ACETYLATION BLOCKS TRANSCRIPTION REPRESSION

by

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ABSTRACT

Acetylation of histones and transcription factors is a primary mechanism of gene regulation. Histone acetylation is thought to facilitate transcription by remodeling nucleosome structure. The mechanisms by which factor acetylation modulates gene expression are poorly understood. Among the major acetylases that have been identified is the coactivator, CBP/p300. CBP/p300 acetylates both histones and non-histone proteins to stimulate transcription. In this thesis, I show that the histone acetylation activity of CBP/p300 is facilitated by its interaction with a histone chaperone protein, RbAp46/48. I demonstrate that RbAp46/48 interacts with the phosphorylated CREB-CBP complex to recruit nucleosomal histones to CBP/p300. Recruitment of nucleosomes to CBP/p300 by RbAp46/48 lowered the K_m of CBP/p300 for histones and facilitated CBP/p300-mediated transcription from in vitro assembled chromatin templates.

CBP/p300-mediated transcription factor acetylation was investigated in the context of nuclear hormone receptor driven genes. I demonstrate that the ligand-dependent corepressor RIP140 represses transcription by its interaction with the corepressor, CtBP. This interaction was disrupted by the specific acetylation of RIP140 within its CtBP binding motif by CBP/p300, abrogating nuclear receptor-dependent transcriptional repression. Acetylation, therefore, not only modifies the core structure of chromatin, but also blocks transcription repression by reconfiguring the composition of repressive complexes.

INTRODUCTION

One of the major concerns in the field of gene regulation is how transcription factors modulate the activation state of a gene in its native conformation. Unlike textbook diagrams of DNA, a gene does not exist in its naked state, with its regulatory elements freely accessible for transcription factor binding. Instead, the native state of a gene is packaged into a highly dense structure that occludes many regulatory DNA elements. A major question in the field, then, is how does transcriptional regulation occur in the context of chromatin? Does the structure of chromatin need to be modified to facilitate formation of transcription regulatory complexes?

Many chromatin remodeling factors have been identified that may aid transcriptional regulation. Among the plethora of factors include enzymes that reconfigure chromatin on many levels—from its highly condensed state to its individual nucleosomal histone form. Characterization of these factors revealed that their enzymatic activities are not restricted to histones and have broad substrate specificity. These discoveries gave rise to questions of how these enzymes selectively modify chromatin and specific transcription factor substrates.

The best-studied histone and non-histone modification is acetylation. The focus of my thesis centers on determining how the activity of coactivator acetyltransferases such as the CREB binding protein (CBP) and its paralog, p300, regulate transcription through their ability to modify both histone and non-histone substrates. In particular, I addressed two issues:

1. How does CBP and p300, proteins that interact with innumerable transcription factors and have a wide variety of substrates, selectively target histone proteins over non-histone proteins? Are there mechanisms by which CBP/p300 targets its acetyltransferase activities towards histones to efficiently stimulate transcription?

and

2. What is the role of factor acetylation in transcriptional regulation? Many hypotheses have been proposed, yet conflicting data has confounded the formulation of a conclusive regulatory role for acetylation of non-histone proteins. To address this question, I examined the nuclear hormone receptor-dependent mechanism of transcriptional regulation to determine how acetylation of a particular coregulator, RIP140, at a specific site, regulates ligand-dependent transcriptional activation or repression.

The General Transcription Machinery

Regulation of transcription is a key step in modulating gene expression. Transcriptional processes can be divided into three steps: initiation, reinitiation, and elongation. All these processes are mediated by the specific recruitment of transcription factor complexes to active sites of transcription. These complexes interact with enhancer and core promoter elements in a combinatorial manner to regulate the expression of a diversity of genes. Binding of activator proteins to select DNA elements may recruit additional coregulators. The sequence composition of these DNA elements can play a crucial role in determining whether activation or repression occurs. For example, recruitment of the transcription factor, NC2/DR1, differentially regulates expression of

TATA element or DPE (downstream promoter element) containing genes (20, 22, 105). NC2/DR1 was originally characterized as a repressor of TATA containing genes (75) but was later discovered to be an activator of DPE containing genes (245). Furthermore, formation of an enhanceosome complex creates stereospecific interaction surfaces with DNA that promote the cooperative recruitment of coactivators and the RNA polymerase II complex (RNAPII) to active sites of transcription (129). In this manner, enhanceosomes increase the kinetics of transcription initiation and reinitiation (reviewed in (129)). Coordination between the enhancers, promoters, and the proteins they recruit generates the specificity required for transcriptional regulation.

The general transcription machinery is composed of RNA polymerase II and a host of general transcription factor complexes (reviewed in (247)). In many genes, the core promoter TATA element is located roughly 35bp upstream of the transcription start site. This TATA box is frequently followed by an initiator (Inr) element. In many classes of genes, however, the Inr element is followed by a DPE, which is located roughly 30bp downstream of the transcription start site. Although not all genes contain a combination of TATA, Inr, and DPE, the presence of each of these three elements provides binding sites for TFIID, the TATA binding protein (TBP) and TBP associated factor (TAFs)-containing complex. Sequential recruitment of the different general transcription factor complexes then leads to phosphorylation of the carboxyl-terminal domain of RNA polymerase II by TFIIH, resulting in gene activation. The combinatorial complexity provided by enhancer-promoter-activator recruitment is just one nuance involved in transcriptional regulation. These select transcription factor binding and unbinding events occur in the context of the native gene, in the context of chromatin.

Chromatin

Rather than being an inert storage unit of genetic information, the structure of the native gene modulates the accessibility of regulatory transcription factors to DNA. Chromatin is a complex of DNA wrapped around histone proteins, resulting in the compaction of two meters of DNA into a single eukaryotic cell nucleus. The series of successively ordered DNA-histone folds is composed of its basic subunit, the nucleosome. Originally described by Kornberg (98) and Oudet et al (162), the nucleosome consists of approximately 146 base pairs of DNA wrapped around a histone octamer that is composed of two dimers of histones H2A-H2B and H3-H4. The crystal structure of the nucleosome reveals that the core histone octamer-DNA interaction is a defined and well-ordered interaction accompanied by relatively disordered amino-terminal (N-terminal) histone tails (Figure 1). These tail domains are thought to mediate higher order nucleosome interactions (118), creating the nucleosome clusters that make up the 10nM nucleosome filament, the 30nM fiber, and the chromosome. Compaction of DNA regulates gene expression by limiting access of sequence specific DNA binding proteins to their select DNA elements.

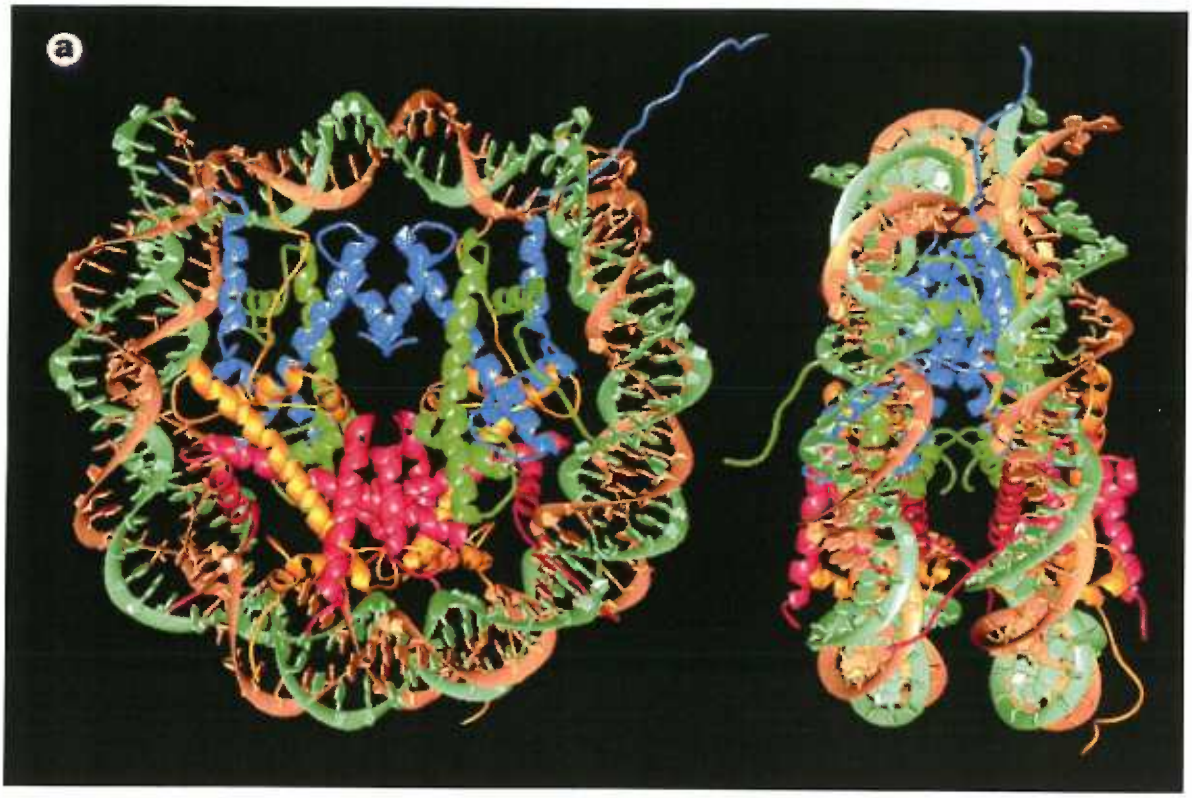

Figure 1. Crystal structure of the nucleosome core particle. DNA is depicted in brown and turqoise, H3 in blue, H4 in green, H2A in yellow, and H2B in red (118).

Two types of chromatin characterize the eukaryotic genome: heterochromatin and euchromatin. Heterochromatin domains are condensed regions of chromatin that are characterized by the high abundance of transcriptionally silenced genes (reviewed in (183)). These regions are primarily inaccessible to DNA binding proteins and are resistant to DNase I digestion. Euchromatin, however, represents genomic domains that are primarily characterized by actively transcribed genes that are highly sensitive to DNase I digestion. Although the molecular mechanisms that create DNase I sensitive sites are unknown, this sensitivity is indicative of "open" or decondensed chromatin. Highly condensed chromatin, such as heterochromatin, however, occludes DNase I hydrolysis of the phosphodiester backbone of DNA. The sensitivity of chromatin to

DNase I digestion is indicative of the transcriptional competence of a given gene. This sensitivity extends beyond the coding regions of DNA. The locus control regions (LCR), for example, are relatively small regulatory chromatin domains that are hypersensitive to DNase I and confer high expression of linked genes. This type of hypersensitivity (vs general DNase I sensitivity) is frequently found in enhancer and promoter regions and may be indicative of the "on" state of a gene. The actions of specific regulatory elements at the LCR, though frequently located many kilobases away from enhancer-promoter elements may regulate the assembly of coactivator and corepressor complexes at enhancer-promoter domains (reviewed in (19)).

Chromatin Remodeling

In order for transcription to occur, the structure of chromatin must be modified to expose the regulatory DNA elements for transcription factor binding. These rearrangements are coordinated by specific enzymatic complexes that act on higher order chromatin, on nucleosomes, and on histones. Binding of activator proteins to DNA stimulates further recruitment of additional chromatin regulatory factors to allow the transcription process to proceed.

Initiation of transcription requires some level of chromatin decompaction at the promoter that allows recruitment of enhancer and core promoter binding proteins to their DNA elements. Although assembly of the preinitiation complex (PIC) is generally thought to follow recruitment of separate chromatin remodeling enzymes, chromatin immunoprecipitation (ChIP) assays indicate that assembly of the PIC on the α_1-AT promoter actually precedes recruitment of these distinct remodeling enzymes (203).

These results suggest that the activities of other regulatory enzymes such as those present in the PIC itself, perhaps the histone modifying enzyme, TAFII250, may be necessary for the remodeling events during PIC assembly.

Following this assembly, the general transcription factors transcribe approximately 15-25bp of DNA before the transcription complex encounters a block to elongation. Recruitment or activation of discrete transcription elongation proteins must then occur for elongation to proceed (reviewed in (160)). Following promoter pausing and reinitiation, the RNAPII complex must still track along the DNA template for elongation of the nascent RNA transcript to occur. The crystal structure of the RNAPII complex and the transcription elongation complex demonstrates that RNA polymerase II initiation (39) and elongation complex (57) forms a clamp and jaw structure that facilitates its procession and eventual exit from DNA. For both initiation and elongation to proceed, the structure of chromatin must be remodeled to facilitate the procession of the RNAPII complex along chromatin.

Chromatin remodeling occurs on many levels, ranging from removal of linker histones to reorganization of histone-DNA contacts. Histone H1 is a linker histone that is absent from actively transcribed chromatin. It promotes folding of chromatin and contributes to the regular spacing of nucleosomes. Depletion of H1 is one mechanism by which chromatin can be remodeled. In addition, many enzymatic complexes are recruited to active sites of transcription to reconfigure the structure of chromatin.

ATP-dependent Remodeling Complexes

An important class of chromatin remodeling proteins is represented in the ATP dependent remodeling complexes. Among these remodeling complexes are the SWI/SNF or SWI/SNF related RSC and ISWI complexes. The catalytic subunits of these complexes use the energy from ATP hydrolysis to remodel the structure of chromatin. Many organisms possess multiple ATP dependent remodeling enzyme complexes that stably alter the structure of chromatin (reviewed in (144, 228)). There are three general classes of ATP-dependent remodeling complexes, the SWI/SNF group, which is composed of the conserved catalytic SWI/SNF component, the ISWI group, in which the ATPase is the ISWI protein, and the Mi-2 chromatin remodeling and deacetylase complex, whose ATPase subunit is a SWI/SNF homolog, CHD4 (or Mi-2ß). Targeting of SWI/SNF complexes to active sites of transcription is mediated by sequence specific DNA binding proteins (146, 147).

Several models have been suggested to explain how chromatin is remodeled. Among these models are nucleosome sliding and repositioning as well as histone removal. In the former mechanism, the activities of ATP-dependent remodeling enzymes allow nucleosomes to slide along the DNA template or translationally reposition themselves as a way of opening up chromatin (10, 82, 244). However, other studies suggest that upon recruitment of chromatin remodeling enzymes, nucleosomal histones are selectively removed from chromatin by either transfer to chaperone proteins for removal (77, 117) from chromatin or by targeting to the 26S proteosome degradation pathway (168). The results of both of these types of remodeling exposes DNA elements for transcription factor binding.

In essence, the decompaction of chromatin promotes transcription activation by allowing transcription factors to compete with histones for binding to DNA. Binding of activators, for example, recruits additional chromatin remodeling enzymes to active sites of transcription. Elongation of the nascent RNA transcript requires recruitment of additional cofactors to facilitate movement of the RNAPII complex along its chromatin template. In the cellular environment, the RNAPII complex has been estimated to track along DNA at 25 nucleotides per second. However, in vitro transcription assays only approach that rate on naked DNA. These results indicate that additional chromatin-specific factors must also be recruited and identified for efficient transcription elongation to occur.

Several such factors have been described in addition to the SWI/SNF related proteins. These factors include the high mobility group proteins such as HMG14, which is found in actively transcribed genes and has been found to increase chromatin-dependent RNAPII elongation in vitro (21). HMG proteins are also components of enhanceosomes and may create the stereoselective structure of this complex (49).

Other Complexes

Other types of chromatin-specific modifying complexes that have been identified alter chromatin structure by selectively removing histone dimers from the nucleosome. The FACT complex (113, 159) contains an HMG like protein, SSRP1, as well as a homolog of the *S. cerevisiae* protein, Spt16. FACT facilitates chromatin remodeling by selectively removing H2A-H2B dimers. These conclusions are supported by studies in which in vitro cross-linking of histones prevents the ability of FACT to facilitate

transcription elongation (161). Removing histones from chromatin has also been demonstrated to follow acetylation of nucleosomal histones, which results in their transfer from chromatin to the histone chaperone protein, NAP1 (77). Furthermore, these types of chromatin rearrangements have been shown to act in cooperation with histone modifying complexes to facilitate RNAPII mediated transcription elongation.

Histone Modifications

In the nucleosome, each histone octamer has its amino terminal portion accessible to enzymatic modification. These modifications occur on histone tails and alter nucleosome structure. The activities of both ATP-dependent remodeling enzymes and histone modifying enzymes generate a specific chromatin environment to allow transcription factor binding and render transcriptional competence to a given gene (Figure 2) (reviewed in (144)).

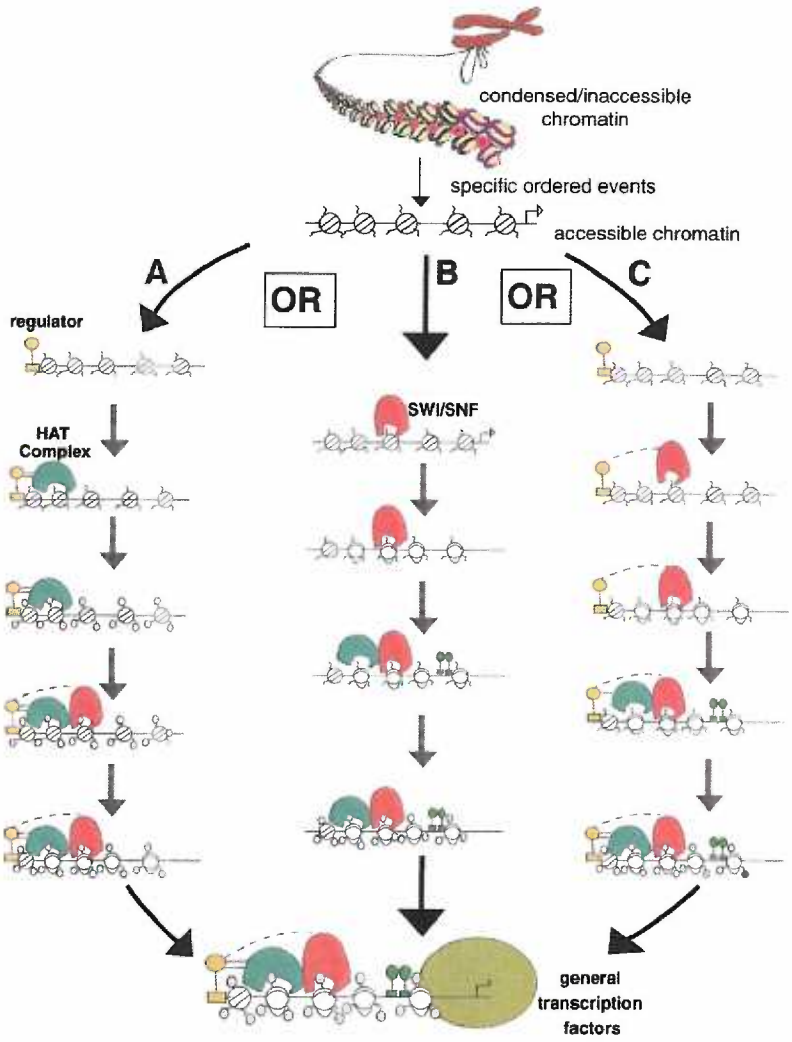

Figure 2. Recruitment of chromatin remodeling complexes by potential sequential pathways (A, B, C) to create a transcriptionally competent gene. DNA binding regulators are depicted in yellow, HAT complex in green, and SWI/SNF complex in red (144).

A major advance in the field of transcription regulation was the recognition that transcription factors had the ability to mediate gene induction through the recruitment of specific enzymatic complexes that modify select residues of the nucleosomal histone. Recent analysis of the crystal structure of the nucleosome demonstrates that the N-terminal tail of these chromatin components is accessible to histone modifying enzymes

(118). Though posttranslational modifications of non-histone proteins have been well documented, modification of select histone residues may alter the charge or hydrophobicity of an amino acid residue, presumably to modify its interaction with DNA. In this manner, transcription factors are able to compete with the largely abundant histone proteins for DNA binding. In addition, modifications of select residues have been postulated to create specific docking sites for recruitment of additional proteins. Furthermore, these modifications have also been reported to alter protein localization and stability. Among the posttranslational modifications that have been identified are ADP-ribosylation, ubiquitination, methylation, and acetylation.

ADP-ribosylation

In general, ADP-ribosylation is induced in response to DNA damage and participates in modulation of telomere length and metabolism of nucleic acids (reviewed in (41)). The addition of multiple ADP-ribose moieties onto proteins modifies their ability to interact with DNA. For example, ADP-ribosylation of the telomere factor, TRF1, by tankyrase 2 inhibits its ability to bind telomeric DNA, facilitating its release from the nucleus (38). Ribosylation of histones is thought to displace them from chromatin in response to DNA damage, freeing access of DNA double strand breaks to the repair machinery. The activities of poly(ADP)glycohydrolase then attack the poly(ADP)-ribose groups, stimulating histone shuttling, a potential mechanism of nucleosome unfolding (reviewed in (2)). In addition, poly(ADP)-ribosylation of histones has also been reported to activate nuclear proteosomes in response to oxidative stress (223) and trigger anoxia-induced apoptosis (31).

Ubiquitination

Ubiquitin is a small, globular 76 amino acid protein that can be singly or multiply conjugated to lysine resides. It has been reported to regulate protein degradation, DNA repair, cell cycle control, stress response, cell differentiation, and transcriptional regulation (reviewed in (84)). Histone H2A, H2B, and H3 are ubiquitinated in vivo, although H2A is the most abundantly ubiquitinated protein in cells. Mono- and poly-ubiquitinated histones are present in chromatin fractions purified from the transcriptionally active Tetrahymena macronuclei (43), suggesting that ubiquitinated histones are correlated with transcriptional activation. In support of this conclusion, the Drosophila homolog of the general transcription factor, TAFII250, has been identified as a histone specific ubiquitin ligase (168). In ubiquitin pull down experiments from mouse extracts, HDAC 6 was identified as one component of the ubiquitin binding complex, linking histone acetylation states with ubiquitination (194). Although it has been suggested that ubiquitination participates in chromatin unfolding, in vitro chromatin reconstitution assays do not support this hypothesis (83). However, ubiquitination of histones may act synergistically with other posttranslational modifications to direct chromatin unfolding (reviewed by (84)).

Phosphorylation

Phosphorylation of histones has been proposed to regulate both mitosis and transcription. Regulation of the phosphorylation state of these histones serves to recruit specific transcription or chromatin factors to a particular histone residue that may

facilitate or prevent further modifications. The primary focus of transcription-related histone phosphorylation centers on histone H3. Serine 10 phosphorylation of H3, for example, is upregulated during mitosis (240, 241) and stimulated in transcriptional activation (33). Several kinases have been proposed to mediate this phosphorylation including the IPL/aurora kinase, which is largely linked to mitosis (72). Enzymes such as PKA and components of the MAP kinase cascade, Rsk2 (189) and Msk1 (216), have also been shown to phosphorylate H3 at Ser10. Phosphorylation by Rsk2 or Msk1 is correlated with transcriptional activation. Chromatin staining of cells from both patients with Coffin-Lowry Syndrome, who lack Rsk2, as well as from Rsk2 knockout mice, exhibit almost no phosphorylation of H3 Ser10 and abrogated transcription activation (189).

Methylation

Unlike other types of posttranslational modifications, the mechanisms underlying regulation of chromatin structure by histone methylation are unclear. In contrast to phosphorylation and acetylation, methylation does not alter the net charge of a residue nor introduce hydrogen bonds or mediate electrostatic interactions (85). It has been suggested, however, that multiple methylation of Lys or Arg residues alters their hydrophobicity, perhaps facilitating recruitment of additional cofactors or altering protein-protein interactions. Two families of histone methyltransferases (HMTases) have been identified, the SET domain containing enzymes that selectively methylate Lys residues and Protein Arginine Methyltransferases (PRMTs), which selectively methylate Arg residues. Unlike other types of modifications, methylation appears to be a stable

modification, and it has been suggested that methylation is not a reversible process. However, further studies are needed to determine whether protein demethylases may participate in transcriptional regulation.

Lysine Methylation

The functional roles of lysine methylation have primarily been studied in the context of transcriptional repression. Methylation of Lys9 of H3, for example, has been shown to recruit the heterochromatin protein, HP1, to histone H3 in order to maintain a transcriptionally repressed state (7, 143, 180). The crystal structure of an H3 peptide containing methylated Lys9 complexed to the chromodomain of HP1 reveals that the methyl groups on the lysine residue stabilize the interaction between H3 and HP1 (80, 152). Since the discovery that the SET domain containing proteins, such as Su(var)3-9, were able to methylate Lys residues in vitro and in vivo (180), understanding the regulatory control mediated by this posttranslational modification has been greatly aided. Methylation of Lys9 has also been correlated with heterochromatin stability and with the methylation state of DNA. For example, loss of the Suv39H HMTase impairs cell viability and generates chromosomal instabilities (165, 166). In *Neurospora Crassa*, methylation of DNA by the DNA methyltransferase, dim-2, requires dim-5-mediated methylation of H3 Lys9 (212). DNA methylation mediated by the enzyme, Kryptonite (79), has also been shown to require Lys9 methylation to regulate heterochromatin formation in *Arabidopsis thaliana.* In addition to methylation of Lys9, methylation of other key histone H3 residues have been identified. For example, Lys27 of H3 is also methylated by the enzyme, G9a (153). In addition, Lys4 of H3 has also been shown to be

methylated in vivo and in vitro. Unlike methylation of Lys9, however, methylation of Lys4 has generally been correlated with transcriptional activation (11, 153). This has been demonstrated in vivo by examining the modification state of the different lysine residues in genes that are actively transcribed as well as genes that are actively repressed (11).

Arginine Methylation

Methylation of arginine residues is an important component of gene regulation. This modification has primarily been characterized in nuclear RNA binding (55, 179, 197) and processing proteins and was widely believed not to occur on histones in vivo. However, the coactivator associated arginine methyltransferase 1 (CARM1) was shown to synergistically activate nuclear hormone receptor dependent transcription by methylating histones in vitro (26). Mass spectral analysis of native histones subsequently confirmed that arginine methylation of histones occurs in native cells. These studies showed that histone H4 is methylated at Arg3 by PRMT1 (206, 235). Though it is unclear exactly how arginine methylation facilitates chromatin remodeling, it has been shown to stimulate acetylation, as methylation of Arg3 promotes H4 acetylation (235). However, pre-acetylation of H4 inhibits Arg3 methylation, suggesting that methylation may regulate transcription by stimulating acetylation-dependent chromatin remodeling.

Arginine methylation of transcription factors has also recently been characterized. The transcription factor, STAT1, is also methylated by PRMT1, a modification that promotes transcription activation (135). Moreover, the coactivator, CBP, was recently shown to be specifically methylated by CARM1. This methylation occurs within the

CREB binding domain of CBP and disrupts the CREB-CBP interaction, abrogating cAMP-dependent transcription (248).

Acetylation

Acetylation is the reversible addition of the acetyl group from acetyl coA onto an ε-amino lysine residue. This reaction is catalyzed by a family of enzymes called histone acetyltransferases (HATs). Removal of the acetyl group is catalyzed by the histone deacetylases (HDACs). Acetylation of conserved lysine residues of histone proteins is perhaps the best-studied posttranslational modification that regulates transcription. Although histone acetylation has long been correlated with chromatin assembly and remodeling, its role in transcriptional regulation was not well understood until the cloning of the first nuclear histone acetyltransferase (HAT) in 1996 (17). This HAT was identified as the yeast transcription adaptor, Gcn5, a protein that was initially characterized as an activator of transcription. Its identification as a HAT linked the field of chromatin remodeling to transcriptional regulation. Since then, an explosion of research has been conducted to identify additional nuclear HATs and to characterize their role in transcription regulation.

The HATs that have been identified can be divided into several families (reviewed in (205)), the GNAT (Gcn5-related N-acetyltransferases) superfamily, the MYST family, the coactivator acetyltransferases, the general transcription factor acetyltransferases, as well as the native nucleosome acetylating complexes.

GNAT and MYST family

Members of the GNAT superfamily of acetyltransferases are classified by the homology of their acetylation related motifs. This class of enzymes is composed of the related proteins, Gcn5 (general control nonderepressible-5) and PCAF (249) as well as more distant homologs, Hat1, Elp3, and Hpa2.

Gcn5 is a highly conserved protein whose role in histone acetylation was first identified by an in-gel HAT assay of nuclear extract fractions (17). Gcn5 is the best characterized and best understood of the HATs. Scanning alanine mutagenesis identified a conserved HAT domain within Gcn5 that can be substituted with the corresponding HAT domains of other species (104, 237). When incubated with a mix of free histones, Gcn5 preferentially acetylates Lys14 of histone H3 over Lys8 or Lys16 of H4 (103). However, though the mammalian isoforms of Gcn5 and PCAF are capable of acetylating both free and nucleosomal histones, recombinant yeast Gcn5 alone is unable to acetylate nucleosomal histones. Instead, studies have shown that this HAT is only capable of acetylating nucleosomes when part of multisubunit complexes such as the SAGA and ADA complexes (59). Indeed, many of the nuclear HATs display differential specificity between free histones versus nucleosomes. Furthermore, in addition to acetylating histones, PCAF is also a primary acetyltransferase for many non-histone transcription factors (see below).

A second class of the identified HATs is the MYST family. Members of this family include MOZ, Ybf2/Sas3, Sas2, and Tip60, as well as yeast Esa1, *Drosophila* MOF, and human HBO1 and MORF. Each of these HATs contain a similar acetyltransferase domain but participate in a variety of biological functions ranging from

transcription silencing, cell cycle progression, HIV modulated histone acetylation, and generation of leukemia (reviewed in (205)).

Coactivator and Transcription Factor HATs

Two of the major coactivator HATs are CBP and p300. Coimmunoprecipitation of CBP from COS cells (6) and affinity chromatography of E1A binding proteins from HeLa nuclear extracts (157) revealed the intrinsic HAT activity of both these proteins. Further analysis of this activity demonstrate that while CBP/p300 acetylates all free core histones at multiple sites, it preferentially acetylates histone H3 and H4 when nucleosomes are substrates. These studies suggest that a major mechanism by which CBP/p300 regulates cell cycle control, differentiation, and apoptosis is through its acetylation function.

Since its initial cloning, CBP/p300 has been demonstrated to interact with a diverse array of transcription factors. Transplantation assays with cells from CBP heterozygotes exhibit tumorigenic potential. Patients with Rubinstein-Taybi Syndrome, who are haploinsufficient for CBP, also exhibit an increased incidence of tumors. Although CBP and p300 are structurally and functionally similar, loss of a p300 allele in mice has not yielded cells with tumorigenic potential. Missense mutations of p300 in humans, however, generate loss of tumor suppressor characteristics (reviewed in (58)). The HAT activity of these coregulators has been correlated with these cancer phenotypes.

The p160/SRC family of nuclear hormone receptor coactivators has also been reported to acetylate histones. As found with CBP/p300, this coactivator can acetylate

multiple histones in vitro, yet preferentially acetylates H3 and H4 within nucleosomes (27). The cAMP responsive transcription factor, ATF2, has also been identified as a HAT (94). It specifically acetylates H2B and H4 in vitro, resulting in an upregulation of cAMP responsive genes.

HAT complexes

As alluded to above, several HATs are present in large, multiprotein complexes. Among these are SAGA and ADA as well as other yeast HAT complexes including NuA4 and NuA3. These latter two were purified by their ability to specifically acetylate nucleosomal histone H4 or H3. Each contains a distinct catalytic subunit, such as Esa1 for NuA4 and Sas3 for NuA3.

Other HAT complexes that have been identified include the Gcn5/PCAF, Tip60, TFIIIC, and HBO1 complexes. Many of these complexes share common subunits. For example, the PCAF complex, which was purified from HeLa nuclear extracts of cells overexpressing FLAG-tagged PCAF, contains several homologs of the yeast SAGA complex (156). Furthermore, the Tip60 complex contains a subunit, TRRAP, which is the yeast homolog of the protein Tra1, a component of yeast SAGA and NuA4. These similarities suggest that the Tip60 complex may be the human homolog of NuA4 (reviewed in (205)). The HAT activities of many of these complexes are enhanced in the context of nucleosomes by incorporation of their catalytic subunits into these distinct multiprotein complexes. Association of these HATs in large, regulatory complexes may not only confer specificity between free versus nucleosomal histones, it may also restrict

acetylation to select enhancer-promoter sites. Such a complex has yet to be purified for CBP/p300.

Regulation of HATs

Both phosphorylation of HATs and hormonal signaling have been shown to regulate enzymatic activity. It has been suggested that the HAT activity of CBP is stimulated by phosphorylation by the cell cycle dependent kinase, cdk2 (1). Phosphorylation of Gcn5 by the DNA dependent protein kinase has been shown to inhibit its HAT activity (9). Phosphorylation of the ATF2 acetyltransferase, in response to irradiation, increases its HAT activity (94). Moreover, binding of nuclear hormone receptor agonists or antagonists regulates HAT activity by directing the recruitment of coactivator HATs to active sites of transcription. Binding of E1A to CBP/p300 has been reported to inhibit its HAT activity by one group (25), although it has also been reported to activate CBP/p300 HAT activity by another group (1).

Histone acetylation is generally thought to be an enhancer-promoter restricted phenomenon. However, analysis of global acetylation patterns of chromatin reveals that histone acetylation is not limited to promoters and enhancers (231). These acetylation patterns can be inherited through epigenetic mechanisms (138). In addition, though acetylation has been correlated with transcriptional activation and deacetylation is correlated with transcription repression, many exceptions to this paradigm exist. In some systems, transcriptional activation has been correlated with histone deacetylation (44) while histone acetylation has been correlated with transcriptional repression (Bresnick et al, 1990 and 1991). These data suggest that the regulatory role of a gene's acetylation

pattern may, in some cases, be promoter specific. Understanding the mechanisms by which the plethora of HATs and their substrates modulate transcription is a key focus of this thesis.

The Histone Code

Recently, a model for a histone code has been proposed which suggests that each posttranslational modification, including histone acetylation, allows the recruitment of specific transcription cofactors to the modified residue (reviewed in (85)). The interaction between the modified residue and its recruited protein are mediated by defined interaction surfaces. As many transcription factors contain conserved domains, such as bromodomains, chromodomains, and chromoshadow domains, these protein substructures have been suggested to form docking sites for specifically modified residues. For example, many of the HATs that have been characterized, including Gcn5, PCAF, CBP/p300, and TAFII250 possess bromodomains that may direct acetyl-lysine interactions. Protein-protein interaction studies demonstrate that histone peptides containing acetylated N-terminal histone tail residues preferentially interact with the bromodomain of PCAF (46). Furthermore, TAFII250, which has two tandem bromodomains, has been shown to preferentially bind di-acetylated histone H4 peptides (81). Though these studies provide compelling evidence for the acetyl-lysine-bromodomain interaction module, interaction studies with in vitro assembled chromatin containing full-length histones, rather than N-terminal peptides, do not show enhanced association of acetylated chromatin with the p300-bromodomain (123). However, support for the histone code hypothesis has been demonstrated through studies of other

histone interaction domains. Methylation of lysine 9 of histone H3 recruits the heterochromatin binding protein, HP1, through its chromodomain (180) (Figure 3). As mentioned above, the crystal structure of a methyl-Lys9 H3 peptide complexed with the HP1 chromodomain indicates that the methyl-lysine binding pocket within HP1 is lined with conserved aromatic residues, creating a conformation that is thought to require methyl groups to stabilize the histone H3-HP1 complex. Molecular modeling suggests that in the absence of the methyl groups, the H3-HP1 interaction may not occur (80, 152).

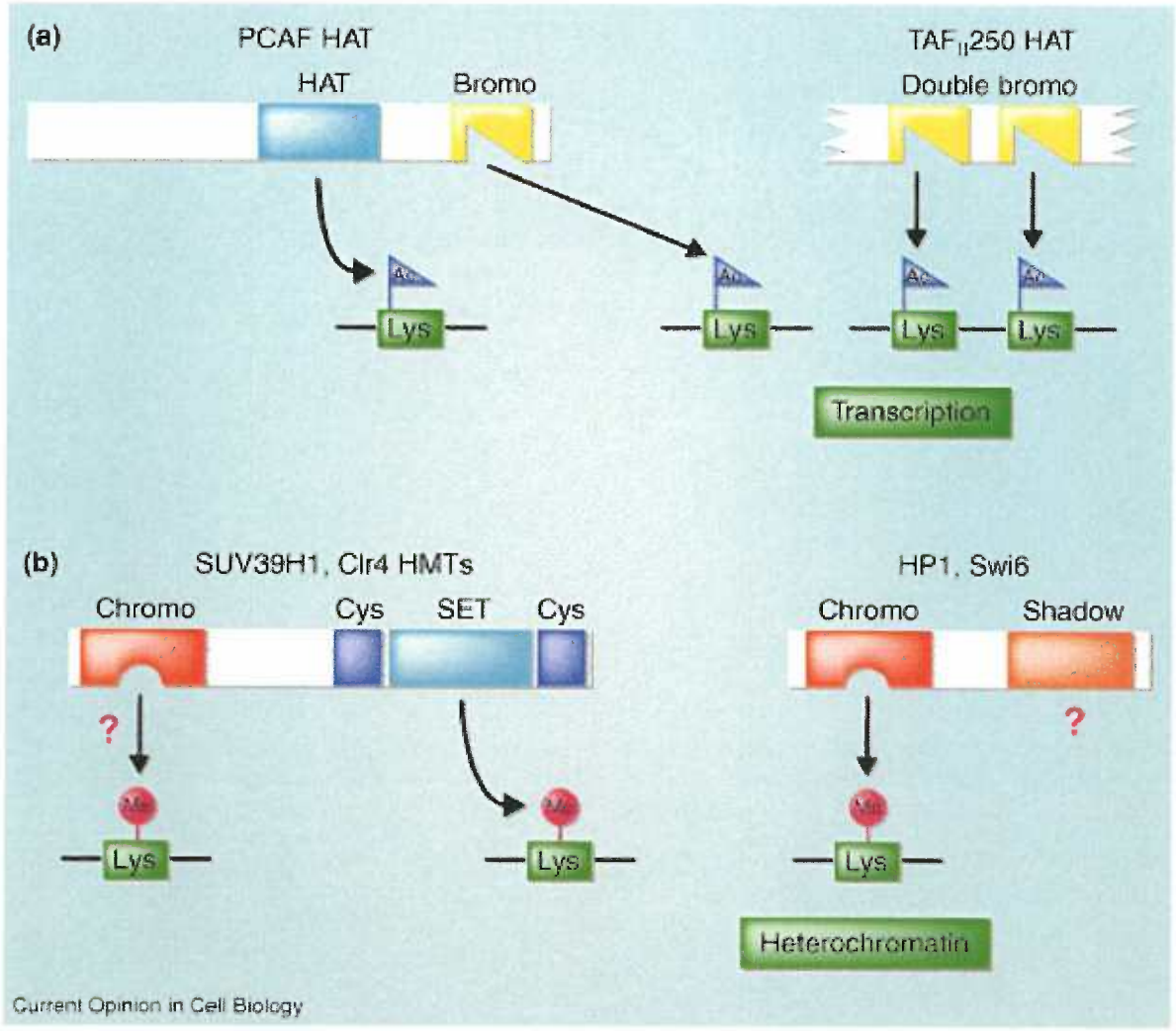

Figure 3. The "histone code." Distinct posttranslational modifications create specific protein-protein interaction surfaces that facilitate recruitment of specific regulatory protein domains. Acetylated lysine residues recruit bromodomain-containing proteins. Methylated lysines recruit chromodomain-containing proteins (182).

Elaboration of the histone code hypothesis illustrates the combinatorial specificity that exists between the different posttranslational modifications identified. Ribosylation of H3 is coupled with phosphorylation (217). Serine 10 phosphorylation of histone H3 reportedly promotes acetylation of lysine 14 by Gcn5, enabling transcriptional activation (33, 213). Using antibodies that selectively recognize the di-modified histone epitope, the Allis group has demonstrated that EGF stimulates the initial Ser10 phosphorylation by the MAP kinase, Rsk2, which then facilitates Lys14 acetylation and promotes transcriptional activation (33, 189) (reviewed in (32)). Conversely, Ser10 phosphorylation inhibits Lys9 methylation, and disruption of Su(var)3-9 activity antagonizes Ser10 phosphorylation, leading to aberrant mitotic division (180).

Consistent with the idea that Lys9 methylation and Ser10 phosphorylation/Lys14 acetylation have opposing consequences, it has been reported that Lys14 deacetylation is required for Lys9 methylation by Clr4, the yeast homolog of Suvar39h1. Recent identification of Set9, an H3, Lys4 specific HMTase, demonstrates that methylation of Lys4 by Set9 perturbs its interaction with the transcription repression complex, NuRD (153). Chromatin immunoprecipitation assays of genes that are transcriptionally silent on the inactive X chromosome contain methylated Lys9. However, transcriptionally active genes located on the same chromosome, such as the Xist gene, have phosphorylated

Ser10, acetylated Lys14, and methylated Lys4 on histone H3 (11). In summary, these cooperative posttranslational modifications mark a particular gene for transcriptional activation or repression. The complexity inherent in the combinatorial recruitment of distinct enhancer-promoter binding proteins is also reflected in the complexity of posttranslational histone modifications required for regulation of transcription.

Transcription Factor Acetylation

Although histones are believed to be the primary target of acetylases and deacetylases, an increasing body of evidence highlights the importance of transcription factor acetylation in transcriptional regulation. Modification of chromatin structure is only one nuance in the complexities of the transcription process. As described above, posttranslational modifications of proteins may not only alter histone-DNA complexes, but these modifications may also disrupt, enhance, or facilitate certain protein-protein interactions. Among the types of nuclear proteins known to be acetylated are DNA-binding proteins, non-DNA binding transcriptional coregulators, as well as proteins that shuttle from the nucleus to the cytoplasm. Acetylation has been proposed to alter the affinity of existing DNA-protein and protein-protein interactions as well as to recruit additional cofactor interactions, alter nucleocytoplasmic localization, or affect protein stability.

The first non-histone substrate identified for CBP/p300 and PCAF was the tumor suppressor protein, p53 (61). Since then, a multitude of non-histone acetylation substrates has been identified. p53 is multiply acetylated at distinct residues within its carboxyl terminus and linker DNA binding and tetramerization domains in vitro and in

vivo. Deacetylation occurs via HDAC1 (89, 120, 137) or by the NAD-dependent deacetylase, Sir2 (119, 224). Moreover, as evidenced by the combinatorial specificity demonstrated for histone modifications, p53 acetylation is influenced by its phosphorylation (187). Electrophoretic mobility shift assays (EMSA) indicate that acetylation enhances the ability of p53 to bind DNA, to recruit coactivators, as well as to alter its subcellular localization (reviewed in (173)). However, examination of p53 acetylation in vivo has generated conflicting data. For example, although its acetylation by p300 has been reported to enhance the ability of p53 to bind a short oligonucleotide from the p21 promoter, no effect was seen with a much longer DNA element (50). These studies were supported by ChIP assays in which mutation of the acetylation sites within p53 from Lys to Arg did not alter the ability of this tumor suppressor to bind DNA (8).

Nevertheless, acetylation of other sequence specific DNA binding proteins such as E2F (125), EKLF (256), and GATA1 (14) also occurs in the DNA binding domain and has been shown to alter protein-DNA interactions. CBP/p300 and PCAF also differentially acetylate the HIV transcription factor, TAT. Acetylation by CBP/p300 occurs within its RNA (TAR) binding domain and decreases its binding to the TAR RNA. However, acetylation by PCAF occurs at a different lysine residue, within the activation domain of TAT, and increases TAT's ability to interact with CDK9/p-TEFb CTD kinase (95). Acetylation within the DNA binding domain of the architectural chromatin protein, HMGI(Y), by CBP/p300 disrupts its ability to bind DNA while its differential acetylation by PCAF stimulates transcriptional activation (136). These studies illustrate the specificity of acetylases and their selective effects on the regulation of transcription.

Acetylation-regulated nucleocytoplasmic shuttling has been demonstrated for NFκB. CBP/p300-mediated acetylation of the p65(RelA) subunit of the NFκB complex both enhances DNA binding and prevents RelA association with the inhibitory IκB protein. Deacetylation of RelA by HDAC3 was also shown to enhance its ability to interact with IκB, leading to its export from the nucleus (30) .

Moreover, members of the general transcriptional machinery can also be acetylated. TFIIE and TFIIF subunits are acetylated by CBP/p300 and PCAF. The ß subunit of TFIIE is acetylated by CBP/p300 as well as by TAFII250. RAP74 and RAP30, components of TFIIF, are acetylated by PCAF (reviewed in (205)). The effects of acetylation of basal transcription activators is unknown.

Although acetylation is generally correlated with transcriptional activation, exceptions to this model have been reported. In vitro acetylation assays and chromatin immunoprecipitation assays demonstrate that acetylation of ACTR by p300 terminates transcription of nuclear hormone regulated genes by disrupting the ability of ACTR to interact with CBP/p300 (28). In this case, the acetylase function of CBP/p300 is correlated with transcriptional repression. CBP/p300 HAT-mediated repression has also been suggested for the *Drosophila* protein, dTCF, a component of the Wnt signaling pathway. Acetylation of dTCF within its Armadillo binding domain, presumably by CBP, disrupts its interaction with this coactivator (234).

In addition, auto-acetylation has also been observed in vitro for some acetylases including CBP/p300, PCAF, Tip60, MORF, Hpa2 and Hpa3. The functional consequences of self-acetylation is unclear.

Given the broad substrate specificities of many of these acetyltransferases, targeting of this enzymatic activity should play a critical role in regulating the accurate acetylation of individual histone and non-histone substrates. Interestingly, studies from the Workman lab demonstrate that targeting of these complexes to specific promoters is regulated by interaction with sequence specific DNA binding activators (107, 145, 147). Targeting of HAT complexes has been demonstrated to occur by the direct association of these complexes, such as Tra1 of the SAGA and NuA4 complexes, with the acidic domains of DNA-binding activator proteins (16). HATs and HDACs have also been shown to interact with the ATP-dependent remodeling complexes (144, 186, 232), indicating that histone modifying enzymes and ATP-dependent remodeling complexes work in collaboration to regulate transcription. In addition to participating in transcriptional initiation, the catalytic subunit of NuA3 complex, Sas3, can also interact with the Spt16 subunit of FACT to facilitate transcription elongation (86). The order of recruitment of HATs or ATP-dependent chromatin remodeling enzymes may be different in different contexts.

Targeting of CBP/p300's acetyltransferase activity has not been characterized in detail. CBP/p300 is a ubiquitously expressed acetyltransferase that has broad substrate specificity and interacts with a wide spectrum of transcription factors. In addition to acetylating itself, histones, and other transcription factors, CBP/p300 frequently acetylates these substrates at multiple sites. Its role as a bridging protein that connects activators with components of the general transcription machinery is one mechanism by which CBP/p300 activates transcription. In addition, its histone acetyltransferase activity similarly regulates transcription. CBP/p300-mediated acetylation of non-histone

substrates can either activate, repress, or terminate transcription. A major focus of this thesis addresses questions of how a multifunctional coregulator, such as CBP/p300, participates in such a diversity of transcription regulatory processes. One question centers on how CBP/p300 targets its enzymatic activity towards histones versus the myriad of non-histone substrates has not been well studied. It is unknown whether CBP/p300 recognizes a specific substrate recognition site, for example, or if it is targeted towards histones rather than non-histones through recruitment of additional regulatory cofactors. This thesis addresses these questions by first asking how histone acetylation may be targeted, perhaps in competition with other substrates, and how acetylation of non-histone binding proteins alters the transcription state of a given gene.

RbAp46/RbAp48

Purification of HDAC containing transcription repression complexes has consistently yielded a common subunit, the histone chaperone proteins, also known as the retinoblastoma (Rb) associated proteins, RbAp46/RbAp48 (176). In fact, RbAp46/48 is present in all identified HDAC1 or HDAC2 complexes with the exception of the CoREST complex (251). Although CoREST binds to HDAC2 and represses transcription, perhaps targeting of this corepressor complex towards histones is mediated by another element. Alternatively, RbAp46/48-meditated recruitment of histones may be associated with transient and dynamic modes of transcriptional regulation. In contrast, repression mediated by the CoREST complex is involved in maintaining the transcriptionally repressed state of neuronal genes in non-neuronal tissues, requiring its

stable association with chromatin in the presence of REST (3, 5). Targeting of the CoREST/HDAC complex by RbAP46/48 in this instance, may not be required.

RbAp46 and RbAp48 were originally cloned by their ability to interact with Rb to regulate cell cycle progression (175, 176). Their homolog in yeast, CAC3, is a negative regulator of Ras/cAMP stimulation and has also been identified as a component of chromatin assembly factor, CAF-1 (93). In addition, cells that are depleted of RbAp48 exhibit a decrease in deacetylase activity (150, 151), most likely due to a defect in HDAC targeting (225).

Although RbAp46/48 has primarily been identified in HDAC rather than HAT complexes, the idea that this histone binding protein, which was previously only characterized as a chaperone protein, plays a role in targeting enzymatic activity to histone proteins suggested a potential mechanism for generating specificity of CBP/p300 HAT activity. If the primary role of RbAp46/48 was to specifically target histones for enzymatic modification, then this histone chaperone may also target other enzymes to histones, such as HATs. The first part of my thesis addresses this possibility and investigates the potential role of CBP/p300 association with RbAp46/48.

Although factor acetylation has been shown to be an essential mechanism of transcriptional regulation, it is unclear how the acetylation state of a particular factor facilitates a switch from a transcriptionally repressed to active state. Does the acetylation state of specific factors regulate their ability to recruit coactivators or corepressors to regulate transcription? I examined the role of transcription factor acetylation in nuclear hormone receptor-dependent transcription. As acetylation of histones disrupts the

repressive nucleosome complex and is required for activation, I postulated that disruption of repressive transcription factor complexes by acetylation may similarly facilitate transcriptional activation.

Nuclear Hormone Receptor Model of Transcriptional Regulation

The nuclear hormone receptor-dependent mechanism of transcription exemplifies the acetylation-dependent regulation of transcription (reviewed in (56, 127)). In general, transcriptional repression occurs in the absence of hormone or the presence of antagonists. In the presence of antagonists, DNA-bound nuclear receptors recruit the corepressors, NCoR (nuclear receptor corepressor) /SMRT (silencing mediator of retinoid and thyroid hormone receptor) (29, 71), which associate with HDAC containing complexes through their interaction with the corepressor, Sin3 (67, 140). Repression of transcription is mediated by deacetylation of histones near the promoter-enhancer elements. The deacetylation activating domain and SANT (SWI3/ADA2/NCoR/TFIIB) motifs within SMRT are essential for conferring enzymatic activity of the associated HDACs (62).

Upon addition of agonist, a conformational change occurs within the nuclear receptors, resulting in dissociation of the NCoR/SMRT complex. Agonist-bound nuclear receptors then recruit the p160 family of coactivators. This family is composed of three members, SRC1 (steroid receptor coactivator 1)/NCoA-1 (nuclear receptor coactivator-1) (70, 218, 230), TIF-2 (transcription intermediary factor-2)/GRIP-1 (glucocorticoid receptor interacting protein-2)/NCoA-2 (nuclear receptor coactivator-2) (70, 230), and pCIP (p300/CBP co-integrator associated protein)/ACTR (activator of the thyroid and

RA receptor)/AIB1 (amplified in breast cancer 1) (4, 27, 218). They are approximately 160 kD, and interact with a broad variety of nuclear hormone receptors. The p160/SRC family of coactivators then recruit additional coactivator HATs, CBP/p300 and PCAF, to initiate transcription (27, 96) (Figure 4). Because the HAT activity of SRC is significantly weaker than that of CBP/p300 and PCAF, acetylation of nucleosomes is thought to be primarily mediated by CBP/p300 and PCAF.

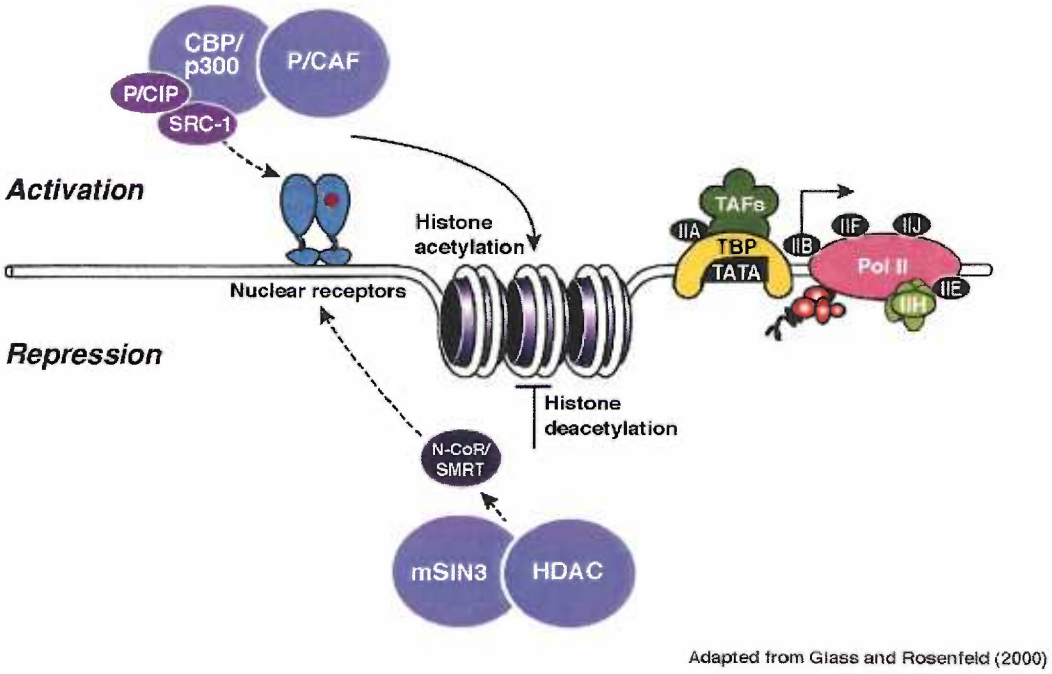

Figure 4. Ligand-mediated recruitment of HATs and HDACs to nuclear hormone receptor complexes. In the presence of agonist, recruitment of coactivator HATs leads to acetylation and disruption of transcriptionally repressive chomatin complexes to turn on gene expression. In the presence of antagonist, coactivator associated HDACs are recruited to deacetylate histones and repress transcription.

The nuclear receptors have conserved structural and functional domains that regulate their ability to activate or repress transcription. For example, estrogen receptor (ER) is divided into six domains. At the N terminus is the A/B domain that mediates transcriptional activation. This domain within ER is also referred to as activation function-1 (AF-1). Immediately adjacent to the A/B domain is the C domain which is responsible for DNA binding, nuclear localization, and perhaps dimerization. The D domain contains the AF-2 domain that is the primary region responsible for nuclear receptor dimerization and hormone binding. The last, F domain, has unknown function.

Binding of NCoR/SMRT or p160/SRC occurs within the AF-2 domain in a ligand dependent manner. These interactions occur through a canonical LXXLL motif present within the coactivators (66) and a LXXI/HIXXXI/L motif present within corepressors (73). The conformational changes induced upon agonist or antagonist binding have primarily been characterized in the AF-2 domain of nuclear receptors.

Structural studies

The conformational changes induced upon ligand binding are well illustrated in the crystal structures of PPARγ, RAR/RXR, ER, and T_3R. These receptors were co-crystallized with the nuclear receptor interaction domains of coactivators and corepressors. The structure of the ligand bound PPARγ complexed with SRC1 reveals that each half of a PPARγ dimer interacts with an individual α-helical LXXLL motif from one molecule of SRC1 (154). In fact, binding of the nuclear hormone receptor with an LXXLL motif cooperatively facilitates their interaction with coactivators. These results were consistent with crystal structures of T_3R and GRIP1 as well as SRC1 and

RAR/RXR heterodimers, indicating that there is a conserved interaction surface between nuclear receptors and coactivators. Recent structural analysis of the RAR/RXR heterodimer demonstrates that each ligand from each RAR or RXR facilitates the synergistic interaction with a single p160 coactivator (54).

Binding of agonists or antagonists to the AF-2 domain of nuclear hormone receptors induces structural changes within this domain to facilitate coactivator or corepressor recruitment. These changes are illustrated in the crystal structure of the ER AF-2 domain (18, 198). The agonists, diethylstilbestrol (DES) or 17ß-estradiol, bind ER within a hydrophobic pocket created by interaction amongst helix 3, 4, 5 with helix 12 of the ligand binding domain (LBD). The LXXLL motif within coactivators is then able to bind this pocket, forming a charge clamp between helix 12 and helix 3. The charge clamp is sensitive to the ligand's chain length such that when antagonists like 4-hydroxy-tamoxifen or raloxifen bind within the hydrophobic pocket of the LBD, the longer side chain of the antagonists positions helix 12 so that coactivator binding is blocked. The interaction between corepressors with the AF-2 domain is independent of the charge clamp.

Examination of the interaction between fragments of ACTR and CBP illustrate that though these individual proteins are intrinsically unstructured in isolation, formation of the ACTR-CBP complex synergistically induces a single, high affinity helical complex (45, 115). This interaction-induced structure is reminiscent of the CREB-CBP/p300 complex that was able to generate α-helicity upon the interaction of the two proteins.

Interaction with corepressors is dependent upon another conserved motif called the CoRNR box motif. This interaction surface is characterized by an LXXI/HIXXXI/L

helix, which resembles an extended version of the LXXLL motif. The CoRNR box binds the same hydrophobic pocket created by helix 12 and, in the process, precludes binding of coactivators. Recognition of specific α-helical chain lengths during coregulator binding facilitates either coactivator or corepressor binding. The conformational change induced upon the AF-2 domain of nuclear receptors provides a molecular switch that governs transcriptional activation or repression.

However, a few exceptions to this mechanism of ligand induced conformational changes in nuclear receptors have been reported. Though the natural ligands for many orphan nuclear receptors have not been identified, this class of transcription factors is generally thought to be regulated similarly to the better characterized nuclear receptors. Recent reports demonstrate that though these orphan nuclear receptors may share structural homology to the classically defined steroid receptors, they also possess unique characteristics. Crystal structure of the orphan nuclear receptor, estrogen related receptor3 (ERR3), for example, shows that it binds to the coactivator, SRC1, in the absence of agonist. In studies examining the ligand binding domain of ERR3 complexed with the second LXXLL motif from SRC1, Greschik et al demonstrated that apo-ERR3 adopts the typical conformation described for transcriptionally active, agonist bound nuclear hormone receptors (60). Molecular modeling indicates that agonists such as E_2 would be unable to fit into the LBD of ERR3. However, a conformational change in ERR3 would allow antagonists such as DES (an ER agonist) and 4-hydroxy tamoxifen to bind the orphan receptor, facilitating binding of corepressors. These studies suggest that some nuclear hormone receptors do not require agonist binding to induce transcriptional activation.

Mediator

In addition to the SRC family, CBP/p300, and PCAF classes of coactivators, recent approaches in proteomics have identified distinct coactivator complexes, termed Mediator, which also facilitate transcriptional activation of nuclear hormone receptors. Mediator is a multi-polypeptide complex that interacts with nuclear receptors in a ligand-dependent fashion (reviewed in (121)). These complexes cooperate with coactivator HATs to coordinate the activities of coactivators with the general transcription machinery. Each of the identified Mediator complexes were purified as distinct functional complexes. For example, thyroid hormone receptor associated proteins (TRAPs) were purified from cells stably expressing FLAG tagged T_3R (53). Vitamin D_3 receptor interacting protein (DRIP) was purified from a vitamin D_3 receptor affinity column (177). Activator recruited cofactor (ARC) was affinity-purified by its interaction with the transcription factor, SREBP (139). In addition, yeast and mouse Mediator complexes have also been purified. Further analysis indicated that each of these supposedly distinct complexes contains many common critical subunits, and genetic analysis illustrated the importance of these subunits to transcriptional regulation. Mice that are null for TRAP220 die early in gestation and have defects in neuronal development. Haploinsufficient animals exhibit a variety of phenotypes including defects in transcription (76). Although TRAP220 has been reported to interact with a class I steroid receptors as well as class II receptors, studies from primary embryonic fibroblasts of TRAPP220 null mutants reveal relatively gene specific defects, namely in

T_3R function. These studies illustrate the important regulatory role imparted by other Mediator subunits in conferring enhancer-promoter specificity.

Though the majority of the Mediator complexes identified regulate chromatin specific transcription, TRAP has also been shown to activate transcription only on naked DNA. As originally proposed by Roeder, the presence of these distinct coactivator complexes may represent a sequential step model of transcription in which coactivator HATs and chromatin specific Mediator isoforms derepress transcription by remodeling chromatin, while complexes such as TRAP are actively involved in transcription initiation (reviewed in(229)). Alternatively, though the p160 coactivators, CBP/p300, and PCAF appear to be general regulatory cofactors mediating nuclear receptor-dependent transcription, distinct Mediator complexes may confer specificity in transcriptional regulation in response to specific extracellular cues. However, to date, it is unclear how Mediator participates in chromatin remodeling.

Unlike coactivator HATs such as p160/SRC, CBP/p300, and PCAF, the Mediator coactivator complex has not definitively been shown to contain intrinsic acetyltransferase activity. Instead, the Mediator complex is thought to link the activator-bound complex with the general transcriptional machinery. However, although no HAT activity has been identified for any of the Mediator subunits, one yeast Mediator complex has recently been purified with the associated HAT, Nut1 (116). The possibility remains that mammalian Mediator activates transcription by association with known coactivator HATs.

Reverse pharmacology

While the classical model of agonist/antagonist-regulated transcription describes many nuclear hormone dependent genes, it does not explain the transcriptional regulation of all genes in this class. For example, some orphan nuclear receptors, such as TR, have been shown to recruit coregulators in the absence of ligand. Moreover, though many steroid hormone receptors only interact with their DNA response element in the presence of ligand, some unliganded nuclear receptors also bind DNA. The retinoic acid (RAR) and thyroid hormone receptor, for example, bind to the retinoic acid response element (RARE) and thyroid hormone (T_3) response element (T_3RE) in the absence of ligand and actively repress transcription of RA- and T_3-responsive genes, respectively. This repression appears to occur through NCoR and SMRT mediated corepressor complexes. Blocking the activities of the individual components of this repression complex by antibodies directed against NCoR or SMRT or by application of HDAC inhibitors results in a loss of repression (67, 140).

Though many nuclear hormone regulated genes conform to this pharmacological paradigm of agonist-mediated activation and antagonist-mediated repression, many exceptions have been described. On one level, it has been demonstrated that certain ER ligands may have either agonist or antagonist actions dependent upon the tissue examined. These pharmacological reagents have been classified as SERMs (selective estrogen receptor modulators) (92). Among these SERMs are the antagonist, 4-hydroxytamoxifen, a pharmacological agent commonly used to treat breast cancer. However, tamoxifen has been demonstrated to activate transcription in some tissues. In some cases, estradiol has been shown to repress rather than activate transcription. This is

true for the quinone oxidoreductase (QR) gene (132, 133). QR is a phase II NAD(H)-dependent enzyme that catalyzes the reduction and detoxification of quinones and its derivatives. Although heterodimerization of ER with the transcription factor AP1 has been proposed to be involved in estradiol-mediated repression, the molecular mechanisms of this mode of repression are not well understood. Regulation of QR by the reversed pharmacology described above may be mediated by previously unidentified cofactors.

This reversed ligand regulation is not specific to estrogen-regulated genes. Some of the classically described T_3-regulated genes are also transcriptionally repressed in the presence of thyroid hormone as a function of the negative feedback regulatory loop of the hypothalamic-pituitary-thyroid axis. The hypothalamic thyrotropin releasing hormone (TRH) stimulates thyrotropin stimulating hormone (TSH) secretion by the pituitary gland. TSH is a glycoprotein composed of two subunits, TSHα and TSHß, which stimulates the synthesis and release of thyroid hormones T_4 and T_3 from the thyroid gland. TRH and TSH synthesis are both repressed in the presence of T_4 and T_3. This mode of transcription repression is mediated by thyroid hormone receptor (34, 35). Furthermore, in the absence of ligand, T_3R can bind to the T_3RE, and activate the transcription of TRH and the two isoforms of TSH. The cofactors responsible for mediating this mode of regulation are still being identified. Several studies demonstrate a role for coregulator associated histone acetylases and deacetylases. However, given the specific conformational changes that occur upon agonist binding to T_3Rs, which creates specific interaction surfaces for coactivators, it is unclear how the absence of ligand can recruit the conventional coactivators to T_3Rs and how the conventional corepressors

interact with agonist bound T_3Rs. It is possible that other coregulatory proteins may mediate agonist-induced repression/antagonist-induced activation.

RIP140

Such a protein may be the nuclear hormone receptor interacting protein, RIP140 (or Nrip1). As exemplified by the crystal structure of the LBD of ER, agonist-bound nuclear receptors recruit coactivators while antagonist-bound nuclear receptors recruit corepressors to differentially regulate transcription activation or repression. RIP140 is an apparent contradiction to this model. Like the p160/SRC family of coactivators, RIP140 is recruited to nuclear hormone receptors and does not bind directly to DNA. However, unlike the p160/SRC family of proteins, RIP140 has been characterized as an agonist-dependent corepressor of transcription (110, 111). This mode of agonist-mediated repression does not fit well with the traditional model of transcriptional regulation and is not well understood. Moreover, the endogenous genes that RIP140 regulates have not been determined. Its unusual mode of transcriptional regulation makes RIP140 an ideal candidate as an agonist-dependent corepressor of E_2-and T_3-responsive genes.

RIP140 is a coregulator of 140 kD in molecular weight and possesses nine LXXLL motifs. It was initially identified from a human breast cancer cell line cDNA library by its ability to interact with the AF-2 domain of the estrogen receptor (24). In vitro interaction assays demonstrate that though estradiol significantly enhances the interaction between RIP140 and ER, RIP140 is also capable of associating with ER in the presence of antiestrogens (167).

Since its initial cloning, RIP140 has been shown to regulate transcription of class I and class II types of nuclear hormone receptors including RAR, RXR (108, 111), T_3R (108), GR (208, 246), the orphan nuclear receptor, TR2 (110), the AhR (102), and the liver X receptor (131). GST pull down assays indicate that though RIP140 has nine LXXLL motifs which are located throughout the protein, only two domains within RIP140, one at the N terminus (aa 1-439) and the other at the C terminus (aa 753-981), mediate interaction with estrogen receptor. These assays demonstrate that RIP140 interacts with nuclear receptors through its LXXLL motifs preferentially in the presence of agonist (108). However, cloning of the mouse isoform of RIP140 revealed the existence of an apo-receptor interaction with TR2 (110). In addition, RIP140 recruitment to nuclear receptors has also been demonstrated for LXR and possibly PPARα (131). These studies suggest that RIP140 may be a dynamic coregulator that interacts with nuclear receptors through a novel interaction surface. Alternatively, some nuclear receptors, such as ERR3, may adopt discrete conformations in the absence or presence of ligand that allows recruitment of coactivators or corepressors in a distinctive ligand-dependent manner not described by the traditional scheme depicted for receptors such as ER.

Initial reports identified RIP140 as a coactivator of transcription (24). Indeed, reporter gene assays of estrogen receptor dependent transcription in yeast demonstrate that addition of RIP140 potentiates transcriptional activation (47, 88). However, subsequent studies in mammalian systems have identified a potent repression function for RIP140. Tethering RIP140 to the Gal4 DNA binding domain demonstrated that Gal4-RIP140 represses rather than activates transcription of reporter genes (110). These

results have been demonstrated for nuclear hormone response element regulated genes as well.

Identification of RIP140 as an agonist-recruited transcription corepressor suggested that it may modulate expression of genes that are negatively regulated by agonist, such as QR and TSH. The molecular mechanism by which RIP140 represses transcription is unclear. Deletion analysis of RIP140 suggests that there are multiple domains within the protein responsible for repressing transcription (110). First, RIP140 may repress transcription by competing with SRC for interaction with nuclear receptors. In addition, RIP140 has been shown to associate with HDACs (238, 239). However, whether this HDAC association mediates histone or transcription factor deacetylation remains unclear. Finally, RIP140 may recruit additional cofactors or it may possess an intrinsic repression activity.

Analysis of the primary sequence of the protein identified the presence of a signature motif within the first amino terminal third of RIP140 that matches a motif required for interacting with a general transcription corepressor, CtBP. Moreover, this PXDLS motif within RIP140 is flanked by a K residue. Previous work from the Goodman lab demonstrated that the consensus CtBP binding motif is frequently followed by a Lys or Arg residue. Acetylation of this Lys residue attenuates the ability of the adenoviral oncoprotein, E1A, to interact with CtBP (255). One possibility, therefore, is that RIP140 represses transcription through CtBP and that this property may be regulated by the acetylation of this critical lysine residue.

CtBP

CtBP is a phosphoprotein of approximately 48 kD initially purified by affinity chromatography against a GST fusion protein spanning the carboxyl terminal 68 residues of E1A. It was subsequently cloned in a yeast two hybrid assay using the C-terminus of E1A as bait (13, 190). This interaction is mediated by a specific 5 residue motif, PLDLS, which when deleted or mutated, abolishes the E1A-CtBP interaction. Although the N terminus of E1A binds to CBP/p300 and PCAF to activate transcription, inclusion of the C terminus of E1A in a Gal4-E1A fusion construct represses transcription. This repression is abrogated by deletion of the PLDLS motif (190), demonstrating that disruption of the E1A-CtBP interaction blocks transcription repression. These reports were confirmed by studies showing that mutation of the PLDLS motif facilitated ras-dependent oncogenic transformation (13, 190).

Three isoforms of CtBP have been identified to date. CtBP1 was first identified by its affinity for E1A. The homolog, CtBP2, was identified by sequence homology to CtBP1 and also later cloned by a yeast two hybrid screen for proteins that interact with the sequence specific DNA binding transcription factor, BKLF (91, 221). Although both CtBP1 and CtBP2 are primarily nuclear proteins, cytoplasmic localization has been identified as well. The carboxyl-terminal domain of RIBEYE, a major component of ribbon synapses, is homologous to CtBP2 (193). The third isoform, CtBP3, also known as BARs, contains an amino-terminal truncation of the conserved CtBP1 and CtBP2 domains. CtBP3, which is essentially the N-terminally truncated isoform of CtBP1, has primarily been characterized as a Golgi protein that participates in protein sorting and processing (242). CtBP has also been shown to interact with nitric oxide synthase (185).

CtBP is most closely related in sequence to the NAD-dependent 2-hydroxy acid dehydrogenases (190) and possesses a conserved NAD binding motif, GXGXXG where G is glycine and X is any amino acid. The recent characterization of the transcription repressor, Sir2, as an NAD-dependent histone deacetylase has stirred interest in studying the transcriptional regulation by metabolic cofactors. The sequence homology of CtBP to NAD-dependent dehydrogenases implies that this corepressor may use NAD to repress transcription. Studies of the CtBP2 portion of RIBEYE have demonstrated that CtBP2 binds NAD in vitro (193). However, demonstration that the nuclear isoforms of CtBP are regulated by these metabolic cofactors did not occur until recently. Studies using in vitro interaction assays and in vivo coimmunoprecipitation assays demonstrate that NAD enhances the ability of CtBP to bind to the PXDLS containing fragment of E1A, requiring approximately 100μM concentration for half-maximal effects on binding. NADH similarly enhances this interaction, but at a half-maximal concentration of 100nM (253). These concentrations of NAD and NADH resemble the physiologically relevant concentrations of available cofactors within the nucleus. Moreover, tryptic digestion assays demonstrate that preincubation of CtBP with NAD and NADH protects its amino terminus from degradation, a protection not seen upon mutation of the NAD(H) binding motif. Moreover, conditions that create a higher ratio of NADH:NAD in the cell were then used to demonstrate that these metabolic cofactors similarly enhance the ability of CtBP binding proteins to repress transcription. The regulation of transcription activation or repression, therefore, is dependent upon the redox state of the cell.

Although RIP140 interacts with a wide variety of nuclear receptors, mice that are null for this gene exhibit relatively subtle phenotypes. RIP140 knockout mice have

reduced female fertility due to failure of oocyte release during ovulation (112, 243). Given the dramatic influence that metabolic cofactors induce upon CtBP interactions, these knockout mice may display more dramatically enhanced phenotypes if subjected to varying states of anabolic and catabolic conditions.

Since its initial cloning, however, CtBP has been identified as a corepressor for many transcription pathways and is involved in regulating growth and differentiation of a diverse array of genes (reviewed in (36)). It has been shown to interact with a wide variety of transcription factors, from sequence specific DNA binding proteins to transcriptional coregulators. In Drosophila, CtBP has been characterized as a general short range corepressor that acts over a distance of several hundred base pairs of DNA to regulate cellular development and differentiation. Short range DNA binding proteins such as Snail, Knirps, and Kruppel interact with CtBP through a motif similar to that found in E1A. Mammalian transcription factors that bind to this class of corepressors include BKLF, TCF, ZEB. Analysis of these and other CtBP interacting proteins confirms that a common, conserved, protein-protein interaction motif mediates binding to this corepressor. This motif has been characterized as the PXDLS motif, which is frequently flanked by a lysine or arginine residue. As demonstrated for E1A, mutation of this consensus CtBP interaction motif disrupts CtBP binding.

The mechanism by which CtBP represses transcription is not known, but CtBP appears to act through HDAC-dependent and independent mechanisms. Reports have shown that CtBP1 interacts with HDAC1 (209), HDAC2, and Sin3 (97) to repress transcription. Although class II HDACs, such as HDAC5, possess a PXDLS-like motif, it appears to interact with CtBP through another domain. Despite these indicated

involvements of HDAC-dependent transcription repression, conflicting reports indicate that in some promoter contexts, CtBP-mediated repression is TSA sensitive (40, 78) while in other promoters, this repression is TSA insensitive (42, 97, 128). Furthermore, CtBP represses transcription through an interaction with the polycomb group proteins in an HDAC independent mechanism, suggesting that CtBP may be involved in maintenance of the transcriptionally repressed states throughout development. The intriguingly high homology between CtBP and NAD-dependent dehydrogenases suggest that CtBP may possess intrinsic enzymatic activity that allows it to repress transcription. CtBP3, for example, has been reported to possess a slow acyl transferase activity. Further studies of the CtBP protein itself may identify key repression domains that may reveal the intrinsic repression properties of this protein.

The presence of a CtBP interaction motif along with a flanking K residue within RIP140 suggested a previously unexplored mechanism for RIP140-mediated repression. Rather than passively competing with coactivators for binding to nuclear receptors (219), formation of the RIP140-CtBP complex may actively repress transcription (Figure 5). If this hypothesis is true, then one mechanism by which transcription factor acetylation actively regulates transcription is through disruption of corepressor complexes. Transcription factor acetylation, though ostensibly distinct from histone acetylation, may similarly disrupt transcriptionally repressive interactions.

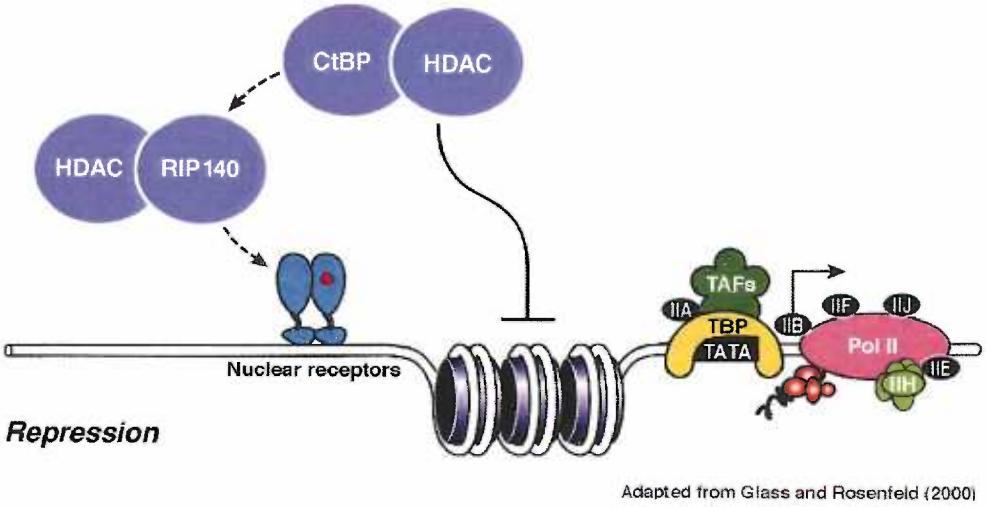

Figure 5. RIP140 may repress transcription by recruiting CtBP to nuclear hormone receptor complexes. Acetylation of RIP140 may regulate formation of this transcription repression complex.

A model in which the acetylation state of RIP140 regulates its ability to activate or repress transcription could reconcile the conflicting early reports of this protein as a coactivator rather than a corepressor of transcription. In its acetylated state, RIP140 may be unable to interact with CtBP, as has been shown for E1A, and perhaps may activate transcription. However, deacetylation of RIP140 may facilitate its interactions with CtBP, resulting in transcriptional repression. The focus of this project centered on identifying and characterizing the acetylation-dependent interaction between RIP140 and CtBP to determine how this interaction regulates transcription.

As part of my thesis work, I demonstrate that the histone chaperone protein RbAp46/48 interacts with the phosphorylated CREB-CBP complex. This interaction

recruited nucleosomes to CBP/p300 and lowered the K_m of CBP/p300 towards histones. Maximal transcriptional stimulation of in vitro assembled chromatin templates is only seen in the presence of CBP/p300, RbAp46/48, and acetyl CoA, suggesting that the HAT activity of this coactivator is facilitated by its association with RbAp46/48.

Investigation of RIP140-mediated repression demonstrated for the first time that RIP140 represses transcription through its interaction with the corepressor, CtBP. Selective acetylation of RIP140 within its CtBP interaction motif disrupts the RIP140-CtBP complex and abrogates transcription repression.

The findings of both these projects emphasize the essential regulation provided by the acetylation state of a gene and illustrates how histone acetylation-dependent mechanisms of transcriptional activation parallels factor-dependent mechanisms of transcriptional regulation. In fact, both processes are intrinsically linked. Acetylation of histones remodels nucleosomes by disrupting histone-DNA interactions; factor acetylation remodels the transcription repression machinery by disrupting the RIP140-CtBP interaction. In this manner, transcriptional activation requires recruitment of coactivator acetyltransferases to terminate transcriptional repression.

CHAPTER 1

The Histone Binding Protein RbAp48 Interacts with a Complex of CBP and Phosphorylated CREB

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Abbreviations: AcCoA, acetyl CoA; BSA, bovine serum albumin; CRE, cAMP-regulated enhancer; CREB, CRE-binding transcription factor; CBP, CREB binding protein; GST, glutathione S-transferase; HDAC, histone deacetylase; MLP, major late promoter; PKA, protein kinase A.

Abstract

A CREB-CBP complex was used as bait to screen a mouse embryo cDNA library in yeast. One of the strongest interactions identified the histone binding protein, RbAp48. RbAp48 also interacted weakly with CBP alone but did not interact with phosphorylated or nonphosphorylated CREB. CBP/p300 from HeLa nuclear extracts co-immunoprecipitated with RbAp48 and its homologue RbAp46 and bound to a glutathione S-transferase (GST)-RbAp48 fusion protein. This interaction was stimulated by the addition of phosphorylated CREB and allowed the association of core histones and mononucleosomes in an acetylation-dependent manner. RbAp48 lowered the Km of the CBP histone acetylase activity and facilitated p300-mediated *in vitro* transcription of a chromatinized template in the presence of acetyl CoA (AcCoA). These data indicate that the association of phosphorylated CREB with CBP/p300 promotes the binding of RbAp48/46, allowing the formation of a complex that facilitates histone acetylation during transcriptional activation.

Introduction

The signaling mechanism responsible for activating genes through the cAMP-regulated enhancer (CRE) represents one of the most intensively studied transcriptional pathways (134). Following the activation of certain G-protein coupled receptors, the catalytic subunit of protein kinase A (PKA) is released from the regulatory subunit and is transported to the cell nucleus where it phosphorylates a unique site in the CRE-binding transcription factor CREB. CREB is phosphorylated at this same site by many additional protein kinases, including those activated by calcium/calmodulin and growth factors. Thus, the transcription factor CREB has been proposed to serve as a fairly general signal-activated transcriptional mediator, linking a variety of signal transduction pathways to genes containing CRE sequences (196).

Phosphorylation allows CREB to interact with the coactivator, CREB binding protein (CBP), or its homologue p300 (37, 48). CBP/p300 associates with a wide variety of additional transcriptional activators as well, suggesting that it may serve as a transcriptional "integrator" (for review, see (200)). Thus, there appears to be a hierarchy of post-transcriptional modifications and protein-protein interactions that permit transcriptional signal integration -- extracellular signals of various types converge on the CREB transcription factor and distinct transcription factors converge by simultaneously interacting with CBP/p300.

How CBP/p300 transmits the activation signal to gene promoters remains unresolved. Evidence from several laboratories has suggested that CBP/p300 interacts with the basal transcription factors TFIIB and TFIID (51, 106, 210). In addition, Nakajima, et al. have shown that CBP contacts the RNA polymerase II holoenzyme through interactions with RNA helicase A (141). Thus, one model for CBP/p300

function is to bridge DNA binding transcription factors to components of the basal transcriptional machinery. Alternatively, CBP/p300 might alter some of these proteins through post-translational modifications (74).

Other evidence suggests that transcriptional activation mediated through CBP/p300 occurs only in the context of chromatin (99, 100). The involvement of chromatin in CBP/p300 function is consistent with the findings that these coactivators, and several associated proteins including PCAF, SRC-1 and pCIP, have the ability to acetylate the amino-terminal tails of histone proteins in a manner that may lead to some, as yet uncharacterized, change in nucleosome structure (6, 27, 158, 204, 218, 249). A multi-step model initially proposed by Roeder and coworkers suggests that CBP/p300 contributes to the first step of transcriptional initiation while other coactivator complexes, such as TRAP, DRIP and ARC, mediate subsequent steps (53, 139, 177). More recent evidence suggests that p300 might function at a stage subsequent to chromatin disruption (114).

Of all the transcription factor-CBP associations, only the interaction with phosphorylated CREB has been studied in detail. NMR analysis has revealed that the interaction of the two proteins introduces structure into both components of the complex (178). After binding to CBP, phosphorylated CREB adopts a bi-helical configuration with the helical axes approximately perpendicular to one another. One prediction from this data is that new protein interaction surfaces might be generated upon CREB-CBP binding. We have taken advantage of this possibility by developing a yeast "three-hybrid" assay that uses a CREB-CBP complex to screen cDNA expression libraries. Such a screen is possible because the PKA site in CREB is phosphorylated in yeast and allows the CREB-CBP interaction (199). In this report, we describe an interaction of the phosphorylated CREB-CBP complex with the histone binding protein, RbAp48.

RbAp48 and its homologue RbAp46 were initially identified as retinoblastoma (Rb) binding proteins (176). Subsequently, these proteins were characterized as components of at least four distinct nucleosome modifying complexes, the nuclear histone deacetylases (HDACs), the Drosophila nucleosome remodeling factor NURF, the chromatin assembly factor CAF-1, and Hat-1, a type B (cytoplasmic) histone acetylase involved in chromatin assembly (65, 93, 126, 164, 215, 222, 227, 257). In general, the functions of the RbAp48/46-like proteins in these complexes remain undetermined. Two exceptions to this generalization are in the context of the human cytoplasmic histone acetyltransferase Hat1 or its yeast homologue Hat1p, where RbAp46/Hat2p appear to link the enzymes to their target, histone H4 (164, 226). Thus, although RbAp48/46 association with nuclear transcriptional coactivators has not been described, there is abundant evidence that these histone binding factors interact with related classes of proteins. Moreover, this function is consistent with the model that a critical function of transcriptional coactivators is to direct the targeting of histone acetyltransferases to specific promoters.

Our studies demonstrate that the association of RbAp48/46 with the coactivator CBP is stimulated by phosphorylated CREB. The binding of RbAp48/46 to the CREB-CBP complex then allows an interaction with core histones and mononucleosomes. The binding of histone particles to the CBP-RbAp48/46 complex depends upon their acetylation state -- acetylation by CBP blocks the ability of histones or mononucleosomes to associate with the coactivator. In addition, RbAp48 increases the histone acetyltransferase activity of CBP, probably by enhancing the affinity of the coactivator for its substrate. This ability of RbAp48/46 to increase CBP/p300 histone acetyltransferase activity is reflected by its capacity to stimulate p300-mediated transcription of a reconstituted chromatin template *in vitro*. Furthermore, this stimulation

is enhanced by the addition of AcCoA, supporting the functional significance of RbAp48/46-targeted histone acetylation.

Materials and Methods

Plasmids: The bait plasmid for the three-hybrid screen was constucted from the pBTMYeA backbone. The parental plasmid pBTM116 was a gift from Stan Hollenberg (Oregon Health Sciences University), and pAD4 was obtained from Michael Wigler (Cold Spring Harbor Laboratory). The plasmid pYeA was created by digesting the ADH promoter, polylinker, and terminator from pAD4 with BamH I and cloning into pYEP24 (New England Biolabs). To create pBTMYeA, the ADH promoter, terminator, and polylinker of pYeA were digested with BamH I, blunt-ended, and cloned ino the Pvu II site of pBTM116. To generate the three-hybrid bait plasmid, VP16-CREB$_{1\text{-}283}$ (199)was digested with BamH I and ligated into the BamH I site of pBTMYeA. LexA-CBP$_{461\text{-}682}$ (199) was digested with EcoR I and BamH I, blunt-ended, and ligated into the LexA-CREBYeA plasmid using a Not I linker, creating LexA-CREB-YeACBP. LexA-CREBM1 encodes aa 1-283 and contains a Ser$_{133}$Ala mutation. The remaining bait plasmids used in secondary screens were generated similarly. An E9.5 mouse embryo VP16 fusion cDNA library was a gift from Stan Hollenberg. The carboxyl-terminal portion of RbAp48 recovered from the library screen, aa 273-425, was subcloned into pGEXKG (Pharmacia) and pET28a (Novagen) by PCR. Full length RbAp48 and RbAp46 in pGEX2T3 were obtained from Bruce Stillman (Cold Spring Harbor Laboratory) and subcloned into pET28a by PCR. pGEXKG-CBP$_{551\text{-}682}$ was kindly provided by Roland Kwok (University of Michigan).

Proteins: GST-RbAp48$_{273\text{-}425}$, GST-RbAp48, and GST-CBP$_{551\text{-}682}$ were expressed in bacteria and purified by glutathione sepharose affinity (Sigma). His-tagged

RbAp48, RbAp46, or RbAp48$_{273-425}$ were expressed in BL21(DE3) and purified by Ni-NTA affinity (Qiagen). HeLa cell nuclear extracts were prepared from HeLa-S3 cells provided by the National Cell Culture Center, Minneapolis, MN(37). CREB containing all cysteines mutated to serines was purified as described previously (184) and phosphorylated with PKA for 1 hr at 30°C according to Laurance, et al. (109). PKA was a gift from Richard Maurer (Oregon Health Sciences University). Flag-tagged CBP and p300 were expressed in baculovirus-infected SF9 cells and purified using an M2 Flag affinity matrix (Sigma). Core histone octamers were isolated from chicken blood (52). Mononucleosomes were generated by digestion of purified chicken chromatin with micrococcal nuclease (Sigma), 2 U/ml, in 0.3 mM $CaCl_2$ at 37°C for 40 min. Precautions were taken to avoid over-digestion of the chicken chromatin to minimize the production of free core histones. The chicken core particles were confirmed to be hypoacetylated by TAU gel analysis according to Tse et al. (220). Gal4-VP16 was expressed in *E. coli* and purified as described (159).

Yeast three-hybrid screen: The LexA-CREB-YeACBP bait plasmid was transformed into the L40 yeast strain using standard small scale transformation protocols (68, 192). This bait strain was then subsequently transformed with approximately 50 μg of the library plasmid. The yeast were allowed to grow at 30°C on leu/trp/his/ura/lys deficient plates, and colonies were picked daily for β-galactosidase assays. Plasmids from yeast that were positive for both histidine and β-galactosidase production were isolated and sequenced. Secondary screens were performed using LexA-CREB, LexA-CBP, LexA-CREBM1, LexA-CREBM1-YeACBP as bait.

GST pull down assays: GST, GST-RbAp48$_{273-425}$, GST-RbAp48, and GST-CBP$_{551-682}$ were coupled to glutathione-sepharose beads (Pharmacia) and blocked with bovine serum albumin (BSA). Equimolar amounts of GST or GST-fusion proteins were

used in the pull down assays. HeLa nuclear extracts, recombinant RbAp48 or RbAp46, purified CREB, phosphorylated CREB, chicken core histones, and mononucleosomes were added to the binding buffer HEG100 (20 mM Hepes, pH 7.6, 10 % glycerol, 100 mM KCl, 1 mM EDTA, 1 mM DTT, 10 μM NaF, 10 μM Na_3VO_4) plus protease inhibitors (Complete™, Boehringer-Mannheim) for 1 hr at 4°C. The beads were washed three times with HEG100, boiled in 15 μl of 5X SDS loading buffer, and electrophoresed on a 6-15% SDS-polyacrylamide gel. After transfer to a PVDF membrane, the bound fraction was detected by western blotting using anti-CBP$_{451-682}$ (which recognizes both CBP and p300), anti-CREB (New England Biolabs), anti-RbAp48/46 15G12 (GeneTex), or anti-histone H4 BWA3 and anti-histone H3 LG2-1 (generous gifts from Marc Monestier, Temple University).

Co-immunoprecipitations: Anti-RbAp48/46 antibody 15G12 was coupled to protein G sepharose (Pharmacia) and blocked with BSA. The beads were used to precipitate RbAp48/46 from HeLa nuclear extracts in HEG100 buffer for 1 hr at 4°C. Normal mouse IgG (Sigma) served as a control for background binding. The beads were washed three times with HEG100, boiled in 15 μl of 5X SDS loading buffer, and electrophoresed on a 6% SDS-polyacrylamide gel. After transfer to a PVDF membrane, the bound fraction was assayed for CBP/p300 by western blotting using anti-CBP$_{451-682}$.

Histone acetylation assays: 0.2 pmole full length CBP was preincubated with 1 pmole His-tagged RbAp48 protein or BSA (as control) in HEG100 on ice for 30 min and then mixed with purified chicken core histones or mononucleosomes in a 30 μl reaction buffer containing 10 mM Tris-HCl, pH 8.0, 10% glycerol, 0.1 mM EDTA, 10 mM sodium butyrate, and ^{3}H-AcCoA (Amersham) and incubated for 30 min at 30°C. The entire reaction was spotted on a phosphocellulose filter (GibcoBRL). The filter was then washed three times with sodium bicarbonate buffer, and the ^{3}H signal from the transfered

acetyl group was quantified by scintillation. CBP autoacetylation was subtracted from the total counts.

Chromatin assembly and *in vitro* transcription assays: S190 extracts and *Drosophila* core histones were generous gifts from W. Lee Kraus (Cornell University). The DNA template containing five Gal4 DNA-binding sites upstream from the adenovirus major late promoter (MLP) encoding a 390 nucleotide G-free transcription cassette (130) and the Gal4-VP16 construct were gifts from Danny Reinberg (University of Medicine and Dentistry of New Jersey). The control DNA template encoding a 170 nucleotide G-free transcription cassette placed downstream from the adenovirus MLP was a gift from Richard Maurer (Oregon Health Sciences University).

Chromatin was assembled as described (90, 159). Gal4-VP16 (200 nM) was added to the reaction mixture subsequent to chromatin assembly and incubated for 30 minutes. Where indicated, full length p300 (25 nM) was added after Gal4-VP16, and this remodeled assembly mixture was incubated for a further 30 minutes at 30°C. The chromatin was then purified over a sepharose CL-4B column (Pharmacia).

In vitro transcription reactions were performed with HeLa cell nuclear extracts as described (159). Briefly, naked or chromatinized DNA was incubated with nuclear extracts in the presence or absence of recombinant RbAp46 (272 nM) and AcCoA (1 μM) for 30 minutes at 30°C. The templates were then transcribed at 30°C for 45 minutes upon the addition of rNTPs and RNaseT1. The adenovirus MLP-driven 170 nt template was added to the transcription mixture as an RNA recovery control. The purified RNA products were resolved on a 5% acrylamide/6 M urea gel and analyzed by autoradiography.

Results

Identification of RbAp48 by a yeast three-hybrid screen. Although the yeast two-hybrid assay has identified many important protein-protein interactions, the high false positive rate often limits its usefulness as a screening approach. This has particularly been the case for the transcriptional coactivator CBP (unpublished observations). One mechanism for generating spurious interactions could result if the bait component is capable of adopting a variety of three-dimensional configurations. As discussed above, the association of phosphorylated CREB with CBP introduces secondary structure into both components of the complex. Thus, this interaction might stabilize structures that are important for specific protein-protein interactions and decrease the occurance of non-physiological protein configurations. Alternatively, the association of phosphorylated CREB and CBP may generate new protein interaction surfaces that are not found in either component in isolation. It is possible that critical transcriptional effectors could interact with CBP only, or preferentially, in the context of these novel induced structures. We have taken advantage of these possibilities by developing a yeast three-hybrid assay that uses a CREB-CBP complex as bait.

We had previously shown that LexA-CREB (containing the activation domain of $CREB_{1-283}$ fused to the LexA DNA-binding domain) becomes phosphorylated at Ser_{133} in yeast, thereby allowing an interaction with VP16-CBP (199). Ser_{133} is the residue that is recognized by PKA and other kinases that have the ability to mediate CREB activation. Mutation of Ser_{133} to Ala (designated LexA-CREBM1) prevented the interaction, supporting the idea that CBP binding to the CREB activation domain in yeast depends upon phosphorylation. The kinase responsible for this phosphorylation event is unknown, although it could be PKA, which is constitutively active in yeast (199). To

develop the yeast three-hybrid assay, we cloned LexA-CREB and a nuclear localized fragment of CBP (representing the CREB-binding domain, aa 461-682, fused to the SV40 Tag nuclear localization signal) into the plasmid BTMYeA, a derivative of pYeA (Fig. 1a). The two fusion proteins were cloned into the same expression vector to reduce the use of selectable markers and to ensure that the expression ratio of both components of the bait remained constant. The two-component bait plasmid (LexA-CREB-YeACBP) was introduced into the yeast strain L40 and was used to screen a VP16-cDNA library representing embryonic day 9.5 mouse mRNAs. In marked contrast to our results using the same fragment of CBP in standard two-hybrid assays, which have shown very little selectivity, virtually all the cDNAs obtained by using the yeast three-hybrid screen encoded proteins involved in transcriptional regulation (unpublished observation).

One of the strongest positives identified in the screen was the histone recognition factor, RbAp48 (Fig. 1b). When subjected to a secondary screen, VP16-RbAp48 was found to bind weakly to LexA-CBP alone but did not interact with LexA-CREB, LexA-CREBM1, or LexA-CREBM1-YeACBP (Fig. 1b). These studies indicate that the interaction requires the CBP component. Because the CBP fragment does not bind to DNA, association with the LexA-CREB component is required to generate an efficient target for VP16-RbAp48. It is not possible in these assays to assess the absolute affinities of the interactions of VP16-RbAp48 with LexA-CBP and LexA-CREB-YeACBP, however, because the two bait plasmids confer different levels of background activity.

Interaction between RbAp48/46 and CBP/p300 in mammalian cells. The portion of RbAp48 isolated in the yeast three-hybrid screen extended from residues 273 to 425, which includes the carboxyl-terminal WD repeats 4 through 7. A GST fusion protein containing this portion of RbAp48, GST-RbAp48$_{273-425}$, and a GST protein containing full length RbAp48 were expressed in bacteria, coupled to glutathione-agarose beads, and

incubated with HeLa nuclear extracts. Both GST-RbAp48 fusion proteins bound to full length CBP/p300, as determined by western blotting using an antibody directed against the CREB-binding domains of CBP and its homologue p300 (Fig. 2a), confirming the interaction identified in yeast. No binding of CBP/p300 was detected in the presence of GST alone. The amino-terminal portion of RbAp48 was incapable of binding to CBP/p300 (data not shown). To determine whether the interaction of CBP/p300 and RbAp48/46 exists *in vivo*, we immunoprecipitated HeLa nuclear extracts with a monoclonal antibody directed against RbAp48/46 and performed western blots using an antibody directed against CBP/p300 (Fig. 2b). These studies showed that RbAp48/46 complexes contain CBP/p300. Although these experiments suggest that the association of CBP/p300 and RbAp48/46 occurs *in vivo*, they do not show that the interaction is direct. Moreover, it is possible that some unknown component of the extracts, such as a transcription factor, could promote the appropriate CBP/p300-interaction interface to allow RbAp48/46 binding.

The association of RbAp48 and CBP is augmented by phosphorylated CREB. We next performed GST pull down assays using GST-CBP$_{551-682}$ and GST alone to determine whether recombinant RbAp48/46 interacted with CBP directly. His-tagged RbAp48 and RbAp46 were expressed in bacteria, purified on a nickel affinity column, and incubated with the two GST fusion proteins. Both RbAp48 and RbAp46 bound directly to GST-CBP$_{551-682}$ (Fig. 3a). The carboxyl-terminal portion of the RbAp48 protein that was identified in the yeast three-hybrid screen was also able to bind directly to GST-CBP$_{551-682}$ (data not shown).

Our initial yeast assays demonstrated that RbAp48 associates more strongly with the phosphorylated CREB-CBP complex than it does with CBP alone. The interaction of RbAp48 with LexA-CBP was easily detectable in yeast, however, and, as indicated

above, purified recombinant RbAp48 bound to GST-CBP551-682 *in vitro*. One explanation for our failure to identify RbAp48 in a standard two-hybrid screen using LexA-CBP as bait may relate to the large number of false positives. The addition of phosphorylated CREB could potentially decrease the abundance of aberrant CBP structures that generate these false interactions. Alternatively, phosphorylated CREB might contribute directly to the RbAp48 interaction interface, either by inducing a necessary structure in CBP or by providing protein interaction sites itself.

Because we are unable to compare the affinities of RbAp48 for the LexA-CBP and LexA-CREB-YeACBP constructs in yeast due to their different background activities, we cannot distinguish between these models. To resolve this issue, we assayed binding of purified recombinant CBP and RbAp48 *in vitro* in the presence of nonphosphorylated or phosphorylated CREB. As previously reported, CREB binds to GST-CBP551-682 only after phosphorylation (Fig 3b). Consistent with our studies in yeast, we did not detect binding of nonphosphorylated or phosphorylated CREB to GST-RbAp48 (data not shown). Surprisingly, we found that the addition of phosphorylated CREB greatly increased the binding of RbAp48 to GST-CBP551-682 (Fig. 3b). Binding of RbAp48 to GST alone was not detected, even in the presence of phosphorylated CREB. This data is consistent with the hypothesis that phosphorylated CREB induces or stabilizes a particular structure in CBP that favors interaction with RbAp48. Alternatively, it is possible that RbAp48 also interacts with CREB structures that have been induced by association with CBP.

RbAp48/46 bridges CBP to histone proteins. The physical association of CBP/p300 and RbAp48/46 and the functional characterization of these proteins as histone acetyltransferases and histone binding factors suggests that RbAp48 may allow CBP to associate with its histone substrates. As described above, a similar function has been

proposed for RbAp46 in the context of the cytoplasmic Hat1 (226). To test whether RbAp48/46 bridges CBP/p300 to histones, we isolated hypoacetylated core histones and mononucleosomes from chicken blood and performed pull down assays using GST-CBP$_{551-682}$ and GST alone. These GST proteins were incubated with chicken core histones or mononucleosomes in the presence or absence of recombinant RbAp48. Bound proteins were detected by western blotting using antibodies specific for RbAp48, H3, or H4. RbAp48 associated with GST-CBP$_{551-682}$ but not GST alone (data not shown). As shown in Fig. 4a, the binding of histone octamers to GST-CBP$_{551-682}$ was dramatically enhanced by the addition of RbAp48. Histone octamer binding was also stimulated by RbAp46 (data not shown). These results suggest that CBP/p300, RbAp48/46, and core histones are capable of forming a stable ternary complex. Relatively little binding of histone proteins to CBP was detected in the absence of RbAp48/46.

Similar results were obtained when experiments were performed using intact mononucleosomes rather than core histones (Fig. 4b). We interpret these data to indicate that the histone binding factor RbAp48/46 targets nucleosomal components to the CBP/p300 histone acetyltransferase. Although the conditions utilized in these studies should maintain mononucleosome integrity, we can not completely rule out the possibility that some of the histone binding might result from the release of core histones from the mononucleosome particles, however.

RbAp48 increases CBP histone acetylation activity. Given the association of CBP, RbAp48, and core histones, one obvious question is whether RbAp48 affects CBP histone acetyltransferase activity. Acetylation assays were performed using purified baculovirus-expressed full length CBP, excess ^{3}H-AcCoA, a five-fold molar excess of RbAp48 or BSA over CBP, and various concentrations of chicken core histones as

substrate. Neither RbAp48 nor BSA had intrinsic histone acetylase activity and neither was a substrate for the CBP acetyltransferase (data not shown). Because CBP acetylates itself, data were corrected by subtracting the self-acetylation activity. RbAp48 treatment did not affect CBP self-acetylation (data not shown).

Results from three independent experiments demonstrated that RbAp48 augmented the acetyltransferase activity of CBP at low histone concentrations but not at higher histone concentrations. When the data were analyzed by the Michaelis-Menton equation, RbAp48 was found to lower the Km by three- to four-fold but had relatively little effect on the Vmax (Table 1). This mode of activation is consistent with a model wherein RbAp48 bridges the histone substrates to the CBP enzyme. RbAp48 also increased the ability of CBP to acetylate intact nucleosomes (data not shown). The ability of RbAp48 to augment CBP histone acetyltransferase activity is reminiscent of its effect on the cytoplasmic Hat1.

Histone binding to the CBP/RbAp48 complex is blocked by acetylation. To assess the specificity of the RbAp48-histone interaction, we examined the effect of acetylation on histone binding. Chicken core histones are largely underacetylated (15) but can be acetylated by incubation with baculovirus-expressed CBP. GST pull down assays were performed using GST-CBP$_{551-682}$ or GST in the presence or absence of RbAp48 and acetylated versus underacetylated histones. As compared to the underacetylated core histones, the acetylated histones bound to the GST-CBP$_{551-682}$-RbAp48 complex relatively poorly (Fig. 5a). Mock-acetylated histones (treated with CBP in the absence of AcCoA) showed the same binding pattern as the underacetylated histone proteins (data not shown). Similar experiments were performed using acetylated versus underacetylated mononucleosomes (Fig. 5b). These experiments showed that only underacetylated mononucleosomes interacted with the GST-CBP$_{551-682}$-RbAp48

complex. Although the CREB-binding motif of CBP did not interact with histones, the possibility exists that full length CBP/p300 might contain an additional domain that makes contacts with histone particles directly. However, because GST-RbAp48 was able to directly bind to either core histones or mononucleosomes in an acetylation-sensitive manner (Fig. 5c), we think that the binding of histone particles to the CBP-RbAp48 complex more likely occurs through RbAp48. Thus, the association of histones with the CBP/p300-RbAp48/46 complex is dependent on their acetylation status, such that the acetylated histone particles fail to associate or readily dissociate from the CBP/p300-RbAp48/46 complexes.

RbAp46 facilitates the p300-mediated transcription of a chromatinized template.

We used a reconstituted chromatin template to examine the functional contributions of RbAp46 to acetylation-dependent, p300-mediated transcription. Productive transcription assays (159) were performed on a chromatinized template that contained five Gal4 DNA-binding sites upstream from the adenovirus MLP and a 390 nt G-free cassette (130). A Gal4-VP16 fusion protein was used to remodel the chromatin templates. We assayed the effects of adding p300 and RbAp46 in the presence and absence of AcCoA. The addition of p300 in the presence or absence of AcCoA did not significantly stimulate transcription above levels obtained with Gal-VP16 alone (Fig. 6a and data not shown). Supplementing the transcription reactions with RbAp46 modestly increased the synthesis of the 390 nt product, in the presence or absence of exogenous p300 (Fig. 6a). It should be noted, however, that the HeLa nuclear extract contains significant levels of both CBP and p300, so it is not possible to assay the contribution of RbAp46 in the complete absence of coactivator. Unfortunately, stripping the extracts of CBP/p300 removes other factors required for transcription (250). A similar increase was detected in the presence of RbAp46 and AcCoA. The highest level of expression was seen in the presence of AcCoA, RbAp46, and p300 (Fig. 6a), which suggests that the activity of RbAp46

depends upon its ability to augment CBP/p300 histone acetyltransferase function. This stimulation of transcription was also observed when RbAp48 was substituted for RbAp46 (data not shown).

As a control, in vitro transcription assays were also performed using naked DNA templates. Neither the 5X GAL4-MLP nor the MLP-driven transcription was significantly stimulated by the addition of AcCoA, RbAp46, and p300 (Fig. 6b). This contrast in the effects of RbAp46 in the presence of naked versus chromatinized templates supports the hypothesis that RbAp46 mediates the selective targeting of the CBP/p300 acetyltransferase acitivity towards histones.

Discussion

As a prototypical transcriptional coactivator, CBP and its homologue p300 have important roles in gene regulation, potentially integrating signals from diverse transcriptional pathways. Consequentially, CBP/p300 have been implicated in a variety of cellular processes, including regulation of the cell cycle, differentiation, DNA repair, and apoptosis. Although there is evidence that CBP and p300 interact with components of the basal transcriptional machinery (51, 106, 142, 210), recent studies suggest that the major activities of these coactivators occur only in the presence of chromatin (99, 100).

Two models for this chromatin-dependant activity can be envisioned. First, the intrinsic histone acetylation activity of CBP/p300 (or the activities of associated histone acetyltransferases) may modify the amino-terminal tails of histone proteins in a manner that may lead to changes in nucleosome structure. These nucleosomal changes could result in chromatin decondensation, which may be required for the initial steps in transcriptional initiation. In support of this model, studies have correlated the histone

acetylation activity of CBP with its ability to activate transcription (124). It should be noted, however, that no direct link between histone acetylation and any structural change in chromatin has been demonstrated. A second model is that CBP/p300 may interact with proteins that have the potential to mediate chromatin remodeling. While such interactions have been proposed (87), there is no evidence that these associations contribute to the chromatin remodeling process. Although neither of these models have been proven, they share a common theme -- that chromatin modifying proteins (histone acetyltransferases, chromatin remodeling factors) may be localized to specific promoters through the CBP/p300 coactivators.

The current report proposes that the nucleosome modifying activities of a coactivator can in fact be augmented by transcription factor binding. We envisage that this occurs through the formation of new protein interaction surfaces. This idea is supported by the NMR analysis of the phosphorylated CREB-CBP complex, which shows that structure is induced into both components of the complex upon their interaction (178). We had previously determined that an uncharacterized kinase in yeast is capable of recognizing Ser_{133} of CREB, the same site that is critical for the interaction with CBP. This allowed us to generate CREB-CBP complexes for use as bait to screen cDNA expression libraries. One of the strongest interactors identified in this screen was the histone binding factor, RbAp48. Subsequent control studies were performed to determine whether both components of the bait were required for the RbAp48 interaction. These experiments indicated that while RbAp48 can bind to CBP alone, the association is much stronger in the presence of phosphorylated CREB. Thus, we conclude that RbAp48 binds preferentially to the complex of the activated transcription factor and its coactivator. This model is somewhat reminiscent of the transcription factor-mediated activation of the peroxisome proliferator-activated receptor γ coactivator, PGC-1, recently reported by Puigserver, et al. (174).

RbAp48 and its homologue RbAp46 have been characterized as components of four distinct complexes involved in nucleosome assembly or modification, the type B histone acetyltransferases, CAF-1, the HDACs, and NURF (65, 93, 126, 164, 215, 222, 227, 257). For example, the type B histone acetyltransferases have been shown to contain two subunits, the catalytic subunit Hat1p and the RbAp48/46-like factor Hat2p (164). Hat2p enhances Hat1p activity by increasing the affinity for histone H4 (164). Hat2p is structurally related to Cac3p, the small subunit of CAF-1 (93). In humans, this activity is provided by RbAp48 (227). In contrast, human Hat1 is associated with RbAp46 (226). Both RbAp46 and RbAp48 copurify with the histone deacetylases HDAC1 and HDAC2 (13, 46, 55). Finally, the ATP-dependent nucleosomal remodeling factor NURF contains a 55 kD (p55) subunit that is highly related to both RbAp proteins (126). Although the NURF complex does not contain histone acetyltransferase activity, recombinant p55 associates with factors in Drosophila nuclear extracts that can acetylate histones H3 and H4 (126). Our studies indicate that the CREB-CBP complex associates with both RbAp48 and RbAp46.

Despite their association with various histone modifying complexes, the functional roles of the RbAp48/46 proteins remain unclear. For example, CAF-1 complexes lacking RbAp48 still can associate with histones, while human Hat1 lacking RbAp46 cannot (226). Perhaps more puzzling, the RbAp48/46 proteins appear to interact with a region of histone H4 (amino acids 31-40) predicted from the crystallographic structure of the nucleosome to be inaccessible for binding (226). The type B acetyltransferases and CAF-1 are involved in *de novo* chromatin assembly, thus the participation of RbAp48/46 in histone recognition in these instances is easy to rationalize. The involvement of RbAp48/46 in transcriptional regulation is obviously more problematic because the target histone sites are embedded within chromatin.

Nonetheless, our data indicates that RbAp48/46 bound to CBP can associate with histone proteins in the context of nucleosomes and, presumably, the same is true for the RbAp48/46 proteins associated with the HDACs. Although we can not conclusively exclude the possibility of a slow partial dissociation of the mononucleosomes in our sample preparation (63), the demonstration that RbAp48/46 stimulates both CBP/p300-mediated acetylation of nucleosomes and transcription from chromatin templates supports our hypothesis that the nucleosome may be capable of adopting an alternative configuration that is permissive for RbAp48/46 binding.

The Vmax and Km values for CBP enzymatic activity were not markedly different from those measured for other histone acetyltransferases. The Vmax for CBP was slightly higher than that of GCN5, for example (s^{-1}= 0.3 vs. 0.08), possibly due to the fact that the GCN5 fragment examined represented only the catalytic domain (214). In the absence of RbAp48, the Km values of GCN5 and CBP were very similar (28 μM vs. 16 μM). The addition of RbAp48 lowered the Km of CBP for the histone substrate to about 6 μM. Somewhat unexpected was the finding that RbAp48/46 bound to CBP recognized underacetylated but not acetylated histones. Although it has not been tested explicitly, the RbAp48/46 proteins in the HDACs would be expected to recognize predominantly acetylated histones. It is possible that the binding specificities of RbAp48/46 could depend upon whether they are associated with histone acetylases or deacetylases.

The ability of RbAp48/46 to augment CBP/p300 histone acetyltransferase function appears to be reflected by an increase in transcriptional activity. Using *in vitro* transcription assays, we were able to demonstrate that transcription from a reconstituted chromatin template was stimulated upon addition of p300, RbAp46 and AcCoA. This enhancement is specific for chromatin templates, suggesting that RbAp48/46 and

CBP/p300 act in concert to alter the acetylation state of nucleosomal histones. These assays utilized a Gal-VP16 activator to remodel the chromatin template. VP16 binds to CBP and p300 (236), and p300 activation of Gal-VP16 has been observed by other investigators (100). The addition of p300 and AcCoA alone did not activate transcription above basal levels achieved by Gal-VP16 in our assays, and the addition of p300 and RbAp46 only modestly stimulated transcription. The requirement for all three components implicates the CBP/p300's histone acetylation function in transcriptional activation. The acetyltransferase acitivity of CBP/p300 is not restricted to histones. It is possible that RbAp48/46 may target the acetylation function of CBP/p300 to histones as opposed to other targets (61, 74).

Finally, it remains to be determined whether the ability of phosphorylated CREB to augment RbAp48 binding to CBP is shared by other transcriptional activators. Many additional transcription factors interact with the CREB-binding domain of CBP, some dependant upon phosphorylation and some in a phosphorylation-independent manner. Three such phosphorylation-independent factors, c-myb, SREBP, and the Drosophila cubitus interruptus, have been proposed to interact with CBP through an alpha helical interface related to that of phosphorylated CREB in the CREB-CBP complex (23, 163), suggesting that similar protein interaction surfaces could be generated. Whether complexes containing these other transcription factors also bind RbAp48/46 is unknown.

Acknowledgements

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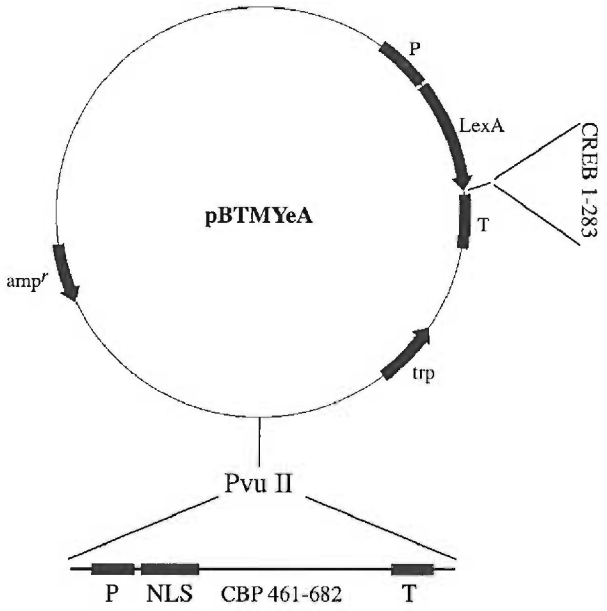
Figure 1. Yeast three-hybrid screen.

a. Illustration of the two-component bait plasmid LexA-CREB-YeACBP. LexA-CREB (containing the activation domain of CREB, aa 1-283, fused to the LexA DNA-binding domain) and a nuclear localized fragment of CBP (representing the CREB-binding domain, aa 461-682) were cloned into the plasmid pBTMYeA, a derivative of pYeA. The bait plasmid is ampicillin resistant (Amp^r) and enables trp production. P, promoter; T, terminator; NLS, nuclear localization signal.

b. VP16-RbAp48 interacts with the CREB/CBP complex and CBP alone but does not interact with CREB. LexA-CBP, LexA-CREB, and LexA-CREBM1 are LexA fusion constructs encoding aa 461-682 of CBP, aa 1-283 of CREB, or 1-283 of CREB with a $Ser_{133}Ala$ mutation, respectively. LexA-CREBM1-YeACBP is a two-component bait plasmid identical to LexA-CREB-YeACBP with the exception of the described $Ser_{133}Ala$ mutation. The levels of interaction with VP16-RbAp48, indicated by the + and – signs, were determined by assaying the growth of the transformants on his-minus background.

a. LexA-CREB-YeACBP



b.

	VP16-RbAp48
LexA-CREB-YeACBP	++
LexA-CREB	-
LexA-CBP	+
LexA-CREBM1	-
LexA-CREBM1-YeACBP	-

Figure 2. Interaction between RbAp48 and CBP/p300 in HeLa cells.

a. GST pull down assay. A GST fusion protein encoding RbAp48$_{273-425}$, which contains the carboxyl-terminal WD repeats 4 through 7 (GST-RbAP48$_{273-425}$), and a GST fusion protein containing full length RbAp48 (GST-RbAP48) were coupled to glutathione-agarose beads and incubated with HeLa nuclear extracts. CBP/p300 bound to the GST-RbAp48 fusion proteins was visualized by western blotting.

b. Association of CBP/p300 and RbAp48/46 *in vivo*. Endogenous RbAp48/46 was immunoprecipitated from HeLa nuclear extracts using a monoclonal antibody against RbAp48/46. CBP/p300 co-immunoprecipitated with RbAp48/46 and was visualized by western blot. Normal mouse IgG was used as negative control.

a.
CBP/p300
Input 10%
GST
GST-RbAp48
GST-RbAp48273-425
b.
Input 5%
Normal Mouse IgG
Anti-RbAp48/46

Figure 3. Augmentation of RbAp48-CBP interaction by phosphorylated CREB.

a. Direct binding of RbAp48 and RbAp46 to CBP. GST pull down assays were performed with GST or GST-CBP$_{551-682}$ and recombinant His-tagged RbAp48 or 46. RbAp48 or 46 bound to GST-CBP$_{551-682}$ was visualized by western blotting.

b. RbAp48 binds to the phosphorylated CREB-CBP complex. GST- or GST-CBP$_{551-682}$-coupled beads were incubated with His-tagged RbAp48 in the presence of nonphosphorylated (+CREB) or phosphorylated CREB (+pCREB). The binding of RbAp48 and CREB to GST-CBP$_{551-682}$ was visualized by western blotting.

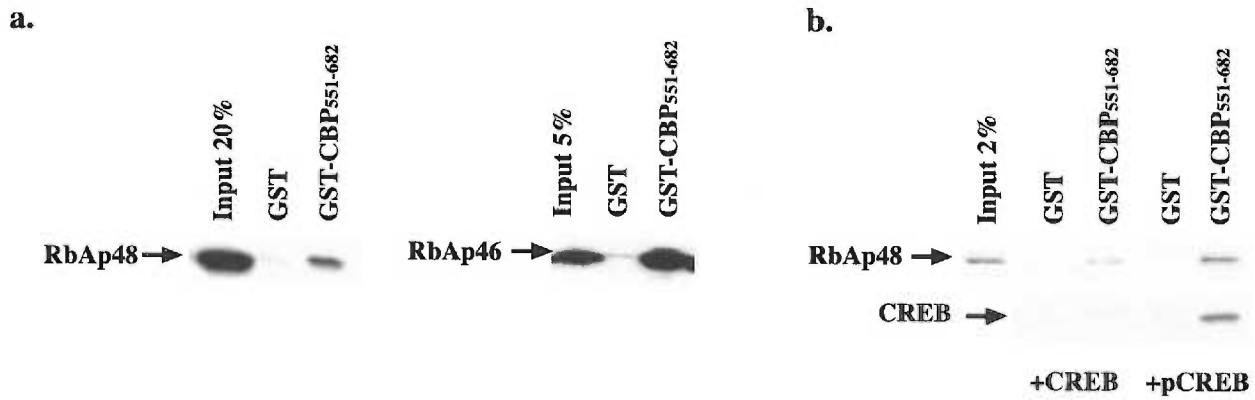
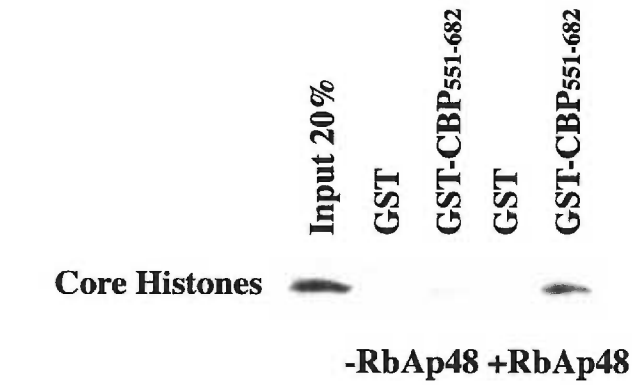

a.
Input 20%
GST
GST-CBP551-682
RbAp48
Input 5%
GST
GST-CBP551-682
RbAp46
b.
Input 2%
GST
GST-CBP551-682
GST
GST-CBP551-682
RbAp48
CREB
+CREB
+pCREB

Figure 4. RbAp48 bridges CBP to histone proteins.

GST- or GST-CBP$_{551-682}$-coupled beads were incubated with chicken core histones (a) or mononucleosomes (b) in the presence or absence of full length RbAp48. Bound core histone octamers were assayed by western blotting using antibody specific for histone H4 (core histones). Bound mononucleosomes were detected by two antibodies against either H3 or H4 (mononucleosomes).

a.


Input 20%
GST
GST-CBP551-682
GST
GST-CBP551-682
Core Histones
-RbAp48 +RbAp48

b.

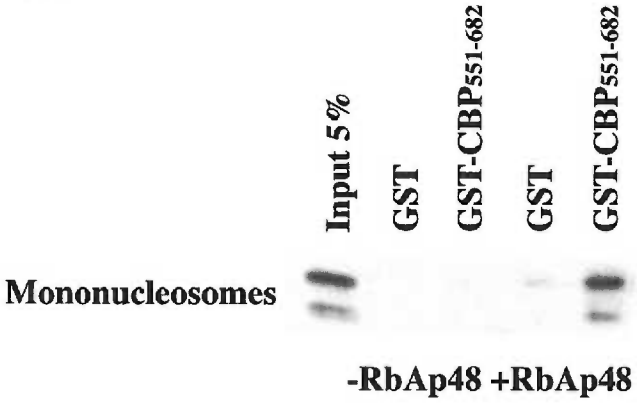

Input 5%
GST
GST-CBP551-682
GST
GST-CBP551-682
Mononucleosomes
-RbAp48 +RbAp48

Figure 5. Histone binding to the CBP-RbAp48 complex is blocked by acetylation.

Core histone octamers (a) and mononucleosomes (b) were acetylated by CBP. GST pull down assays were performed using GST or GST-CBP$_{551-682}$ in the presence or absence of RbAp48 with acetylated versus underacetylated histone particles. c. Both hypoacetylated (underacetylated) and CBP-acetylated (acetylated) core histones or mononucleosomes were incubated with GST- or GST-RbAp48-coupled beads directly. Bound core histone octamers were assayed by western blotting using antibody specific for histone H4 (histones). Bound mononucleosomes were detected by two antibodies against either H3 or H4 (mononucleosomes).

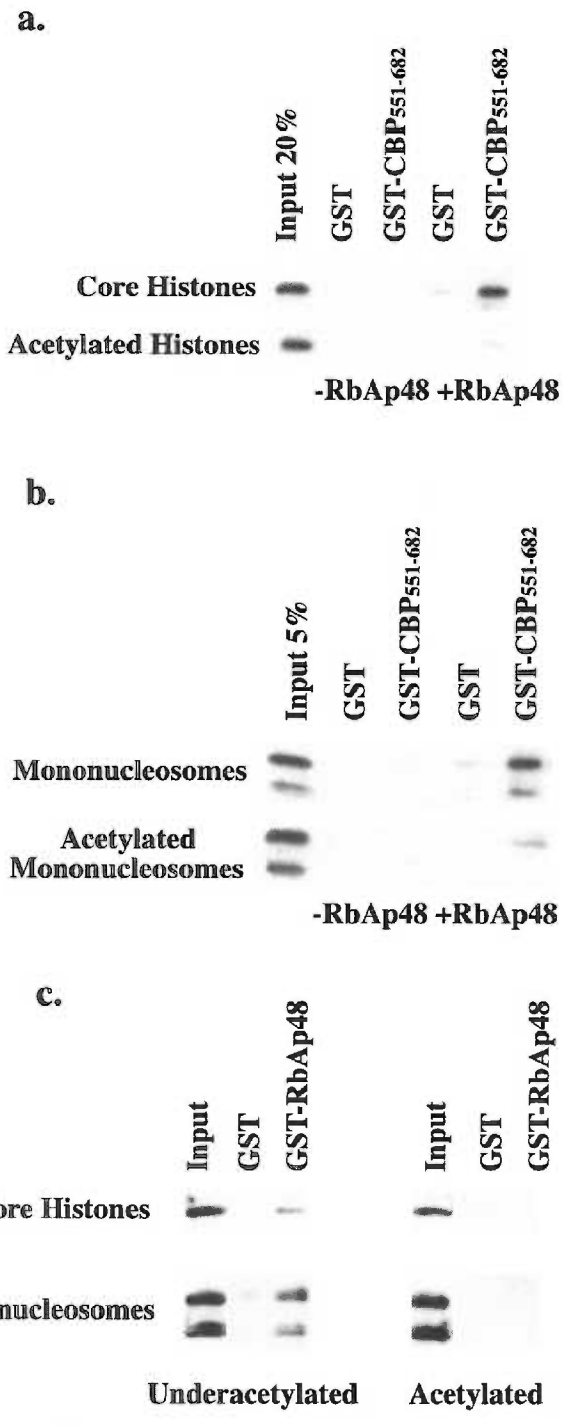
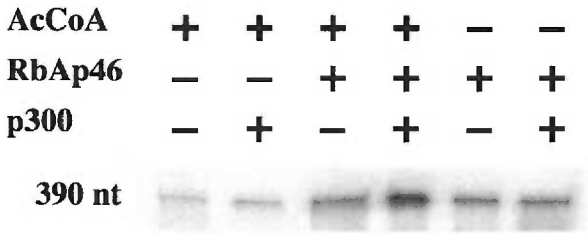

a.
Input 20%
GST
GST-CBP551-682
GST
GST-CBP551-682
Core Histones
Acetylated Histones
-RbAp48 +RbAp48
b.
Input 5%
GST
GST-CBP551-682
GST
GST-CBP551-682
Mononucleosomes
Acetylated Mononucleosomes
-RbAp48 +RbAp48
c.
Input
GST
GST-RbAp48
Input
GST
GST-RbAp48
Core Histones
Mononucleosomes
Underacetylated
Acetylated

Figure 6. RbAp46 facilitates the p300-mediated transcription of a chromatinized template.

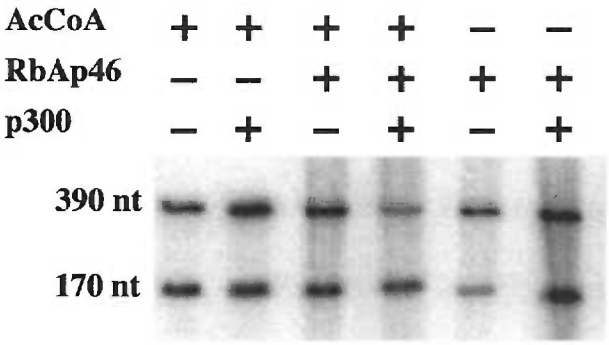
a. *In vitro* transcription assays were performed using Gal-VP16 remodeled chromatin templates. The DNA template contains five Gal4 DNA-binding sites upstream from the adenovirus MLP and a 390 nucleotide G-free transcription cassette (390 nt). The addition of AcCoA alone or p300 and AcCoA did not signifcantly enhance transcription. The addition of p300 and RbAp46 modestly activated transcription while the addition of p300, RbAp46, and AcCoA significantly increased the transcription levels of the 390 nt transcript. Similar results were obtained in four independent assays.

b. *In vitro* transcription assays using a naked DNA template. The DNA template contains five Gal4 DNA-binding sites upstream from the adenovirus MLP and a 390 nucleotide G-free transcription cassette (390 nt). As RNA recovery control, a DNA template encoding a 170 nucleotide G-free transcription cassette (170 nt) placed downstream from the adenovirus MLP was used in the productive transcription assays. When normalized with the MLP control, no significant increase in transcription was detected among the different manipulations. These results were obtained in three independent assays.

a.



b.



	CBP HAT Activity	
	Vmax (1/s)	Km (μM)
CBP + BSA	**0.38**	**16.8**
	0.29	**13.9**
	0.31	**15.0**
CBP + RbAp48	**0.25**	**5.1**
	0.26	**9.4**
	0.23	**4.0**

Table 1. RbAp48 increases CBP histone acetylation activity.

Various concentrations of chicken core histones were acetylated by baculovirus-expressed full length CBP that had been pretreated with a five-fold molar excess of RbAp48 (CBP+RbAp48) or an equivalent amount of bovine serum albumin (CBP+BSA) in the presence of excess ^{3}H-AcCoA. Data were corrected by subtracting the self-acetylation of CBP and analyzed by the Michaelis-Menton equation. The Km (μM) and the Vmax (1/s) from three independent experiments are shown.

CHAPTER 2

Acetylation of nuclear hormone receptor interacting protein RIP140 regulates binding of the transcriptional corepressor CtBP

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Abstract

CtBP (carboxyl-terminal binding protein) participates in regulating cellular development and differentiation by associating with a diverse array of transcriptional repressors. Most of these interactions occur through a consensus CtBP binding motif, PXDLS, in the repressor proteins. We previously showed that the CtBP binding motif in E1A is flanked by a Lys residue and suggested that acetylation of this residue by the p300/CBP-associated factor P/CAF disrupts the CtBP interaction. In this study, we show that the interaction between CtBP and the nuclear hormone receptor corepressor RIP140 is regulated similarly, in this case by p300/CBP itself. CtBP was shown to interact with RIP140 *in vitro* and *in vivo* through a sequence, PIDLSCK, in the amino-terminal third of the RIP140 protein. Acetylation of the Lys residue in this motif, demonstrated *in vivo* by using an acetylated RIP140-specific antibody, dramatically reduced CtBP binding. Mutation of the Lys residue to Gln resulted in a decrease in CtBP binding *in vivo* and a loss of transcriptional repression. We suggest that p300/CBP-mediated acetylation disrupts the RIP140-CtBP complex and derepresses nuclear hormone receptor-regulated genes. Disruption of repressor-CtBP interactions by acetylation may be general mode of gene activation.

Introduction

CtBP (carboxyl-terminal binding protein) was initially identified through its ability to interact with a five-residue motif, PLDLS, in the carboxyl-terminus of the adenoviral transforming protein E1A (190, 191). Mutation of this motif increases the level of E1A-directed cellular transformation (13, 190), suggesting that the CtBP interaction normally serves to dampen E1A function. In keeping with this model, mutation of the CtBP-binding motif decreases the ability of E1A to repress transcription (191). Like other transcriptional corepressors, CtBP does not interact with DNA directly but blocks gene expression when fused to a heterologous DNA binding domain (202). Precisely how CtBP blocks transcription has not been resolved, however. Interactions have been demonstrated between CtBP and the class I and class II histone deacetylases (HDACs), as well as with other proteins believed to be involved in transcriptional silencing (195, 209, 252).

Like the better-characterized E1A-binding proteins CBP/p300 and Rb (retinoblastoma protein), CtBP has been shown to have critical functions in the regulation of cellular genes involved in growth and differentiation. Perhaps the most definitive demonstrations of these functions have been elucidated in *Drosophila*. These studies have shown that CtBP is essential for "short-range" repression, a process involved in the establishment of localized stripes, bands, and tissue-specific expression in the syncytial embryo (122). Examples of CtBP-binding transcriptional repressors in *Drosophila* include snail, knirps, zhf-1, and kruppel (148, 149, 170). In general, these factors each interact with CtBP through a motif, PXDLS, that is highly related to that in E1A. In mammalian cells, transcription factors that function through CtBP include BKLF (basic kruppel-like factor), ZEB (zinc finger, E box binding factor, a homolgue of zhf-1), Ikaros, and Net (28, 97, 172, 221). The effects of CtBP on gene expression are

frequently cell- and context-dependent, however, suggesting that CtBP interactions with particular repressors may be subject to additional levels of regulation (169). We recently provided evidence that one such mode of regulation involves acetylation (255). The CtBP binding motif in E1A is flanked by a Lys residue that we have shown is acetylated *in vitro* and *in vivo* by the p300/CBP-associated factor, P/CAF. Of note, acetylation of this residue was found to decrease the CtBP interaction. Thus, in the context of E1A, acetylation disrupts CtBP binding and leads to a loss of transcriptional repression. Whether this model pertains to other CtBP complexes is unknown.

In this paper, we examine the interaction of CtBP with the nuclear hormone receptor interacting protein, RIP140. We first identified RIP140 by searching a protein database for sequences containing an extended consensus CtBP-binding sequence, PXDLSXK/R. This sequence includes the core PXDLS motif in addition to the flanking basic residues (K/R) found in E1A and certain other known CtBP-binding repressors (171, 191, 221). RIP140 was also identified as a CtBP-binding partner in a yeast two-hybrid screen.

RIP140 is perhaps the most enigmatic of the nuclear hormone receptor-interacting proteins. In the unliganded or antagonist-bound state, nuclear hormone receptors are bound by corepressor complexes containing NCoR/SMRT, Sin3, and one or more of the HDACs (reviewed in Ref. (56)). Upon ligand binding, nuclear receptors undergo a conformational change, resulting in displacement of the Sin3/ NCoR complex, followed by recruitment of ligand-dependent coactivators in the p160 family, p300/CBP, and P/CAF (12, 18, 181, 198, 233). The intrinsic acetyltransferase activities of these coactivators are believed to modify the amino-terminal tails of nucleosomal histone proteins in a manner that facilitates transcription. In the sequential step model of transcriptional activation initially proposed by Roeder and coworkers, other coactivators,

such as TRAP/ARC/DRIP, are then recruited to mediate RNA polymerase II-dependent transcription (for review, see Ref. (229)). RIP140 does not appear to conform to the standard model because it associates with receptors in a ligand-dependent manner but is a corepressor rather than a coactivator (111, 131, 208, 219, 239).

Because of its ligand-dependent association with the nuclear hormone receptors, RIP140 was initially classified as a transcriptional coactivator (24). Subsequent studies refuted this role, however, and RIP140 is now generally acknowledged to function as a corepressor (110, 219). Deletional analyses have identified three domains within RIP140 that are capable of inhibiting gene expression (111) but the mechanisms of repression remain unclear. Initially, it was proposed that RIP140 competed with the p160 family of coactivators for binding to the ligand-activated conformation of the receptor (208, 219). More recent studies have suggested that HDACs might also contribute to repression. In support of this idea, a portion of the gene repression activity of RIP140 has been shown to be sensitive to trichostatin A (TSA), an HDAC inhibitor, and direct binding of RIP140 to the class I HDACs has been demonstrated (238, 239).

The studies in this paper indicate that the amino-terminal repression function of RIP140 is mediated by the corepressor CtBP. Of interest, the CtBP binding site in RIP140 is flanked by a Lys residue which, like the motif in E1A, is a potential site of acetylation. We demonstrate that RIP140 is acetylated at this site by CBP/p300 and that this modification abrogates the CtBP interaction, resulting in a loss of repression. These studies support the hypothesis that acetylation of transcriptional repressors may be a general mode of disrupting CtBP corepressor complexes.

Materials and Methods

Plasmids

pEFRIP140 was a gift from F. Schaufele (University of California, San Franscisco). RIP140 (1-495), (623-951), and (977-1158) were cloned by PCR into pET23b (Novagen) or pGEXKG (Promega). The PIDLSCK motif in RIP140 is located between amino acids 440 and 446. The K446Q, PIDL->AIAL, and PIDL->AAAA mutations were constructed by the Quikchange™ method (Stratagene). His-tagged human CtBP1 (hCtBP1) was cloned by PCR into pET23b (Novagen). Rc/CMV-hCtBP1 was a gift from G. Chinnadurai (St. Louis University Health Sciences Center). Gal4-RIP140 (386-470) was cloned by PCR into a pcDNA3 vector lacking the SV40 origin. The VP16-hCtBP1 construct was subcloned into pcDNA3-VP16 (provided by R. Maurer, Oregon Health Sciences University). FLAG-hCtBP1-pcDNA3 was cloned by PCR into pcDNA3. LexA-hCtBP1 was cloned by PCR into pBTM116 (69). The $(Gal4)_5$-E1b-luciferase vector was from M. Green (University of Massachusetts Medical Center). The $(Gal4)_5$-TK-luciferase vector and SV40-lacZ were gifts from L.-N. Wei (University of Minnesota Medical School). The Gal4-SV40-CAT vector and Gal4-ZEB (700-776) construct were gifts from D. Dean (Washington University School of Medicine). The $(ERE)_2$-pS2-CAT reporter gene was a gift from W. L. Kraus (Cornell University).

Protein purification

GST-RIP140 (1-495), (623-951), (977-1158) wild type and mutant constructs were expressed in bacteria and purified by glutathione sepharose affinity chromatography (Sigma). His-tagged hCtBP1 was expressed in bacteria and purified by Ni-NTA affinity chromatography (Qiagen). Full length FLAG-tagged p300 was expressed in Sf9 cells and purified using the M2 FLAG affinity matrix (Sigma).

Antibodies

The acetylated K446 RIP140 antibody (αAcK446) was generated against the peptide, PIDLSCacKHGTE. The antibody was affinity-purified against the acetylated peptide and cross-absorbed against the nonacetylated peptide (Research Genetics, Huntsville, AL). A commercially available RIP140 antibody (αRIP140) (Santa Cruz Biotechnology) was used for immunoprecipitation of endogenous RIP140. A monoclonal tetra-His antibody (Qiagen) was used to detect His-tagged hCtBP1. FLAG-tagged hCtBP1 was detected by using an antibody directed against the FLAG epitope (Upstate Biotechnology).

Yeast two-hybrid assay

L40 yeast cells expressing LexA-hCtBP1 were transformed with an E9.5 mouse VP16 fusion cDNA library (69) as previously described (254). Yeast positive for histidine auxotrophy and ß-galactosidase production were identified and confirmed by a second round of transformations. LexA-lamin was used as a negative control.

In vitro acetylation assays

Recombinant His-tagged RIP140 fragments or GST-RIP140 fragments were incubated with full length, baculovirus purified p300 for 30 minutes at 30 ˚C in a 30μl reaction containing acetylation buffer (10 mM Tris-HCl, pH 8.0, 10% glycerol, 1 mM EDTA, 10 mM sodium butyrate, 1 mM DTT) and [^{3}H] acetyl coenzyme A (AcCoA; Amersham Pharmacia Biotech). Reactions were electrophoresed on a 10% polyacrylamide gel and analyzed by fluorography. For GST pull down assays, acetylation reactions were performed as described using unlabeled AcCoA (10 μM). Mock acetylation reactions were performed using 1 pmol p300 in the absence of 10 μM AcCoA or with AcCoA in the absence of p300.

GST pull down assays

Binding assays. GST or GST-RIP140 fragments were acetylated or mock acetylated prior to immobilization onto glutathione beads. Equimolar amounts of GST or GST fusion proteins were linked to glutathione beads (Amersham Pharmacia Biotech) in the presence of binding buffer (20 mM Hepes, pH 7.6, 300 mM KCl, 10% glycerol, 0.5% NP40, 1 mM DTT, 10 μM Na_3VO_4, 10 μM NaF, Complete^{TM} protease inhibitors (Roche Molecular Biochemicals)) for 1 hour at room temperature. BSA was then added to a final concentration of 0.5% for 30 minutes at 4°C. The beads were washed once with binding buffer and incubated for 1 hour at room temperature with ^{35}S-labeled *in vitro* translated hCtBP1 (Promega) or recombinant his-tagged hCtBP1. The samples were washed 3 times with binding buffer, electrophoresed on a 10% polyacrylamide gel, and processed for autoradiography or western analysis.

Peptide competition assays. Recombinant His-tagged hCtBP1 (11.6 nmol) was preincubated with 20 μM or 40 μM of nonacetylated (SNCVPIDLSCKHGT) or acetylated (SNCVPIDLSCacKHGT) peptides in binding buffer for 30 minutes on ice and subsequently incubated with the immobilized GST or GST fusion proteins as described. After washing in binding buffer, the proteins were eluted with 5X SDS-PAGE buffer and electrophoresed on 10% polyacrylamide gels. The reactions were analyzed by western blotting using a tetra-His antibody (Qiagen).

Cell culture and transfection assays

Mammalian two-hybrid assays. HepG2 cells were cultured in DMEM (BRL) supplemented with 10% FBS (Hyclone). Transfections were performed with the Fugene reagent (Roche Molecular Biochemicals) as directed by the manufacturer. The cells were grown in 24-well plates and transfected with 100 ng reporter, 50 ng Gal4-RIP140 (386-470), and 50 ng VP16-hCtBP1. The total amount of DNA per well (500 ng) was kept

constant by the addition of an empty pcDNA3 vector. Luciferase assays were performed as described (255). Each condition was assayed in triplicate a minimum of 3 times.

Repression assays. COS7 and HepG2 cells were cultured in DMEM supplemented with 10% FBS. Cells were cultured in 6 well plates and transfected using the Fugene reagent with 300 ng Gal4-SV40-CAT reporter, 20 ng SV40-lacZ, and 700 ng Gal4 fusion construct. The total concentration of DNA was maintained at 1.5 µg using empty pcDNA3 vector. Protein levels of the Gal4-RIP140 constructs were determined by immunoprecipitation followed by western analysis of cell extracts.

For studies of estrogen receptor-dependent transcription, HepG2 cells were cultured in phenol red free DMEM (BRL) supplemented with charcoal/dextran stripped FBS (Hyclone) for a minimum of two weeks before culturing in 24 well plates. Transfections of 100 ng $(ERE)_2$-pS2-CAT reporter, 20 ng SV40-lacZ, 100 ng full length pEFRIP140 wild type and mutants, and 100 ng pCMV-hCtBP1 were performed using the Fugene reagent. Cells were treated with 100μM $CoCl_2$ for 16 hours and either 10 nM 17ß-estradiol or ethanol for 4-6 hours. The total DNA concentration was kept constant by addition of empty pcDNA3 vector. CAT ELISAs (Roche Molecular Biochemicals) were performed 48 hours after transfection as directed by the manufacturer. ß-galactosidase assays were performed using the Emerald II reagent (TROPIX™, PE Biosystems). Each condition was assayed in triplicate a minimum of 3 times.

In vivo acetylation assays

COS7 and HepG2 cells were cultured in 10% FBS-supplemented DMEM and grown on 6 cm plates for transfection with 2.5 μg full length CBP. After 48 hours, the cells were washed with cold PBS and lysed by incubation on ice for 10 minutes in 500 μl lysis buffer (20 mM Hepes, pH7.6, 300 mM KCl, 10% glycerol, 0.1 mM EDTA, 0.2 mM

$ZnAc_2$, 1% NP40, 1 mM DTT, 10 μM Na_3VO_4, 10 μM NaF, and Complete™ protease inhibitors). Cell debris was cleared by centrifugation at 14,000 rpm for 10 minutes at 4 °C. Approximately 0.8 μg polyclonal RIP140 antibody (Santa Cruz Biotechnology) or normal rabbit IgG (Sigma) were bound to protein G sepharose beads (Amersham Pharmacia Biotech) and incubated with the cell lysates for 1 hour at 4 °C. The beads were washed with lysis buffer and eluted with 5X SDS PAGE loading buffer. The immunoprecipitates were subjected to western analysis using the affinity-purified acetylated K446 RIP140 antibody (αAcK446)or a commercially available RIP140 antibody.

Co-immunoprecipitation assays

COS7 cells were grown to 50% confluency on 100 mm plates and transfected with 4 μg FLAG-hCtBP1-pcDNA3. Cells were washed with cold PBS 48 hours post-transfection, collected in lysis buffer (PBS, 0.1% NP40, 1 mM DTT, 50 mM ß-glycerophosphate, 10 mM NaF, 10 μM Na_3VO_4, and Complete™ protease inhibitors), and sonicated. The cell lysates were then centrifuged, and the supernatants were added to an M2 FLAG affinity matrix (Sigma). Co-immunoprecipitations were performed for 1 hour at 4°C. Subsequently, the beads were washed 4 times in lysis buffer and eluted by boiling for 5 minutes in 5X SDS-PAGE loading buffer. After gel electrophoresis and transfer to PVDF membrane (Millipore), the immunoprecipitates were probed with an anti-RIP140 antibody (Santa Cruz Biotechnology).

Results

Identification of potential CtBP-interacting proteins

To determine whether acetylation might be a general mechanism of regulating CtBP interactions, we identified a family of CtBP binding partners using a yeast two-hybrid screen. Approximately 68% of the 41 positive clones identified from a mouse embryonic day 9.5 cDNA library contained a sequence that was closely related to the PXDLS motif (Table 1). Of these, 75% contained a flanking Lys or Arg residue. Of the DNAs containing a PXDLS motif whose identities were known, 72% corresponded to known or suspected transcriptional repressors. Among this group were CtIP (CtBP-interacting protein), BKLF, and ZEB, which have previously been shown to repress transcription by binding to CtBP. The interaction of RIP140 with CtBP had not been described before.

Characterization of the RIP140-CtBP interaction

To identify the CtBP binding site in RIP140, we generated GST-RIP140 constructs spanning the three previously characterized repression domains ((111); Fig. 1). GST pull down assays verified that only the fragment containing the PXDLS motif, GST-RIP140 (1-495), was capable of interacting with CtBP (Fig. 2A). This fragment overlapped with the portion of RIP140 detected in the yeast two-hybrid assay. Mutation of the PXDLS motif completely abolished the interaction (Fig. 2B). Neither the portion of RIP140 containing the central cluster of LXXLL motifs, GST-RIP140 (623-951), nor the C-terminal portion, GST-RIP140 (977-1158), were capable of interacting with CtBP (Fig. 2A), suggesting that these domains may repress transcription through an alternate mechanism. Indeed, we showed that GST-RIP140 (977-1158) interacted with HDAC1 from HeLa nuclear extracts (data not shown).

To determine whether the full length RIP140 and CtBP proteins interact *in vivo*, we performed co-immunoprecipitation assays in COS7 cells, which contain low but detectable levels of RIP140. Cells were transiently transfected with FLAG-tagged hCtBP1 and lysates were incubated with an M2 FLAG affinity matrix. Immunoprecipitates containing FLAG-hCtBP1 were analyzed by western blotting using an antibody directed against the amino-terminal portion of RIP140. These assays confirmed that endogenous RIP140 associates with CtBP *in vivo* (Fig. 3). Treatment of cells with estradiol did not affect the amount of CtBP that co-immunoprecipitated with RIP140 (data not shown).

RIP140 is acetylated in vitro and in vivo

Previous studies from our lab have demonstrated that acetylation of a Lys residue (Lys239) near the carboxyl-terminus of E1A regulates the interaction with CtBP (255). The modified Lys residue in E1A is adjacent to the CtBP-binding site, an arrangement that is conserved in RIP140. We thus tested whether RIP140 could similarly become acetylated. Our previous studies showed that P/CAF and GCN5 were equally capable of acetylating E1A. To our surprise, neither enzyme was able to acetylate RIP140 *in vitro* (data not shown). In contrast, *in vitro* acetylation reactions using baculovirus expressed, full length mouse p300 and [^{3}H] AcCoA demonstrated that GST-RIP140 (1-495) is readily acetylated (Fig. 4A). Mutation of the Lys residue adjacent to the CtBP binding motif markedly attenuated acetylation (Fig. 4A). Although acetylation was not completely abolished, our results suggest that Lys446 is a major RIP140 acetylation site. Neither GST-RIP140 (623-951) nor GST-RIP140 (977-1158) could be acetylated by p300 (data not shown).

Commercially available antibodies do not recognize acetylated Lys residues in all contexts, and this appears to be the case for RIP140 (data not shown). Consequently, to determine whether acetylation of RIP140 at Lys446 occurs *in vivo*, we generated an antibody against a RIP140 peptide, PIDLSCacKHGTE, containing the acetylated Lys (αAcK446). After affinity-purification, the αAcK446 antibody recognized the acetylated form of RIP140 but did not detect RIP140 subjected to a mock acetylation reaction (Fig. 4B, top). Specificity of the αAcK446 antibody was also demonstrated by showing that it did not recognize other acetylated proteins, such as p53 (data not shown). Under the conditions used, the αRIP140 and αAcK446 antibodies appear equally capable of recognizing their epitopes in the recombinant GST-RIP140 fusion proteins (Fig. 4B).

To determine whether RIP140 was acetylated *in vivo*, we transiently transfected COS7 and HepG2 cells, both of which contain endogenous RIP140, with a full length mouse CBP expression vector. The cell lysates were immunoprecipitated using an antibody that recognizes RIP140 (αRIP140) or IgG fractions from non-immunized rabbits. Western analysis of the immunoprecipitates demonstrates that in the absence of exogenous CBP, only low levels of endogenous acetylated RIP140 were detected (Fig. 4C, top panels). However, in the presence of exogenous CBP, RIP140 is readily acetylated *in vivo* in both cell types (compare Fig. 4C middle and bottom panels).

Acetylation of RIP140 abrogates its interaction with CtBP

To determine whether the Lys residue adjacent to the CtBP-binding site participates in the RIP140-CtBP interaction, we mutated this residue to Gln, which resembles acetylated Lys in that it is uncharged at neutral pH. Mutation of Lys446 to Gln abolished the interaction (Fig. 5A), confirming the importance of the Lys residue for CtBP interaction and suggesting that acetylation of this residue might also regulate CtBP

binding. To test this hypothesis, GST-RIP140 (1-495) was acetylated or mock acetylated (treated in the absence of AcCoA) with p300 and incubated with *in vitro* translated hCtBP1. Acetylation abolished interaction of RIP140 with CtBP, while mock acetylation did not (Fig. 5A). These studies indicated that the ability of RIP140 to interact with CtBP was regulated by acetylation.

To confirm these results, we performed a series of peptide competition assays. Because only the non-acetylated form of RIP140 is able to interact with CtBP, the non-acetylated peptide, but not the acetylated form, should be able to compete with GST-RIP140 (1-495) for CtBP binding. Peptide competition assays were performed using an acetylated RIP140 peptide (SNCVPIDLSCacKHGT) or a non-acetylated peptide (SNCVPIDLSCKHGT). Preincubation of the non-acetylated peptide completely blocked GST-RIP140 (1-495) binding to hCtBP1 while incubation with the acetylated peptide did not, even at the higher concentrations (Fig. 5B). These studies confirm the potent effects of RIP140 acetylation of CtBP binding.

It is not possible to manipulate the level of RIP140 acetylation very precisely *in vivo*, but evidence for the effect of Lys446 acetylation on CtBP binding can be obtained by examining the Lys446Gln mutant. Mammalian two-hybrid assays were performed using Gal4-RIP140 (386-470) and VP16-hCtBP1. Wild type Gal4-RIP140 is able to interact with VP16-hCtBP1 in HepG2 cells, a cell line that normally expresses RIP140, and activate the E1b or thymidine kinase (TK) promoters (Fig. 5C). Mutation of the PILD sequence in CtBP to AIAL completely blocked the interaction, while mutation of Lys446 to Gln reduced the interaction by 70-80%.

RIP140 represses transcription through its association with CtBP

Our studies suggest that one of the mechanisms by which RIP140 represses transcription is through its interaction with the corepressor, CtBP. Disrupting this interaction by acetylation or mutation of Lys446 should result in a loss of repression. To test this hypothesis, we analyzed the ability of wild type Gal4-RIP140 (386-470), which contains sequences spanning the CtBP interaction domain, as well as the Lys446Gln and AIAL mutants, to repress transcription of a Gal4-SV40-CAT reporter gene. As a control for these experiments, we analyzed Gal4-ZEB (700-776), a known CtBP-dependent transcriptional repressor (172). In both COS7 and HepG2 cells, wild type Gal4-RIP140 (386-470) represses transcription to a similar level as Gal4-ZEB (700-776) (Fig. 6). Alteration of the consensus CtBP interaction motif, either by mutation of Lys446 to Gln or alteration of the PIDL sequence to AIAL, restores transcription levels to those seen in the absence of repressor (Fig. 6). We conclude from these studies that modification of Lys446 contributes to the regulation of RIP140 repressor function.

RIP140 represses nuclear hormone receptor dependent transcription

We next examined the ability of full length RIP140 to repress transcription of an estrogen receptor-dependent reporter gene, $(ERE)_2$-pS2-CAT. This gene contains a duplicated estrogen response element (ERE) upstream from the pS2 promoter and has been used extensively to examine estrogen-responsive transcription (101). In the absence of exogenous RIP140, CtBP did not significantly repress transcription (Fig. 7). However, in the presence of exogenous wild type RIP140 or a form in which Lys446 had been mutated to Arg, transcription was dramatically repressed. Mutation of Lys446 to Gln or alteration of the PIDL motif to AIAL severely attenuated RIP140-mediated transcription repression. These results support the conclusion that RIP140 represses transcription in large part through its association with CtBP.

Discussion

RIP140 associates in a ligand-dependent manner with a variety of nuclear hormone receptors, including the estrogen, glucocorticoid, retinoic acid, retinoid X, thyroid, and liver X receptors (24, 111, 131, 208, 219). Unlike other proteins that interact with ligand-bound nuclear hormone receptors, RIP140 is a transcriptional corepressor. In this study, we have analyzed the regulation of RIP140 corepressor function by acetylation. Previous work from our lab has demonstrated that the E1A-CtBP interaction is similarly modulated by acetylation (255). In the case of E1A, the acetylation activity of P/CAF was much more efficient than that of p300/CBP. With RIP140, p300/CBP was able to mediate acetylation while P/CAF was completely ineffective. The common theme of both studies was that Lys acetylation regulated the binding of CtBP and, hence, repressor function. Our yeast two-hybrid assays suggest that this mechanism may be a fairly common mode of gene derepression, in that many of the putative CtBP binding sequences were flanked by a Lys residue (see Table 1, Group I). Whether the repressors in Group III can be acetylated and whether this modification blocks CtBP binding has not been addressed. In several instances, however, this residue is an Arg (shown in Group II), which is not a known target of the coactivator acetyltransferases. Indeed, we have shown in the context of E1A that a Lys to Arg substitution actually increases CtBP binding *in vivo* (255), although it is possible that this reflects a basal level of Lys acetylation rather than a true difference in the affinity of Lys- or Arg-containing proteins. Nonetheless, it appears that individual transcriptional repressors may be differentially responsive to coactivator acetyltransferases depending upon the identity of this single amino acid.

Why acetylation of the flanking Lys residue is so critical for CtBP interaction is unknown. Presumably, this issue will become clarified once the structure of one of the CtBP-binding repressors has been solved. Although such information is not yet available, clues to the nature of the CtBP interaction site may possibly be gleaned from other known structures. For example, the prohormone processing carboxypeptidase Kex1 contains a PXDLTXK sequence that might also be expected to bind CtBP. Indeed, we have shown that CtBP interacts with Kex1 in GST pull down assays (N. Vo, unpublished observations.) Structural analysis of Kex1 indicates that the motif is located on the surface of the protein and forms a pocket that is filled by the positively charged Lys residue (201). Thus, neutralization of this positively charged Lys residue by acetylation would be predicted to perturb the CtBP-binding interface. In keeping with this model, we have shown that mutation of the Lys to Gln prevents CtBP binding. It is possible, therefore, that the flanking Lys is ideally positioned to regulate CtBP binding. In contrast, the results of the two-hybrid assay suggest that the Lys is not essential for binding (see Group II, where the flanking Lys is not present). We suggest, therefore, that although the Lys is not essential, its acetylation prevents the CtBP interaction.

Our model is somewhat reminiscent of that described for the coactivator, ACTR, which, when acetylated by p300, disrupts the coactivator complex and terminates transcription (28). Unlike the situation with ACTR, however, acetylation of RIP140 leads to gene activation rather than repression. Coactivator histone acetyltransferases recruited by particular transcription factors are believed to modify specific nucleosomes in the vicinity of activated genes (reviewed in Ref. (207)). Recent studies demonstrate that histone modifications may occur globally as well (231). The idea that the basal state of nucleosomes contains both acetylated and deacetylated histones suggests that chromatin can be "primed" for transcription. Thus, the ability of a transcription factor such as RIP140 to function as an activator or repressor may depend on the global acetylation state

of a given gene. Alternatively, RIP140 could be acetylated locally through recruitment of p300/CBP. Whether nuclear hormone receptors can associate simultaneously with p300/CBP and RIP140 is unknown, however.

In conclusion, the acetylation state of CtBP binding proteins such as E1A and RIP140 directly modulates their ability to mediate transcriptional repression. In the unacetylated state, these proteins can act as transcriptional repressors through their interaction with CtBP but, in the acetylated state, the interaction is prevented. Although the mechanism by which CtBP represses transcription remains unknown, this corepressor has clearly been shown to be critical for the regulation of a large number of developmental and differentiation processes. Potentially, these activities can also be modulated by the acetylation state of the CtBP-interacting transcription factors. It is widely accepted that the level of histone deacetylation is correlated with transcriptional repression while histone acetylation is correlated with transcriptional activation. Our studies suggest that disruption of repressor-corepressor complexes might be an equally plausible mechanism to explain how coactivator acetyltransferases activate selected genes.

Acknowledgements

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Figure 1. RIP140 contains a consensus CtBP binding site. The nine LXXLL motifs present in RIP140 are represented by the vertical bars. The fragment of RIP140 (amino acids 403-521) isolated from an E9.5 mouse embryo cDNA library is indicated by the gray bar. Fragments of RIP140 used in GST pull down assays are also depicted. Mutations in the consensus CtBP binding motif (amino acids 440-446) are indicated.

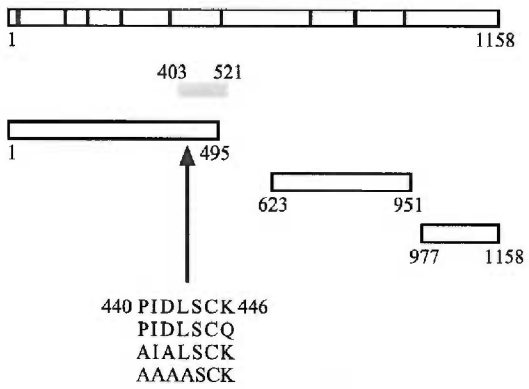

1
1158
403
521
1
495
623
951
977
1158
440 PIDLSCK 446
PIDLSCQ
AIALSCK
AAAASCK

Figure 2. RIP140 binds to hCtBP1 *in vitro*. (A) Equimolar amounts of GST-RIP140 (1-495), GST-RIP140 (623-951), GST-RIP140 (977-1158), and GST alone were incubated with *in vitro* translated hCtBP1. The samples were electrophoresed on a 10% polyacrylamide gel and analyzed by autoradiography. (B) Mutation of the consensus hCtBP1 binding motif abolishes the RIP140-hCtBP1 interaction. Equimolar concentrations of GST-RIP140 (1-495) wild type (WT), PIDL->AIAL, PIDL->AAAA, and GST alone were incubated with recombinant, His-tagged hCtBP1. The samples were electrophoresed on a 10% polyacrylamide gel and analyzed by western blotting using a monoclonal His-tag antibody.

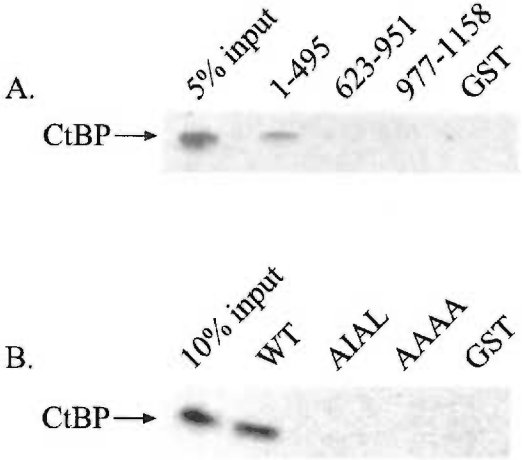

A.
5% input
1-495
623-951
977-1158
GST
CtBP
B.
10% input
WT
AIAL
AAAA
GST
CtBP

Figure 3. RIP140 interacts with hCtBP1 *in vivo*. COS7 cells were transiently transfected (+) or mock transfected (-) with FLAG-hCtBP1. Immunoprecipitations were performed using an M2 FLAG affinity matrix. Western analyses were performed using a polyclonal RIP140 (left panel) or FLAG (right panel) antibody, as indicated at the top of the figure.

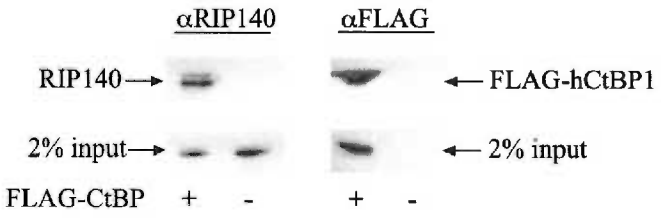
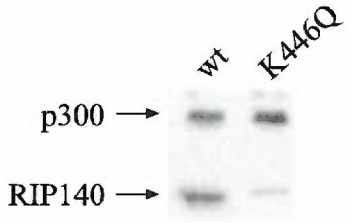
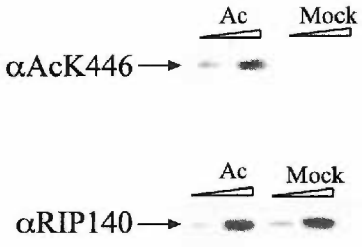

αRIP140
αFLAG
RIP140
FLAG-hCtBP1
2% input
2% input
FLAG-CtBP + - + -

Figure 4. RIP140 is acetylated at lysine 446. (A) 11 nmol of recombinant, GST-RIP140 (1-495) wild type or K446Q mutant were incubated with full length, baculovirus purified p300 (1 pmol) in the presence of [^{3}H] acetyl CoA. Reactions were analyzed by SDS-PAGE and visualized by fluorography. (B) Recombinant GST-RIP140 (1-495), 25 ng and 300 ng, was acetylated or mock acetylated with p300. Samples were electrophoresed on a 10% polyacrylamide gel and subjected to western analysis using an antibody directed against acetylated (top) or unacetylated (bottom) RIP140. (C) In the top panels, endogenous RIP140 was immunoprecipitated from COS7 and HepG2 cells with a RIP140 antibody and subjected to western analysis using the acetylated K446 RIP140 antibody. Middle panels show cells transfected with full length CBP before immunoprecipitation. Total RIP140 protein immunoprecipitated from these cells are shown in the bottom panels.

A.


wt
K446Q
p300
RIP140

B.


Ac
Mock
αAcK446
Ac
Mock
αRIP140

C.

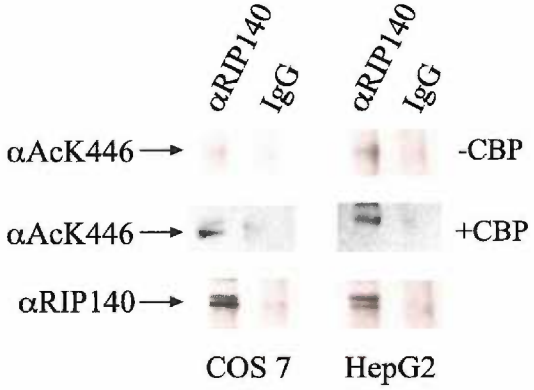
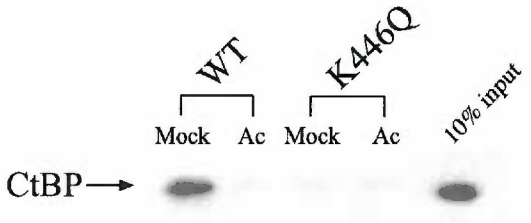
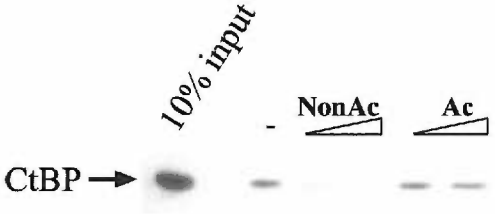

αRIP140
IgG
αRIP140
IgG
αAcK446
-CBP
αAcK446
+CBP
αRIP140
COS 7
HepG2

Figure 5. Acetylation of RIP140 disrupts its interaction with hCtBP1. (A) Mutation or acetylation of K446 disrupts the interaction of RIP140 with hCtBP1. GST pull-down assays were performed using acetylated or mock acetylated GST-RIP140 (1-495) WT or K446Q. Samples were incubated with *in vitro* translated hCtBP1, electrophoresed on a polyacrylamide gel, and analyzed by autoradiography. (B) Peptide competition assays. GST-RIP140 (1-495) was incubated with recombinant, His-tagged hCtBP1 (11.6 nmol) in the presence or absence of 20 μM or 40 μM nonacetylated (NonAc) or acetylated (Ac) peptides. Bound hCtBP1 was analyzed by western blotting using a His-tag antibody. (–) designates binding in the absence of peptide competitor. (C) K446Q mutation attenuates the RIP140-hCtBP1 interaction in a mammalian two-hybrid assay. Gal4-RIP140 (386-470) and VP16-hCtBP1 were cotransfected with either $(Gal4)_5$-E1b-luc or $(Gal4)_5$-TK-luc reporter constructs into HepG2 cells. Luciferase activity was measured 48 hours post-transfection.

A.


WT
K446Q
Mock
Ac
Mock
Ac
10% input
CtBP

B.


10% input
-
NonAc
Ac
CtBP

C.

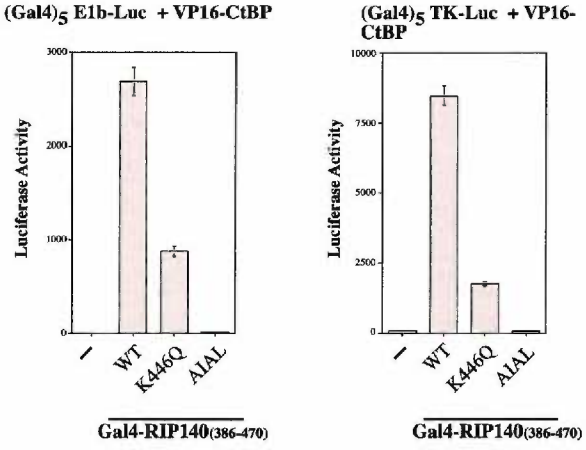

(Gal4)5 E1b-Luc + VP16-CtBP
(Gal4)5 TK-Luc + VP16-CtBP
Luciferase Activity
3000
2000
1000
0
10000
7500
5000
2500
0
–
WT
K446Q
A1AL
Gal4-RIP140(386-470)

Figure 6. RIP140 represses transcription by binding to hCtBP1. Gal4-RIP140 fusion protein containing the CtBP interaction motif was analyzed for its ability to represss a Gal4-SV40-CAT reporter. Wild type, K446Q, or the AIAL mutants were compared to Gal4-ZEB (700-776). COS7 and HepG2 cells were transiently transfected with 300 ng reporter, 700 ng Gal4 fusion constructs, and 20 ng SV40-lacZ. CAT protein produced in the absence of repressor (-) is indicated. The total amount of DNA was kept constant using empty pcDNA3 vector. Protein levels of the RIP140 constructs are shown in the insets. CAT protein production was measured 48 hours after transfection using an ELISA system (Roche Molecular Biochemicals) and values are normalized to ß-galactosidase activity.

Gal4-SV40-CAT

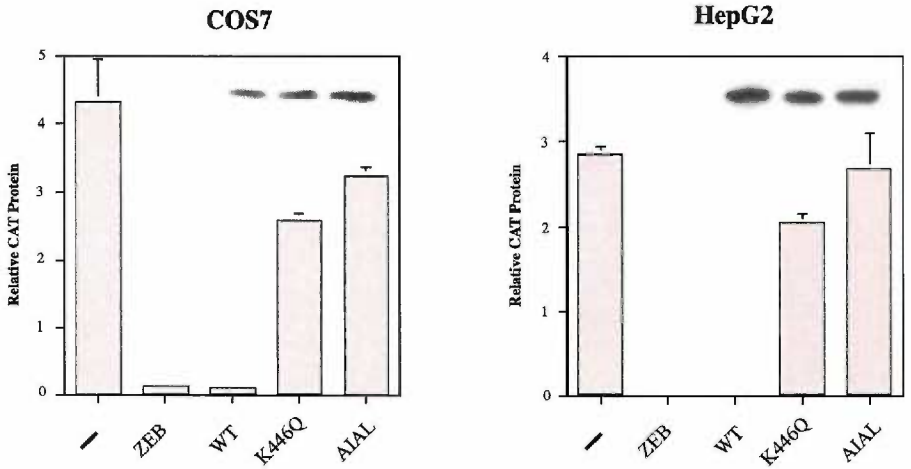


Figure 7. RIP140 represses estrogen receptor-dependent transcription. The ability of full length RIP140, wild type and mutants, to repress transcription of an $(ERE)_2$-pS2-CAT reporter gene was analyzed in HepG2 cells treated with 10 nM estradiol. Cells were transfected with 100 ng reporter, 20 ng SV40-lacZ, 100 ng pEFRIP140 wild type and mutants, in the absence or presence of pCMV-hCtBP1. The total concentration of DNA was kept constant by addition of empty pcDNA3 vector. CAT protein production was measured 48 hours after transfection and values are normalized to ß-galactosidase activity.

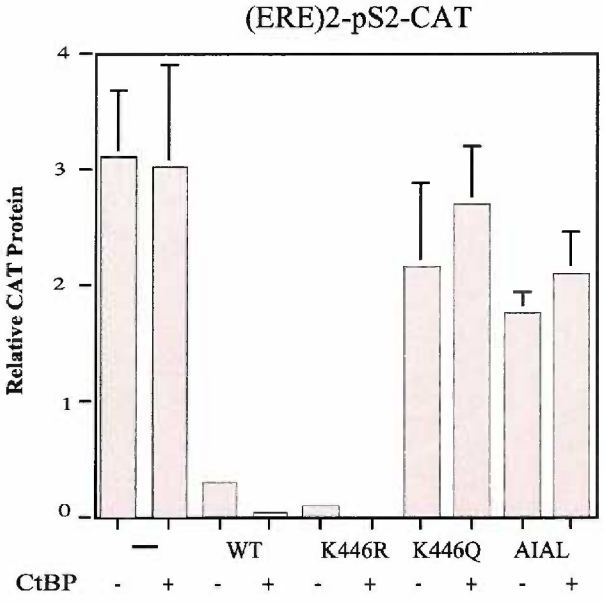

(ERE)2-pS2-CAT
Relative CAT Protein
4
3
2
1
0
—
WT
K446R
K446Q
AIAL
CtBP
- + - + - + - + - +

Table 1. CtBP-interacting proteins identified in a yeast two-hybrid assay are grouped into four categories, as indicated in bold letters. Proteins shown previously to interact with CtBP are indicated by asterisks. Two potential CtBP binding motifs in MyT1 and RIZ are listed. Thirteen clones did not contain an identifiable PXDLS motif.

Table 1. Yeast Two-Hybrid CtBP Interaction Proteins

GROUP	MOTIF								NAME
I	**P**	**X**	**D**	**L**	**S**	**X**	**K**		
	P	I	D	L	S	C	K		RIP140
	P	L	D	L	S	S	K		BCoR
	P	T	D	L	S	M	K		unknown
	P	L	D	L	S	A	K		unknown
	P	E	N	L	S	T	K		MyT-1
	P	Q	D	L	P	S	K		MyT-1
	V	L	D	L	S	T	K		unknown
	V	L	D	L	S	V	K		RP58
II	**P**	**X**	**D**	**L**	**S**	**X**	**R**		
	P	L	D	L	S	D	R		CtIP*
	P	L	D	L	S	M	R		unknown
	P	L	D	L	S	L	R		Zinc finger protein 219
	P	L	D	L	T	V	R		unknown
	P	L	N	L	S	S	R		SRp55-3
III	**P**	**X**	**D**	**L**	**S**	**X**	**X**	**K**	
	P	L	D	L	S	L	P	K	ZEB*
	P	V	D	L	T	V	N	K	BKLF*
	P	I	D	L	T	K	S	K	Teashirt2
	P	L	D	L	S	P	V	K	unknown
	P	M	D	L	S	T	V	K	RING 3, and unknown
	P	L	D	I	L	Q	S	K	Retinoic acid induced protein
	V	V	D	L	T	L	P	K	Ku70
	V	L	D	L	S	V	H	K	RIZ
IV	**P**	**X**	**D**	**L**	**S**	**X**	**X**	**X**	
	P	L	D	L	S	S	G	V	RIZ
	V	V	D	L	S	K	A	S	unknown
	P	L	D	L	S	C	G	S	unknown
	P	L	N	L	S	L	G	P	Zinc finger protein 217
	P	T	D	L	S	V	N	P	unknown
	V	I	D	L	T	I	E	S	Miz1
	P	L	N	L	R	I	P	S	NAB1

CONCLUSIONS AND FUTURE DIRECTIONS

The regulation imparted by factor acetylation mirrors the regulation afforded by histone acetylation. In both cases, transcription repression is relieved by the acetylation activities of coactivator proteins. Acetylation of lysines neutralizes the positive charge of this residue. At the N-terminal tail of histones, the addition of an acetyl group onto conserved lysines weakens the interaction between histones and DNA, resulting in the remodeling of nucleosomes necessary for activation of transcription. Acetylation of these residues may also block or promote key protein-protein interactions involved in transcriptional regulation. A major acetylase responsible for mediating histone acetylation is the coactivator, CBP/p300. However, the enzymatic activity of this coactivator is not limited to histones as CBP/p300 also acetylates non-histone proteins, conferring either activation or repression of transcription. In this thesis, I have investigated the role of CBP in modifying histones and the non-histone transcriptional corepressor RIP140. I propose that the underlying mechanism in both cases involves an acetylation-mediated block of transcriptional repression through disruption of macromolecular complexes.

While a great deal of work has addressed the potential consequences of histone modification by coactivator HATs, little is known about factor acetylation. The histone code predicts that posttranslational modifications of histones create specific protein-protein interaction motifs that recruit additional transcription factors. This proposal is supported by crystal structure analysis of both acetyl-lysine: bromodomain interactions as well as methyl-lysine: chromodomain interactions that illustrate how these posttranslational modifications create stable interactions between the modified residues

with specific, conserved protein interaction domains. Factor acetylation has also been proposed to disrupt or enhance protein-nucleic acid interactions, disrupt or recruit protein-protein interactions, as well as alter nucleocytoplasmic localization and protein stability. There are few examples, however, of how this occurs. In this thesis, I have also investigated the regulation imparted by transcription factor acetylation, particularly, acetylation of the corepressor RIP140, to determine how the acetylation state of one particular factor regulates transcription.

1. How does CBP/p300 selectively target its acetyltransferase activity towards histones?

To answer this question, I investigated the ability of CBP/p300 interaction partners to regulate its HAT activity. An ideal system to identify protein-protein interactions is the yeast two-hybrid assay. However, yeast two-hybrid screens frequently identify a high number of false positives that can be difficult to sift through. The solution structure of the CBP interaction domain of CREB (residues 121-143) and the CREB binding domain of CBP (residues 586-666) revealed that these two domains are relatively unstructured in isolation yet form defined α-helical structures upon interaction (178). This data suggested that perhaps one reason why traditional yeast two-hybrid assays that have used portions of CBP yielded so many false positives was due to the unstructured conformation of the CBP fragment that could support non-specific protein-protein interactions. To circumvent this problem, the Goodman lab created a yeast bait vector that could simultaneously express both the activation domain of CREB and the CREB binding domain of CBP. Previous studies from the lab demonstrated that an endogenous kinase in yeast phosphorylates CREB at Ser133 to mediate CBP interaction. This yeast

three-hybrid vector would be ideal in identifying proteins that specifically interacted with the pCREB/CBP complex. I used this vector to screen an embryonic mouse VP16 fusion cDNA library. This screen identified an interaction between pCREB/CBP with the histone chaperone protein, RbAp48. Subsequent analysis using GST pull down assays and coimmunoprecipitation assays confirmed this interaction and demonstrated that the homolog, RbAp46, also specifically interacted with pCREB/CBP. Through its association with histones, RbAp46/48 specifically recruits both unacetylated free core histones and mononucleosomes to CBP/p300. Formation of the CBP/p300-RbAp46/48-nucleosome complex facilitated histone acetylation by lowering the K_m of CBP/p300 for histones, increasing the specificity of the acetyltransferase activity of CBP/p300 for its substrate. Upon acetylation, histones are released from the CBP/p300-RbAp46/48 complex, perhaps to facilitate recruitment of additional unacetylated histones for further acetylation and chromatin remodeling. The transient and dynamic association between CBP/p300 and histones was also shown to stimulate transcription of an in vitro assembled chromatin template in a p300, RbAp46, and acetyl coA dependent manner. These studies showed that the CBP/p300-RbAp46/48 complex increases the specificity of chromatin remodeling events required for transcription to proceed. In a sequential step model of transcription originally proposed by Roeder (121), recruitment of specific transcription factor complexes such as the pCREB-CBP/p300-RbAp46/48 complex may follow the enhanceosome and mediator complexes to regulate the activation of transcription.

To determine whether this complex is formed on actively transcribed genes in vivo, I would like to perform ChIP assays to examine the acetylation state of histones

upon recruitment of both the coactivator HAT, CBP/p300, and the histone chaperone or targeting protein, RbAp46/48. An ideal system to study CBP/p300 recruitment to endogenous promoter elements is the estrogen receptor-dependent mechanism of transcription regulation. Future experiments include ChIP analysis of the transcription factor complexes bound at the pS2 gene enhancer-promoter elements in MCF7 cells in the presence or absence of estradiol. This gene is turned on in the presence of estradiol and turned off in the presence of anti-estrogens so that estradiol should induce recruitment of CBP/p300 to DNA-bound estrogen receptors and stimulate histone acetylation. As I demonstrated using in vitro transcription reactions of in vitro assembled chromatin templates, CBP/p300-mediated histone acetylation should be stimulated by recruitment of RbAp46/48. Chromatin immunoprecipitation of RbAp46/48 should detect this histone chaperone at the pS2 promoter.

2. What is the role of factor acetylation in transcriptional regulation?

In general, regulation of nuclear receptor-dependent genes is a ligand-dependent process. The primary paradigm described for the regulation of this class of genes is based on a model of agonist-mediated activation and antagonist-mediated repression. Although many of the coregulators that have been identified adhere to this paradigm, RIP140, in contrast, regulates transcription in an unusual manner. RIP140 is recruited to nuclear receptors in the presence of agonist, but represses rather than activates transcription. RIP140 thus provides a paradox for the classical model of nuclear hormone receptor regulated genes, and the molecular mechanism for its mode of transcription regulation has not been well described. I have investigated potential mechanisms of

RIP140-mediated repression to elucidate its role in the expression of nuclear hormone dependent genes.

Examination of the primary sequence of RIP140 revealed that it contains a conserved CtBP interaction motif which was later demonstrated in a LexA-CtBP yeast two-hybrid screen. Subsequent in vitro and in vivo assays demonstrated that RIP140 represses transcription through its interaction with CtBP. Moreover, in agreement with previous studies from the Goodman lab (255), this interaction is regulated by the acetylation state of the Lys residue adjacent to the core CtBP consensus interaction motif. Acetylation of this lysine, Lys446, by CBP/p300 abolished the interaction between RIP140 and CtBP and abrogated repression of estrogen receptor-dependent genes.

These studies show that acetylation of RIP140 blocks its ability to function as a transcriptional repressor. Because ligand-activated nuclear hormone receptors interact with CBP/p300, a coactivator that has the ability to acetylate and block the repressive actions of RIP140, I propose that RIP140 may not, in fact, function as a repressor in vivo. Just as histone acetylation disrupts the repressive structure of nucleosomes, acetylation of RIP140 disrupts the repressive association of RIP140 and CtBP. In both cases, transcription repression is abrogated.

In future experiments, I would like to examine the levels of acetylated RIP140 at endogenous gene promoters by performing ChIP assays from both MCF7 cells to examine E_2-regulated genes and TαT1 cells to examine T_3-regulated genes. I will use my acetylated K446 specific antibody to determine whether genes that are positively regulated by agonist, such as the pS2 gene, recruit acetylated RIP140 and not CtBP to DNA bound nuclear receptors. I will also use the ChIP assay to determine whether

RIP140 is recruited to enhancer-promoter elements of the TSHß gene, which is known to be repressed by agonist. In the presence of T_3 and the absence of CBP/p300, RIP140 should be associated with CtBP. It is possible that RIP140, through its ability to interact with CtBP in an acetylation-dependent manner, provides part of the answer to why some nuclear hormone-regulated genes are activated and others are inhibited by the addition of ligand. For those genes that are associated with CBP, RIP140 is acetylated, does not bind CtBP, and cannot act as a repressor. However, in the absence of CBP, RIP140 is able to bind to CtBP and repression transcription. Activation or repression, in this model, is regulated by recruitment of coactivator acetylases to transcription factor complexes in addition to histones. If, as many believe, large chromatin domains can be induced to be in an acetylated or nonacetylated state, it is possible that these "global" effects could also influence the repressor functions of proteins like RIP140.

As shown in my studies and work from many other laboratories, coactivator HATs do not exclusively target histones. Another implication of these studies is not so obvious. The ability of CBP/p300 to disrupt complexes of repressors and their effects supports the idea that a complex at one promoter site could influence the activity of adjacent regulatory elements. In other words, the regulatory complexity generated by specific combinations of transcription factor interactions at select DNA elements is perhaps enhanced by the regulation that one complex can exert over another. Many genes have multiple enhancer elements that cooperatively or synergistically nucleate the formation of distinct regulatory complexes. One corollary of my results is that individual signals that stimulate the formation of these complexes at specific regulatory DNA elements could regulate the formation or composition of transcription factor complexes

bound at adjacent elements. Genes such as TRH and TSH, for example, are regulated by cAMP as well as by thyroid hormone (64, 188, 211). Recruitment of CBP/p300 to a cAMP response element (CRE) may facilitate disruption of the RIP140-CtBP complex bound to nearby nuclear hormone receptor response elements (Figure 6). This type of regulation exerted by multiple enhancer-promoter elements has been described in the context of the locus control region. The LCR contains multiple regulatory DNA elements that can recruit specific transcription factors capable of exerting long-range regulation of linked genes. ChIP assays could be used to determine whether CBP/p300 may be recruited to cAMP response elements within the TRH or TSH enhancer and whether this recruitment influences the acetylation state of RIP140. These assays will allow me to ask whether RIP140 acetylation requires CBP/p300 to be recruited to the nuclear hormone receptor complex or whether recruitment to an adjacent CRE is sufficient.

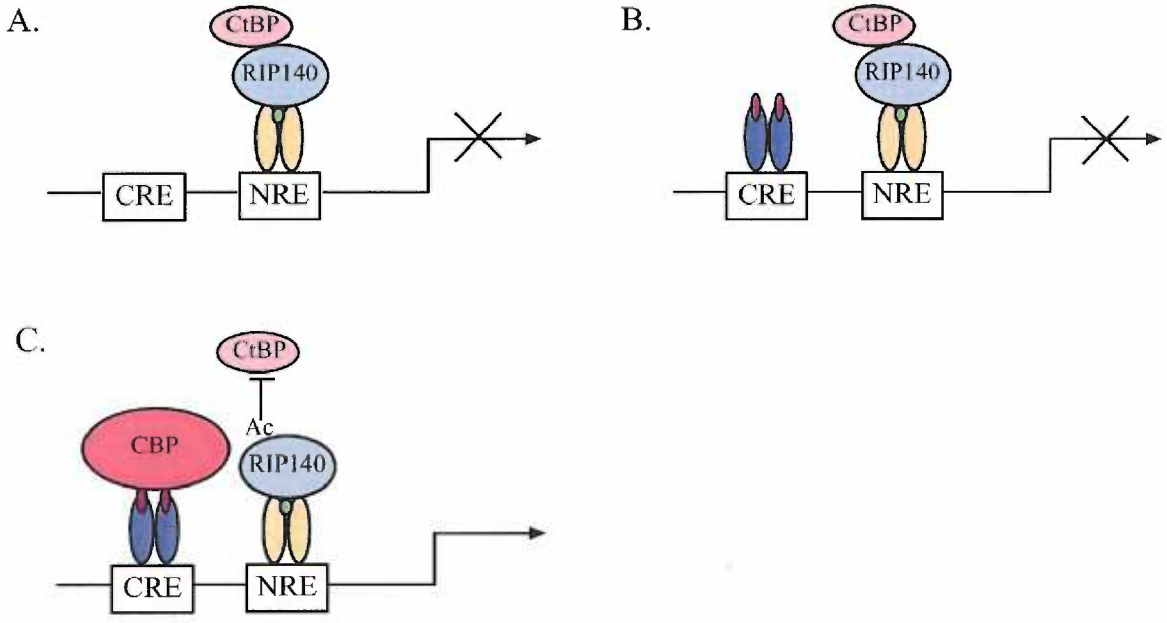


Figure 6. Multiple signaling events exert control over transcription factor complex assembly. (A) Ligand-bound nuclear receptors recruit RIP140 and CtBP to the NRE. (B) Stimulation of the cAMP pathway facilitates recruitment of the coactivator acetyltransferase, CBP, to an adjacent enhancer element. (C) Acetylation of RIP140 by CBP disrupts its interaction with CtBP, abrogating transcription repression. cAMP response element (CRE), nuclear response element (NRE)

Given the recent finding that CtBP-mediated transcriptional regulation is significantly enhanced in the presence of the metabolic cofactors, NADH/NAD (253), with NADH being the more potent enhancer of CtBP-mediated repression, I would also like to assay the contributions of the RIP140-CtBP complex to transcription repression under conditions that increase cellular NADH/NAD ratios. In the liver, for example, metabolism of ethanol significantly increases the levels of NADH. Under these conditions, the RIP140-CtBP interaction should be enhanced by the high levels of NADH, stabilizing the transcription repression machinery.

Perhaps one reason that RIP140 knockout mice exhibit relatively subtle phenotypes (112, 243) is that these mice are maintained under optimal conditions. If placed in conditions that states that alter cellular NADH/NAD ratios, these mice may exhibit more dramatic defects in cellular growth and differentiation.

Under certain hypoxic and metabolic conditions (such as diabetes), ratios of NADH/NAD are also elevated (155). The anabolic actions of steroid hormones, such as estrogens, in these cases may be deleterious to the cell if it is in a catabolic state. One possibility is that the NADH enhancement of CtBP-mediated repression may prevent the

potentially deleterious anabolic effects of estrogens and other nuclear hormones. For example, NADH or NAD may strengthen the RIP140-CtBP interaction such that recruitment of acetylases may not be sufficient to activate transcription. To address these questions, I would again perform ChIP assays on cells that are grown under normal and hypoxic conditions to determine whether metabolic cofactors regulate the acetylation-dependent regulation of RIP140-mediated transcription. If NADH/NAD negates the effects of RIP140 acetylation on CtBP binding, I would like to determine whether NAD-dependent deacetylases, such as Sir2, act to deacetylate Lys446 of RIP140 to maintain transcription repression.

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APPENDIX I

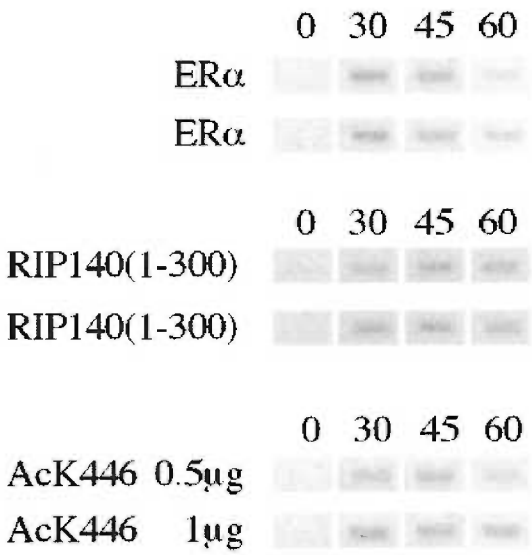

Figure 7. Acetylated RIP140 is recruited ER at the pS2 promoter in the presence of estradiol. The MCF7 breast cancer cell line, which expresses endogenous ER, RIP140, CBP, and pS2, was treated with ethanol (time "0") or estradiol for the indicated time points. Immunoprecipitation of the pS2 promoter using the acetylated Lys446 antibody demonstrates that acetylated RIP140 (AcK446) is recruited to genes that are positively regulated by ligand. ChIP assays using commercially available ER (ERα) and RIP140 (RIP140(1-300)) antibodies were conducted in duplicate. In addition, two different concentrations of the acetylated RIP140 antibody were used to determine whether acetylated RIP140 is recruited to actively transcribed genes.

APPENDIX II

CBP and p300 in Transcriptional Regulation

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CBP (CREB-binding protein) and p300 are believed to participate in the activities of hundreds of different transcription factors (see Fig. 1). Current models suggest that the binding of these coactivators to transcription factor activation domains positions histone acetyltransferases (HATs) near specific nucleosomes in target gene promoter regions (for review, see (40). Interactions with components of the general transcriptional machinery, such as TFIID, TFIIB, and the RNA polymerase II holoenzyme (RNAPII) have also been suggested to contribute to CBP/p300 function. The simultaneous interaction of multiple transcription factors with CBP/p300 has been proposed to contribute to transcriptional synergy. Conversely, competition for CBP/p300 binding has been suggested to mediate some examples of signal-induced transcriptional repression. An overview of CBP/p300 in cellular growth and differentiation has recently been published (10), but many questions regarding their role in transcriptional regulation remain unanswered. This review deals with some of the more controversial aspects of CBP/p300 function. In particular, we will ask whether CBP and p300 have distinct functions, review the evidence for their regulation by phosphorylation, and ask whether they function primarily by acetylating histones or other proteins. We will also revisit the evidence for the role of CBP/p300 as transcriptional "integrators." Finally, we will attempt to localize CBP/p300 function within the complex series of processes involved in transcriptional activation.

Are CBP and p300 redundant?

Although CBP and p300 are highly related and share many functional properties, there is evidence that these factors are not really interchangeable. Subtle differences in the expression of CBP and p300 during development (34) may explain why knockouts of the two coactivators in mice result in somewhat distinct phenotypes. For example,

heterozygosity for CBP causes certain hematological defects and a predisposition to cancer that is not seen in mice lacking one allele of p300 (25). Studies of specific transcription factor pathways provide additional evidence of differences between the functions of CBP and p300. For example, fibroblasts derived from homozygous p300 knockouts are defective for retinoic acid receptor (RAR) but not CREB signaling (49). Similarly, ribozyme-mediated ablation of p300, but not CBP, blocks the RAR response (18). Other differential functions of CBP and p300 have been revealed by their distinct interactions with viral transforming proteins. For example, the Kaposi sarcoma-associated herpes virus protein vIRF has been reported to be stimulated by CBP and repressed by p300 (17). Distinct roles for CBP and p300 have also been suggested in the differentiation of muscle and F9 teratocarcinoma cells (37), (43). On the other hand, homozygous mutations in CBP and p300 both result in lethality and a similar constellation of phenotypic defects (25), (49). Moreover, CBP/p300 double heterozygotes are invariably lethal, suggesting that functions of CBP and p300 must overlap, at least to some degree. This complexity is not shared by simpler metazoans, such as *Drosophila* and *C. elegans*, which express only a single isoform of CBP/p300 (reviewed in (10)).

How does phosphorylation regulate CBP/p300 function?

Although cell cycle dependent phosphorylation of p300 was reported almost a decade ago (48), it is still not entirely clear how phosphorylation regulates CBP/p300 function. In large part, this lack of understanding is due to the fact that the specific phosphorylation sites in CBP/p300 have never been precisely identified. Phosphorylation of p300 and CBP by cyclin E/Cdk2 was reported by Perkins et al (35) and Ait-Si-Ali, et al (1), respectively. In the case of p300, cyclin E/Cdk2 was shown to negatively regulate coactivator function in a manner that can be blocked by the cyclin-dependent kinase

inhibitor, p21. In this model, p21 was proposed to participate in a positive feedback loop, whereby activators such as p53, which depend upon p300 for function, induce p21 which then alleviates the block in p300 action mediated by cyclin E/Cdk2. In contrast, cyclin E/Cdk2 was reported to increase the intrinsic HAT activity of CBP, potentially activating expression of S-phase genes that are repressed in early G1 (1). Because the phosphorylation sites in p300 and CBP have not been mapped, however, it may be premature to conclude that the two coactivators are differentially regulated by cyclin-dependent kinases.

CBP and p300 both contain a consensus protein kinase A (PKA) site adjacent to their third zinc finger domains and several groups have proposed that phosphorylation by PKA may contribute to CBP/p300 regulation. For example, Xu, et al have argued that phosphorylation of CBP is responsible for the PKA-mediated augmentation of the transcription factor Pit-1 (47). This is an intriguing model because Pit-1 itself cannot be phosphorylated by PKA. Using microinjection assays, these workers demonstrated that the activation of Pit-1 by PKA was lost in the presence of CBP containing a point mutation at the consensus PKA site. This model was not confirmed by Zanger, et al (52), however, and Swope et al (42) have suggested that the PKA-responsive domain in CBP resides near its amino-terminus. At this point, the mechanism of PKA-activation of CBP/p300 remains enigmatic.

Other kinases proposed to regulate CBP/p300 function include calcium/calmodulin (CaM) kinase IV, MAPK, and pp90Rsk. Although several reports suggested that CBP cannot mediate its transcriptional functions in the absence of CaM kinase IV stimulation (4), (12), (14), later studies showed that recruitment of CBP, by itself, was sufficient for transcriptional activation (3), (7). It remains possible, however,

that phosphorylation of CBP/p300 by CaM kinase IV could contribute to signaling by augmenting the transcriptional response.

Phosphorylation and activation of CBP by MAPK was first reported by Janknecht and Nordheim (16). Activation of MAPK through the ras pathway by insulin or nerve growth factor was reported to recruit pp90Rsk to the third zinc finger domain of CBP in a manner that prevents the binding of essential CBP effectors such as RNAPII (33). Interestingly, modulation of CBP by pp90Rsk does not appear to require its catalytic kinase activity. This inhibitory effect of pp90Rsk has not been seen by other investigators, however, so it is possible that it is cell type specific (46). Clearly, the understanding of CBP/p300 regulation by phosphorylation remains a major topic for future study.

Are CBP and p300 primarily HATs or FATs?

In addition to their intrinsic acetyltransferase functions, CBP and p300 are known to associate with additional HATs, including P/CAF, SRC-1, and p/CIP. Why so many different HATs are required for transcriptional regulation is unknown, but the answer may lie in the differing preferences of these enzymes for free histones as compared to nucleosomes and their distinct targets within the histone substrates (for review, see (41)). While it has been suggested that the HAT domains in CBP/p300 are highly related to those in P/CAF and GCN5 (29), the primary sequences of these domains are actually quite different. Moreover, these differences are significant enough to allow the development of specific inhibitors of the P/CAF and CBP/p300 enzymatic activities (26). Kraus, et al have shown that the p300-mediated activation of estrogen receptor (ER) function on reconstituted chromatin depends upon the coactivator's intrinsic acetyltransferase activity, demonstrating that this enzymatic function is essential in the

context of chromatin (23). Because histone acetylation is not required for transcription of naked DNA templates, these results imply that some component of chromatin is the acetylation target. These conclusions are supported by the results of Ludlam, et al (submitted for publication) which show that flies containing an acetyltransferase-deficient form of CBP are incapable of activating specific target genes in vivo. While confirming the importance of the CBP enzymatic function, these studies do not identify the acetylation target. Recent studies have shown that CBP/p300's HAT activity is directed towards nucleosomes through interactions with the histone chaperone, RbAp 48 (53). Moreover, Ito et al have found that histone acetylation by p300 facilitates the transfer of H2A-H2B from nucleosomes to the chaperone protein NAP-1 (15). In this model, the recruitment of p300 and the subsequent histone acetylation follows a chromatin remodeling step mediated by ATP-dependant proteins in the ISWI family. These results are consistent with in vivo chromatin immunoprecipitation experiments in yeast showing that the association of SWI/SNF components on the HO promoter is required for the subsequent HAT recruitment (6), (24). Whether the release of H2A-H2B results from the acetylation of these proteins directly or whether other nucleosomal components are the primary targets of the acetyltransferases remains to be determined.

Acetylation of transcription factors (through FAT, factor acetyltransferase activities) by CBP/p300 may provide an equally important mode of regulation. First identified in the context of the tumor suppressor p53 (11), acetylation of transcription factors has been increasingly recognized as a mechanism of gene regulation. In some instances, acetylation has clearly been shown to increase the binding of transcription factors to DNA (11). In most cases, however, the mechanism of activation is unknown. Recent evidence suggests that coactivator acetyltransferases might also serve to disrupt activator and repressor complexes. For example, Evans and coworkers have shown that the recruitment of p300 to the ligand-activated ER leads to the acetylation of ACTR (an

associated acetyltransferase), disruption of the ACTR-p300-ER complex, and the termination of transcription (5). Another possibility, consistent with the multistep model of transcription proposed by Roeder (reviewed in (39) and (28)), is that CBP/p300-mediated acetylation of the complex may promote the transition from a CBP/p300-dependent to a mediator-dependent stage of transcription (see below). A converse mechanism was proposed by Zhang, et al, (in press). In these studies, interaction of the histone deacetylase-binding corepressor, CtBP, (Carboxy-Terminal Binding Protein) to a variety of transcriptional repressors was shown to be blocked by acetylation of the CtBP interaction sites. In this instance, as in the classical histone acetylation model, acetylation is proposed to activate transcription by disrupting protein complexes involved in repression. Paradoxically, acetylation by CBP can also cause transcriptional repression in some systems. For example, in flies, CBP has been shown to inhibit wingless signaling by acetylating the Drosophila homologue of the high mobility group protein, LEF/TCF-1 (44). Acetylation of a specific residue in LEF/TCF-1 is believed to block the binding of the coactivator beta-catenin/Armadillo, one of the intermediates in the wingless signaling pathway. In support of this model, CBP loss-of-function mutants have been found to suppress the effects of an Armadillo mutation.

Does CBP/p300 function as a transcriptional integrator?

It is somewhat surprising that CBP and p300, which mediate the activities of so many different transcription factors, might be present in the cell at limiting concentrations. Nonetheless, there is considerable evidence that this is the case. Even discounting experiments involving transcription factor overexpression, which would perhaps be expected to exceed the capacity of the endogenous CBP/p300, studies have shown that relatively small decreases in the concentrations of coactivator are deleterious. For example, in the human Rubinstein-Taybi syndrome, loss of a single CBP allele

results in severe developmental defects (36). The idea that CBP/p300 levels are limiting is also supported by tissue culture experiments, as exemplified by the studies of Hottiger, et al (13), which examined the ability of interferon-α (IFN-α) to inhibit TNF-α stimulated HIV gene expression. This inhibition was shown to be mediated by competition between STAT-2 (stimulated by IFN-α) and the p65 subunit of NF-κB (stimulated by TNF-α) for a shared binding site within the first zinc finger domain of CBP/p300. It is not certain that two transcription factors must compete for the same binding site to be mutually antagonistic, however. If CBP/p300 levels are truly limiting, it is possible that they could be directed toward specific genes to the exclusion of others. Testing this hypothesis will require the use of experimental paradigms that do not involve the overexpression of exogenous transcription factors.

The idea that CBP/p300 contributes to transcriptional synergy is probably best supported by studies of the IFN-β enhanceosome (see below), but other complex promoters have also been shown to contain binding sites for multiple CBP/p300-interacting transcription factors. (Indeed, given the large number of factors that bind CBP/p300, it is difficult to imagine a promoter where this would not be the case.) Nonetheless, although transcriptional synergy through CBP/p300 is an appealing model, it has not been shown conclusively that these coactivators interact with multiple transcription factors simultaneously. In addition, while the recruitment of coactivators to the enhanceosome appears to be required for synergistic activation, tethering CBP/p300 to the promoter through a heterologous DNA-binding domain is not sufficient. As suggested by Merika, et al (30), the activation domains of the individual transcription factors comprising the enhanceosome may contribute critical interactions with basal factors. Alternatively, CBP/p300 may only participate in a transient (albeit required) step in the transcriptional process. It may be equally important for CBP/p300 to be replaced by other factors, such as the mediator complex, for transcription to proceed. In support

of this idea, Kraus and Kadonaga (22) have demonstrated that while both the ER and p300 are necessary for transcriptional initiation from chromatin templates, only the ER is required for reinitiation.

How do CBP/p300 fit into the complex series of events that mediate transcriptional activation?

Transcriptional processes are regulated through the sequential interactions of a large number of modulatory multiprotein complexes. Assembly of basal transcription factors at the promoter represents the end result of these interactions. Regulation is imparted by additional components such as enhanceosomes and mediator complexes which, along with coactivators, integrate specific extracellular events and intracellular signals.

Enhanceosomes are stable multiprotein complexes that promote the cooperative recruitment of coactivators and the RNAPII complex to active sites of transcription. In one well-characterized example, formation of the enhanceosome involves recruitment of NFκB, ATF-2/c-jun, IRFs, and HMGI (Y) to enhancer elements in the IFN-β promoter to create stereospecific interaction surfaces between the enhancer binding proteins and the CBP/p300-associated RNAPII complex (Fig. 2) (45), (20). The critical role of CBP/p300 in this context is to promote the rapid formation of the preinitiation and reinitiation complex to facilitate multiple rounds of transcription (50). Depletion of CBP/p300 from this complex decelerates the rate of transcription (51). CBP/p300 may also participate in terminating IFN-ß gene transcription by acetylating HMG1(Y), decreasing its affinity for DNA and disrupting the enhanceosome (50), (31), (2)). Whether enhanceosomes actually rely on the HAT activity of CBP/p300 has not been determined, however. Recent evidence demonstrating an intrinsic, phosphorylation-dependent HAT activity in ATF-2,

one of the DNA binding proteins found in the IFN-β enhanceosome, suggests that the CBP/p300 HAT function could be redundant (19).

Mediator complexes provide the penultimate step in the activation process, leading to the recruitment of the general transcriptional machinery (Fig. 3). These mediators, ARC/DRIP/TRAP/SMCC, NAT, CRSP, SRB/Med, and mouse Mediator, share a subset of common components (reviewed in (21) and (28)). The relationships between these complexes and CBP/p300 have not been entirely resolved, however. TRAP, the first of the mammalian mediators to be characterized, does not contain CBP/p300 and lacks detectable HAT activity (8), (9). Consistent with its absence of associated HAT activities and with its relationship to the yeast Mediator, TRAP shows potent coactivator functions with diverse activators on naked DNA templates, whereas additional functions with chromatin templates remain to be tested. In contrast, ARC- and DRIP-mediated transcription have been observed on chromatin templates, possibly reflecting the presence of some TRAP components in the assays with DNA templates and/or the loose association or copurification of CBP/p300 (or other HATs) with ARC and DRIP complexes (32), (38). Might TRAP components also exhibit additional essential (or enhanced) functions with chromatin templates, indicating a potential need for additional protein-protein interactions for formation of the preinitiation complex in this context? What is the role of CBP/p300 in ARC and DRIP? Does CBP/p300 bridge transcriptional activators to the mediator complex, or is its role to alter nucleosome structure in a manner that allows the mediators to function at a subsequent stage of transcription?

Part of the ambiguity regarding the association of CBP/p300 with mediator complexes may stem from the different methods used to purify these mediators. TRAP was purified using a functional assay (8), while ARC and DRIP were identified through

their binding to activated transcription factors (38). It is likely that the fusion proteins used to purify ARC and DRIP interact with CBP/p300 and the mediators in a mutually exclusive manner. This explanation would be consistent with the multistep interaction model proposed by Roeder (28). In this model, activated transcription factors have the capacity to interact with both CBP/p300 and the mediator complexes, but the mediator interactions might be nonfunctional until appropriate nucleosomal modifications have been induced by CBP/p300.

Nonetheless, it may be premature to conclude that mediators do not contain HATs. Lorch et al (27) have determined that the yeast mediator forms direct interactions with nucleosomes and contains a subunit, Nut1, that specifically acetylates nucleosomal histone H3. Therefore, at least in yeast (which does not contain CBP/p300), mediator complexes do have intrinsic HAT activity. These studies reopen the issue of whether the mammalian mediator complexes might also contain loosely associated HATs or whether this activity must be provided by a distinct complex containing CBP/p300.

Summary and Future Perspectives

In the sequential step model of transcriptional regulation, coactivator HATs such as CBP/p300 are key regulators in the assembly and mobilization of the basal transcription machinery. Precisely how CBP/p300 prepares the template for subsequent steps in the transcriptional process remains to be determined. Understanding the positioning, timing, activation, and termination of CBP/p300 functions will shed light on how cells use common transcriptional complexes to mediate specific genetic responses to diverse cellular signals.

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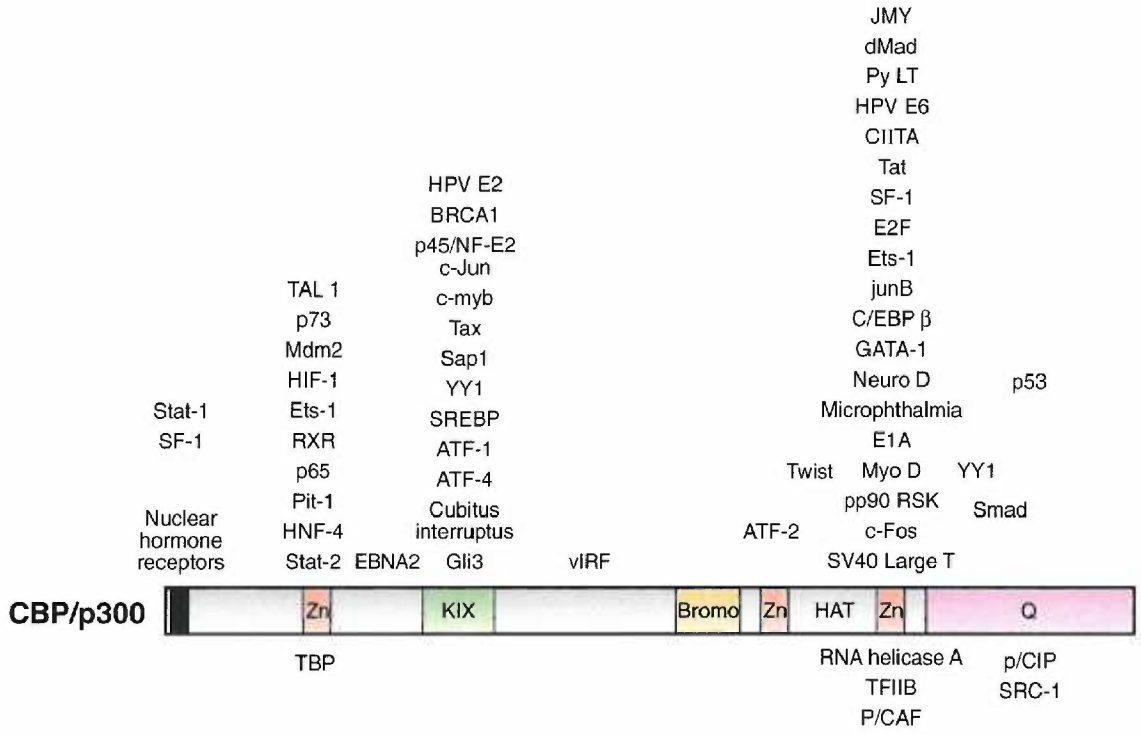

Figure 1. Organization of CBP/p300 binding proteins. Association of CBP/p300 with transcriptional activators (top) and basal transcription factors and HATs (below). The zinc fingers (Zn), CREB binding domain (KIX), bromodomain (Bromo), HAT domain and glutamine-rich domain (Q) are indicated.

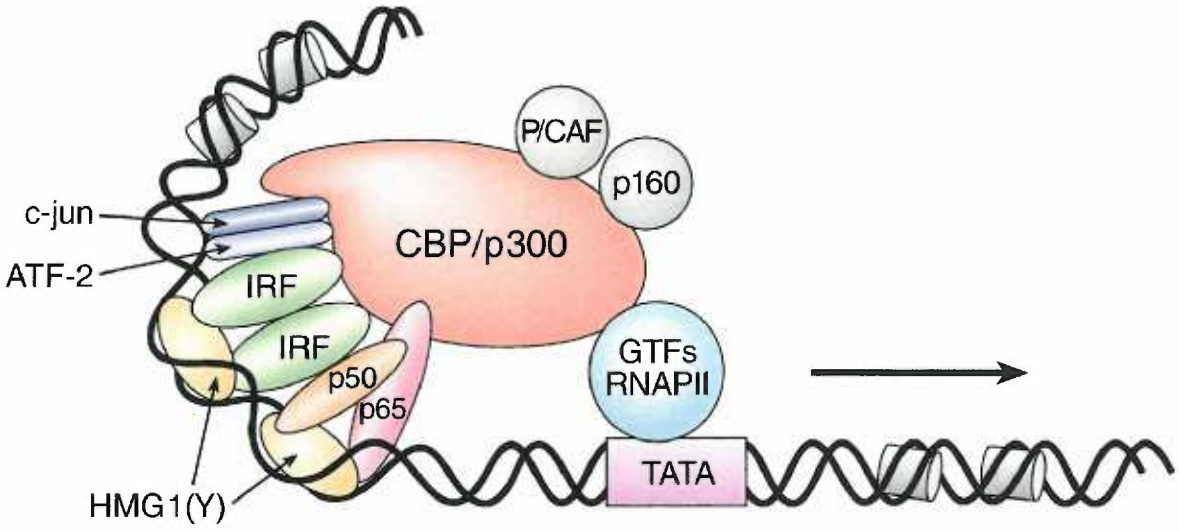

Figure 2. The IFN-ß enhanceosome complex. Assembly of the IFN-ß enhanceosome creates a stereospecific interaction surface for recruitment of CBP/p300 and the basal transcription machinery to allow multiple rounds of transcription. GTFs indicate general transcription factors. p160 refers to the SRC/TIF/pCIP family of coactivators.

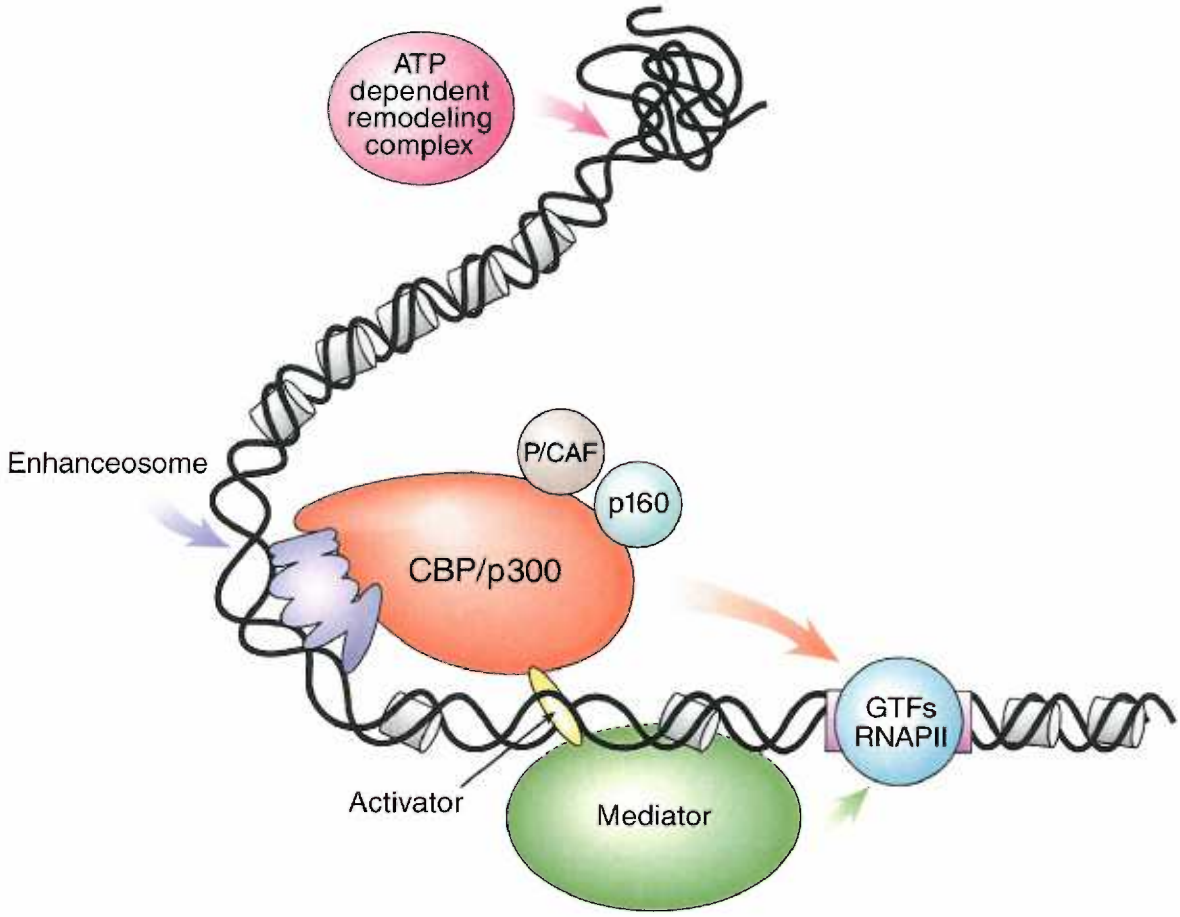

Figure 3. The multistep model of transcription. First, ATP-dependent remodeling complexes alter the structure of chromatin. Second, coactivator HATs facilitate the formation of enhancesomes and permit the actions of mediator complexes. CBP/p300 may facilitate the recruitment of the mediator complex to active sites of transcription. Mediator, in turn, regulates transcription through interactions with components of the basal transcription machinery. The dashed line indicates an unknown association with DNA.