integrated into the yeast genome to better reflect cellular conditions. Although a mammalian environment was not used and as there was limitation by overexpression of factors, we believed that the yeast environment would be better reflective of the eukaryotic intracellular environment as compared to the traditional southwestern filter hybridization or affinity chromatography techniques. Our studies interestingly resulted in the isolation of KLF6/GBF, a novel KLF factor which shows similar GC-rich binding properties as Sp1 (Suzuki et al, 1998). This was the only Sp/KLF factor identified in our screen thus suggesting the possibility that distinct factors may bind GC-rich sites in the cellular environment. Therefore, at present, while biochemical studies do show that Sp/KLF factors bind similar GC-rich sites, the actual intracellular environment especially in the context of chromatin may allow for preferential binding of different factors. This issue on effect of intracellular context remains to be further explored.

IV. Regulation through chemical modifications and/or differential protein-protein interactions

Regulation through differential protein-protein interactions and/or chemical modifications (e.g. acetylation) are further likely to contribute to the differential functions of Sp/KLF factors. We have focused our attention on the role of the DNA-binding domain (DBD) because it is most reasonable, if not optimal, for regulating DNA-associated events such as promoter access and topological changes given its ability and activity to bind DNA (**Figure 1**). Amino acid differences are evident in the zinc finger DNA-binding domain of Sp/KLF factors, although there is extensive conservation overall. Aside from the likelihood of affecting DNA-binding properties, these differences in primary structure and quite possibly in the overall conformation of the folded protein may have a profound effect on post-translational modifications in addition to protein-protein interactions.

A. Regulation by chemical modification

Focusing on the regulatory role of acetylation on Sp/KLF transcription factors, we have shown differential regulation through interaction and acetylation on the DNA-binding domain by the coactivator/acetylase p300 (Suzuki et al, 2000). Acetylation is an important nuclear regulatory signal which regulates transcriptional processes, importantly with biological implications which include regulation of development, differentiation and oncogenesis (Brownell and Allis, 1996; Cheung et al, 2000; Nakatani 2001; Freiman and Tjian, 2003) which closely resembles the roles of Sp/KLF family members.

We thought that the Sp/KLF-factors might be differently regulated by acetylation and showed that the coactivator/acetylase p300 but not the MYST-type acetylase Tip60 specifically interacts and acetylates Sp1 but not KLF6 through the zinc finger DNA-binding domain, and further that DNA binding inhibits this interaction and acetylation (Suzuki et al, 2000). Interaction of p300 acetyltransferase region and the Sp1 zinc finger DNA-binding domain stimulates the DNA-binding activity of the latter, while acetylation *per se* has only marginal effects. While much is known of acetylation in general, its regulation and implications are still poorly understood.

A similar mechanism has been shown for KLF13/FKLF2. KLF13 is acetylated both by PCAF and CBP, as well as interact through the zinc finger DNA-binding domain of KLF13. The acetyltransferase regions of PCAF and CBP stimulate KLF13 binding to its cognate DNA-binding site. These findings suggest and further support that acetyltransferase interaction with the zinc finger DNA-binding domain of at least KLFs affects DNA-binding activity (Song et al, 2002). Acetylation of KLF13 by CBP has been further shown to inhibit KLF13 DNA-binding activity, and that PCAF

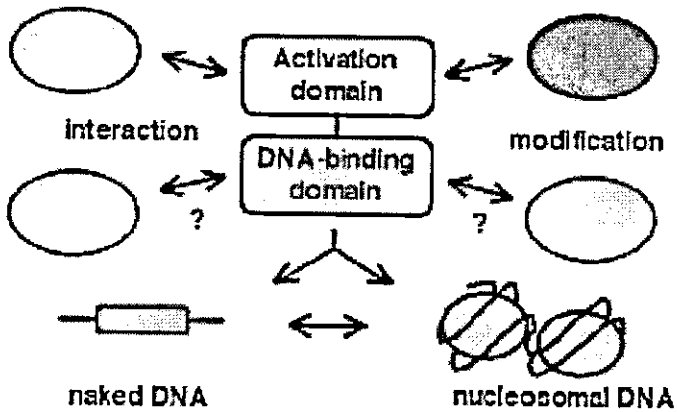

Figure 1. Regulation of DNA-binding transcription factors in general. Note that there are modular activation and DNA-binding domains. Regulation through interaction and modification of DNA-binding domains is poorly understood. We have focused our studies on the role of the zinc finger DNA-binding domain for Sp/KLF factors. The active role of the DNA-binding domain is suggested in DNA-binding processes not only for naked DNA but also in the context of nucleosomal DNA.

blocks CBP acetylation and its disruption of DNA binding (Song et al, 2003).

Our findings on Sp1 and further those on KLF13 provide an attractive model of promoter access by cooperative action of DNA-binding activator with coactivator/acetyltransferase. Important here is that there is a concerted interaction between these two factors which facilitates promoter access (**Figure 2**). The regulatory and activation domains likely play an additional role. This is in contrast to the extant model of recruitment of coactivator/acetyltransferase to the DNA-binding activator involving specific binding by the latter to its cognate binding site with subsequent recruitment of the former to the promoter (Ogryzko et al, 1996). Our interpretation and model explains one of the limitations of this prior model on how the DNA-binding activator accesses its cognate site or how interaction with coactivator/acetyltransferases affects this reaction which were issues which remained unclear.

Other Sp/KLF factors are also acetylated in the zinc finger DNA-binding domain. EKLF/KLF1 is acetylated by p300 and its homologue CBP at two lysine residues, one residing in the DNA-binding zinc finger domain and the other in the transactivation domain. The mutation of the zinc finger acetylated residue does not affect DNA-binding activity and the individual role of its acetylation is unclear, but mutation of the transactivation domain lysine residue results in decreased transactivation and acetylation collectively increased affinity for the SWI/SNF chromatin remodeling factors (Zhang and Bieker, 1998; Zhang et al, 2001). Sp3 is acetylated in its inhibitory domain lying between the glutamine-rich activation domain and zinc finger DNA-binding domain. Acetylation of this lysine residue regulates transcriptional activity (Braun et al, 2001).

There are other modifications such as phosphorylation, methylation, glycosylation, ubiquitination, and SUMOylation (SUMO; small ubiquitin-related modifier) among others. From the perspective of the DNA-binding domain, cell-cycle dependent phosphorylation by a putative kinase has been reported for Sp1 (Black et al, 1999). Casein kinase II also phosphorylates the second zinc finger of Sp1 resulting in a reduction in DNA-binding activity (Armstrong et al, 1997). PKC-zeta also binds and phosphorylates the zinc finger region of Sp1 which is suggested to result in transcriptional activation (Pal et al, 1998). Sp1 is also glycosylated (Jackson and Tjian, 1988). Much of our knowledge on the regulatory mechanisms of the Sp/KLF factors at present are centered on Sp1 as it was one of the first eukaryotic DNA-binding regulatory transcription factors ever identified and serves as an excellent molecular model to dissect and understand mechanisms of transcriptional activation.

A recent report has further shown that Sp3 is SUMOylated at the same residue that is acetylated (Sapetschnig et al, 2002). While we still have much to learn on post-transcriptional modifications, cross-talk and co-regulation of signaling pathways not only for lysine modifications but also for coupling of pathways such as a phosphorylation-acetylation cascade will likely show the complex nature of regulation by chemical modifications.

B. Regulation by protein-protein interaction

The zinc finger DBD motif, while binding DNA, is also an interface for protein-protein interaction such as homo- and hetero-dimerization in addition to protein-protein interactions with heterologous proteins (MacKay and Crossley 1998)

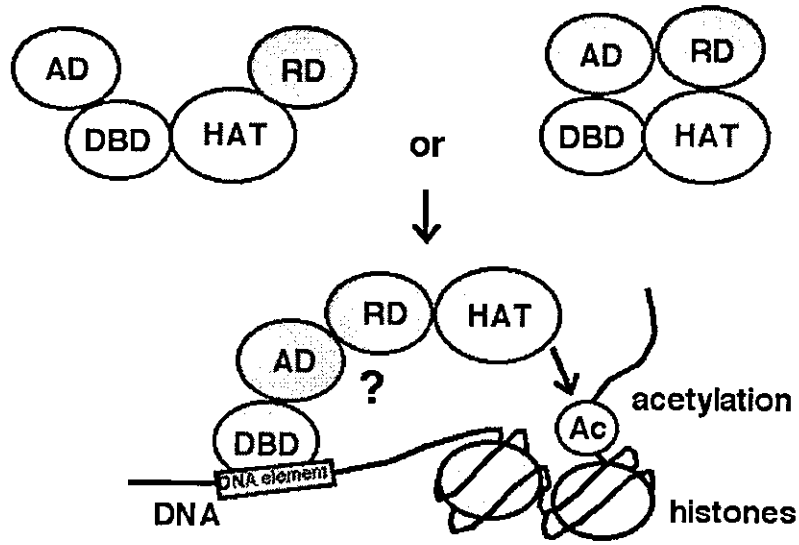

Figure 2. Model of promoter access as mediated by interaction betweeen the zinc finger DNA-binding domain (DBD) of the Sp/KLF transcription factor and catalytic region of acetyltransferase (HAT) (e.g. p300 for Sp1 and PCAF for KLF13). Interaction between the activation domain (AD) of the DNA-binding factor and regulatory domain (RD) of the acetyltransferase is unknown but is likely to play an additional role to retain the DNA-binding factor and HAT on the promoter.

which results in specific regulation. In general, while much research on transcription factors has focused on the role of the activation domain to mediate regulation (e.g. activation, repression, ligand-dependent modulation, etc.) (Horikoshi et al, 1988a,b; Roeder, 1996; Lemon and Tjian, 2000), functions of the DBD other than its DNA-binding activity have received little attention (Wagner and Green, 1994). Here the discussion will focus on the fact that numerous chromatin remodeling factors and other factors which act on transcription at the level of higher-order DNA interact and regulate through the zinc finger DBD (**Figure 1**).

As mentioned in the above section on acetylation, Sp1 and KLF13 catalytically interact with acetyltransferase (e.g. p300 with Sp1, and PCAF and CBP with KLF13). Importantly, they also stably interact through the zinc finger DBD which results in stimulation of DNA-binding activity of the DNA-binding transcription factor. These findings allow for the model of promoter access as shown in **Figure 1**. While we assume *a priori* that DNA-binding factors recruit acetyltransferase and other chromatin remodeling factors to DNA after they are pre-bound to DNA, these results suggest that they in fact show interaction in solution and that DNA binding is inhibitory to interaction. This suggests that interaction promotes access of the DNA-binding factor to DNA but is released once bound to DNA.

Deacetylases also bind Sp/KLF factors through the zinc finger DNA-binding domain. Both Sp1 and EKLF/KLF1 have been shown to associate with HDAC1. Both Sp1 and EKLF bind HDAC1 through the zinc finger DNA-binding domain. Interaction of Sp1 and HDAC1 is thought to be repressive on Sp1 transcription because coexpression of E2F1, which interferes with HDAC1 binding to Sp1, abolishes Sp1-mediated transcriptional repression (Doetzlhofer et al, 1999). EKLF also binds HDAC1 through its zinc finger DNA-binding domain which results in transcriptional regulation (Chen and Bieker, 2001). From within the HDAC-associated corepressor complex, sin3A also binds EKLF through the zinc finger DNA-binding domain.

Further, the zinc finger DNA-binding domains of Sp1 and that of EKLF interact with the ATP-dependent nucleosome remodeling enzyme Swi/Snf (Kadam et al, 2000). Two SWI/SNF subunits (BRG1 and BAF155) are required for targeted chromatin remodeling and transcriptional activation by EKLF *in vitro*. Remodeling is achieved with only the BRG1-BAF155 minimal complex and the EKLF zinc finger DBD, whereas transcription additionally requires an activation domain.

We have recently shown that the zinc finger DNA-binding domain of Sp1 mediates interaction with the histone chaperone TAF-I (template activating factor)(Suzuki et al, 2003). Interaction is specific, as different subsets of DNA-binding factors do not bind TAF-I and as other ATP-independent nucleosome remodeling enzymes do not bind Sp1. TAF-I negatively regulates Sp1 activity by inhibiting DNA binding, and likely as a consequence of this, regulates Sp1-mediated promoter activation.

Based on these findings, the Sp1 DBD interacts with all three major chromatin-related factors consisting of chemical modification enzymes (e.g. acetyltransferase p300), ATP-dependent nucleosome assembly factor (e.g. SWI/SNF) and histone chaperone (e.g. TAF-I)(**Figure 3**). This finding is of particular interest because it implicates the DBD to play a likely role in mediating transcriptional regulatory processes in eukaryotes at the chromatin level. Although interaction with individual chromatin remodeling factors has been documented for numerous proteins, as interaction with all three chromatin remodeling factors has only been reported previously for histones, the DNA-transcription factor, and importantly its DNA-binding domain, may, therefore, represent a vital target for chromatin-related transcriptional processes

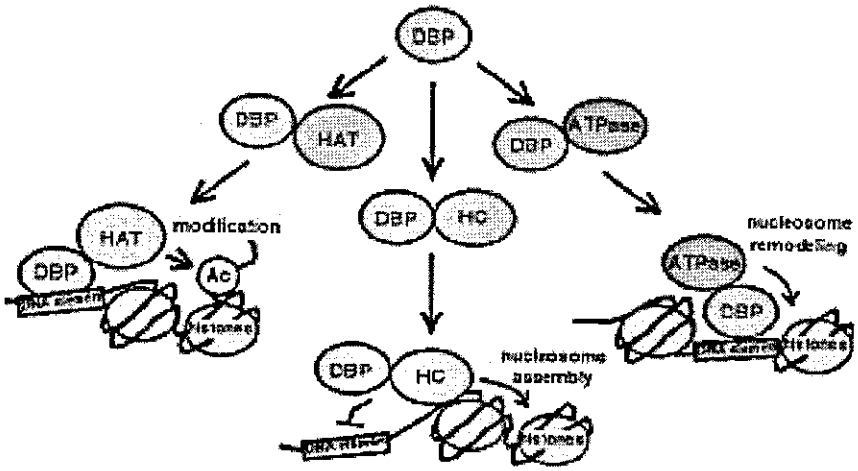

Figure 3. Model (deducted from Sp1 interactions) explaining how the DNA-binding domain of the transcription factor (DBP) interacts with all three classes of chromatin remodeling enzymes which has only been known for histones. Interactions include the chemical modification enzyme acetyltransferase (HAT)(Suzuki et al, 2000), the ATP-independent nucleosome remodeling enzyme histone chaperone (HC)(Suzuki et al, 2003), and the ATP-dependent nucleosome remodeling enzyme (ATPase)(Kadam et al, 2000).

through cooperative interaction with chromatin-remodeling factors.

The zinc finger transcription factors are the most widely evolved family of transcription factors in eukaryotes. Given that this biological diversification was coupled with the evolution of nuclear structure in eukaryotes, it is conceivable that regulation of chromatin is a necessary process to further allow for efficient use and access of factors to the tightly packaged DNA genetic information. Important mechanisms of transcriptional regulation in the context of chromatin have been shown as discussed in this review. The mechanism that the DBD mediates important regulation of the DNA-binding transcription factors through interaction and modification with chromatin factors can certainly be generalized to DNA-binding transcription factors other than the described zinc finger factors. Selectivity may be found between interaction of subsets for chromatin factors and DBD motifs. Furthermore, although only three types of chromatin factors were described including modification enzymes (e.g. acetyltransferase), ATP-independent (e.g. histone chaperones) and ATP-dependent (Swi/snf) factors, other chromatin factors are likely also to participate in regulatory interactions. Understanding the hierarchy and network of regulation among DNA-binding transcription factors and chromatin factors will likely play an important role in understanding the complexity of eukaryotic transcriptional regulation. As the Sp/KLF factors are a key family important in mammalian biological processes ranging from development, differentiation, to oncogenic processes, further studies aimed at understanding the temporospatial regulation of chromatin centered on Sp/KLF factors will surely advance our understanding of eukaryotic transcriptional mechanisms of chromatin activation in a biological context. Future gene therapy approaches could use strategies of expressing such activator, modifier or factor genes individually or in complexed form to facilitate regulation of therapeutically important genes at the physiologically relevant chromatin DNA level.

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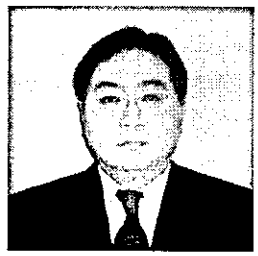

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Functional Interaction of the DNA-binding Transcription Factor Sp1 through Its DNA-binding Domain with the Histone Chaperone TAF-I*

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Transcription involves molecular interactions between general and regulatory transcription factors with further regulation by protein-protein interactions (*e.g.* transcriptional cofactors). Here we describe functional interaction between DNA-binding transcription factor and histone chaperone. Affinity purification of factors interacting with the DNA-binding domain of the transcription factor Sp1 showed Sp1 to interact with the histone chaperone TAF-I, both α and β isoforms. This interaction was specific as Sp1 did not interact with another histone chaperone CIA nor did other tested DNA-binding regulatory factors (MyoD, NFκB, p53) interact with TAF-I. Interaction of Sp1 and TAF-I occurs both *in vitro* and *in vivo*. Interaction with TAF-I results in inhibition of DNA-binding, and also likely as a result of such, inhibition of promoter activation by Sp1. Collectively, we describe interaction between DNA-binding transcription factor and histone chaperone which results in negative regulation of the former. This novel regulatory interaction advances our understanding of the mechanisms of eukaryotic transcription through DNA-binding regulatory transcription factors by protein-protein interactions, and also shows the DNA-binding domain to mediate important regulatory interactions.

Transcription involves a collective milieu of molecular interactions between general transcription initiation factors, regulatory DNA-binding transcription factors, and regulators that act through protein-protein interactions (*e.g.* transcriptional cofactors) (1, 2). Because of our increasing knowledge of the role of protein-protein interactions, we now know that single or complexed interacting factor(s) play an important role in regulating transcription. Regulators that function through protein-protein interactions are comprised of a diverse range of factors with varying activities and effects on transcription ranging from regulation of gene- and promoter-specific transcription (*e.g.* BOB-1/OBF-1/OCA-B, FOG-1) (3–6) to higher-ordered chromatin-related events alone or in complexed form (*e.g.* ARC, DRIP, TRAP) (7–9). Deciphering the regulatory interactions as mediated by protein-protein interactions is a prerequisite for our better understanding of the complex regulation of transcription.

The DNA-binding regulatory transcription factor that plays a central role in dictating specific transcription (*e.g.* cell cycle, cell differentiation) consists of an activation/regulatory domain, which interacts with the basal transcription machinery and other protein-protein interactors (*e.g.* transcriptional cofactors), and the DNA-binding domain (DBD),[1] which specifies the target promoter (10–16). The DBD has been generally thought to possess a passive role to tether the activation/regulatory domain to the transcription machinery on the promoter, and functions other than its DNA binding activity have received little attention (17).

We have, however, focused on the role of the DBD as a target of regulation, and have shown in the past for Sp1 (18), the best studied and founding factor of the Sp/KLF (Sp1 and Krüppel-like factor) family (19–21) of C_2H_2-type zinc finger transcription factors, to show differential regulation by protein-protein interaction and chemical modification through the DBD (22). Much research on the activation mechanisms through Sp1 has focused on interaction through the activation domain (*e.g.* transcription machinery, chromatin factors) (8, 23). Recently, we have shown that the Sp1 DBD interacts with acetyltransferase (22), and others have shown interaction of the Sp1 DBD with other factors (*e.g.* cell cycle regulatory factor E2F, Refs. 24 and 25; deacetylase HDAC1, Ref. 26; ATP-dependent nucleosomal remodeling enzyme SWI/SNF, Ref. 27; as well as other zinc finger transcription factors including Krüppel-like factors and nuclear receptors, Ref. 28). The DBD of Sp1, therefore, mediates protein-protein regulation important for transcription. To further understand functional regulation of Sp1 through its

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[1] The abbreviations used are: DBD, DNA-binding domain; GST; glutathione *S*-transferase; CBB, Coomassie Brilliant Blue; HA, hemagglutinin; TOF-MS, time-of-flight mass spectrometry; MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight.

DBD, we have in the present study affinity-purified interacting factors and analyzed their functional effects on Sp1.

Here we show interaction of Sp1 through its DBD with the histone chaperone TAF-I. Interaction between these proteins is specific. This interaction inhibits Sp1 DNA binding, and also likely as a result of such, inhibits promoter activation by Sp1; thus TAF-I functions as a negative regulator of Sp1. This novel regulatory interaction between DNA-binding regulatory transcription factor and histone chaperone adds to our understanding of the mechanisms of how DNA-binding regulatory transcription factors are regulated by protein-protein interactions.

EXPERIMENTAL PROCEDURES

Preparation of Recombinant Proteins—The hexahistidine-tagged and GST-fusion Sp1 DBD constructs and purification have been described (22). The purification protocol for the hexahistidine-tagged protein was slightly modified. Bacterial supernatant was applied to HiTrap Heparin column (Amersham Biosciences), washed with buffer A (20 mM Tris-HCl, pH 7.9, 0.2 M KCl, 20% glycerol, 5 mM 2-mercaptoethanol, 100 μM $ZnSO_4$, 1 mM phenylmethylsulfonyl fluoride, 0.5 μg/ml leupeptin, and 1 μg/ml pepstatin), and then eluted with a linear salt gradient of 0.2–2 M KCl. Pooled fractions were next applied to HisTrap column (Amersham Biosciences), washed with buffer A containing 500 mM KCl and 20 mM imidazole, and then eluted with a stepwise gradient up to 0.5 M imidazole. Pooled fractions were dialyzed against buffer B (25 mM HEPES-KOH, pH 7.9, 10% glycerol, 150 mM KCl, 100 μM $ZnSO_4$, 5 mM 2-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride, 0.5 μg/ml leupeptin, and 1 μg/ml pepstatin). For GST fusion proteins, glutathione-Sepharose resin (Amersham Biosciences) was added to the lysate, incubated, followed by repeated washes with Buffer A, and then eluted using Buffer A containing 20 mM reduced glutathione (Wako). The GST fusion full-length Sp1 construct was a gift from Dr. Hans Rotheneder.

TAF-Iα, β, and mutant constructs (gifts of Dr. Kyosuke Nagata) were expressed in bacteria and purified using Probond resin (Invitrogen) with buffer C (20 mM Tris-HCl, pH 7.4, 10% glycerol, 0.5 M KCl, 50 mM β-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride, 2 μg/ml leupeptin, and 1 μg/ml pepstatin A). Washes were done with buffer C containing 20 mM imidazole, and then eluted with buffer C containing 0.2 M imidazole. All protein procedures were done at 4 °C.

Preparation of Nuclear Extract—Nuclear extract from HeLa S3 cells was prepared as described (29). Briefly, cells were lysed by glass Dounce homogenizer in Dignam's buffer A, then centrifuged for 10 min at 700 × *g* to pellet nuclei. The pellet was suspended in Dignam's buffer C, rotated for 60 min to extract nuclear protein, and then centrifuged for 15 min at 18,000 × *g*. Buffer B was used for final dialysis. Dialysate was centrifuged for 15 min at 18,000 × *g*, and the supernatant was used as nuclear extract.

Isolation of Factors Interacting with Sp1 DNA-binding Domain—50 μg of hexahistidine-tagged Sp1 DBD was bound to 50 μl of Probond resin (Invitrogen), and after washing, incubated with 725 μg of HeLa S3 nuclear extract. Proteins were eluted with buffer B containing 0.5 M imidazole. Samples were resolved by SDS-PAGE analysis and stained with Coomassie Brilliant Blue.

Protein Identification by MALDI/TOF Mass Spectrometry—Protein bands were excised, dehydrated with acetonitrile, and after removing acetonitrile, dried, and then in-gel digested with trypsin in 25 mM ammonium bicarbonate, pH 8. After soaking in 50% acetonitrile/5% tetrahydrofuran, the supernatant was collected, dried, and then reconstituted by adding 50% acetonitrile, 0.1% tetrahydrofuran and mixed with α-cyano-4-hydroxycinnamic acid. Mass spectrometry (MALDI-TOF MS; Voyager-DE STR, Applied Biosystems) was used to analyze proteins. Masses were calibrated internally with peptides derived from trypsin autolysis, and accuracy was within 10 ppm. Data base searches were done against a nonredundant protein sequence data base of NCBI using the Protein Prospector program V.3.2.1 (UCSF mass spectrometry facility).

Protein-Protein Interaction Assay—GST fusion proteins were immobilized to glutathione-Sepharose 4B resin (Amersham Biosciences) and incubated with hexahistidine-tagged proteins in buffer of 20 mM HEPES (pH 7.6 at 4 °C), 20% glycerol, 0.2 mM EDTA, 0.1% Triton X-100, 100 mM NaCl, and 100 μM $ZnSO_4$. Reactions were carried out at 4 °C for 2 h and washed twice with the same buffer. Bound proteins were resolved on a 10% SDS-PAGE gel, transferred to nitrocellulose membrane, immunoblotted with anti-HIS-probe (G-18) antibody (Santa Cruz Biotechnology), and then visualized by chemiluminescence (ECL, Amersham Biosciences). Commercially available recombinant proteins were used for p53, MyoD, and NFκB (Santa Cruz Biotechnology).

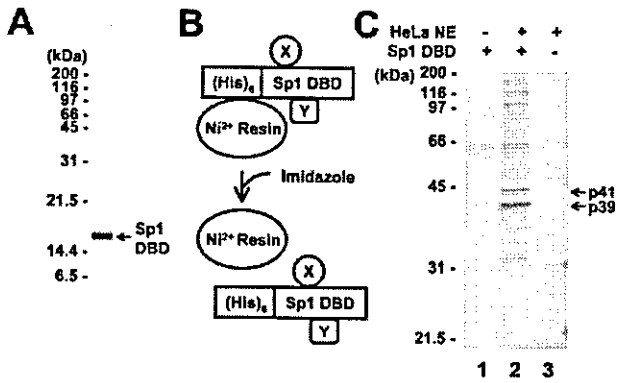

FIG. 1. **Affinity purification of proteins interacting with Sp1 DBD.** *A*, silver-stained gel of the Sp1 DNA-binding domain (*Sp1 DBD*) protein. Molecular weight markers are shown on the *left*. *B*, schematic illustration of the affinity purification procedure. Hexahistidine-tagged protein (*Sp1 DBD*) and interacting proteins are bound to nickel-charged resin and then eluted with imidazole. *C*, SDS-PAGE analysis of interacting proteins in HeLa S3 nuclear extract (HeLa NE) with the DNA-binding domain of Sp1 (Sp1 DBD). Binding reaction of Sp1 DBD and HeLa NE is shown in *lane 2*. *Lane 1* is Sp1 DBD alone and *lane 3* is HeLa NE alone reacted with resin. Molecular weight markers are shown on the *left*.

Co-immunoprecipitation Assay—1 μg of anti-Sp1 antibody (PEP-2, Santa Cruz Biotechnology) or control IgG (sc-2027, Santa Cruz Biotechnology) was bound to protein G-Sepharose (Amersham Biosciences) followed by incubation with 1 mg of HeLa S3 cell extract. After washing with radioimmune precipitation assay buffer (50 mM Tris-HCl, pH 7.9, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM phenylmethylsulfonyl fluoride, 0.5 μg/ml leupeptin, and 1 μg/ml pepstatin), immunoprecipitates were subjected to SDS-PAGE, and then immunoblotted using TAF-Iα (KM1715) and TAF-Iβ (KM1720) specific antibodies (gifts from Dr. Kyosuke Nagata).

Gel-shift DNA Binding Assay—Gel-shift DNA binding assays were done essentially as described (22). Briefly, the Sp1 consensus binding sequence (top 5′-ATTCGATCGGGGCGGGGCGAGC-3′) was used as the probe (underlined nucleotides GG were substituted by TT for mutant analysis). Annealed double-strand probe was gel-purified, and then kinase-labeled by using [γ-^{32}P]ATP (222 TBq(6000 Ci)/mmol, PerkinElmer Life Sciences) and T4 polynucleotide kinase (Stratagene). Unincorporated radiolabeled ATP was separated by NucTrap purification column (Stratagene). Specific activity of the radiolabeled probe was adjusted by adding cold double-stranded probe. Binding reactions were done in binding buffer of 20 mM HEPES (pH 7.6 at 4 °C), 20% glycerol, 0.2 mM EDTA, 0.1% Triton X-100, 100 mM NaCl, and 100 μM $ZnSO_4$. Recombinant proteins were incubated at 30 °C for 15 min in binding buffer prior to addition of 1.0×10^4 cpm (1 ng) of labeled probe, followed by further incubation at 30 °C for 15 min before separation on nondenaturing polyacrylamide gels. Gels were dried and analyzed using BAS 1500 (Fuji Photo Film).

Co-transfection Reporter Assay—25,000 HeLa cells were seeded and transfected 24 h later with the SV40 early promoter reporter (100 ng) and effector expression vectors by liposome-mediated transfer (Tfx-20; Promega). Full-length human Sp1 cDNA (a gift of Dr. James Kadonaga) was inserted into the expression vector pcDNA3 (Invitrogen). pCHA-TAF-Iα and β were gifts of Dr. Kyosuke Nagata. p53-Luc, which contains 15 copies of a p53-binding sequence upstream of the luciferase reporter and the expression vector pFC-p53, were purchased from Stratagene. The total effector DNA amount in transfection reactions was corrected to 1 μg by addition of empty vector. Cells were harvested after 48 h and assayed for luciferase activity (luciferase assay system, Promega). Luciferase activity was normalized against protein concentration of cell lysates. Protein expression was examined by Western blot using anti-HA antibody (Roche Diagnostics) for TAF-I, and anti-Sp1 antibody (PEP2, Santa Cruz Biotechnology) for Sp1. Error bars denote S.E.

RESULTS

The Histone Chaperone TAF-I Associates with Sp1 DNA-binding Domain—To isolate protein factors that interact with the DBD of Sp1, hexahistidine-tagged Sp1 DBD was bacterially

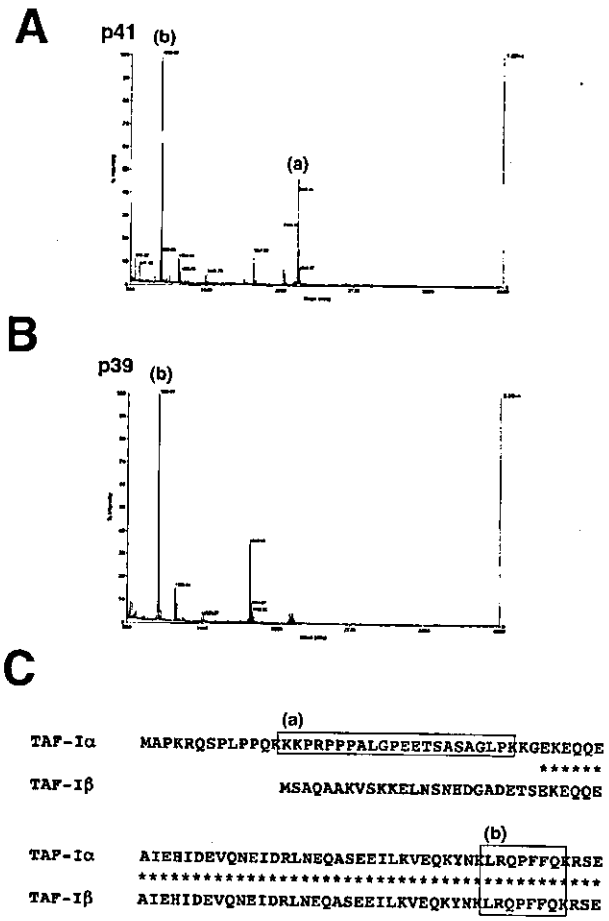

FIG. 2. **Identification of proteins interacting with Sp1 DBD.** *A*, TOF-MS analysis/charts of tryptic fragments of p41. *B*, TOF-MS analysis/charts of tryptic fragments of p39. *C*, primary amino acid sequences of TAF-Iα and β with identifying tryptic fragments (*a*) and (*b*)(*boxed*).

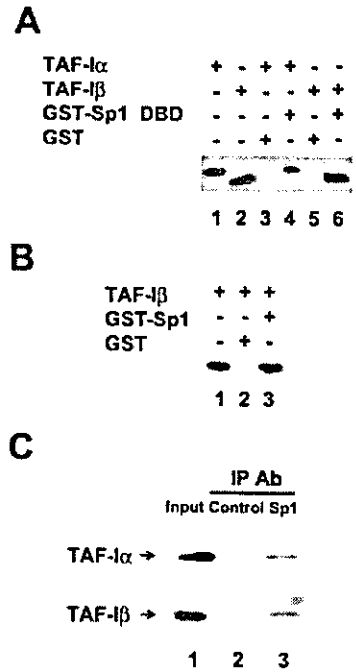

FIG. 3. **Interaction of Sp1 and TAF-I *in vitro* and *in vivo*.** *A*, *in vitro* binding assay for direct interaction between Sp1 DBD and TAF-I. Hexahistidine-tagged TAF-Iα and β were pulled-down with GST fusion Sp1-DBD (*lanes 4*, *6*; input, *lanes 1* and *2*) and GST alone (*lanes 3* and *5*). *B*, *in vitro* binding assay for direct interaction between full-length Sp1 and TAF-I. Hexahistidine-tagged TAF-I β was pulled-down with GST fusion full-length Sp1 (*lane 3*; input, *lane 1*) and GST alone (*lane 2*). *C*, co-immunoprecipitation assay for interaction of TAF-I and Sp1. Immunoprecipitation by antibody (IP Ab) against Sp1 (*lane 3*; input, *lane 1*) and control (normal IgG, *lane 2*) was followed by immunoblot with antibodies against TAF-Iα and β.

expressed and purified to near homogeneity using heparin and Ni^{2+}-chelating columns (Fig. 1*A*). HeLa S3 nuclear extract was applied to hexahistidine-tagged Sp1 DBD immobilized on Ni^{2+}-chelating resin, and then proteins bound to the peptide were eluted with imidazole (Fig. 1*B*).

Coomassie Brilliant Blue (CBB) staining of the bound proteins as resolved by SDS-gel electrophoresis revealed several bands spanning molecular mass from 30 to 200 kDa, which were seen only when the binding reaction between the Sp1 DBD and HeLa nuclear extract was done (Fig. 1*C*, *lane 2*) and not when Sp1 DBD alone (*lane 1*) or HeLa nuclear extract alone (*lane 3*) was reacted with resin. Two major bands, which were the most abundant on CBB staining were of apparent molecular masses of 41 and 39 kDa and are hereafter referred to as p41 and p39, respectively.

To identify the proteins, the p41 and p39 bands were excised, trypsinized, and then subjected to MALDI-TOF mass spectrometry. The proteins were identified by peptide mass fingerprinting with a computer search of the nonredundant protein sequence NCBI data base as available for the mammalian proteome, and then further subjected to post-source decay peptide sequencing. Surprisingly, mass spectra of p41 and p39 identified them to be the products of a single gene, template-activating factor-I (TAF-I) (Fig. 2, *A–C*) (30, 31). p41 and p39 were TAF-Iα and β, respectively. TAF-Iα and β are the alternative splicing products of a single gene and differ only in a short region of their amino-terminal ends (Fig. 2*C*) (31).

TAF-I was originally identified as a cellular factor which stimulates adenovirus core DNA replication (30, 31), and has been shown to be a histone chaperone which is a factor which can displace and/or assemble nucleosomal histones in an ATP-independent manner (32–35). TAF-Iβ is identical to the SET oncogene whose translocation has been implicated in leukemia (36).

Sp1 and TAF-I Interact in Vitro and in Vivo—To examine whether Sp1 DBD directly binds the TAF-I proteins, GST pull-down binding assays were done with recombinant TAF-Iα, β, and Sp1 DBD (Fig. 3*A*). Under the described binding conditions, both bacterially expressed hexahistidine-tagged TAF-Iα and β (*lanes 1* and *2*) bound to GST fusion Sp1-DBD (*lanes 4* and *6*) but not to GST alone (*lanes 3* and *5*) showing that Sp1 DBD directly binds both TAF-Iα and β. We have reproducibly seen that TAF-Iβ binds Sp1 DBD with a slightly higher affinity than TAF-Iα (*lanes 4* and *6*).

To next see if TAF-I also binds full-length Sp1, GST pull-down binding assays were done with recombinant TAF-Iβ and full-length Sp1 (Fig. 3*B*). Under the described binding conditions, bacterially expressed hexahistidine-tagged TAF-Iβ (*lane 1*) bound to GST fusion full-length Sp1 (*lane 3*) but not to GST alone (*lane 2*) showing that full-length Sp1 directly binds TAF-I. TAF-I can therefore directly bind Sp1.

To further see whether these proteins interact in the cell, immunoprecipitation was done using specific antibodies against TAF-Iα, β, and Sp1 (Fig. 3*C*). Sp1 was immunoprecipitated from HeLa cells followed by immunoblotting using TAF-Iα- and β-specific antibodies. TAF-Iα and β (*lane 1*) both bound Sp1 (*lane 3*) as compared with the control immunoprecipitation

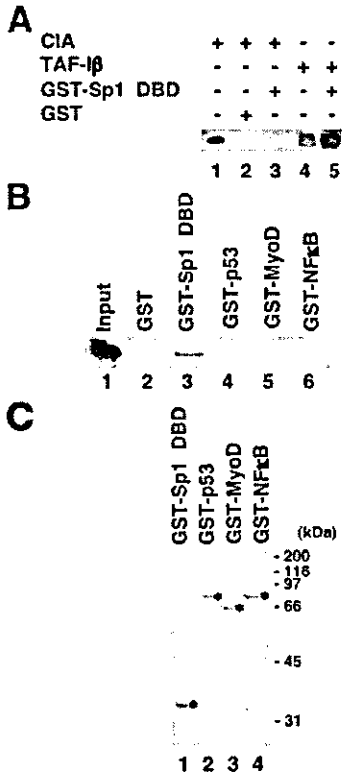

FIG. 4. **Specific interaction between Sp1 and TAF-I.** *A, in vitro* binding assay for interaction between Sp1 DBD and the ATP-independent nucleosome assembly factor CIA and TAF-Iβ. CIA was pulled-down with GST Sp1 DBD (*lane 3*; input, *lane 1*; GST alone, *lane 2*) under conditions in which TAF-Iβ bound to GST-fusion Sp1 DBD (*lane 5*; input, *lane 4*). *B, in vitro* binding assay for interaction between DNA-binding transcription factors and TAF-Iβ. Under conditions in which TAF-Iβ was pulled-down with GST Sp1 DBD (*lane 3*; input, *lane 1*; GST alone, *lane 2*), binding of TAF-Iβ to GST fusion proteins for p53, MyoD, and NFκB were similarly examined (*lanes 4–6*). *C*, CBB stain of the GST fusion proteins with an *asterisk* denoting respective protein.

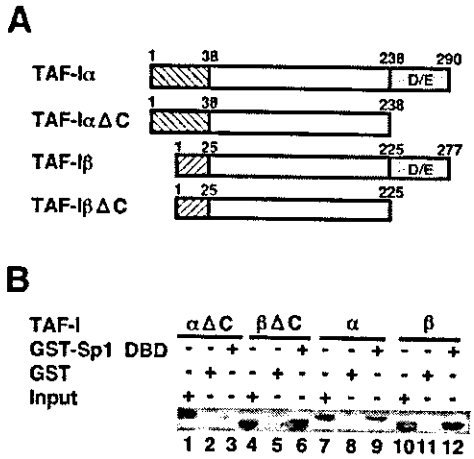

FIG. 5. **Effects of the acidic carboxyl-terminal regions of TAF-I on interaction with Sp1.** *A*, carboxyl-terminal mutants for TAF-Iα (TAF-IαΔC) and TAF-Iβ (TAF-IβΔC). Amino acid residues are shown in *numbers above*. *B, in vitro* binding assay for TAF-I acidic carboxyl-terminal region truncated proteins with Sp1 DBD. Carboxyl-terminal mutant for TAF-Iα (TAF-IαΔC) was pulled-down with GST fusion Sp1 DBD (*lane 3*; input, *lane 1*; GST alone, *lane 2*). Carboxyl-terminal mutant for TAF-Iβ (TAF-IβΔC) was similarly pulled-down with GST fusion Sp1-DBD (*lane 6*; input, *lane 4*; GST alone, *lane 5*). Simultaneous pull-down assays of wild-type TAF-Iα and β proteins are shown for comparison (*lanes 7–12*).

using normal IgG antibody (*lane 2*) confirming that these proteins do indeed interact in the cell. Collectively, TAF-I interacts with Sp1 *in vitro* and *in vivo*.

Specific Interaction between Sp1 and TAF-I—To see whether interaction of Sp1 DBD with TAF-I is specific or common for histone chaperones, a GST pull-down binding assay of Sp1 DBD was done with the histone chaperone CIA (also known as ASF1 in *Saccharomyces cerevisiae* and RCAF in *Drosophila* complexed with histones) (37–39) (Fig. 4*A*). Under conditions in which Sp1 DBD bound TAF-Iβ (*lanes 4* and *5*), Sp1 DBD did not bind CIA (*lanes 1–3*) showing that interaction between Sp1 DBD and TAF-I is specific. Therefore, interaction between Sp1 DBD and TAF-I is a specific property of these factors, and not a property common for histone chaperones.

To next see whether interaction with TAF-I is specific for Sp1 or common for DNA-binding factors, a GST pull-down binding assay with the DNA-binding transcription factors p53, MyoD, and NFκB was performed (Fig. 4*B*). Under conditions in which Sp1 DBD bound TAF-Iβ (*lanes 1–3*), TAF-Iβ did not bind p53, MyoD, or NFκB (*lanes 4–6*) thus showing that interaction between Sp1 DBD and TAF-I is specific. A CBB stain of the GST fusion proteins is shown (Fig. 4C). Therefore, interaction between Sp1 DBD and TAF-I is a specific property of these two factors.

Effects of the Acidic Carboxyl-terminal Regions of TAF-I in Interaction with Sp1—Histone chaperones including TAF-I have in common an acidic region (32, 34, 38, 40), but as Sp1 DBD did not bind CIA (Fig. 4*A*), we thought that the acidic region may not mediate interaction between TAF-I and Sp1 DBD. To address the effects of the acidic region of TAF-I on interaction with Sp1 DBD, GST pull-down binding assays using mutants of TAF-Iα and β, which lack the common acidic carboxyl-terminal end, TAF-IαΔC and TAF-IβΔC, respectively, were done (Fig. 5*A*). We have reproducibly seen that TAF-IαΔC binds Sp1 DBD with less affinity than TAF-IβΔC (Fig. 5*B*, *lanes 3* and *6*), which may suggest that the acidic carboxyl-terminal end participates in regulation of binding of TAF-Iα with Sp1 DBD greater than for TAF-Iβ. The acidic region of TAF-I may therefore be involved in modulation of binding affinity.

TAF-I Inhibits DNA Binding Activity of Sp1—As TAF-I binds the DNA-binding domain of Sp1, we examined the effects of TAF-I proteins on the DNA binding activity of Sp1 by gel mobility shift analysis (Fig. 6*A*). Under conditions in which TAF-Iα and β did not show DNA binding activity (*lanes 2* and *3*), incubation of TAF-I with Sp1 DBD resulted in inhibition of specific DNA binding activity of Sp1 DBD to its cognate binding sites (*lanes 4–6*) as shown by the dose-dependent decrease in intensity of the shifted DNA-protein complex for TAF-Iα (*lanes 7* and *8*) and TAF-Iβ (*lanes 9* and *10*). TAF-Iβ inhibited the DNA binding activity of Sp1 DBD to a slightly greater extent than TAF-Iα (*lanes 7* and *8 versus 9* and *10*). Under identical conditions, the acetyltransferase p300 stimulates DNA binding activity of Sp1 DBD and thus this is not a nonspecific effect of TAF-I (data not shown and Ref. 22). Therefore, TAF-I inhibited the DNA binding activity of Sp1 DBD.

TAF-I Inhibits Sp1-dependent Promoter Activation—As interaction of TAF-I proteins and Sp1 DBD inhibited the DNA binding activity of Sp1 DBD, we examined whether TAF-I would inhibit Sp1-dependent promoter activation as would be expected as a secondary result. Co-transfection analysis was performed with a luciferase-reporter construct harboring the SV40 early promoter which contains six Sp1 binding sites (Fig. 7*A*). As expected, under conditions in which transfection of an expression plasmid harboring full-length Sp1 showed dose-de-

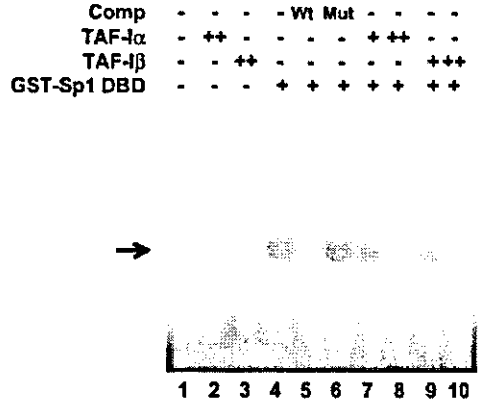

FIG. 6. **Effect of TAF-I on Sp1 DNA binding activity.** Gel-shift analysis for effects of TAF-I on DNA binding activity of Sp1. Sequence-specific DNA binding by Sp1 DBD to the consensus binding site probe (DNA-protein complex indicated by *arrow*; Sp1 DBD 1.9 pmol; *lane 1*, probe alone) as shown by competition experiments with 100-fold excess wild-type binding site (*Wt*, *lane 5*) and mutant site (*Mut*, *lane 6*) oligonucleotides is seen, and TAF-Iα and β do not bind DNA (*lanes 2* and *3*; 2.5 pmol) under these conditions. TAF-Iα (*lanes 7* and *8*; *lane 7*, 0.83 pmol; *lane 8*, 2.5 pmol) and TAF-Iβ (*lanes 9* and *10*; *lane 9*, 0.83 pmol; *lane 10*; 2.5 pmol) were reacted with Sp1 DBD and then subjected to gel-shift analysis.

pendent activation of the SV40 early promoter reporter construct in HeLa cells (*lanes 1–3*) and in which TAF-I did not show activation of this reporter (TAF-Iα, *lanes 10–12*; TAF-Iβ, *lanes 13–15*), co-transfection of TAF-I with Sp1 resulted in inhibition of Sp1-dependent promoter activation (TAF-Iα, *lanes 4–6*; TAF-Iβ, *lanes 7–9*). Protein expression from the expression vectors was confirmed by Western blot. Both TAF-Iα and β showed increasing amounts of expression. Sp1 also showed increased expression, which shows that the effects of TAF-I on Sp1 are not due to inhibition of Sp1 expression from its expression vector. Further, as a control, we tested effects of TAF-I on p53 promoter activation as our binding studies showed that p53 does not associate with TAF-I. Under our tested conditions, TAF-I did not inhibit p53 promoter activation (Fig. 7*B*). TAF-I therefore negatively regulates Sp1-mediated promoter activation likely as a result of inhibition of DNA-binding.

DISCUSSION

Here we have described a novel interaction between a DNA-binding transcription factor and histone chaperone. Specifically, we have described functional interaction between the DNA-binding transcription factor Sp1 and the histone chaperone TAF-I.

Novel Activity of Histone Chaperone—Histone chaperones are a class of factors that possess activity to mediate assembly and/or disassembly of nucleosomal histones in an ATP-independent manner and include the factors TAF-I, CIA, nucleoplasmin, and NAP-1 among others (30, 31, 38–43). While interaction with histones and their activity to assemble/disassemble nucleosomal histones has been well addressed, their interaction with DNA-binding transcription factors has not been explored. The present study shows that the histone chaperone TAF-I functionally interacts with the DNA-binding transcription factor Sp1 (Figs. 3, 6, and 7). This interaction is specific as another histone chaperone examined did not bind Sp1, and as other tested DNA-binding transcription factors did not bind TAF-I (Fig. 4). TAF-I acts to negatively regulate the DNA binding and likely as a result of such also promoter activation by Sp1 (Figs. 6–8). TAF-I has been similarly shown to negatively regulate promoter activation by the retinoic acid receptor using cell co-transfection studies (43). Importantly,

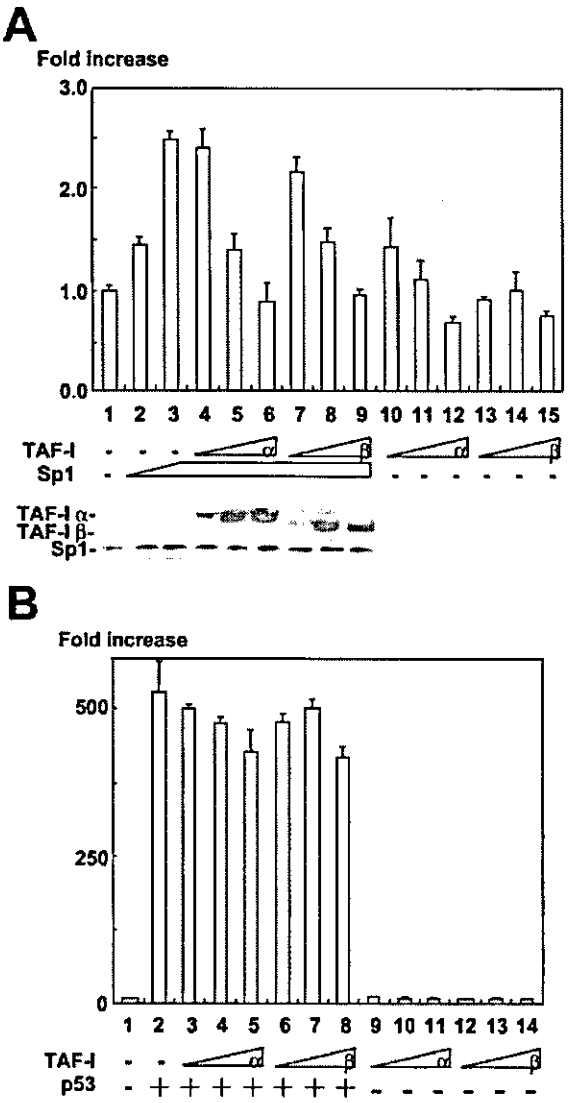

FIG. 7. **Effect of TAF-I on Sp1 promoter activation.** *A*, Co-transfection reporter analysis for effects of TAF-I on promoter activation by Sp1. An expression plasmid harboring full-length Sp1 and an SV40 early promoter reporter construct containing six consensus Sp1-binding sites were co-transfected into HeLa cells (*lanes 1–3*: *lane 1*, vector only; *lane 2*, 0.017 pmol; *lane 3*; 0.15 pmol). Sp1 (0.15 pmol) was co-transfected with TAF-Iα (*lanes 4–6*: *lane 4*, 0.025 pmol; *lane 5*, 0.075 pmol; *lane 6*, 0.15 pmol) and TAF-Iβ (*lanes 7–9*: *lane 7*, 0.025 pmol; *lane 8*, 0.075 pmol; *lane 9*, 0.15 pmol) with the reporter construct. TAF-Iα (*lanes 10–12*: *lane 10*, 0.025 pmol; *lane 11*, 0.075 pmol; *lane 12*, 0.15 pmol) and TAF-Iβ (*lanes 13–15*: *lane 13*, 0.025 pmol; *lane 14*, 0.075 pmol; *lane 15*, 0.15 pmol) were transfected alone with the reporter construct as controls. Western blot of expressed proteins are shown below. TAF-I was blotted using anti-HA antibody, and Sp1 using anti-Sp1 antibody. *B*, effects of TAF-I were similarly tested under conditions in which a multimerized p53 reporter construct was co-transfected with p53 expression vector (*lanes 2–8*, 0.15 pmol).

TAF-I may act to negatively regulate a subset of DNA-binding transcription factors that includes at least the zinc finger-type factors, which both Sp1 and nuclear receptors are.

We note that TAF-I has been shown to stimulate transcription from *in vitro* chromatin template (35). The cell co-transfection assay used by us and others differs from the *in vitro* transcription reaction as the former is a cellular experiment in which a transfected episomal plasmid reporter is activated by forced expression of a transcription factor likely in the cyto-

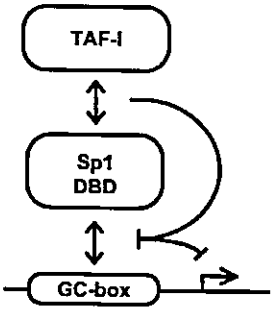

FIG. 8. **Schematic representation of results.** TAF-I interaction with Sp1 inhibits Sp1 DNA binding, which then leads to reduced promoter activation as a resultant effect.

plasm, in contrast to the latter biochemical study, which uses reconstituted components. The latter better reflects the involved fundamental reactions and allows for dissection of mechanisms of action; however, the former in contrast better reflects the collective surrounding regulatory reactions as seen in the cell albeit possible inherent limitations associated with compartmentation (*e.g.* cytoplasmic reaction) and concentration (*e.g.* effect of forced expression in contrast to basal endogenous levels). The apparent discrepancy in results between *in vitro* and *in vivo* experiments will be a topic needed to be addressed in further studies.

The mechanism of how histone chaperones are involved in reactions associated with specific promoters has also remained elusive. Functional interaction between DNA-binding transcription factor and histone chaperone may play a role in specifying the site of the reaction as dictated by the gene- and site-specific targeting properties of the DNA-binding factor. In reference, the centromeric proteins (CENP-A,B,C) through its DNA-binding component (CENP-B) shows centromere sequence-specific binding and localization allowing the CENP complex to modulate centromeric nucleosomes (44). This is one example in which concerted action between sequence-specific DNA-binding factor with histone-associated catalytic protein(s) results in specific and localized chromosomal/nucleosomal processes. Functional sequelae of the interaction between DNA-binding transcription factor and histone chaperone may be dictation of site-specificity by DNA-binding transcription factors for catalytic events to be mediated by the histone chaperone (*e.g.* nucleosome assembly/disassembly).

Regulatory Role of the DNA-binding Domain—Of additional importance, interaction of Sp1 with TAF-I was mediated through the DBD. Much focus on regulation of Sp1 through protein-protein interaction has focused on the role of the activation domain (*e.g.* interaction with the basal machinery dTAF110/hTAF130 and transcriptional complex ARC) (8, 23) in contrast to the role of the DNA-binding domain, which has been poorly addressed. However, past studies by ourselves and others have shown that the DBD of Sp1 mediates important regulatory interactions such as with the cell cycle regulator E2F (24, 25), the acetyltransferase p300 (22), the histone deacetylase HDAC1 (26), the ATP-dependent nucleosomal remodeling enzyme SWI/SNF (27) as well as other zinc finger transcription factors including Krüppel-like factors (28).

Interestingly, the Sp1 DBD interacts with all three major chromatin-related factors consisting of chemical modification enzymes (*e.g.* acetyltransferase p300), ATP-dependent nucleosome assembly factor (*e.g.* SWI/SNF) and histone chaperone (*e.g.* TAF-I), which is a finding which has only been shown for histones. This finding is of particular interest because it implicates the DBD to play a likely role in mediating transcriptional regulatory processes in eukaryotes at the chromatin level. Combined regulation of the transcription factor by interaction with chromatin-related complexes through its activation domain (*e.g.* ARC, DRIP, TRAP) and the three factors through the DBD likely results in coordinated transcriptional regulation at the chromatin level. Importantly, as the DBD specifies the target gene or DNA sequence, selective and ordered interaction of chromatin-related factor with the DBD of the transcription factor may play a role in gene- and factor-selective regulation. Selective interaction between histone chaperones with DNA-binding factors such as interaction of TAF-I with zinc-finger type transcription factors is further suggestive of a specific regulatory role in transcription.

Cooperative Interaction of Histone Chaperone and DNA-binding Transcription Factor—Functional interaction between DNA-binding factor and histone chaperone is the most noteworthy new molecular mechanism, which results from our present study. As the interaction is specific, and as the histone chaperone negatively regulates activities of the DNA-binding factor, it is tempting to envision that TAF-I plays an important role to negatively regulate subsets of DNA-binding factors to affect selective gene expression. We, however, do not rule out the possibility that TAF-I may also participate in activation processes under certain regulatory conditions in consideration of the fact that TAF-I has been shown to possess stimulatory effects on transcription *in vitro* (35).

The next important questions which need to be answered are whether TAF-I contributes to continuous regulation/inactivation or if this is a triggered event, and how histones which also bind histone chaperones contribute to this process. It is noteworthy that the chaperone proteins including the Hsp90-co-chaperone p23, Hsp90, and Hsp70 modulate assembly as well as disassembly of transcriptional complexes as shown for nuclear receptors (45–47). It is tempting to envision that histone chaperones also contribute to DNA-binding transcription factor regulation by mediating inactivation processes. Although the mechanisms of interaction of histone chaperone on DNA-binding factor are yet unclear, TAF-I may inhibit the activities of Sp1 by competitive interaction with the DNA-binding surface, but alternatively binding to the non-DNA-binding surface of Sp1 DBD may induce an allosteric/conformational change to Sp1 DBD making it transcriptionally incompetent/competent for further regulatory interactions (*e.g.* DNA binding, transcriptional activation).

Based on our data centered on Sp1 DBD, we have shown in the past that p300 acetyltransferase facilitates promoter access (22), therefore TAF-I as a negative regulator may act in concert with the acetyltransferase to mediate a balance of promoter activation and inactivation. Given that acetyltransferase and TAF-I have been shown to regulate acetylation and its inhibition on histones (43), respectively, this may be one of the signal modifications regulated by this concerted interaction (22, 43). As we have shown that Sp1 DBD is acetylated (22), further experiments to investigate whether TAF-I regulates inhibition of acetylation of DNA-binding transcription factor will also add to our understanding of transcriptional regulation. Further, although it would seem that inactivation/activation is an energy-consuming process, as histone chaperones are essentially non-ATP-dependent factors, their contribution would likely facilitate this process and allow for efficient transcriptional regulation.

Collectively, we have shown that the histone chaperone TAF-I negatively regulates a DNA-binding transcription factor. Our results provide an initial step in understanding the role of histone chaperones in the regulation of DNA-binding transcription factors.

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Positive and Negative Regulation of the Cardiovascular Transcription Factor KLF5 by p300 and the Oncogenic Regulator SET through Interaction and Acetylation on the DNA-Binding Domain

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Here we show a novel pathway of transcriptional regulation of a DNA-binding transcription factor by coupled interaction and modification (e.g., acetylation) through the DNA-binding domain (DBD). The oncogenic regulator SET was isolated by affinity purification of factors interacting with the DBD of the cardiovascular transcription factor KLF5. SET negatively regulated KLF5 DNA binding, transactivation, and cell-proliferative activities. Down-regulation of the negative regulator SET was seen in response to KLF5-mediated gene activation. The coactivator/acetylase p300, on the other hand, interacted with and acetylated KLF5 DBD, and activated its transcription. Interestingly, SET inhibited KLF5 acetylation, and a nonacetylated mutant of KLF5 showed reduced transcriptional activation and cell growth complementary to the actions of SET. These findings suggest a new pathway for regulation of a DNA-binding transcription factor on the DBD through interaction and coupled acetylation by two opposing regulatory factors of a coactivator/acetylase and a negative cofactor harboring activity to inhibit acetylation.

The Sp/KLF (for Sp1- and Krüppel-like factor) family of zinc finger transcription factors has received recent attention due to important roles in developmental, differentiation, and oncogenic processes, among others (2, 3, 35). It is comprised of over 15 mammalian family members which have in common three similar C_2H_2-type zinc fingers at the carboxyl terminus which comprises the DNA-binding domain (DBD). Sp/KLF family members include the founding ubiquitous factor Sp1 (9), the erythroid differentiation factor EKLF/KLF1 (27), and the tumor suppressor gene KLF6/GBF/Zf9/COPEB, which we and others identified as a cellular factor possibly involved in human immunodeficiency virus type 1 transcription (18, 32, 44). It was recently shown by gene knockout studies that the proto-oncogene KLF5/BTEB2/IKLF (40, 42) is important for cardiovascular remodeling in response to stress (41). Contrary to initial expectations that this family of factors would likely have redundant functions, they in fact have important individual biological functions. However, the underlying mechanisms governing their specific functions and regulation are poorly understood.

We have studied the regulatory mechanisms of action of Sp/KLF family members in the past and have shown differential regulation through interaction and acetylation on the DBD by the coactivator/acetylase p300 (45). Acetylation is an important nuclear regulatory signal which regulates transcriptional processes with biological implications, including regulation of development, differentiation, and oncogenesis (5, 10, 31), which closely resembles the roles of Sp/KLF family members. We therefore thought that the Sp/KLF factors may be differently regulated by acetylation and showed that the coactivator/acetylase p300, but not the MYST-type acetylase Tip60, specifically interacts and acetylates Sp1 but not KLF6 through the zinc finger DBD and that DNA binding inhibits this interaction and acetylation (45). While much is known of acetylation in general, its regulation and implications are still poorly understood, especially its negative regulation.

Studies on negative regulation of acetylation have been centered mainly on the role of histone deacetylases, which are categorized into three classes based on sequence characteristics, subcellular localization, and catalytic properties (17, 33). We have shown an additional pathway involving negative regulation by DNA binding (45), and others have shown that a set of molecules inhibit the acetylation of histones by masking the protein from acetylation (e.g., inhibitors of histone acetylation [INHAT]) (39). Acetylation is therefore regulated at multiple levels by both catalytic and noncatalytic processes.

Here we show a regulatory pathway of acetylation involving the oncogenic regulator SET, a subunit of a complex previously shown to inhibit histone acetylation by masking the protein (INHAT) (39), through interaction with the DNA-binding transcription factor KLF5. Our findings suggest a new tran-

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scriptional regulatory pathway through the DBD by convergence of two opposing regulatory pathways involving p300 and SET through coupled interaction and acetylation.

MATERIALS AND METHODS

Preparation of recombinant epitope-tagged protein. The zinc finger region/DBD of human KLF5 (KLF5 ZF/DBD) (40) (a kind gift of C. Teng) was PCR amplified and subcloned into *Bam*HI-digested 6His-pET11d (45). Protein expression and purification were done essentially as described previously, with modification by use of HiTrap heparin and HisTrap columns (Amersham Pharmacia Biotech) (45). Glutathione *S*-transferase (GST)-tagged constructs KLF5 wt (wild type), KLF5-ΔDBD, KLF5-DBD, KLF5-zinc finger 1, KLF5-zinc finger 2, and KLF-zinc finger 3 were similarly PCR amplified and inserted into pGEX vectors (Amersham Pharmacia Biotech) (44, 45). Hexahistidine-tagged SET/TAF-Iβ construct (29) (a kind gift of K. Nagata) was transformed into the BL21-Gold(DE3)pLysS strain, induced, and then purified with Probond resin (Invitrogen) and buffer C containing 20 mM imidazole for washing and 200 mM imidazole for elution similar that described previously (45). SET/TAF-Iβ deletion constructs were in part a generous gift from K. Nagata, otherwise they were constructed by PCR mutagenesis. The p300 histone acetyltransferase (HAT) domain constructs have been described previously (45) (a kind gift of Y. Nakatani). All procedures were done at 4°C. Zinc finger peptides were synthesized commercially by Hokkaido System Science Co. Ltd. Product purity was more than 95%, and the molecular weights of synthesized peptides were confirmed by mass spectral analysis.

Cell culture and preparation of nuclear extract. C2/2 rabbit vascular smooth muscle cells (VSMCs) (48) were maintained in Dulbecco's modified Eagle medium (Sigma), and HeLa S3 cells were maintained in Joklik's modified Eagle medium (Sigma) supplemented with 10% fetal bovine serum with 100 μg of streptomycin/ml and 100 U of penicillin G/ml. Nuclear extract was prepared as described previously (7). The final supernatant was dialyzed against buffer B (25 mM HEPES [pH 7.9], 10% glycerol, 150 mM KCl, 100 μM $ZnSO_4$, 5 mM 2-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride, 0.5 μg of leupeptin/ml, and 1 μg of pepstatin/ml), centrifuged for 15 min at 18,000 × *g*, and used as nuclear extract.

Isolation of factors associating with KLF5 ZF/DBD. Fifty micrograms of hexahistidine-tagged KLF5 ZF/DBD was bound to 5 μl of equilibrated Probond nickel-chelating resin (Invitrogen) by rotating for 6 h. Following five washes with buffer B, the resin bound with KLF5 ZF/DBD was then incubated for 6 h with 725 μg of C2/2 nuclear extract. After 10 washes with buffer B containing 20 mM imidazole, the bound proteins were eluted with buffer B containing 500 mM imidazole. Samples were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and analyzed by Coomassie brilliant blue staining. All procedures were done at 4°C.

Protein identification by MALDI-TOF (MS). Protein bands were excised from a Coomassie brilliant blue-stained SDS-PAGE gel and washed three times in 50% acetonitrile–25 mM ammonium bicarbonate (pH 8), and 100% acetonitrile was added. Following removal of acetonitrile, the gel slices were dried and the protein band was in-gel digested with 15 μg of trypsin/ml in 25 mM ammonium bicarbonate (pH 8) at 37°C overnight. Gel slices were then soaked in 50% acetonitrile–5% tetrahydrofuran, and the supernatant was collected and dried up. The dried digest was reconstituted by the addition of 50% acetonitrile–0.1% tetrahydrofuran, mixed with α-cyano-4-hydroxycinnamic acid, and analyzed by matrix-assisted laser desorption–ionization time of flight (mass spectrometry) [MALDI-TOF (MS)] (Voyager-DE STR; Applied Biosystems). Database searches were performed against the nonredundant National Center for Biotechnology Information database by using Protein Prospector programs, version 3.2.1, developed by the University of California—San Francisco MS facility. MS analysis of peptide acetylation was done essentially as described previously (16).

Protein-protein interaction assay. GST fusion proteins were immobilized to glutathione-Sepharose 4B resin and incubated with histidine-tagged proteins in a buffer containing 20 mM HEPES (pH 7.6 at 4°C), 20% glycerol, 0.2 mM EDTA, 0.1% Triton X-100, 100 mM NaCl, and 100 μM $ZnSO_4$. Reactions were carried out at 4°C for 3 h, and the mixtures were washed two times in the same buffer. Bound proteins were resolved on an SDS-PAGE (10% polyacrylamide) gel, transferred to a nitrocellulose membrane, immunoblotted with anti-HIS probe (G-18) antibody (Santa Cruz Biotechnology) or anti-FLAG (M2) antibody (Sigma), and detected with enhanced chemiluminescence Western blotting detection reagents essentially according to the manufacturer's instructions (Amersham Pharmacia Biotech). Histidine tag pull-down assays were done with Probond resin (Invitrogen) and blotted with anti-GST antibody (Santa Cruz) when appropriate.

Coimmunoprecipitation assay. One microgram of anti-KLF5 rat monoclonal antibody (KM1785) (41) or 1 μg of control rat immunoglobulin G (IgG) (sc-2026; Santa Cruz Biotechnology) was bound to 10 μl of protein G-Sepharose (Amersham Pharmacia Biotech) by rotating for 6 h in buffer B at 4°C. Following three washes with buffer B, protein G-Sepharose with antibody was then rotated with 1 mg of cell extract protein for 6 h. After 10 washes with radioimmunoprecipitation assay buffer, washed immunoprecipitates were subjected to SDS-PAGE and immunoblotting with SET/TAF-Iβ-specific antibody (a generous gift of K. Nagata) (30).

Immunoconfocal fluorescence microscopy. Murine VSMCs were prepared from the thoracic aortas of 8-week-old mice according to the enzyme digestion method (4). Cells were plated on glass coverslips, fixed with 4% formaldehyde, blocked with 3% bovine serum albumin in phosphate-buffered saline–Tween 20, incubated with anti-SET/TAF-Iβ mouse monoclonal antibody (a kind gift of K. Nagata) (30) and anti-KLF5 rat monoclonal antibody (KM1785), exposed to anti-mouse IgG antibody conjugated with fluorescein isothiocyanate and anti-rat IgG antibody conjugated with rhodamine isothiocyanate as the secondary antibodies, and then examined by confocal microscopy with a Leica TCS 4D equipped with an argon-krypton laser.

Gel shift DNA-binding assay. The gel shift DNA-binding assay was done essentially as described previously (45). A DNA oligomer containing the KLF5 binding sequence, 5′-ATGGGCATGAGGGCCAGCCTATGAGA-3′ (SE1), was used to analyze the DNA binding of KLF5 ZF/DBD (48). For mutant analysis, the underlined nucleotides GGGCC were replaced by TTTAA. For control NF-κB gel shifts, commercially available NF-κB probe (Promega) and recombinant NF-κB p50 protein (Promega) were used. The details (e.g., protein combinations) of individual experiments are given in the figure legends.

Cotransfection reporter assay. Plasmid constructs for the reporters, SMemb/NMHC-B and PDGF-A chain, and the effector pCAG-KLF5 have been described previously (41, 48). pCHA-SET/TAF-Iβ was a kind gift of K. Nagata (30). pCI-p300 and pCI-p300ΔHAT constructs were kind gifts of V. Ogryzko. Transient transfection assays were done by seeding cells (50,000 cells/24-well plate) 24 h prior to transfection and then transfected with 100 ng of the reporter plasmid and a total of 1 μg of either vector, pCAG-KLF5, or pCHA-SET/TAF-Iβ 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 then subjected to an assay of luciferase activity (luciferase assay system; Promega) (Lumat LB9501; Berthold). The luciferase activity was normalized to the protein concentration of the cell lysates measured according to the Bradford method (Bio-Rad). For NF-κB control experiments, commercially available NF-κβ reporter was used (Stratagene), with coexpression of NF-κβ p50 and p65 expression plasmids (K. Aizawa, T. Suzuki, N. Kada, A. Ishihara, K. Kowase, T. Matsumura, K. Sasaki, Y. Munemasa, I. Manabe, M. Kurabayashi, T. Collins, and R. Nagai, submitted for publication).

Construction of point mutant expression construct. Site-directed mutagenesis was used to construct the mutant pCAG/KLF5-K369R, which involves a lysine-to-arginine mutation at amino acid residue position 369 of KLF5. PCR was done with the primers 5′-GACGACCATCCACTACTGCGATT-3′ and 5′-TCTCCA AATCGGGGTTACTCCTT-3′, with pCAG/KLF5 as a template and KOD Plus (Toyobo) polymerase. The construct was sequenced for verification.

Construction of recombinant adenovirus vectors. KLF5, the KLF5 K369R mutant, and SET cDNA were subcloned into the adenovirus cosmid vector pAxCAwt (Takara) at the *Swa*I site. 293 cells were cotransfected by the cosmid vector and restriction enzyme-treated DNA-terminal protein complex, with subsequent selection of plaques as a result of homologous recombination. The protein expression was confirmed by Western blot analysis. The titer was determined by the plaque method.

Production of stable transformant cell lines. KLF5 and KLF5 K369R cDNA inserts were subcloned into 3× FLAG expression vectors (Sigma). Constructs were transfected into cells (3T3-3) and then selected on the basis of G418 resistance for 2 weeks.

BrdU incorporation assay. Cells were plated on 96-well plates. Following adenovirus-mediated transfection in given experiments, bromo-2′-deoxyuridine (BrdU) incorporation was examined after 24 h over a span of 2 h by using the Biotrak cell proliferation enzyme-linked immunosorbent assay system, version 2 (Amersham Pharmacia Biotech), essentially according to the manufacturer's instructions. Experiments were done in triplicate.

Phorbol ester-induced expression of KLF5 and SET. Cell lysates from C2/2 VSMCs stimulated with phorbol 12-myristate 13-acetate (PMA) (100 ng/ml) following 24 h of starvation (0% fetal bovine serum) were resolved on an SDS–12% PAGE gel, transferred to a nitrocellulose membrane, and then im-

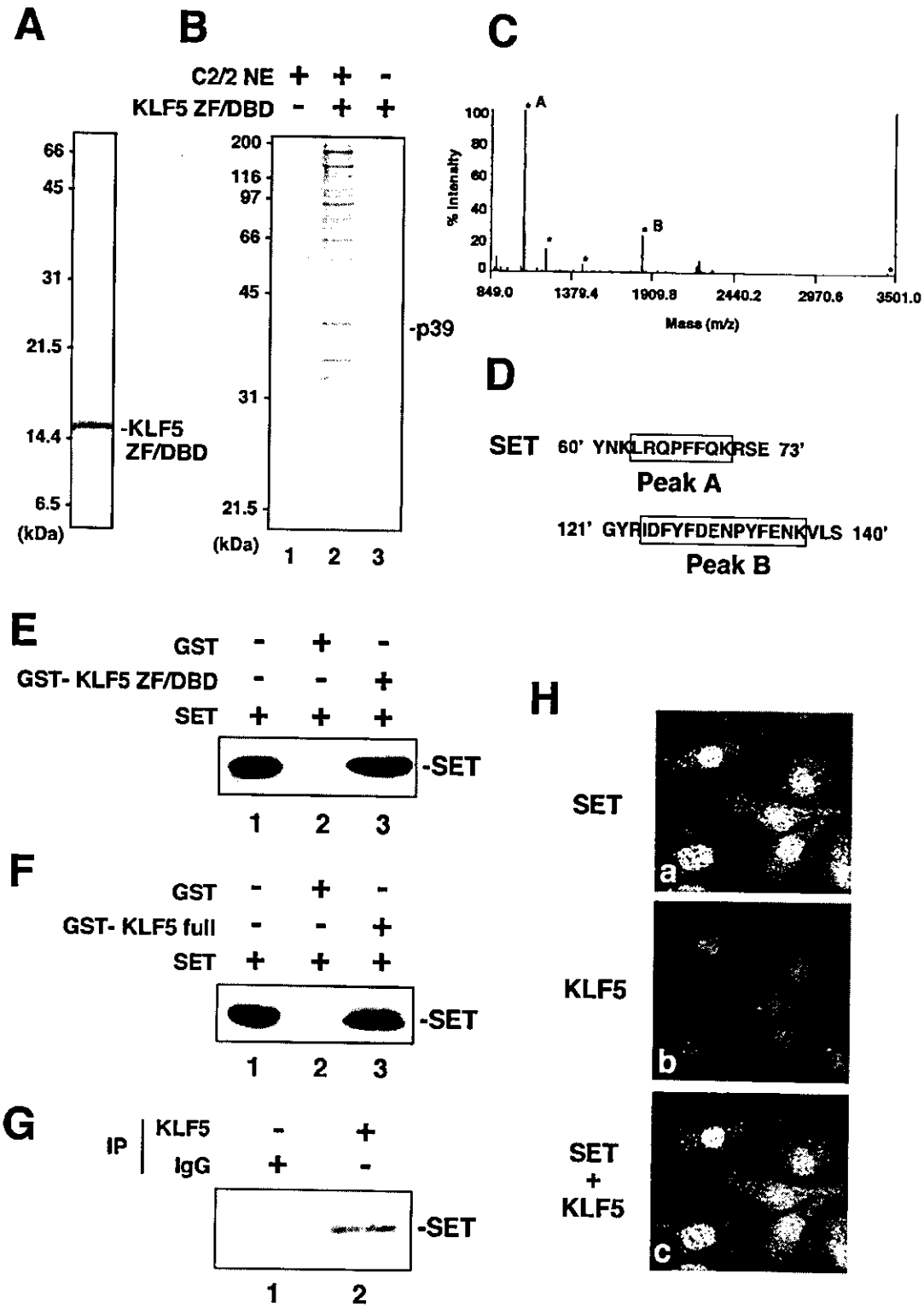

FIG. 1. Isolation of interactors of KLF5. (A) Silver-stained gel of the histidine-tagged KLF5 ZF/DBD recombinant used for interaction studies. Molecular mass markers are shown on left. (B) Isolation of factors associating with KLF5 ZF/DBD. Histidine-tagged KLF5 ZF/DBD was bound to nickel-chelating resin and subjected to VSMC C2/2 nuclear extract (NE) (lane 2). Lane 1 is nuclear extract alone, and lane 3 is recombinant protein immobilized on resin alone. Eluate was resolved by SDS-PAGE (12% polyacrylamide) and stained by Coomassie brilliant blue. The stained band, which was excised and subjected to further analysis, is indicated p39. (C) MALDI-TOF mass spectra obtained from tryptic peptides of p39.

munoblotted with anti-KLF5 monoclonal antibody (KM1785) or anti-SET/TAF-Iβ antibody (30). The relative intensity of KLF5 or SET protein in reference to Coomassie brilliant blue stain was calculated by using National Institutes of Health Image software. Total RNA was obtained by the RNeasy preparation kit (Qiagen) and reverse transcribed, and then quantitative PCR was performed with a platelet-derived growth factor A (PDGF-A) chain gene-specific primer set (5′-CAGCATCCGGGACCTCCAGCGACTC-3′ and 5′-TCGTAAATGACCG TCCTGGTCTTGC-3′) and a QuantumRNA 18S internal standard primer set (Ambion) as previously described (22). The relative intensity of the PDGF-A chain in reference to internal 18S rRNA was calculated by National Institutes of Health Image software.

Rat arterial injury model and immunohistochemistry. After 4 weeks on their respective diets, rats weighing 400 to 450 g were anesthetized with chloral hydrate (370 mg/kg of body weight, intraperitoneally). Balloon denudation of the left common carotid artery was performed. The right common carotid artery served as a control. Fourteen days after operation, rats were euthanized with a lethal dose of anesthetic, after which the carotid arteries were perfused with 4% paraformaldehyde and phosphate-buffered saline. Each injured left carotid artery was excised from the proximal edge of the omohyoid muscle to the carotid bifurcation. The middle third of the segment was then fixed in 4% paraformaldehyde for 12 h and embedded in paraffin. Serial cross sections (6 μm thick) were cut from each sample and stained with hematoxylin-eosin or prepared for immunohistochemistry. For immunohistochemical analysis, tissue sections were preincubated with 2% bovine serum albumin and then serially treated with SET-specific antibody (KM1720) (30) or KLF5-specific antibody (KM1785). Specimens were then treated with biotinylated goat anti-mouse IgG antibody (Vector Laboratories) or biotinylated goat anti-rat IgG antibody (Chemicon) followed by avidin-biotinylated horseradish peroxidase (Vectastain ABC kit; Vector Laboratories) and developed with 0.004% H_2O_2 and 0.02% diaminobenzidine tetrahydrochloride.

RESULTS

Isolation of interactors of KLF5. To isolate factors which regulate KLF5 by protein-protein interaction, we affinity purified interacting factors by using the ZF/DBD region, which is a potent protein-protein interface (21) that has been previously shown to mediate differential interaction with acetylase (45). Nuclear extract obtained from C2/2 VSMCs which express KLF5 were applied to hexahistidine-tagged recombinant KLF5 ZF/DBD immobilized on Ni^{2+}-chelating resin (Fig. 1A). Bound proteins were released by imidazole and then analyzed by SDS-PAGE with Coomassie brilliant blue staining (Fig. 1B). Approximately 20 bands ranging from 30 to 200 kDa were seen when the binding reaction between the KLF5 ZF/DBD and nuclear extract was done (Fig. 1B, lane 2) and not when either nuclear extract or KLF5 ZF/DBD alone (Fig. 1B, lanes 1 and 3) was used.

One band with an apparent molecular mass of 39 kDa was found in abundance and easily discernible from other nearby bands. MALDI-TOF (MS)–peptide mass fingerprinting, with a computer search of the National Center for Biotechnology Information mammalian database with further confirmation of the amino acid sequence by postsource decay peptide sequencing, revealed this protein to be the SET oncoprotein, the product of the SET oncogene whose translocation has been implicated in leukemia (Fig. 1C and D) (1, 47). SET is identical to the cellular factor template activating factor-Iβ which stimulates adenoviral core DNA replication (24, 29) and has been shown to be a histone chaperone, which is a factor that can displace and/or assemble nucleosomal histones in an ATP-independent manner (15, 25, 26, 34). While interaction of histone chaperones with histones and their activities to assemble and disassemble nucleosomal histones have been well addressed, their interaction with DNA-binding transcription factors has not been explored. Further, their cellular functions are poorly understood. Thus, characterization of this new interaction would add to our understanding of how histone chaperones may be involved in specific transcription by cooperative interaction with transcription factors as well as their cellular functions.

In vitro and in vivo interactions of KLF5 and SET. To determine whether SET directly interacts with KLF5 ZF/DBD, a GST pull-down assay was done (Fig. 1E). SET bound KLF5 ZF/DBD (Fig. 1E, lane 3) but not GST alone (Fig. 1E, lane 2), showing that SET and KLF5 ZF/DBD bind directly. To next see whether full-length KLF5 binds SET, a similar GST pull-down assay with full-length KLF5 was done (Fig. 1F), which showed that SET also binds full-length KLF5 (Fig. 1F, lane 3). To further see whether KLF5 and SET interact in the cell, immunoprecipitation was done with specific antibodies against KLF5 and SET (Fig. 1G). Immunoprecipitation of KLF5 followed by immunoblotting with SET antibody showed detection of SET when antibody against KLF5 was used (Fig. 1G, lane 2) but not for control IgG antibody (Fig. 1G, lane 1). SET therefore interacts with KLF5 in vitro and in vivo.

To further examine the expression and cellular localization of SET, immunohistochemistry was done (Fig. 1H). SET (Fig. a) and KLF5 (Fig. b) colocalized to the nucleus in an overlapping pattern (Fig. c), which is supportive of functional interaction.

Functional effects of interaction of KLF5 and SET. To next address the functional implications of the interaction of SET and KLF5, we first examined the effects on the DNA-binding activity of KLF5 (Fig. 2A). Gel shift analysis under conditions in which KLF5 ZF/DBD showed sequence-specific binding and SET did not bind the probe DNA (Fig. 2A, lanes 2 to 4) showed inhibition of KLF5 DNA-binding activity by the addition of SET (Fig. 2A, lanes 7 and 8). SET did not inhibit the DNA-binding activity of the control NF-κB p50 subunit (Fig. 2B).

To further examine the effects of SET on KLF5-dependent

Fragment peaks assigned to SET are marked (asterisks). Peaks indicated A and B were subjected to postsource decay sequencing. (D) Partial peptide sequences of human SET. Numbering is from the initiation methionine of SET. The peptide sequences of peaks A and B obtained by postsource decay are boxed. (E) In vitro binding of KLF5 ZF/DBD and SET. Immobilized GST-KLF5 ZF/DBD fusion protein was reacted with histidine-tagged SET protein, separated by SDS-PAGE, and analyzed by immunoblotting with anti-HIS probe antibody (lane 3). Lane 1 is the input. GST protein was used as the control (lane 2). (F) In vitro binding of KLF5 full-length protein and SET. Immobilized GST-KLF5 full-length fusion protein was reacted with histidine-tagged SET protein, separated by SDS-PAGE, and analyzed by immunoblotting with anti-HIS probe antibody (lane 3). Lane 1 is the input. GST protein was used as a control (lane 2). (G) Coimmunoprecipitation of SET with KLF5. Cell lysate was immunoprecipitated with anti-KLF5 rat monoclonal antibody (lane 2) or normal rat IgG (lane 1) as a control. Bound materials were separated by SDS-PAGE and analyzed by immunoblotting with anti-SET antibody. (H) Intracellular localization of KLF5 and SET. Endogenous SET (a) (green) and KLF5 (b) (red) were detected mainly in nuclei. Confocal microscopy double-staining analysis indicates colocalization of SET and KLF5 (c) (yellow). All experiments were done at least twice with consistent findings. +, present; −, absent.

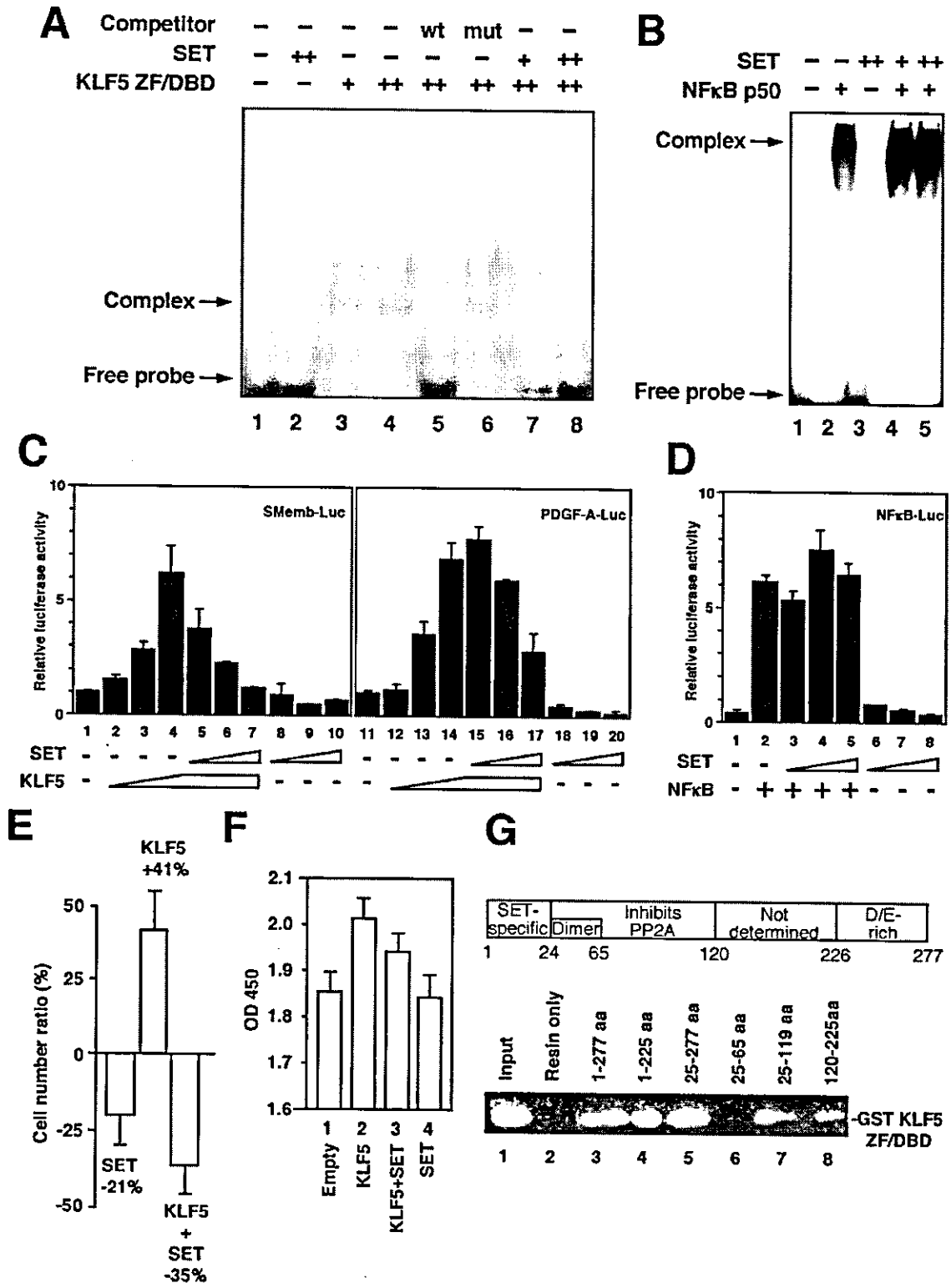

FIG. 2. Effects of SET on KLF5 activity. (A) Effects of SET on KLF5 DNA-binding activity. A gel shift assay with recombinant KLF5 ZF/DBD and SET was performed. Wt and mut represent wild and mutant oligonucleotide competitors (lanes 5 and 6). The amount of recombinant protein is as follows: 150 (+) and 450 (++) ng for SET (lanes 2, 7, and 8) and 10 (+) and 50 (++) ng for KLF5 ZF/DBD (lanes 3 to 8). (B) Gel shift assay of control NF-κB p50 subunit and SET. Gel shift units (0.1) of NF-κB p50 (lanes 2, 4, and 5) and 100 (+) and 300 (++) ng of SET (lanes 3, 4, and 5) were used. (C) Effects of SET on KLF5 transactivation. Cotransfection analysis of effects of SET on KLF5 transcriptional activation.

transcriptional activation, cotransfection reporter assays were done (Fig. 2C). Using the originally identified KLF5-responsive embryonic vascular smooth muscle (SMemb) promoter (48) and the PDGF-A chain promoter, which is an endogenous target of KLF5 (41), under conditions in which KLF5 showed dose-dependent transactivation of the SMemb and PDGF-A chain promoter activities (Fig. 2C, lanes 2 to 4 and 12 to 14) and SET did not show activation of the respective promoters (Fig. 2C, lanes 8 to 10 and 18 to 20), cotransfection of SET showed dose-dependent inhibition of KLF5-mediated transactivation (Fig. 2C, lanes 5 to 7 and 15 to 17). SET did not inhibit transactivation by NF-κB (Fig. 2D). SET therefore negatively regulates both KLF5 DNA-binding and transactivation activities in a specific manner.

The effects of SET on KLF5 cellular activity were further addressed given that KLF5 is a proto-oncogene which accelerates cell growth (Fig. 2E) (43). Under conditions in which adenovirus-mediated forced expression of KLF5 stimulated the growth of C2/2 cells, expression of SET inhibited cell growth in KLF5-expressing cells to almost basal levels, suggesting coordinated effects of SET and KLF5 on cell growth. To note, SET alone also showed inhibitory effects on cell growth. A BrdU assay further confirmed that SET inhibited cell growth as induced by KLF5 (Fig. 2F).

To determine the region of SET which interacts with KLF5, deletion mutants were made and subjected to a pull-down assay (Fig. 2G). Results collectively showed that a 40-amino-acid stretch (amino acids 25 to 65) known as a coiled-coil dimerization domain of SET (28) did not interact with KLF5, but otherwise, a broad region of SET interacted with KLF5. Initial results with a SET mutant which does not interact with KLF5 did not show effects on cell growth, suggesting that interaction is important for cooperative effects of SET and KLF5 on cell growth (data not shown).

Biological implications of interaction of KLF5 and SET. To further examine the biological implications of the interaction of KLF5 and SET, we assessed the effects of SET on KLF5-dependent gene expression. KLF5 and its downstream gene PDGF-A chain are induced by various stimuli (e.g., phorbol ester, angiotensin II, and serum) (14, 48). We examined whether SET expression could also be similarly regulated. Expression levels of KLF5, SET, and PDGF-A chain were assessed at various time intervals after PMA stimulation by Western blotting for KLF5 and SET, and reverse transcription-PCR analysis for the PDGF-A chain (Fig. 3A to D). An induction of KLF5 protein was seen at 2 h with a 1.9-fold increase after PMA stimulation, with a coinciding 2.5-fold decrease in SET protein levels (Fig. 3A and B). PDGF-A mRNA levels showed a 2.2-fold increase at 4 h (Fig. 3C and D). KLF5, SET, and PDGF-A chain levels returned to basal levels at 24 h. Collectively, reciprocal increased KLF5 and coinciding decreased SET correlated with increased PDGF-A chain expression after PMA stimulation.

We further examined expression of KLF5 and SET in pathological states by histopathological analysis. An experimental atherosclerosis model (balloon injury) was used in which KLF5 is activated in proliferating neointimal smooth muscle cells after injury (11, 41). Balloon-injured aortas and controls at 2 weeks were examined for expression of KLF5 and SET by immunohistochemistry (Fig. 3E). In contrast to low basal levels of either SET or KLF5 in the nucleus of noninjured control aortic medial cells, proliferating neointimal cells, which form after injury and consist mainly of proliferating smooth muscle cells, showed marked expression of both KLF5 and SET in the nucleus. These findings suggest correlation and colocalization of KLF5 and SET in pathological states and support a functional interaction at the tissue or animal level.

Inhibition of KLF5 acetylation by SET. SET regulates the actions of KLF5 as determined from these studies, but the underlying mechanisms were still not fully understood. One of the functions of SET has been recently shown to be inhibition of histone acetylation (39). It has been shown in the past that the KLF5 family member Sp1 is acetylated by p300 in the ZF/DBD region (45), and as Sp1 and KLF5 have similar ZF/DBDs, and further because SET interacted with the ZF/DBD of KLF5, we reasoned that KLF5 may also be similarly acetylated, and if so, be inhibited by SET. By such, SET may negatively regulate the actions of KLF5 by blocking acetylation.

First, we examined whether KLF5 can be acetylated by pulse-chase experiments (Fig. 4A). Using [^{3}H]acetate and the histone deacetylase inhibitor trichostatin A, cells expressing KLF5 showed a clear uptake, thus showing that KLF5 can be acetylated in vivo. We next examined which region of KLF5 is acetylated by an in vitro acetylation assay by using acetyl [^{14}C]coenzyme A (acetyl-[^{14}C]CoA) (Fig. 4B). Using a catalytic recombinant protein of p300 which acetylates the similar factor Sp1, full-length KLF5 and the KLF5 ZF/DBD region were acetylated but the non-ZF/DBD region was not, showing

One hundred nanograms of reporter was used in each lane. Effectors are as follows: lanes 2, 3, and 4 through 7, and 12, 13, and 14 through 17 were 83, 250, and 750 ng of KLF5 expression plasmid (pCAG-KLF5), respectively; lanes 5 and 8, 6 and 9, 7 and 10, 15 and 18, 16 and 19, and 17 and 20 were 28, 83, and 250 ng of SET expression plasmid (pCHA-SET/TAF-Iβ). The total amount of effector plasmid was adjusted to 1 μg with the respective control vector. (D) Effects of SET on control NF-κB transactivation. NF-κB transactivation (lanes 2 to 5) was done by transfection of equal amounts (250 ng) of p50 and p65 subunit expression vectors. (E) Effects of SET on KLF5-induced cell growth. SET was transiently transfected into cells stably expressing epitope-tagged (3× FLAG) KLF5 or mock vector in 3T3-3 cells. The cell count on day 5 after transfection compared with mock vector-treated cells is shown. Error bars denote standard errors. (F) BrdU assay showing effects of KLF5 and SET on cell growth by use of adenovirus-mediated transfer (multiplicity of infection, 100) of KLF5 and SET adenoviruses. Empty (lane 1) denotes empty vector alone. Error bars denote standard errors. OD_{450}, optical density at 450 nm. (G) In vitro binding of KLF5 ZF/DBD and SET deletion mutants. Immobilized histidine-tagged SET protein was reacted with GST-KLF5 ZF/DBD fusion protein, separated by SDS-PAGE, and analyzed by immunoblotting with anti-GST antibody. SET deletion mutants are shown by their amino acid numbers in reference to the schematic diagram of functional domains shown above. Lane 1 is GST KLF5 ZF/DBD input, and lane 2 shows that GST KLF5 ZF/DBD does not bind Probond nickel-chelating resin. Lanes 3 to 8 show GST KLF5 ZF/DBD binding to respective resin-bound deletion mutants. Amino acids (aa) 1 to 24 comprise the SET-specific N-terminal region, amino acids 25 to 65 comprise the coiled-coil dimerization domain, amino acids 25 to 119 comprise a region known to inhibit phosphatase PP2A, amino acids 120 to 225 comprise a region with unknown function, and amino acids 226 to 277 comprise the acidic C-terminal region. All experiments were done at least twice with consistent findings.

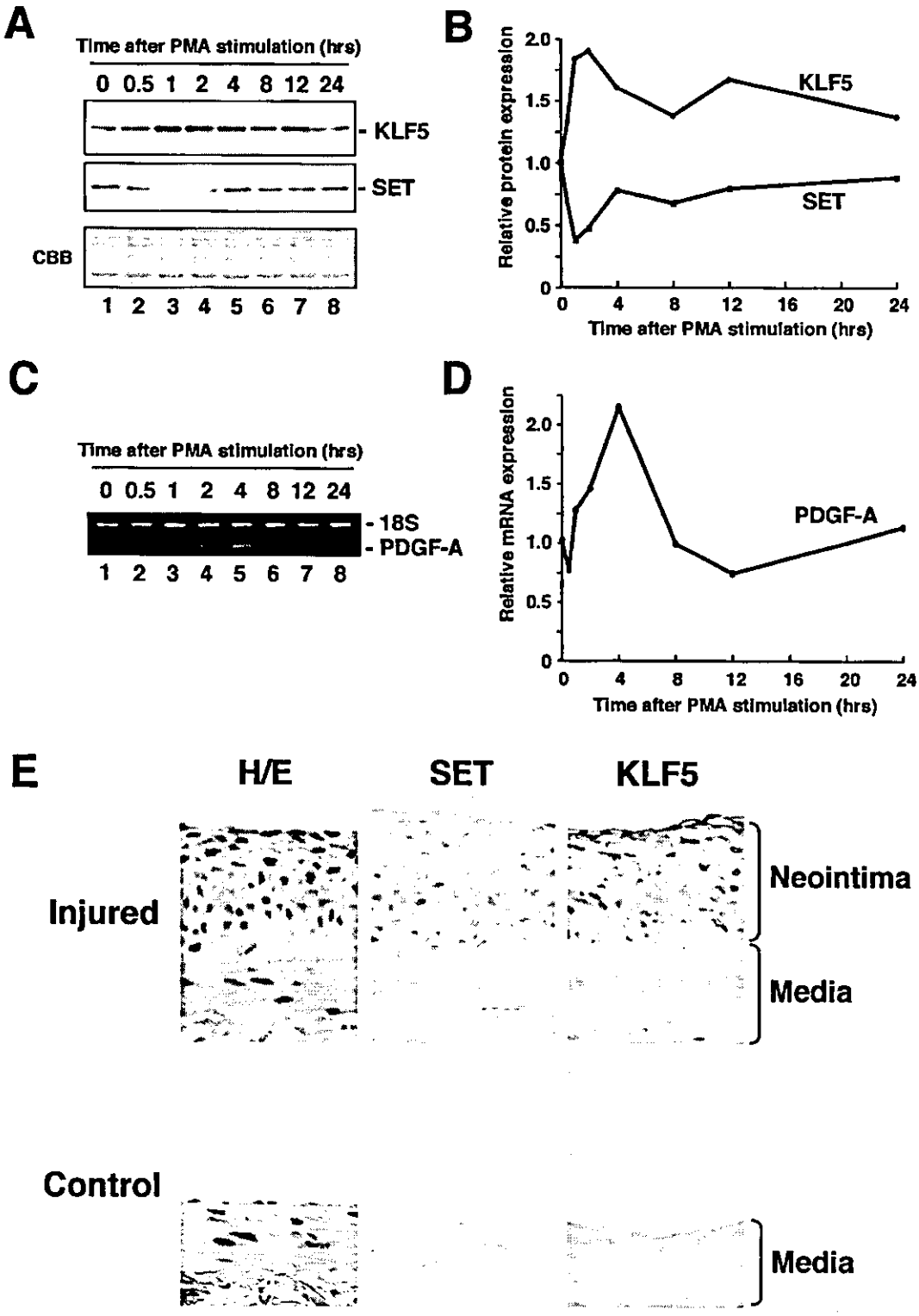

FIG. 3. Effects of SET on KLF5 downstream gene expression and pathological states. (A) Induction of KLF5 protein and repression of SET protein after mitogenic stimulation. Cells were starved at the times shown for 24 h, incubated with 100 ng of PMA/ml for the indicated times, and then harvested. Cell lysate was resolved by SDS-PAGE and subjected to Western blotting or Coomassie brilliant blue staining. (B) Quantification of KLF5 and SET protein levels. KLF5 and SET protein levels were normalized by the corresponding Coomassie brilliant blue staining pattern. The relative expression level was shown as the level at 0 h. (C) Induction of PDGF-A chain mRNA expression. Cells were starved at the times shown for 24 h, incubated with 100 ng of PMA/ml for the indicated times, and then harvested. The quantitative reverse transcription-PCR fragment

that the ZF/DBD region is acetylated similar to Sp1 (Fig. 4C). This was specific, as neither acetylase Tip60 (MYST domain containing Tat-interacting protein) nor GCN5 (homologue of mammalian PCAF) acetylated KLF5 ZF/DBD (data not shown).

To see whether acetylation affects the DNA-binding activity of KLF5, a gel shift assay was done under acetylation conditions which showed that acetylation of KLF5 has no effect on its DNA-binding activity (Fig. 4D). As p300 acetylated KLF5, we next examined whether SET could inhibit the acetylation of KLF5 (Fig. 4E). As expected, SET inhibited the acetylation of KLF5 ZF/DBD by p300. Order of addition experiments showed that SET was able to inhibit acetylation when reacted with KLF5 prior to the addition of acetylase, but it was unable to react if KLF5 and p300 were reacted beforehand, suggesting that SET inhibits acetylation of KLF5 by masking the protein or inducing a conformational change which does not allow for subsequent acetylation.

p300 as a transcriptional cofactor of KLF5. As the coactivator/acetylase p300 acetylated KLF5, we examined whether p300 acts as a coactivator of KLF5, that is, if it interacts with KLF5 and is able to potentiate KLF5-mediated transcriptional activation. First, to examine the interaction between KLF5 and p300, by use of immunoprecipitation with specific antibodies against KLF5 and p300, we show that, under conditions in which p300 is pulled down, immunoblot against KLF5 shows interaction of KLF5 with p300 (Fig. 5A). To next examine whether interaction is direct and by which region interaction is mediated, GST pull-down assays were done with KLF5 deletion mutants and the p300 acetylase catalytic region (Fig. 5B). Results showed that KLF5 ZF/DBD and full-length KLF5, but not non-ZF/DBD region KLF5, interact with p300. Thus, importantly, the ZF/DBD is the interacting domain with p300 in addition to the substrate for acetylation.

To further see whether p300 potentiates transactivation of KLF5, a cotransfection reporter assay was done (Fig. 5C). Under conditions in which KLF5 activated the PDGF-A chain reporter, the addition of p300 resulted in a dose-dependent increase in transactivation but transactivation did not occur with the addition of p300 alone, thus showing that p300 coactivates KLF5 transcription. p300 is therefore a coactivator of KLF5. A mutant of p300 with the acetylase catalytic region deleted showed reduced activation, suggesting that acetylation and/or interaction through this region with KLF5 is important for transactivation of PDGF-A chain reporter activity by KLF5.

Mapping of the acetylation site of KLF5. As SET inhibited acetylation of KLF5, we reasoned that understanding the effects of KLF5 acetylation would lead to a better understanding of the actions of SET. For this, we determined the acetylated residue(s) in KLF5 and used nonacetylated point mutants to understand the implications of acetylation in vivo. To determine the acetylation site within the KLF5 ZF/DBD, peptides of each of the three zinc fingers were prepared and subjected to acetylation reactions (Fig. 6A). The first zinc finger was the only peptide acetylated by p300 for both GST proteins and synthetic peptides (Fig. 6B and data not shown). MS of the peptides further showed that the first zinc finger peptide is monoacetylated, as shown by a single shifted peak of 42 *m/z* (Fig. 6C). The second and third zinc finger peptides did not show a shifted peak and were thus not acetylated in vitro (data not shown).

To next identify the acetylated lysine residue, the acetylated first zinc finger peptide was digested with Lys-C endopeptidase, which cleaves after nonacetylated but not acetylated lysine residues. Analysis of expected fragment masses against actual masses showed that the lysine residue at amino acid number 369 was acetylated (Fig. 6D). To further confirm that this is the only acetylated residue, this lysine was mutagenized to an arginine residue (hereafter referred to as a K369R substitution) to preserve the similar basic charge and then the peptide was subjected to an acetylation assay which showed that the arginine substitution resulted in loss of acetylation (Fig. 6E). SET can bind both K369R and wild-type KLF5, suggesting that interaction is not impaired by this mutation (Fig. 6F).

Functional implications of KLF5 acetylation. To examine the effects of acetylation on KLF5 function, the transcriptional activity and cellular effects of the K369R mutation were tested. Transcriptional activation examined by cotransfection reporter analysis showed that the K369R mutation showed no effects on PDGF-A chain promoter activity compared to wild-type KLF5 when transfected alone (Fig. 7A). SET also inhibited transactivation by KLF5 similarly for the wild type and the K369R mutant (Fig. 7B), suggesting that interaction is important for the inhibitory effects of SET on KLF5 transactivation. Masking acetylation is a likely result of this interaction. Further, a 20% decrease in PDGF-A chain promoter activity was seen when p300 was cotransfected with the K369R mutant KLF5 construct compared to the KLF5 wild-type construct (Fig. 7C), showing that acetylation is important for transactivation of PDGF-A chain reporter activity by p300 on KLF5.

To further examine the effects of acetylation on KLF5 cellular activity, effects on cell growth were investigated. In cells transfected with the wild type or the K369R mutant by adenovirus-mediated transfer, the K369R mutant-transfected cells showed a reduction in cell number (Fig. 7D). A BrdU assay also showed reduced uptake in the K369R mutant-transfected cells, further showing that cell replication is decreased in these cells (Fig. 7E). Interestingly, results of the effects of the nonacetylated K369R mutation of KLF5 closely mimicked the effects of SET on KLF5, suggesting

was resolved on a 2% agarose gel. (D) Quantification of mRNA expression level for the PDGF-A chain. The expression level of the PDGF-A chain, an endogenous target gene of KLF5, was normalized to that of 18S. (E) KLF5 and SET expression in the pathological neointima. The immunohistochemistry of SET and KLF5 in a balloon injury model of atherosclerosis was examined. The left common carotid artery was denudated by balloon injury, and the neointima was observed 2 weeks after the balloon injury (Injured). The right common carotid artery served as a control (Control). The rat aorta was stained with anti-SET and anti-KLF5 antibodies. Cells in the neointima were clearly positive for SET and KLF5. All experiments were done at least twice with consistent findings. H/E, hematoxylin and eosin staining.