from naked DNA to chromatin remodeling to thus affect gene expression. Given that zinc finger transcription factors greatly evolved in genomic complexity in eukaryotes (46), it is likely that they play an important role in specific transcription associated with biological diversification. A better understanding of the actions and regulation of these factors and pathways will add to our understanding of eukaryotic transcriptional regulation.

Functional implications of KLF5-SET interaction. Our results show a biological setting in which the described mechanisms may contribute to transcriptional regulation of gene expression. The temporospatial biological activity of KLF5 may be dictated by the coordinated regulation of its induced expression and the reduced expression of its negative regulator SET. KLF5 was induced and activated by mitogenic stimulation. In contrast, SET was repressed in response to mitogenic stimulation, and importantly, the repression of SET coincided with the induction of KLF5, which is coupled with expression of a downstream gene. In pathological states, SET and KLF5 were both highly expressed, as examined in an experimental model of atherosclerosis. Expression of SET and KLF5 were both increased in neointimal hyperplasia cells, which are proliferative cells induced in response to a pathological stimulus. Given that their coexpression was seen in late stages of pathogenic evolution, we envision that increased SET expression acted to limit the actions of KLF5 so that uncontrolled inappropriate cell proliferation (e.g., oncogenesis) did not occur.

Interestingly, p300 is also induced by phorbol ester stimulation (23). Together with our findings, our proposed mechanism of coupled positive regulation of KLF5 by p300 and negative regulation by SET may play a role in the biological setting of pathophysiological induction of KLF5 as exemplified by the actions of the model agonist, phorbol ester. Induction of KLF5 activation and of its downstream genes are coupled with upregulation of its coactivator p300 and down-regulation of its repressor SET, thereby likely resulting in amplification of KLF5 actions. Another interesting issue on the regulation of SET may be that there is possible regulation of SET by its splicing variant and dimerization partner TAF-Iα. We have seen that while SET alone is expressed in KLF5-active smooth muscle cells, TAF-Iα is also expressed in stoichiometric amounts in other cells. Given that TAF-Iα has been suggested to be a negative regulator of SET (28), we believe that the amount of SET relative to TAF-Iα may be critical in regulating SET actions. SET may therefore act only in the absence of TAF-Iα or when SET is found in relative abundance; therefore allowing for dissection of the unique actions of SET as a negative regulator in the TAF-Iα-deficient KLF5-expressing smooth muscle cell. Collectively, our findings suggest a new transcriptional regulatory pathway of a DNA-binding transcription factor by combined use of inducible positive and negative cofactors centered on interaction and acetylation which likely reflects the mechanisms underlying triggered activation by stimuli.

Importantly, the regulatory pathway by p300 and SET allows for bimodal regulation of KLF5, which will efficiently allow for a rapid amplification of coordination and fine tuning of gene regulation such as in gene- and cell type-specific transcriptional regulation (e.g., temporal as well as spatial regulation). Future studies aimed at understanding the regulation and generality of the described mechanisms will further advance our understanding of the biological role of the involved factors and reactions.

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S. Miyamoto, T. Suzuki, S. Muto, and K. Aizawa contributed equally to this work.

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Purification, crystallization and preliminary X-ray diffraction analysis of human oncoprotein SET/TAF-1β

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The human oncoprotein SET/TAF-1β has been crystallized by the sitting-drop vapour-diffusion method using ammonium sulfate as a precipitant. The crystal belongs to space group $C2$, with unit-cell parameters a = 119.6, b = 62.8, c = 61.0 Å, β = 89.7°, and contains two molecules in the asymmetric unit. A complete data set was collected to 2.8 Å resolution using synchrotron radiation.

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1. Introduction

SET/TAF-1β is a multifunctional molecule which is involved in many biological phenomena. The *set* gene was originally identified as a fusion gene with *can* in acute undifferentiated leukaemia (AUL). The *set–can* fusion gene is the product of the translocation (6;9)(p23q34) that is a hallmark of acute myeloid leukaemia. This translocation usually results in the formation of a *dek–can* fusion gene on chromosome 6p−. In the case of AUL, one of the classes of acute myeloid leukaemia, *set* is fused to *can* instead of *dek* (Von Lindern *et al.*, 1992; Adachi *et al.*, 1994). The *set* gene is suggested to play a key role in the leukaemogenesis of AUL.

TAF-1β was independently characterized as a host factor that stimulates adenovirus core DNA replication (Matsumoto *et al.*, 1993). Cloning of TAF-1β revealed that it is encoded by the *set* gene (Nagata *et al.*, 1995). Further studies showed that SET/TAF-1β is a multifunctional factor which is involved in transcription (Matsumoto *et al.*, 1995), silencing (Cervoni *et al.*, 2002) and apoptosis (Fan *et al.*, 2003). SET/TAF-1β was also characterized as a histone chaperone that has been suggested to be involved in alteration of chromatin structure (Kawase *et al.*, 1996). Since SET/TAF-1β inhibits the acetylation of histones (Seo *et al.*, 2001) and DNA-binding transcription factors (Miyamoto *et al.*, 2003) as well as the methylation of DNA (Cervoni *et al.*, 2002), SET/TAF-1β may therefore function as a regulator of transcription and replication by affecting the chemical modifications of nucleosome and transcription factors.

We recently isolated SET/TAF-1β as an interacting factor of the DNA-binding domain of transcription factors Sp1 and KLF5 (Suzuki *et al.*, 2003; Miyamoto *et al.*, 2003). SET/TAF-1β inhibits the DNA binding of Sp1 and KLF5, consequently down-regulating their transcriptional activities, contrary to the case of the coactivator/acetyltransferase p300 (Suzuki *et al.*, 2000). Furthermore, SET/TAF-1β inhibits the acetylation of KLF5 by p300. Therefore, SET/TAF-1β is suggested to function as a multifunctional factor by interacting with DNA-binding proteins.

To analyze the molecular action of SET/TAF-1β at the atomic level, we have initiated structural studies of SET/TAF-1β. We utilized the protein without its acidic stretch (amino acids 1–225) because the acidic stretch is difficult to crystallize; importantly, however, the protein is still functional and shows negative effects on DNA binding, acetylation and transcriptional activity of KLF5 (Miyamoto *et al.*, 2003). Here, we report the purification, crystallization and preliminary crystallographic analysis of human SET/TAF-1β.

2. Materials and methods

2.1. Protein expression and purification

To overexpress SET/TAF-1β, *Escherichia coli* BL21 (DE3) pLysS (Stratagene) cells were transformed with the pET14b-SET/TAF-1β (amino acids 1–225) recombinant plasmid (Nagata *et al.*, 1995). Transformed cells were grown at 300 K in TBG-M9 medium containing 100 μg ml^{-1} ampicillin and 34 μg ml^{-1} chloramphenicol until OD_{595} reached 0.6. Overexpression of SET/TAF-1β was induced by the addition of 0.4 m*M* IPTG. After a 3 h culture at 300 K, the cells were harvested by centrifugation (3000 rev min^{-1}, 10 min, 277 K), resuspended in buffer *A* containing 20 m*M* Tris–HCl (pH 7.9 at 277 K), 10% glycerol, 500 m*M* NaCl, 50 m*M* 2-mercaptoethanol, 0.5 m*M* PMSF, 10 μg ml^{-1} leupeptin and 10 μg ml^{-1} pepstatin A and then lysed by EmulsiFlex-C5 (Avestin). The cell lysate was centrifuged at 24 000 rev min^{-1} for 30 min at 277 K. The supernatant was then applied to ProBond resin (Invitrogen); after washing the resin, the protein was eluted with buffer *A* containing 0.2 *M* imidazole. For further purification, the eluted protein was concentrated by

Centriprep YM-30 (Millipore) and then fractionated by gel filtration on a HiLoad 26/60 Superdex 200 column (Amersham Pharmacia). The pooled SET/TAF-1β protein was concentrated to 70 mg ml^{-1} by Centriprep YM-30 (Millipore). The purity of SET/TAF-1β was examined by SDS–PAGE (Fig. 1). Although the molecular weight of SET/TAF-1β is calculated to be 28 kDa from the sequence, the SET/TAF-1β protein was detected as a molecule of around 38 kDa by SDS–PAGE.

2.2. Crystallization

Crystallization trials were initially performed by the hanging-drop vapour-diffusion method at 293 K. Hampton Crystal Screen and Crystal Screen 2 kits (Hampton Research) were used to determine the initial crystallization conditions. The drop was prepared by mixing 1 µl of protein solution with 1 µl of reservoir solution. Twinned thin plate-like crystals were grown within one month using a solution containing 2.0 *M* ammonium sulfate, 0.1 *M* sodium citrate buffer pH 5.4 and 0.2 *M* potassium/sodium tartrate. Further screenings to obtain single crystals were accomplished by varying the pH, the concentration of both the precipitant and potassium/sodium tartrate, and by adding different additives. The best large crystals were obtained using 5 µl of 70 mg ml^{-1} protein solution (20 m*M* Tris–HCl, 100 m*M* NaCl, 10 m*M* 2-mercaptoethanol), 5 µl reservoir solution (2.75 *M* ammonium sulfate, 0.1 *M* sodium citrate buffer pH 5.4, 0.2 *M* potassium/sodium tartrate and 30 m*M* $MgCl_2$). At 293 K, crystals grew to approximate dimensions of 0.3 × 0.2 × 0.02 mm in one week (Fig. 2). A solution containing artificial mother liquor (2.75 *M* ammonium sulfate, 0.1 *M* sodium citrate buffer pH 5.4, 0.2 *M* potassium/sodium tartrate and 30 m*M* $MgCl_2$) and 30%(*w*/*v*) trehalose was used as a cryoprotectant. Instead of trehalose, 30% glycerol and 30% glucose were also tried, but yielded poor results.

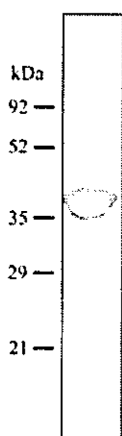

Figure 1
SDS–PAGE of SET/TAF-1β stained with Coomassie Brilliant Blue.

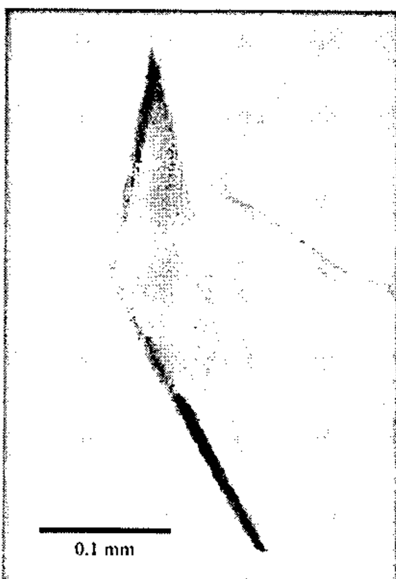

Figure 2
Crystal of human oncoprotein SET/TAF-1β.

2.3. Data collection

Initial inspection of the crystals was performed on an R-AXIS IV^{++} imaging-plate system mounted on a Rigaku rotating-anode X-ray generator (FR-D) operated at 50 kV and 60 mA. The crystal diffracted to around 3.5 Å resolution. In order to obtain a better data set, the data were collected from a single crystal on beamline 6A using an ADSC Quantum 4R CCD detector at the Photon Factory, Tsukuba, Japan. The wavelength used was 0.9780 Å and the incident beam was collimated to a diameter of 0.2 mm. The crystal-to-detector distance was set to 250 mm. A complete data set was collected to a maximum resolution of 2.8 Å (Fig. 3). All data were processed and scaled using the programs *MOSFLM* and *SCALA* from the *CCP*4 suite (Collaborative Computational Project, Number 4, 1994).

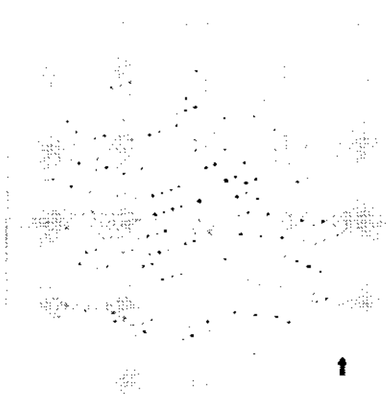

Figure 3
A 1° oscillation diffraction pattern from a crystal of SET/TAF-1β. The arrow indicates a resolution of 2.80 Å.

Table 1
Data-collection and processing statistics.

Values in parentheses are for the last shell, 2.95–2.80 Å.

Space group	*C*2
Unit-cell parameters (Å)	*a* = 119.6, *b* = 62.8, *c* = 61.0, β = 89.7°
Resolution range (Å)	42.64–2.80
No. measured reflections	67843
No. unique reflections	11065
R_{merge}† (%)	6.1 (27.7)
Completeness (%)	98.4 (98.2)
Average $I/\sigma(I)$	7.2 (2.7)

† $R_{merge} = \sum|I(\mathbf{h}) - \langle I(\mathbf{h})\rangle|/\sum I(\mathbf{h})$, where $I(\mathbf{h})$ is the observed intensity and $\langle I(\mathbf{h})\rangle$ is the mean intensity of reflection **h** over all measurements of $I(\mathbf{h})$.

3. Results

Examination of diffraction data from human SET/TAF-1β crystals revealed that the crystals diffracted to beyond 3 Å resolution and belonged to space group *C*2, with unit-cell parameters *a* = 119.6, *b* = 62.8, *c* = 61.0 Å, β = 89.7°. Data-collection statistics are summarized in Table 1. A total of 67 843 measured reflections were merged into 11 065 unique reflections with an R_{merge} of 6.1%. The merged data set is 98.4% complete to 2.8 Å resolution. A value for the Matthews coefficient of 2.3 Å^3 Da^{-1} and a solvent content of 46% were obtained assuming two molecules in the asymmetric unit and a molecular weight of 24 750 Da. An attempt to solve the structure using the MAD method is in progress.

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The Deacetylase HDAC1 Negatively Regulates the Cardiovascular Transcription Factor Krüppel-like Factor 5 through Direct Interaction*

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Transcription is regulated by a network of transcription factors and related cofactors that act in concert with the general transcription machinery. Elucidating their underlying interactions is important for understanding the mechanisms regulating transcription. Recently, we have shown that Krüppel-like factor KLF5, a member of the Sp/KLF family of zinc finger factors and a key regulator of cardiovascular remodeling, is regulated positively by the acetylase p300 and negatively by the oncogenic regulator SET through coupled interaction and regulation of acetylation. Here, we have shown that the deacetylase HDAC1 can negatively regulate KLF5 through direct interaction. KLF5 interacts with HDAC1 in the cell and *in vitro*. Gel shift DNA binding assay showed that their interaction inhibits the DNA binding activity of KLF5, suggesting a property of HDAC1 to directly affect the DNA binding affinity of a transcription factor. Reporter assay also revealed that HDAC1 suppresses KLF5-dependent promoter activation. Additionally, overexpression of HDAC1 suppressed KLF5-dependent activation of its endogenous downstream gene, platelet-derived growth factor-A chain gene, when activated by phorbol ester. Further, HDAC1 binds to the first zinc finger of KLF5, which is the same region where p300 interacts with KLF5 and, intriguingly, HDAC1 inhibits binding of p300 to KLF5. Direct competitive interaction between acetylase and deacetylase has been hitherto unknown. Collectively, the transcription factor KLF5 is negatively regulated by the deacetylase HDAC1 through direct effects on its activities (DNA binding activity, promoter activation) and further through inhibition of interaction with p300. These findings suggest a novel role and mechanism for regulation of transcription by deacetylase.

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Transcription is regulated by a network of regulatory transcription factors and coregulatory proteins (cofactors) that collectively act in concert with the general transcription machinery (1–5). Cofactors, as coactivators or corepressors, exert their activities in main through protein-protein interaction and/or chemical modification (*e.g.* phosphorylation, acetylation), thus allowing for noncatalytic and/or catalytic regulatory processes. Understanding the molecular mechanisms underlying transcriptional regulation, especially with a focus on protein-protein interaction with coupled chemical modification, is a recent topic of interest.

Acetylation is a chemical modification that is linked to transcription and is regulated in main by the catalytic enzymes, acetylase and deacetylase. Recent research has identified the factors bearing acetylase activity (*e.g.* p300, p300/ CREB-binding protein-associated factor) as well as deacetylase activity (HDACs) and their catalytic roles in transcriptional regulation (6–10). We have, however, shown that the acetylase p300 not only regulates a DNA binding transcription factor, Sp1, by catalytic means (acetylation) but also that its effects are importantly, as well as unexpectedly, mediated by direct noncatalytic effects through interaction. That is, in addition to specific acetylation of Sp1 by p300, interaction stimulates the DNA binding activity of Sp1; further, its DNA binding inhibits both acetylation by and interaction with p300. Interestingly, these regulatory interactions were mediated by the catalytic acetylase domain of p300 and the DNA-binding domain of Sp1 (11).

Sp1 is the founding and most studied member of the Sp1 and Krüppel-like factor (Sp/KLF)[1] family of zinc finger factors that have in common three contiguous C_2H_2-type zinc fingers at the carboxyl terminus. The family has over 20 members and has received recent attention because of important roles in cell proliferation, apoptosis, and oncogenic processes (12–19). In the process of dissecting the regulatory mechanisms of this family, we further showed that the histone chaperone TAF-I/SET through its interaction with KLF5, a transcription factor of the Sp/KLF family of zinc finger factors and a key regulator of cardiovascular remodeling as shown by null mutation in mice (20), represses the DNA binding, promoter activation, and growth stimulation activities of KLF5 by interaction as well as by non-catalytically "masking" it from acetylation by p300. Intriguingly, these interactions were also mediated by the DNA-binding domain of the transcription factor (21, 22).

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[1] The abbreviations used are: KLF, Krüppel-like factor; GST, glutathione *S*-transferase; DBD, DNA-binding domain; HAT, histone acetyltransferase; PDGF, platelet-derived growth factor; PMA, phorbol 12-myristate 13-acetate; CREB, cAMP-response element-binding protein.

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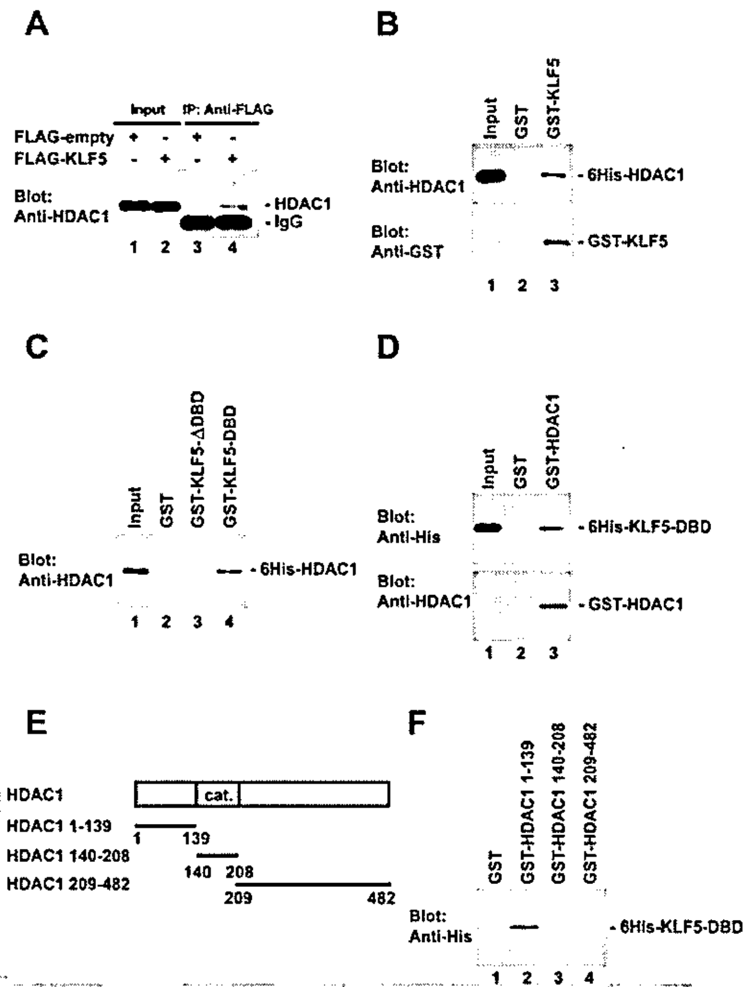

FIG. 1. **Interaction of KLF5 and HDAC1 in the cell and *in vitro*.** *A*, co-immunoprecipitation of HDAC1 with KLF5. Cell lysate with FLAG-tagged KLF5 overexpression (*lane 4*) and without overexpression (*lane 3*) was immunoprecipitated with anti-FLAG affinity gel. Immunoblot with anti-HDAC1 antibody showed interaction between KLF5 and HDAC1. *Lanes 1* and *2* were input (2.5%), confirming that the applied protein amounts were the same. The bands of IgG below those of HDAC1 in *lanes 3* and *4* were derived from anti-FLAG affinity gel. *B*, *in vitro* binding of KLF5 and HDAC1. Immobilized GST-tagged KLF5 fusion protein was reacted with hexahistidine-tagged HDAC1, separated by SDS-PAGE, and then analyzed by immunoblotting with anti-HDAC1 antibody (*lane 3*, *upper*). *Lane 1* is input (2.5%). GST protein was used as a control (*lane 2*). *C*, *in vitro* binding of KLF5-DBD and HDAC1. Hexahistidine-tagged HDAC1 was pulled down with GST-tagged KLF5-DBD (*lane 4*), but not with GST-tagged KLF5-ΔDBD (*lane 3*) or GST protein alone (*lane 2*). *Lane 1* is input (1%). *D*, immobilized GST-tagged HDAC1 fusion protein was reacted with hexahistidine-tagged KLF5-DBD, separated by SDS-PAGE, and analyzed by immunoblotting with anti-His probe antibody (*lane 3*, *upper*). *Lane 1* is input (10%). GST protein was used as a control (*lane 2*). *E*, schematic representation of GST-tagged deletion mutants of HDAC1. GST-tagged HDAC1 (140–208) contains the central catalytic region, and GST-tagged HDAC1 (1–139) and GST-tagged HDAC1 (209–482) contain the remaining amino- and carboxyl-terminal regulatory regions, respectively. *Cat*, catalytic region. *F*, hexahistidine-tagged KLF5-DBD was pulled down with GST-tagged HDAC1 (1–139) (*lane 2*), but not with GST-tagged HDAC1 (140–208), HDAC1 (209–482), or GST protein alone (*lanes 1*, *3*, and *4*).

Collectively, we have shown that non-catalytic direct actions of the chemical modification enzyme acetylase as well as the regulation of modification (*e.g.* acetylation) are important mechanisms underlying transcriptional regulation and that these important regulatory interactions are mediated by the DNA-binding domain of the transcription factor. This has led us to further extend our working hypothesis that chemical modification enzymes regulate transcription through non-catalytic, in addition to catalytic, means. We have addressed in this report whether deacetylase (HDAC) shows direct non-catalytic effects with a particular focus on the DNA-binding domain.

Here, we have shown that deacetylase through direct effects negatively regulates the transcription factor KLF5 by inhibiting its DNA binding activity and also, likely as a secondary result, its promoter activation activity. Importantly, deacetylase (HDAC1) can inhibit interaction with acetylase (p300) on the transcription factor, thus showing that deacetylase negatively affects transcription at multiple stages, including direct effects on the activities of the transcription factor (DNA binding activity, promoter activation) as well as by inhibition of interaction with its opposing coactivator/acetylase, all through the DNA-binding domain. The present findings on deacetylase add to our understanding of how protein-protein interaction coupled with chemical modification, and how catalytic and non-catalytic regulation by modification enzymes, regulate transcription.

EXPERIMENTAL PROCEDURES

Preparation of Plasmid Constructs, Recombinant Epitope-tagged Protein, and Recombinant Adenovirus—The KLF5 expression vector pCAG-KLF5 was previously described (20). Full-length KLF5 was subcloned into pExchange-3b vector (Sigma) at the EcoRV site to construct a mammalian expression vector for FLAG-tagged KLF5. Glutathione *S*-transferase (GST)-tagged constructs for KLF5 wild type, KLF5-ΔDBD, KLF5-DBD, the zinc finger peptides of KLF5, and protein expression and purification were described previously (22). The zinc finger region/DBD of human KLF5 (KLF5 ZF/DBD) (23) was PCR amplified and subcloned into BamHI-digested His_6-pET11d. HDAC1 (a kind gift from C. Seiser) was excised by EcoRI and BamHI and then ligated into pGEX4T-1 (Amersham Biosciences), pET-30a (Novagen), or pcDNA3 (Invitrogen) to construct GST-tagged HDAC1, hexahistidine-tagged HDAC1, or mammalian expression vector, respectively. To construct GST-tagged HDAC1 deletion mutants, the respective inserts were amplified by PCR and then subcloned into pGEX4T-1 at the BamHI and EcoRI sites. GST-tagged HDAC1 constructs were transformed into the BL21-(DE3)pLysS or BL21-Gold(DE3)pLysS strains and then induced by isopropyl 1-thio-β-D-galactopyranoside (0.4 mM). The recombinant protein was purified with glutathione-Sepharose 4B resin (Amersham Biosciences), buffer A (10 mM Tris-HCl, pH 7.9 at 4 °C, 150 mM NaCl, 10% glycerol, 0.1% Nonidet P-40, 1 mM dithiothreitol, and 0.5 mM phenylmethylsulfonyl fluoride) for washing, and buffer A containing 20 mM reduced glutathione for elution. Hexahistidine-tagged HDAC1 construct was transformed into the HMS174-(DE3)pLysS strain and induced by isopropyl 1-thio-β-D-galactopyranoside (0.4 mM). Bacterial extracts were added to Probond resin (Invitrogen), washed, and then eluted with buffer 250 (10 mM Tris-HCl, pH 7.9 at 4 °C, 250 mM NaCl, 10% glycerol, 50 mM 2-mercaptoethanol, 0.1% Nonidet P-40, and 0.5 mM phenylmethylsulfonyl fluoride) contain-

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ing 200 mM imidazole. A hexahistidine- and FLAG-tagged p300-histone acetyltransferase (HAT) domain construct was previously described (11). The platelet-derived growth factor A (PDGF-A) chain luciferase promoter construct (PDGF-900) was previously described (24). Recombinant adenoviruses were prepared with the AdEasy system using homologous recombination in bacteria as previously described (25).

Cell Culture—Human HeLa cells were maintained in Dulbecco's modified Eagle's medium (Sigma) supplemented with 10% fetal bovine serum with 100 μg/ml streptomycin and 100 units/ml penicillin G. The culture methods for rat aortic smooth muscle cells and C2/2 rabbit vascular smooth muscle cells were described previously (22, 26).

Co-immunoprecipitation Assay—HeLa cells were transfected with 5 μg of either pExchange-3b-empty vector or pExchange-3b-KLF5 and 5 μg of pcDNA3-HDAC1 vector/10-cm dish by liposome-mediated transfer (Tfx-20; Promega) according to the manufacturer's instructions. Cells were harvested after 48 h with lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton-X, 100 μM ZnSO4, 0.5 mM phenylmethylsulfonyl fluoride, 0.5 μg/ml leupeptin, and 1 μg/ml pepstatin). 500 μl of cell lysate were incubated with 10 μl of anti-FLAG M2 affinity gel (Sigma) in lysis buffer containing 0.5% bovine serum albumin for 2 h at 4 °C. After four washes with IP wash buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 100 μM ZnSO4, 0.5 mM phenylmethylsulfonyl fluoride, 0.5 μg/ml leupeptin, and 1 μg/ml pepstatin), immunoprecipitate was subjected to SDS-PAGE analysis and then immunoblotted with anti-HDAC1 mouse monoclonal antibody (Upstate Biotechnology).

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In Vitro "GST Pulldown" Binding Assay—1 μg of GST fusion protein was incubated with 10 μl of glutathione-Sepharose 4B resin for 2 h in binding buffer (20 mM HEPES, pH 7.9, 40 mM KCl, 0.1 mM EDTA, 0.1% Nonidet P-40, and 100 μM ZnSO4). After washing with binding buffer, 1 μg of each hexahistidine-tagged protein was added and then incubated for 2 h in the same buffer, followed by three to five washes with wash buffer (20 mM Tris-HCl, pH 8.0, 100 mM KCl, 10% glycerol, 0.1 mM EDTA, 0.5% Nonidet P-40, and 100 μM ZnSO4). For binding assay with p300-HAT domain, buffer containing 20 mM HEPES, pH 7.6, 100 mM NaCl, 20% glycerol, 0.1 mM EDTA, 100 μM ZnSO4, and 0.1% Triton X-100 was used for binding and washing. For competition assay, the details of protein combinations and the order of incubation are given in the figure legends. All reactions were carried out at 4 °C. Bound proteins were resolved by SDS-PAGE analysis and then immunoblotted with anti-HDAC1 (Upstate Biotechnology), anti-His probe (G-18), anti-GST (B-14) (Santa Cruz), or anti-FLAG antibody (Sigma).

Gel Shift DNA Binding Assay—A DNA oligomer containing the KLF5 binding sequence, 5′-ATGGGCATGAGGGCCAGCCTATGAGA-3′ (SE1), was used to analyze the DNA binding of KLF5 ZF/DBD (27). For mutant analysis, the underlined nucleotides GGGCC were replaced by TTTAA. Recombinant proteins were incubated for 15 min at room temperature with 1 ng of ^{32}P-labeled DNA probe in 20 μl of buffer (10 mM Tris-HCl, pH 7.9 at 4 °C, 150 mM NaCl, 10% glycerol, 0.1% Nonidet P-40, 0.2 mM EDTA, and 100 μM ZnSO4). DNA-protein complexes were resolved by electrophoresis on a 6% polyacrylamide gel. Supershift assay was done using anti-His probe (G-18) antibody (Santa Cruz). The details (*e.g.* protein combinations) of individual experiments are given in the figure legends.

Co-transfection Reporter Assay—Transient transfection assays were done by seeding HeLa cells (50,000 cells/24-well plate) 24 h prior to transfection. The cells were then transfected with 90 ng of the reporter plasmid, 240 ng of pCAG-empty vector and/or pCAG-KLF5, and 90 ng of pcDNA3-empty vector and/or pcDNA3-HDAC1 in combination, as described in the figure legends, by liposome-mediated transfer (Tfx-20; Promega) according to the manufacturer's instructions. Cells were harvested after 48 h and subjected to an assay of luciferase activity (luciferase assay system; Promega) (Lumat LB9501; Berthold). Assays were done in duplicate, and error bars denote S.D. Expression levels of KLF5 were confirmed by immunoblotting with anti-KLF5 rat monoclonal antibody (KM1785).

Phorbol Ester-induced Expression of KLF5 and PDGF-A—After 48 h of growth arrest with serum-free medium, subconfluent rat smooth muscle cells (26) were transfected with 4 μg/6-well plate of either pcDNA3-empty vector or pcDNA3-HDAC1 expression vector using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. After 24 h, rat smooth muscle cells were stimulated with phorbol 12-myristate 13-acetate (PMA) (Sigma) (100 ng/ml) and harvested. For assays with C2/2 rabbit vascular smooth muscle cells, cells were starved for 24 h and then stimulated with PMA at 36 h after adenoviral infection. Total RNA was obtained by the RNeasy preparation kit (Qiagen) and reverse transcribed, and then quantitative PCR was performed with a gene-specific primer set and a QuantumRNA 18 S internal standard primer set (Ambion). The sequences of gene-specific primer sets were: rat KLF5, 5′-GGTTGCACAAAAGTTTATAC-3′ and 5′-GGCTTGGCACCCGTGTGCTTCC-3′; rat HDAC1, 5′-ACGGCATTGATGATGAGTCC-3′ and 5′-CTGAGCCGCACTGTAGGACC-3′; PDGF-A chain, 5′-CAGCATCCGGGACCTCCAGCGACTC-3′ and 5′-TCGTAAATGACCGTCCTGGTCTTGC-3′. The relative intensity in reference to internal 18 S rRNA was calculated by National Institutes of Health Image software.

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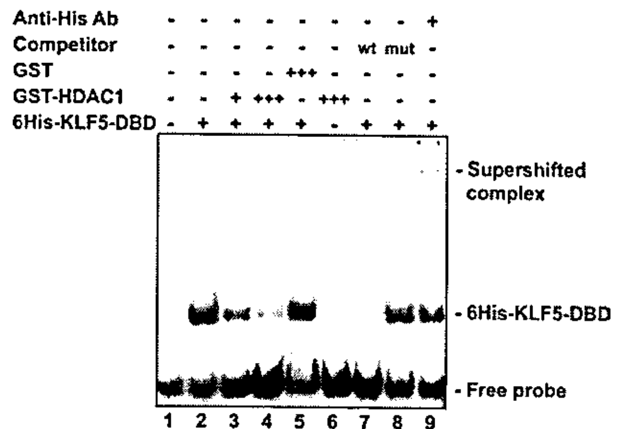

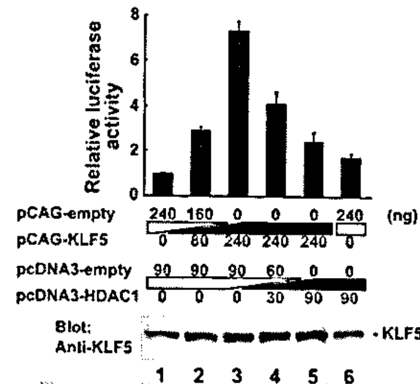

FIG. 2. **Effect of HDAC1 on DNA binding affinity of KLF5 and its promoter activation.** *A*, gel shift assay with hexahistidine-tagged KLF5-DBD and GST-tagged HDAC1. *Wt* and *mut* represent wild and mutant oligonucleotide competitors (*lanes 7* and *8*). The amount of recombinant protein is as follows: 90 (+) ng for KLF5-DBD (*lanes 2-5* and *7-9*) and 90 (+) or 270 (+++) ng for GST-HDAC1 (*lanes 3, 4*, and *6*) and GST protein (*lane 5*). Under conditions in which GST-tagged HDAC1 alone did not bind to DNA probe (*lane 6*) and cold wild type and mutant probes confirmed sequence specificity (*lanes 7* and *8*), GST-tagged HDAC1 impaired the DNA binding affinity of KLF5-DBD in a dose-dependent manner (*lanes 2-4*), as shown by decreased intensity of shifted bands. The KLF5-DBD protein is supershifted by anti-His probe antibody (*lane 9*). *B*, results of co-transfection reporter assay with PDGF-A chain showing dose-dependent inhibition of KLF5-dependent promoter activation by HDAC1. Effectors are as follows: 80 or 240 ng of pCAG-KLF5 (*lanes 2-5*), 160 or 240 ng of pCAG-empty (*lanes 1, 2*, and *6*), 30 or 90 ng of pcDNA3-HDAC1 (*lanes 4-6*), and 60 or 90 ng of pcDNA3-empty (*lanes 1-4*). Note that the total amount of effector plasmid was adjusted to a total of 330 ng with the respective control vectors. Under conditions in which HDAC1 alone did not show activation or suppression (*lane 6*), HDAC1 showed dose-dependent inhibition of KLF5-dependent promoter activity (*lanes 3-5*). Results of immunoblotting with anti-KLF5 antibody are shown below, confirming that expression level of KLF5 was not affected by HDAC1 overexpression.

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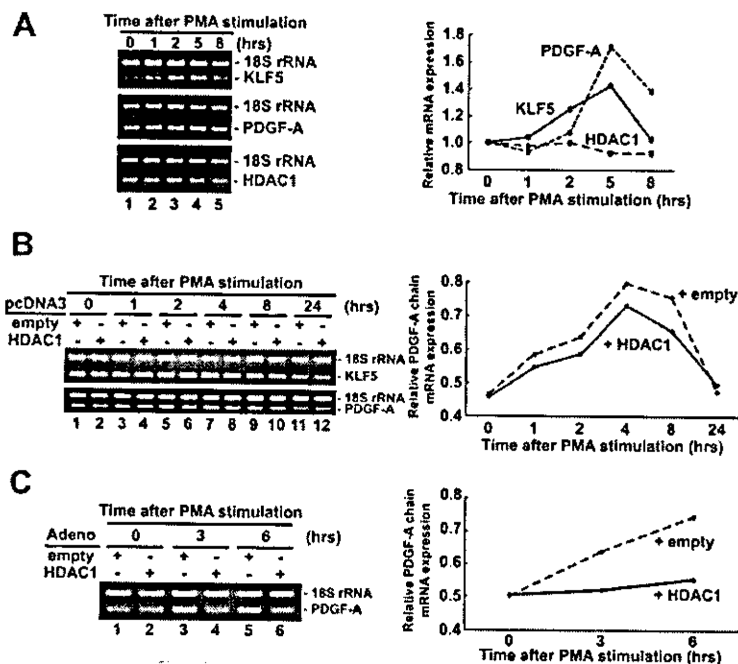

FIG. 3. **Effect of HDAC1 on phorbol ester-stimulated activation of PDGF-A chain.** *A*, induction of KLF5 and its downstream gene, PDGF-A chain, by phorbol ester. The expression level of HDAC1 was not affected. The *graph* on the *right* shows quantification of mRNA expression levels for KLF5, PDGF-A chain, and HDAC1. The expression level was normalized to that of 18 S rRNA and subsequently to that at 0 h. *B*, effect of overexpression of HDAC1 on phorbol ester-stimulated PDGF-A chain. HDAC1 was overexpressed in *even lanes*, and a control vector was transfected in *odd lanes*. Note that the expression level of KLF5 was not affected by HDAC1 and that of PDGF-A chain was suppressed by HDAC1. The *graph* on the *right* shows quantification of mRNA expression levels of PDGF-A chain. The expression level of PDGF-A chain was normalized to that of 18 S rRNA. HDAC1 overexpression suppressed PDGF-A chain promoter activation (*solid line*) compared with control vector (*dotted line*). *C*, effect of adenoviral-mediated forced expression of HDAC1 on phorbol ester-stimulated PDGF-A chain expression. Adenovirus expressing HDAC1 was infected in *even lanes*, and control empty adenovirus was infected in *odd lanes* to C2/2 vascular smooth muscle cells. Note the difference of PDGF-A chain expression levels in *lanes 6 versus 5* and *4 versus 3*, which correspond to HDAC1 and empty vector-transfected cells at 3 and 6 h, respectively. The *graph* on the *right* shows quantification of mRNA expression levels of PDGF-A chain similar to that of *panel B*.

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RESULTS

KLF5 and HDAC1 Can Interact in the Cell and Directly Bind in Vitro—First, to see whether KLF5 and HDAC1 can interact in the cell, immunoprecipitation was done with HeLa cells overexpressing FLAG-tagged KLF5 and HDAC1 (Fig. 1*A*). HDAC1 was immunoprecipitated from cell lysate in which FLAG-tagged KLF5 was overexpressed (*lane 4*), but not from cell lysate in which FLAG-empty vector was transfected (*lane 3*), which shows that these proteins can interact under cellular conditions.

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Next, to see whether KLF5 directly binds to HDAC1, an *in vitro* GST pulldown binding assay was done (Fig. 1*B*). Hexahistidine-tagged HDAC1 bound to GST-tagged KLF5 (*lane 3*), but not to GST alone (*lane 2*), demonstrating that KLF5 can interact directly with HDAC1.

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KLF5-DNA-binding Domain Binds to the Amino-terminal Region of HDAC1—To further see which domain of KLF5 interacts with HDAC1, an *in vitro* binding assay was done using two deletion constructs, GST-tagged amino-terminal KLF5 regulatory/activation domain (KLF5-ΔDBD) and GST-tagged carboxyl-terminal KLF5 DNA-binding domain (KLF5-DBD) (Fig. 1*C*). GST pulldown binding assay revealed that hexahistidine-tagged HDAC1 interacts with GST-tagged KLF5-DBD (*lane 4*), but not with GST-tagged KLF5-ΔDBD (*lane 3*) or GST alone (*lane 2*).

To confirm that KLF5-DBD is sufficient to interact with HDAC1, a reciprocal experiment was done using GST-tagged HDAC1 and hexahistidine-tagged KLF5-DBD (Fig. 1*D*). As expected, GST-tagged HDAC1 bound to hexahistidine-tagged KLF5-DBD (*lane 3*). Thus, HDAC1 binds to KLF5 through its DBD.

Next, we examined which region of HDAC1 interacts with KLF5-DBD. The central region of HDAC1 is essential for its deacetylase activity and considered to be a putative deacetylase motif (8, 28–30). Accordingly, we constructed three deletion mutants of HDAC1, GST-tagged HDAC1 (1–139) corresponding to the amino-terminal regulatory region, GST-tagged HDAC1 (140–208) corresponding to the central catalytic region, and GST-tagged HDAC1 (209–482) corresponding to the carboxyl-terminal regulatory region (Fig. 1*E*). GST pulldown binding assay showed that GST-tagged HDAC1 (1–139) corresponding to the amino-terminal regulatory region is the main region required for the binding to KLF5-DBD (Fig. 1*F*). This amino-terminal region also includes the binding site for Tax (30) and thus may be a common protein-protein interaction surface. Collectively, KLF5-DBD binds to the amino-terminal region of HDAC1.

HDAC1 Inhibits DNA Binding Affinity of KLF5—As KLF5 is a DNA binding factor and HDAC1 interacts with the DBD, we tested the effect of their interaction on the DNA binding affinity of KLF5 by gel shift DNA binding assay (Fig. 2*A*). Strikingly, under conditions in which KLF5 showed sequence-specific binding to its cognate DNA-binding site as confirmed by use of cold wild type and mutant probes (*lanes 7* and *8*), HDAC1 inhibited the DNA binding activity of KLF5 in a dose-dependent manner (*lanes 2–4*). Incubation with KLF5-DBD and control GST protein alone had no effect on the binding affinity of KLF5 (*lane 5*) nor did HDAC1 show DNA binding activity (*lane 6*) under these conditions, showing the specificity of this reaction. Therefore, we have shown that interaction of KLF5 with HDAC1 impairs the DNA binding affinity of KLF5, revealing an unexpected but important property of HDAC1.

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HDAC1 Inhibits KLF5-dependent Promoter Activation—As a secondary result of inhibition of DNA binding activity, it was expected that HDAC1 would inhibit KLF5-dependent promoter activation. To test this, reporter assay was done using a platelet-derived growth factor-A chain (PDGF-A chain) luciferase construct, as PDGF-A chain has been previously shown to be an endogenous target of KLF5 (Fig. 2*B*) (24). As expected, under conditions in which transfection of KLF5 showed dose-dependent activation of the promoter (*lanes 1–3*) and in which HDAC1 alone showed no effect on activation of this promoter (*lane 6*), co-transfection of HDAC1 with KLF5 resulted in dose-dependent inhibition of promoter activity (*lanes 3–5*). The expression level of KLF5 was not affected by HDAC1 overexpression, as shown by Western blot. Thus, reporter assay further revealed that HDAC1 suppresses KLF5-dependent promoter activation in a dose-dependent manner without affecting the expression level of KLF5.

HDAC1 Suppresses Phorbol Ester-stimulated Activation of PDGF-A Chain—Next, to see the cellular implications of the negative regulatory effect of HDAC1 on KLF5-dependent transcription, we examined whether HDAC1 can suppress cooperative regulation of PDGF-A chain by KLF5 and phorbol ester (PMA) stimulation. PMA, a model agonist to investigate inducible pathophysiological stimulation, is known to transcriptionally activate KLF5, which in turn up-regulates its endogenous downstream gene, PDGF-A chain (Fig. 3*A*) (24). We assessed whether overexpression of HDAC1 can inhibit PMA-stimulated activation of PDGF-A chain in rat smooth muscle cells. Under conditions in which KLF5 was induced by PMA and in which overexpression of HDAC1 had no effect on KLF5 expression, HDAC1 showed partial but reproducible suppression of PDGF-A chain promoter activation in a sustained manner (Fig. 3*B*). We further used adenoviral-mediated forced expression of HDAC1 to confirm attenuation of PDGF-A chain expression. As shown in Fig. 3*C*, adenoviral transfer of HDAC1 significantly inhibited PMA-stimulated expression of PDGF-A chain. Therefore, HDAC1 suppresses promoter activation of an endogenous downstream gene of KLF5 as stimulated by a model agonist of pathophysiological stimulation.

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HDAC1 Binds to the First Zinc Finger of KLF5, the Same Region that Interacts with p300—To further dissect the mechanism of interaction between KLF5 and HDAC1, we investigated which region of KLF5-DBD interacts with HDAC1. KLF5-DBD has three zinc finger motifs, and we compared their individual binding affinities with HDAC1 using GST-tagged constructs containing each of the three zinc finger peptides (Fig. 4*A*). Interestingly, GST pulldown assay revealed that the first zinc finger of KLF5 is the only peptide that HDAC1 can bind to, despite their apparent similarities (Fig. 4*B*).

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Furthermore, this is intriguing because we previously showed that p300, which coactivates and acetylates KLF5, interacts with KLF5-DBD and that the first zinc finger of KLF5 is the only region that p300 acetylates (22). These findings led us to speculate that the first zinc finger of KLF5 may be the region of interaction for p300 and that HDAC1 and p300 interact with KLF5 through the same region. To confirm this, GST pulldown assay was done with the FLAG-tagged p300-HAT domain (Fig. 4*C*). As expected, the FLAG-tagged p300-HAT domain bound only to the first zinc finger of KLF5 (*lane 1*). Thus, HDAC1 and p300 bind to KLF5 through the same region, the first zinc finger of KLF5, which underscores a specific functional role of this region.

HDAC1 Competes with p300 for Binding to the First Zinc Finger of KLF5—We further asked whether HDAC1 competes with p300 for interaction with the first zinc finger of KLF5 (Fig. 5). Preincubation of HDAC1 with the first zinc finger of KLF5 inhibited the subsequent binding of the p300-HAT domain to KLF5 in a dose-dependent manner (*lanes 3–5*). In contrast, order-of-addition experiments showed that addition of HDAC1 after incubation of the first zinc finger of KLF5 with the p300-HAT domain showed no or marginal inhibitory effect on their binding affinity (*lanes 7–9*), indicating the HDAC1 can inhibit, but not displace, interaction of p300 with this region. HDAC1 and p300 therefore show competitive binding to the first zinc finger of KLF5.

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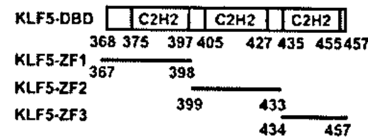

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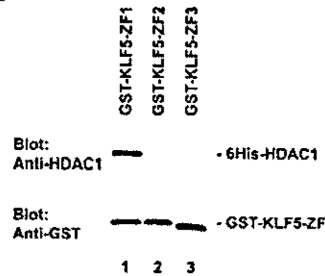

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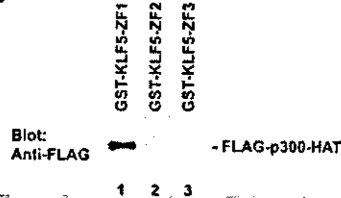

FIG. 4. **In vitro binding of HDAC1 and p300 to KLF5 through its first zinc finger.** *A*, schematic representation of GST-tagged zinc finger peptides of KLF5. GST-tagged zinc finger peptides of KLF5 cover each of the zinc fingers, respectively, from the amino terminus. *B*, hexahistidine-tagged HDAC1 was pulled down with the GST-tagged first zinc finger of KLF5 (*lane 1, upper*), but not with the GST-tagged second or third zinc finger of KLF5 (*lanes 2* and *3*). Results of immunoblotting with anti-GST antibody (*lower*) confirmed that the same amounts of GST-tagged peptides were used. *C*, the FLAG-tagged p300-HAT domain was pulled down with the GST-tagged first zinc finger of KLF5 (*lane 1*), but not with the GST-tagged second or third zinc finger (*lanes 2* and *3*).

DISCUSSION

Through studies to understand the regulatory mechanisms underlying KLF5, we have shown here a novel regulatory action of deacetylase, that is, that the deacetylase HDAC1 interacts directly with the transcription factor KLF5 and thus impairs the DNA binding affinity and promoter activation conferred by the latter. We further showed that HDAC1 and acetylase p300, which we previously showed coactivates and acetylates KLF5 (22), interact directly with KLF5 through the same region, the first zinc finger of KLF5, and that HDAC1 can inhibit interaction of p300 to KLF5 (Fig. 6). The deacetylase therefore negatively regulates transcription not only by its catalytic activity but also by inhibiting the activities of its interacting transcription factor (DNA binding activity, promoter activation) as well as interaction with coactivator/acetylase (p300).

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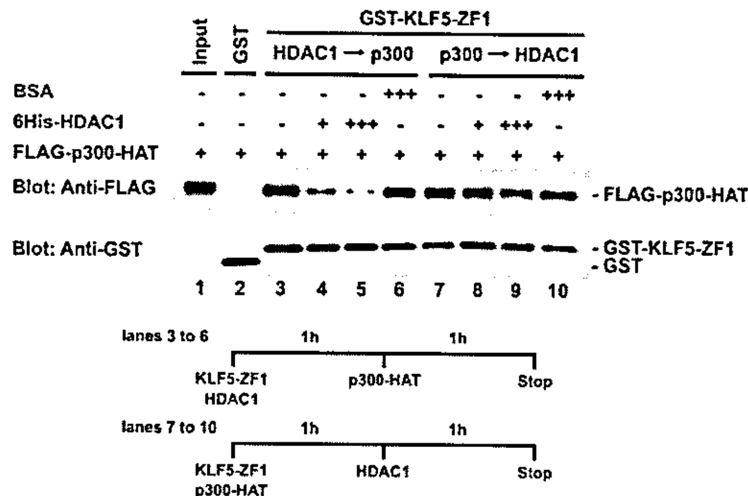

FIG. 5. **In vitro competition of HDAC1 and p300 for binding to KLF5.** *In vitro* binding assay was performed using 0.75 μg of the immobilized GST-tagged first zinc finger of KLF5 (*lanes 3-10*) or GST protein alone (*lane 2*). In *lanes 3-6*, 1.5 μg of the FLAG-tagged p300-HAT domain was added 1 h after the GST-tagged first zinc finger of KLF5 and 0.5 (+) or 1.5 (+++) μg of hexahistidine-tagged HDAC1 or 1.5 (+++) μg of bovine serum albumin as a control. The reaction was stopped 1 h after the FLAG-tagged p300-HAT domain was added. In *lanes 7-10*, the GST-tagged first zinc finger of KLF5 and 1.5 μg of the FLAG-tagged p300-HAT domain were preincubated 1 h before 0.5 (+) or 1.5 (+++) μg of hexahistidine-tagged HDAC1 or 1.5 (+++) μg of bovine serum albumin was added. *Lane 1* is input (2.5%). Under conditions in which the FLAG-tagged p300-HAT domain bound to the GST-tagged first zinc finger of KLF5 (*lane 3*), but not to GST alone (*lane 2*), preincubation of HDAC1 inhibited the binding of the p300-HAT domain to the first zinc finger of KLF5 in a dose-dependent manner (*lanes 3-5*). Preincubation of bovine serum albumin in the same amount as HDAC1 showed no effect on the binding affinity between the first zinc finger of KLF5 and p300-HAT domain (*lane 6*), confirming that this is a specific effect of HDAC1. Adding HDAC1 after preincubation of the first zinc finger of KLF5 and the p300-HAT domain showed no or marginal inhibitory effect on their binding (*lanes 7-9*), indicating the importance of order of addition. Results of immunoblotting with anti-GST antibody (*lower*) confirmed that the amounts of applied GST fusion proteins were the same.

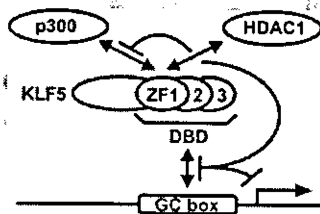

FIG. 6. **Schematic representation of results.** HDAC1 interacts directly with the first zinc finger of KLF5, impairs its DNA binding affinity, and negatively regulates KLF5-dependent promoter activation. In addition, HDAC1 can exert its negative regulatory function by interfering with the binding of p300 to KLF5.

Insight into Mechanism of Negative Regulatory Effect on KLF5 by HDAC1—Though HDAC1 is a widely recognized corepressor (8–10, 31, 32), the mechanisms of direct effects of HDAC1 on the DNA binding affinity of a transcription factor are poorly known. Our results of gel shift DNA binding assay (Fig. 2A) clearly indicated that the binding of HDAC1 to KLF5 itself is sufficient to inhibit the DNA binding affinity of KLF5 *in vitro*, which is a hitherto unknown property of HDAC1, presumably because of interference and/or induced conformational change of KLF5. Inhibition of promoter activation was likely a secondary result of this impaired DNA binding activity.

Additionally, the results of competition assay between deacetylase and acetylase (Fig. 5) led us to propose another regulatory mechanism of the deacetylase HDAC1. HDAC1 can suppress KLF5 promoter activation by inhibiting interaction with p300. The plausible underlying mechanism is interference and/or induced conformational change of KLF5, although the former is more likely given that the interaction surface is a single zinc finger motif whose surface can likely only be spatially occupied directly by a single protein. This mechanism is indeed rational given the opposing activities of deacetylase and acetylase, that is, that they not only counter-regulate each other catalytically but also non-catalytically (interaction). Although a recent study suggested competition between CREB-binding protein and HDAC1 for binding to a transcription factor using the lysate of cells overexpressing HDAC1 (30), this is the first description of direct competition for binding on a specific interaction region by deacetylase and acetylase.

Regulatory Pathway Targeted on the DBD and Different Functions among Zinc Finger Motifs—The DBD has been classically thought to play a passive role, only anchoring the protein to its binding site of DNA, whereas the activation and regulatory domain of transcription factors plays an important role in regulatory interactions (33, 34). However, our recent studies as well as those of others revealed that the DBD, particularly that of the Sp/KLF family, not only binds DNA but also mediates important protein-protein interaction as well as chemical modifications and is thus an important target for regulatory interaction (11, 19, 21, 22, 35). Our present findings, which show that the DBD is the target of regulation by deacetylase as well as its interplay with acetylase, further support a key role of the DBD in regulation.

Additionally, our results provide insight into the individual functions of zinc finger motifs in a multiple zinc finger protein. Members of the Sp/KLF family have a highly conserved DBD consisting of three C_2H_2-type zinc fingers (12–19). Despite their apparently similar structures, we clearly showed that the first zinc finger of KLF5 has a specific role in mediating protein-protein interaction. We speculate that, at least for the Sp/KLF family of zinc finger factors given their similarities, the first zinc finger of other Sp/KLF family members may also be an interface for protein-protein interaction.

Biological Implication of Interaction between KLF5 and HDAC1—An important question that remains to be addressed is the understanding of the coordinated regulation of transcription factor by multiple cofactors in the cell to affect gene expression. For the transcription factor KLF5, one possible mechanism is regulation of interaction as conferred through a signaling-coupled modification similar to that shown in a re-

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cent study in which phosphorylation of the nuclear factor-κB p65 subunit by protein kinase A determines whether it associates with either CREB-binding protein/p300 or HDAC1 (36). Another possibility is regulation by the relative expression levels of these cofactors. We previously showed reciprocal increase of KLF5 and decrease of SET protein, a negative regulatory cofactor of KLF5, after phorbol ester stimulation, suggesting their correlation and possible amplification of KLF5 actions (22). Further, p300 is similarly induced by phorbol ester stimulation (37). In contrast to these factors that show regulated expression levels, our studies on HDAC1 show that its expression levels are not regulated by pathological stimulation (phorbol ester) in the examined cell lines and also that it shows constitutive expression in pathological tissues (ubiquitous distribution similar to neointima and media in balloon injury model) (data not shown). Thus, whereas p300 and SET are inducible cofactors, HDAC1 is a relatively ubiquitous and constitutive factor, presumably because of its high basal expression level and/or possible negative feedback regulation as suggested by the findings that protein expression of endogenous HDACs 1, 2, and 3 were significantly reduced in HDAC1-overexpressing cells (38). Whereas inducible cofactors like p300 and SET control transcriptional activity stimulated with pathological stimuli, the deacetylase HDAC1, with ubiquitous and constitutive properties, may prevent uncontrolled and inappropriate promoter activation as a baseline inhibitor.

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In conclusion, we have shown that KLF5 is negatively regulated by the deacetylase HDAC1 through direct effects on the transcription factor as well as by inhibiting interaction with the coactivator p300. These findings suggest a novel role and mechanism for the deacetylase HDAC1 in negative regulation of transcription.

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