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クロマチン転写制御を目的とした人工酵素の開発に関する研究

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クロマチン転写制御を目的とした人工酵素の開発に関する研究

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研究要旨

真核生物における転写制御を理解するためには、クロマチン構造を解除する機構を解明しない限り、転写調節の制御機構論を理解することはできない。クロマチンの制御には３種類の酵素（化学修飾酵素、ATP非依存ならびに依存のクロマチン構造変換因子）が重要な役割を果たすことが近年明らかになった。クロマチン構造変換酵素は、DNA結合型転写因子と相互作用することにより特定のプロモーターへ誘導されるという真核遺伝子転写の選択性、特異性を説明しうる新しいメカニズムを我々は提唱してきた。

本計画では、クロマチン構造変換酵素とDNA結合型転写因子の相互作用に着目し、協調性を生み出す酵素・基質両者の機能上重要な領域を特定し、その制御（増減）を可能にすることを目的としている。DNA結合蛋白とクロマチン構造変換因子の相互作用は細胞や個体分化において重要な働きがある事を示唆する知見が得られており、本研究は細胞分化制御の理解、さらに細胞分化誘導をはじめとした疾患（癌、臓器再生）の新しい治療法の開発につながると期待できる。最終的にはヒトに代表される真核細胞のクロマチンからの選択的な転写制御を可能にし、人工転写酵素（クロマチン構造変換酵素）を用いたピンポイントでのナノ治療（ナノミセル等の導入）の基盤となる情報及び技術を開発する。

A．研究目的

本研究の目的は、ヒトにおけるクロマチン状態からの遺伝子転写を制御することを可能にするための基盤情報の集積及び技術の開発であり、最終的にはナノ技術を応用したクロマチンからの転写の操作を可能にする新しい治療法を開発することである。

転写因子をターゲットとする治療戦略の開発は受容体や酵素に比し、遅れている状況にある。その理由は、従来の転写研究は裸のDNAの状態を想定した研究が中心に発展したためと考えられる。すなわち、ヒトでは遺伝子転写を解明するうえで、クロマチン状態での制御の理解が不可欠であるが、過去の研究はin vitroでの裸のDNAの状態を対象としてきため、クロマチン状態からの転写を十分に説明できなかった。そのため、真核転写を対象とした治療戦略が発展しなかったと考えられる。しかしながら、遺伝子転写制御は病態をはじめ、あらゆる生命現象において中心的な役割を担っている。遺伝子転写制御を解明することは極めて重要な分野であり、クロマチンからの転写制御の理解が真核転写を理解する鍵になると考えられる。本計画で真核転写の基盤を明らかにし、真核細胞のクロマチンをターゲットとする遺伝子発現転写調節の新しい治療戦略を可能にすることが目的である。クロマチン構造変換酵素とDNA結合型転写因子の相互作用の制御は、細胞分化や癌化と密接に関わっていることは既に知られており、幅広く生命現象の制御に関わっている。この相互作用の制御が可能になれば、細胞分化誘導をはじめとする疾患（癌、臓器再生）の新しい治療法の開発につながると期待できる。具体的な例としては、ホメオボックス蛋白を標的とした臓器再生、E2Fによる細胞周期制御あるいはオンコジーン転写因子の不活化を通した抗癌療法などが考えられる。特定組織、細胞への導入には人工的なナノ技術が理想と考えられる研究課題である。このように、クロマチンレベルでの遺伝子発現転写調節を通した普遍的な治療法は、ナノマシンによるピンポイント・デリバリーとの併用により、癌から特定臓器疾患（心血管疾患等）に幅広く応用できる新しい治療法の開発につながると期待できる。

B．研究方法

遺伝子発現転写制御におけるクロマチンの役割及びその制御は約５年前から内外において注目されている分野である。DNAとともにクロマチンの基本コンポーネントを構成するヒストンの役割が主に研究されてきた。しかしながら、ヒストンは普遍的に存在するため、遺伝子特異性・選択性は説明できないといち早く注目した我々は、DNA結合蛋白とクロマチン構造変換因子の相互作用が真核生物での転写活性化の機構論の解明の糸口になると考え、転写反応の特異性を決定する上でもっとも重要なDNA結合型転写因子との協調的な相互作用を通した制御機構に注目した。

具体的には、以下のような実験を行った。

１。クロマチン構造変換酵素とDNA結合方転写因子の相互作用の単離・同定

・相互作用因子単離同定法：Sp/KLF因子の6HIS (hexahistidine)エピトープを付加したリコンビナント蛋白質を調製し、細胞核抽出液からSp/KLF因子と相互作用する因子をHISエピトープに対するアフィニティ精製を用いて単離した。SDS-PAGEゲル電気泳動にて展開後、Coomassie Brilliant Blueで染色し、分離の良いバンドは切り出した。分離の悪いバンドについては、pH勾配で二次元電気泳動を行い、切り出した。切り出したバンドは、酵素消化後質量分析器(MALDI TOF-MS)にて質量パターンを解析し、さらにフィンガープリント法でアミノ酸配列を推定した。

２。クロマチン構造変換酵素とDNA結合方転写因子の相互作用の機能的意義の解析

・相互作用の検討：リコンビナント蛋白質を用いた直接相互作用の解析には、GST pull-down法を用いた。具体的には、リコンビナント蛋白質にはGSTを付加し、GSTに対するアフィニティ・レジンで固定した。相互作用因子側（エピトープに対するウエスタン、His等）で検出した。細胞免疫沈降にはProtein-A/G sepharoseに抗体を固定し、細胞抽出液を添加後、相互作用因子側に対する抗体でイムノブロットした。
・DNA結合能の検討：ゲルシフトアッセイをSuzuki et al. 2000 の方法にしたがって行った。^{32}Pでラベルした DNAプローブにリコンビナント蛋白質を反応後、アクリルアミドゲルにて電気泳動し、オートラジオグラフィーにて結合の有無と程度を検出した。Sp1はSV40のGC配列、KLF5はSE1配列をプローブに用いた。
・転写活性の検討：レポーター・トランスフェクション・アッセイを行った。4x10^{5}個の細胞を24穴プレートにまき、24時間後にリポフェクチン法を用いてプラスミドを細胞に導入し、さらに48時間後に細胞を回収し、ルシフェラーゼアッセイのプロトコール通り(Promega)にルシフェラーゼ・レポーター活性を測定した。Sp1はSV40のearly プロモーターを、KLF5にはSMembとPDGF-A鎖両遺伝子のプロモーターのレポーター・コンストラクトを用いた。
・アセチル化能の検討：アセチル化反応をSuzuki et al. 2000の方法にしたがって行った。具体的には、^{14}Cでラベルした acetyl CoAをアセチル化酵素反応に加え、SDS-PAGE電気泳動後、オートラジオグラフィにてアセチル化の有無と程度を評価した。
・細胞内遺伝子発現調節の検討：フォルボル・エステルをはじめとする増殖刺激剤を添加した培養細胞株を経時的にサンプリングし、RT-PCR解析(PDGF-A鎖遺伝子) 用にRNAを、ウエスタンブロット解析用に蛋白質をそれぞれ調製した。
・血管病態モデルでの検討：ラットでのバルーン障害モデルは、頸動脈をバルーンで拡張後、２週後に病理切片を作製し、免疫組織評価を行った。
・安定発現細胞株の作製並びに細胞増殖能の検討：安定発現細胞株は、G418カセットを含む発現ベクターを構築後、ネオマイシンに対する耐性を基に安定発現細胞を株化した。細胞増殖の検討は、細胞の経時的な細胞数の計測の他、BrdUの導入効率を測定した。
・点変異ならびにアデノウイルスのコンストラクトの作製：アセチル化残基の点変異はPCR法を用いて導入した。アデノウイルスはDNA-TPC法を用いて作製した。

３。クロマチン構造変換酵素の結晶構造解

析

・結晶作製、結晶構造解析：リコンビナントを大腸菌内で発現後、アフィニティ精製した。濃縮とサイズ分画を行い、高純度の標品を得た。hanging drop法で結晶を作製し、X線ビームで回折像を得た。

（倫理面への配慮）
本計画は、本学の組み替え実験計画の承認を得ている。生化学、細胞生物学的実験は、当施設の実験ガイドラインを遵守する。また、動物実験については、動物愛護の観点から配慮する。

C．研究結果

クロマチン構造を解除する機構を解明しない限り、真核生物における転写調節の制御機構論を理解することはできない。クロマチンの制御には３種類の酵素（化学修飾酵素、ATP非依存ならびに依存のクロマチン構造変換因子）が重要な役割を果たすことが近年明らかになった。クロマチン構造変換因子は化学修飾酵素（アセチル化、メチル化、燐酸化、ユビキチン化）及びATP依存（SWI/SNF等）とATP非依存（ヒストンシャペロンASF/CIA/RCAF、TAF-1等）のクロマチン構造変換因子の３群に大別されるが、これらの因子の酵素活性の機能ならびに構造は十分に明らかにされていない。しかしながら、酵素活性がクロマチン構造変換にとって必須であるため、活性制御がクロマチン構造変換の制御の鍵になると考えられる。そのため、酵素活性領の制御（増減）を通したクロマチンへのアクセスの調節の視点からの研究を進めることは、真核転写を解明し、さらに調節を可能にするうえで重要と考えられる。実際に、世界でもっとも解析が進んでいるアセチル化酵素p300の酵素活性領域をマップするための点変異ミュータントは我々自身が世界に先駆けて作製した(Suzuki et al., 2000)。

クロマチン構造変換酵素は、DNA結合型転写因子と相互作用することにより特定のプロモーターへ誘導されるという真核遺伝子転写の選択性、特異性を説明しうる新しいメカニズムを我々は提唱してきた。今までに、DNA結合蛋白とクロマチン構造変換因子の相互作用及び制御の機能的な意義を明らかにしてきた。まず、アセチル化酵素とDNA結合蛋白の相互作用を解析した。過去に我々が単離同定したDNA結合蛋白Sp/KLFファミリー因子間(Suzuki et al., 1998) にアセチル化酵素との相互作用に特異性があり、また相互間の制御を明らかにした(Suzuki et al., 2000)。ファミリー因子間の特異的な制御におけるアセチル化酵素との相互作用の役割、またDNA結合蛋白によるアセチル化酵素活性の制御を明らかにした世界ではじめての例であった。

研究期間中に、同DNA結合蛋白Sp/KLFファミリー因子の相互作用因子を単離・同定し、ATP非依存のクロマチン構造変換因子との相互作用を明らかにした。その結果、世界ではじめてDNA結合蛋白ファミリー因子とATP非依存クロマチン構造変換因子間の相互作用とその特異性を示し、さらにその機能的な意義を示した(Suzuki et al., 2003; Miyamoto, Suzuki et al., 2003)。

具体的には、Sp/KLFのリコンビナント蛋白質を用いて細胞核抽出液から相互作用因子をアフィニティ精製後、バンドをTOF-MS法にて同定した。今回は、ATP非依存のクロマチン構造変換因子TAF-Iの単離に成功した。Sp/KLF因子とTAF-Iの相互作用を確認するために、リコンビナント蛋白質を用いたin vitroでのGST pull-downアッセイで直接結合を確認後、抗体を用いた免疫沈降を施行し、細胞内で実際に相互作用することを確認した。Sp1はHeLa細胞からアフィニティ精製の結果、TAF-Iのαとβの両アイソフォームと相互作用したが(Suzuki et al., 2003)、KLF5はTAF-Iβ/SETのみと相互作用した(Miyamoto, Suzuki et al., 2003)。また、TAF-Iはp53, MyoD, NFkappaB等他のDNA結合型転写因子と相互作用しなかった。さらに、TAF-Iと同様にATP非依存クロマチン構造変換因子（ヒストンシャペロン）であるASF/CIA/RCAFはSp/KLFと相互作用しなかったため、DNA結合型転写因子Sp/KLFとATP非依存クロマチン構造変換因子（ヒストンシャペロン)TAF-Iの相互作用に特異性を認めた。

次に、相互作用の機能的な意義を検討するために、Sp/KLF因子のDNA結合能ならびに転写活性化能への影響を検討した。ゲルシフトアッセイ、レポーター・コトランスフェクションアッセイを施行した結果、TAF-IはSp/KLF因子(Sp1, KLF5) のDNA結合

活性及び転写活性化能を抑制し、リプレッサーとして作用することを明らかにした。具体的には、TAF-Iαとβは両方ともSp1のDNA結合配列(SV40遺伝子配列) への結合を抑制し、Sp1によるSV40遺伝子プロモーターの転写活性化を阻害した。また、SETはKLF5のDNA結合配列(SMemb遺伝子SE配列) への結合を抑制し、KLF5によるSMemb、PDGF-A鎖両遺伝子のプロモーターの活性化を阻害した。よって、TAF-IはSp/KLF因子の複数の活性を抑制した。

TAF-Iの細胞ないし個体のレベルでの機能を解析するために、細胞機能が明らかなKLF5を中心に解析した。KLF5はprotooncogeneであり、心血管系を中心に細胞レベルでは細胞増殖を促進し、病態形成に関わる。組織レベルでは、心血管系臓器の病態刺激に対するリモデリング反応を制御する (Shindo et al., 2002)。一方、TAF-Iはtumor suppressor因子と考えられており、それに対してKLF5はprotooncogeneであることから、TAF-IはKLF5の細胞機能を負に制御する可能性があると考えた。KLF5を発現する細胞株を樹立した結果、細胞増殖が促進された。TAF-1を同細胞にトランスフェクトした結果、細胞増殖が抑制された。また、病態においてもKLF5とTAF-Iは興味深くカップリングした発現パターンを認めた。KLF5とTAF-Iはともに正常血管ではほとんど発現を認めないが、バルーン障害後の新生内膜で両者とも発現が誘導された。病態における発現を検討するために、モデルアゴニストとしてphorbol ester刺激を用いた細胞実験の結果、刺激後KLF5は発現が誘導されるのに対して、TAF-Iは発現が抑制された。これらの知見より、TAF-IはKLF5の抑制因子であり、刺激(phorbol ester)によりTAF-I自身の発現が抑制され、さらにKLF5の発現が亢進することにより、KLF5が活性化されるメカニズムが考えられた。TAF-Iのアデノウイルス等の発現コンストラクトを用いて、遺伝子導入による治療効果を検討した結果、KLF5導入により血管病態モデルでの新生内膜が増大するのに対し、TAF-Iの導入によりその増大の阻害が確認された。遺伝子治療の可能性が示された。

同時に、KLF5とアセチル化酵素p300が相互作用し、その結果、後者が前者をアセチル化し、さらに転写を活性化することを示した。アセチル化修飾を受ける残基を質量分析器で特定した。点変異を作成した結果、アセチル化されない点変異体は、KLF5 wild typeでみられる細胞増殖促進作用を有さず、また転写活性化を示さなかった。アデノウイルスコンストラクトを血管病態モデルに投与した結果、新生内膜の増大を認めず、KLF5の作用においてアセチル化が重要であることを示した。さらに、TAF-Iはp300によるKLF5のアセチル化を阻害し、KLF5に対するTAF-Iの作用は、アセチル化の阻害も介することを示した。DNA結合転写因子に対して、p300とTAF-Iがそれぞれ正負に作用し、そのメカニズムが相互作用並びに化学修飾のカップリングを介することをはじめて示した。

また、脱アセチル化酵素によるDNA結合転写因子への直接作用を明らかにした (Matsumura, Suzuki et al., 2005)。具体的には、KLF5が脱アセチル化酵素HDAC1と相互作用し、その結果前者のDNA結合活性ならびに転写活性を阻害することを示した。さら、KLF5との相互作用において、HDAC1とp300が競合することを示した。p300とTAF-Iによる協調的な制御と併せて、複数のクロマチン構造変換因子によるDNA結合転写因子への協調的制御をはじめて明らかにした。

これらの新規のクロマチンレベルでの遺伝子発現の制御に関わる分子メカニズムに対して創薬を試みた。まず、前述のアデノウイルスを用いた実験を通して、遺伝子治療の可能性を示した。相互作用を制御すっる化合物をスクリーニングし、現在も進行中である。また、DNA結合型転写因子Sp/KLFとクロマチン構造変換酵素TAF-Iの相互作用の物理化学的な基盤を明らかにし、ピンポイント制御薬を開発する目的で、TAF-Iの結晶構造を解析した。その結果、2.8オングストロームの解像度のX線回折像を得た(Muto et al., in press)。最終的には、複合体解析を行い、相互作用の作用点をピンポイントで制御するコンパウンドをデザインする。

D. 考察

我々は、クロマチン構造変換酵素とDNA結合型転写因子が相互作用することにより特定のプロモーターへ誘導されるという真核遺伝子転写の選択性、特異性を説明しうる新しいメカニズムを提唱してきた。今まで

に、我々の仮説に基づき、DNA結合蛋白とクロマチン構造変換因子の相互作用及び制御の機能的な意義を明らかにしてきた。アセチル化酵素とDNA結合蛋白の相互作用及び、相互間の制御さらにDNA結合蛋白によるアセチル化酵素活性の制御を世界ではじめて明らかにした研究実績に立脚し、今回新たにATP非依存クロマチン構造変換因子との相互作用を明らかにした。

E．結論

本研究の基礎となるクロマチン構造変換酵素とDNA結合型転写因子の協調的な相互作用のメカニズムは我々が世界に先駆けて見出したことであり(Suzuki et al., 2000)、このメカニズムの応用である本計画は極めて独創性が高く、また内外において他に研究されていない状況にある。

本計画の特徴のひとつは、クロマチン構造変換因子の酵素活性に着目した点にある。すなわち、クロマチン構造変換因子は化学修飾酵素（アセチル化、メチル化、燐酸化、ユビキチン化）及びATP依存（SWI/SNF等）とATP非依存（ヒストンシャペロンASF/CIA/RCAF、TAF-1等）のクロマチン構造変換因子の３群に大別されるが、これらの因子の酵素活性の機能ならびに構造は十分に明らかにされていない。しかしながら、酵素活性がクロマチン構造変換にとって必須であるため、活性制御がクロマチン構造変換の制御の鍵になると考えられる。そのため、酵素活性の制御（増減）を通したクロマチンへのアクセスの調節の視点からの研究を進めることは、真核転写を解明し、さらに調節を可能にするうえで重要と考えられる。

F．健康危険情報

特になし

G．研究発表

１．論文発表

Muto S, Senda M, Adachi N, Suzuki T, Nagai R, Horikoshi M. Purification, crystallization and preliminary X-ray diffraction analysis of human oncoprotein SET/TAF-1β. Acta Crystallogr D Biol Crystallogr. 2004;60:712-4.

Matsumura T, Suzuki T, Aizawa K, Munemasa Y, Muto S, Horikoshi M, Nagai R. The deacetylase HDAC1 negatively regulates the cardiovascular transcription factor Kruppel-like factor 5 through direct interaction. J Biol Chem. 2005 in press.

２．学会発表

・アメリカ心臓病学会 American Heart Association（平成１６年１１月９日ー同１３日、米国ニューオーリンズ）

Takayoshi Matsumura, Toru Suzuki, Kenichi Aizawa, Yoshiko Munemasa, Kana Iimuro, Ryozo Nagai. The Cardiovascular Remodeling Transcription Factor KLF5 is Regulated by the Histone Deacetylase HDAC1 through Direct Interaction.

・第１０回放射光医学研究会講演会(平成１７年１月２２日、大阪）。

鈴木亨、武藤真祐、千田俊哉、堀越正美、永井良三。心血管転写因子KLF5及びそれの相互作用制御因子の結晶構造解析を通したピンポイント創薬

H．知的財産権の出願・登録状況（予定を含む）。

１．特許取得
なし

２．実用新案登録
なし

３．その他
なし

研究成果の刊行に関する一覧表

書籍

著者氏名	論文タイトル名	書籍全体の編集者名	書籍名	出版社名	出版地	出版年	ページ
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雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Muto S, Senda M, Adachi N, Suzuki T, Nagai R, Horikoshi M	Purification, crystallization and preliminary X-ray diffraction analysis of human oncoprotein SET/TAF-1β	Acta Crystallogr D Biol Crystallogr	60	712-714	2004
Matsumura T, Suzuki T, Aizawa K, Munemasa Y, Muto S, Horikoshi M, Nagai R	The deacetylase HDAC1 negatively regulates the cardiovascular transcription factor Kruppel-like factor 5 through direct interaction	J Biol Chem		in press	2005

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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, $\beta = 89.7°$, and contains two molecules in the asymmetric unit. A complete data set was collected to 2.8 Å resolution using synchrotron radiation.

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.

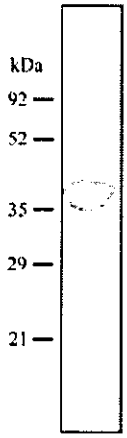

Figure 1
SDS–PAGE of SET/TAF-1β stained with Coomassie Brilliant Blue.

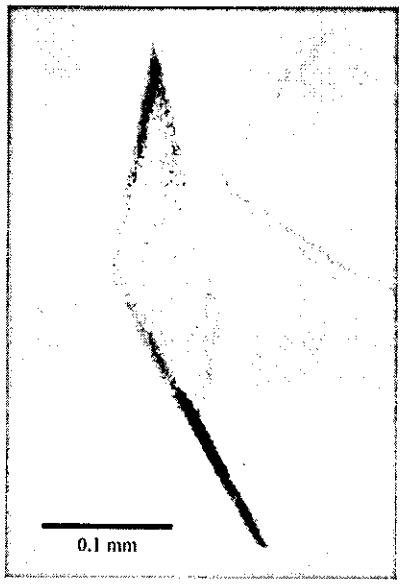

Figure 2
Crystal of human oncoprotein SET/TAF-1β.

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).

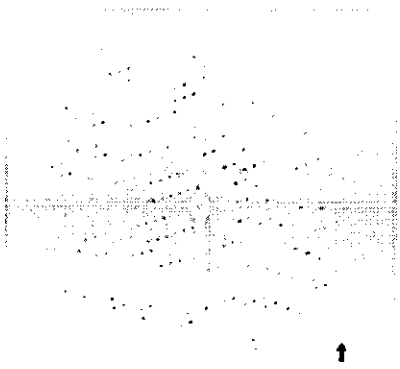

Figure 3
A 1° oscillation diffraction pattern from a crystal of SET/TAF-1β. The arrow indicates a resolution of 2.80 Å.

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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(NEDO) and the Exploratory Research for Advanced Technology (ERATO) of the Japan Science and Technology Corporation (JST).

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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.

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.

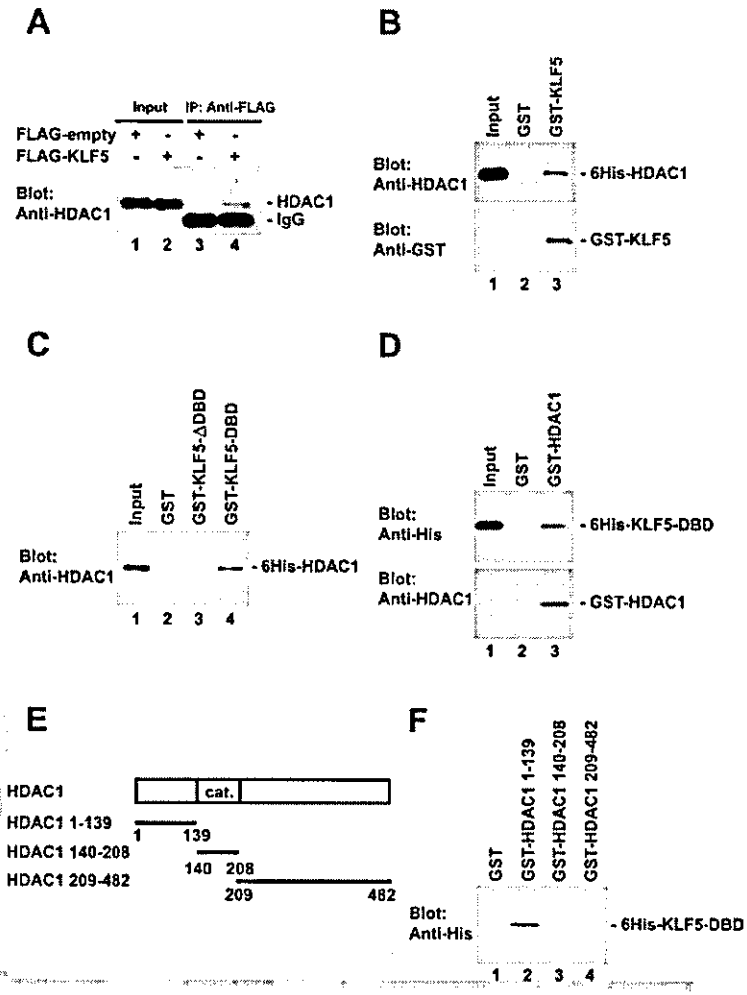

FIG. 1. **Interaction of KLF5 and HDAC1 in the cell and *in vitro*.** *A*, co-immunoprecipitation of HDAC1 with KLF5. Cell lysate with FLAG-tagged KLF5 overexpression (*lane 4*) and without overexpression (*lane 3*) was immunoprecipitated with anti-FLAG affinity gel. Immunoblot with anti-HDAC1 antibody 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). AQ: E

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. AQ: F

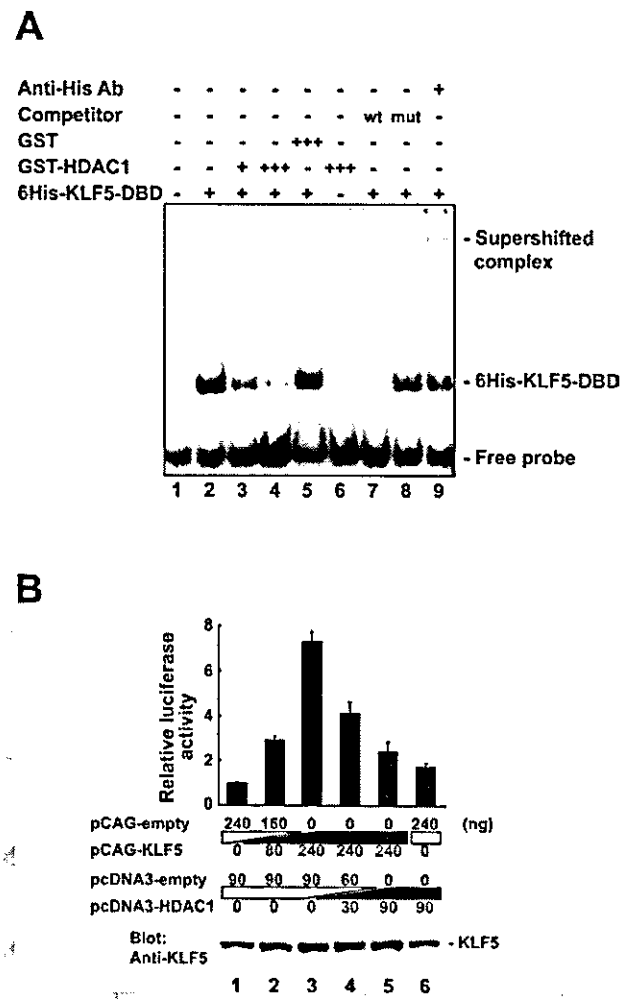

FIG. 2. **Effect of HDAC1 on DNA binding affinity of KLF5 and its promoter activation.** *A*, gel shift assay with hexahistidine-tagged KLF5-DBD and GST-tagged HDAC1. *Wt* and *mut* represent wild and mutant oligonucleotide competitors (*lanes 7* and *8*). The amount of recombinant protein is as follows: 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. AQ: O

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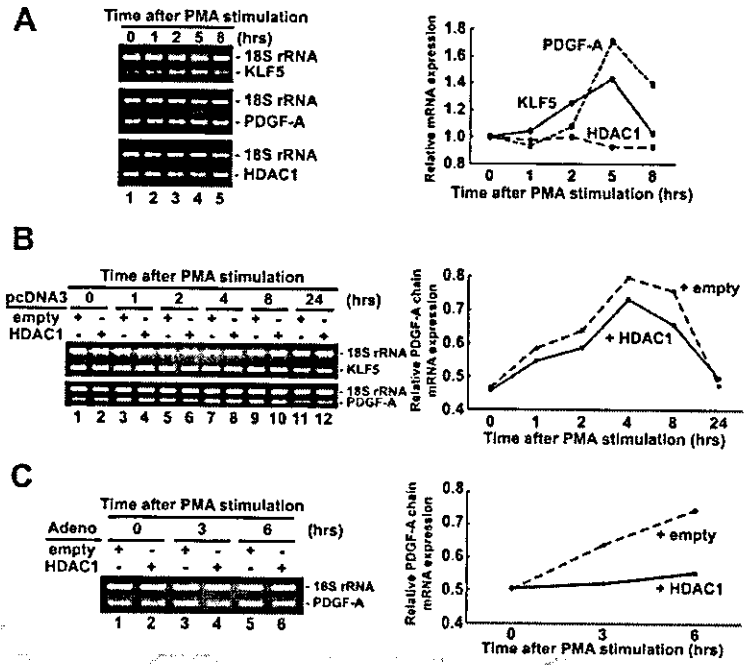

FIG. 3. **Effect of HDAC1 on phorbol ester-stimulated activation of PDGF-A chain.** *A*, induction of KLF5 and its downstream gene, PDGF-A chain, by phorbol ester. The expression level of HDAC1 was not affected. The *graph* on the *right* shows quantification of mRNA expression levels for KLF5, PDGF-A 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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AQ: I *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
F3 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.

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
F4 (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*).

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.
F5 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.

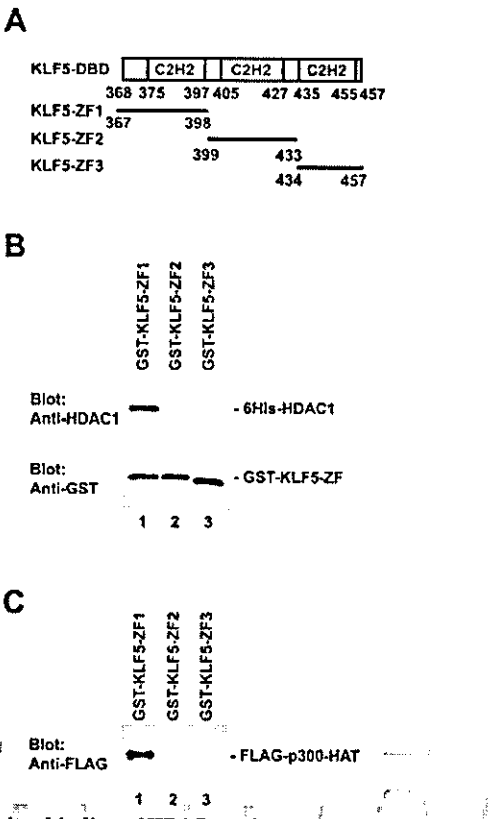

FIG. 4. **In vitro binding of HDAC1 and p300 to KLF5 through its first zinc finger.** *A*, schematic representation of GST-tagged zinc finger peptides of KLF5. GST-tagged zinc finger peptides of KLF5 cover each of the zinc fingers, respectively, from the amino terminus. *B*, hexahistidine-tagged HDAC1 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 ac- AQ: J
tion 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 F6
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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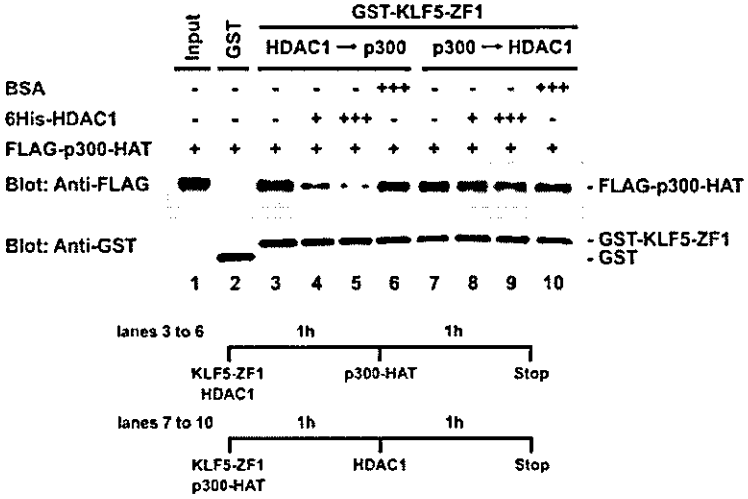

FIG. 5. **In vitro competition of HDAC1 and p300 for binding to KLF5.** *In vitro* binding assay was performed using 0.75 μg of the immobilized GST-tagged first zinc finger of KLF5 (*lanes 3-10*) or GST protein alone (*lane 2*). In *lanes 3-6*, 1.5 μg of the FLAG-tagged p300-HAT domain was added 1 h 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.

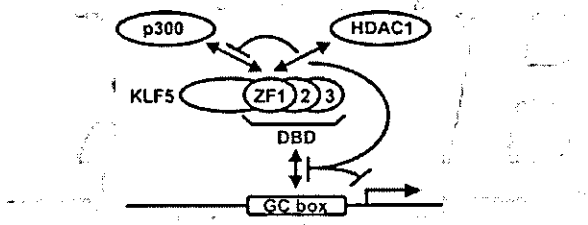

FIG. 6. **Schematic representation of results.** HDAC1 interacts directly with the first zinc finger of KLF5, impairs its DNA binding affinity, and negatively regulates KLF5-dependent promoter activation. In addition, HDAC1 can exert its negative regulatory function by interfering with the binding of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