AN ABSTRACT OF THE THESIS OF

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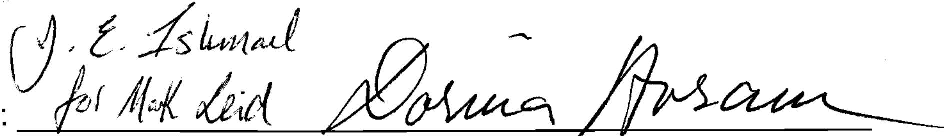
Abstract Approved: 
Mark Leid / Dorina Avram

COUP-TF interacting protein 1 (CTIP1) is a novel C_2H_2 zinc finger protein that mediates transcriptional repression of ARP1, a member of the COUP-TF subfamily of orphan nuclear receptors [1]. Here we show that CTIP1 interacts with heterochromatin-associated protein 1 (HP1) *in vitro* and in cells. This interaction suggests the existence of a novel mechanism of transcriptional repression for COUP-TFs, which may involve association with heterochromatin. Analysis of *in vitro* interaction of different CTIP1 deletion mutants with HP1 reveals that the amino terminal region of CTIP1 as well as the central core interact with HP1. These two domains were previously shown to mediate transcriptional repression when fused to GAL4 DBD [1] suggesting that association with HP1 proteins may be implicated in the repression function.

Abbreviations

ARP1	Apolipoprotein Regulator Protein 1
COUP-TF	Chicken Ovalbumin Upstream Promoter Transcription Factor
CTIP1	COUP-TF Interacting Protein 1
CBP	CREB Binding Protein
CREB	Cyclic-AMP Response Element Binding Protein
GST	Glutathione-S-Transferase
HAT	Histone AcetylTransferase
HDAC	Histone DeACetylase
HEK 293	Human Embryonic Kidney cells, 293
HP1	Heterochromatin associated Protein 1
NCoR	Nuclear receptor CoRepressor
P/CAF	p300/CBP Associated Factor
PCR	Polymerase Chain Reaction
TBP	TATA Binding Protein
TSA	Trichostatin A

Characterization of CTIP1 Interaction with HP1

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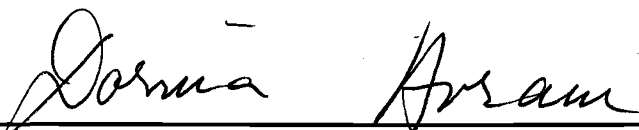

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Dr. Mark Leid is the principle investigator of the project and was involved in the design, analysis, and writing of the thesis. Dr. Dorina Avram was involved in the experimental design of several individual experiments, analysis, and writing of the thesis.

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Characterization of CTIP1 Interaction with HP1 Proteins

Literature Review

Introduction

Transcription of eukaryotic genes by RNA polymerase II is a complex process that involves the cooperation of several protein complexes. In a schematic way, the TATA binding protein (TBP) binds the TATA box, located in the promoter region of a gene and recruits TBP associated factors (TAFs) and transcription factors TFIID, TFIIB, TFIIF, TFIIE, and TFIIH to form a preinitiation complex (PIC) [1] (fig. 1). As a result RNA polymerase II is recruited to the promoter region and initiation of transcription occurs [1]. The RNA polymerase II core machinery, including TBP, general transcription factors, and RNA polymerase II, is prevented from randomly binding DNA and initiating transcription by a highly ordered chromatin structure [2]. The basic unit of chromatin, the nucleosome, is composed of the core histones (2 molecules each of H2A, H2B, H3, and H4, and one molecule of H1) that wraps 160 base pairs of DNA tightly around its surface [3]. Core histones are organized into a nucleosome fiber, which is further organized into a chromosome [4]. The core transcriptional machinery relies on transcriptional regulators to modify chromatin and thus allow gene expression [5] (fig. 1).

Nuclear Receptors

Nuclear receptors are ligand dependent transcriptional regulators that bind short sequences of DNA called response elements located upstream in the promoter region of target genes [6] (fig. 1). The nuclear receptor superfamily includes several receptors for hormones such as estrogen, thyroid, and glucocorticoids, non-hormone receptors such as retinoic acid receptors, and also receptors that bind products of lipid metabolism such as fatty acids and prostaglandins [7], [8]. Nuclear receptors contain highly conserved DNA binding domains (DBD), a hinge region, and a carboxyl terminus ligand binding domain (LBD) [9]. Ligand binding to a nuclear receptor results in conformational changes and recruitment of transcriptional coactivator complexes to the promoter region [3].

The function of coactivators is to remodel the chromatin structure by modifying the amino termini of histones [4]. Some nuclear receptor coactivators such as CBP/p300 [10] and P/CAF [11] (fig. 1) harbor intrinsic histone acetyltransferase activity [2]. These enzymes add acetyl groups to lysines on histone tails, neutralizing the positively charged amino acids [2]. An accumulation of HAT activity alters one or more nucleosomes, which leaves the associated DNA significantly more accessible to non-histone proteins [4]. As a result promoter regions of target genes are more accessible to general transcription factors and initiation of transcription may occur [6].

In the absence of a ligand, some nuclear receptors remain bound to DNA and function as transcriptional repressors [3] (fig. 1). Nuclear receptor

corepressor (NcoR) and a homologous factor, silencing mediator of retinoid and thyroid hormone receptor (SMRT) are transcriptional corepressors that are able to associate with unliganded receptors such as retinoid acid receptor (RAR) or thyroid hormone receptor (TR). Antagonist-bound nuclear receptors such as estrogen receptor (ER)-bound tamoxifen can also associate with nuclear receptor corepressor complexes that minimally contain a histone deacetylase complex (HDAC) and, in some cases, Sin3a [5]. HDACs remove acetyl groups from histone tails, restoring the positive charge of the lysine residues. This results in a more compact chromatin structure that is less accessible to the RNA polymerase II complex and, therefore, repression of transcription [4].

COUP-TF Subfamily of Orphan Nuclear Receptors

Orphan nuclear receptors are a subfamily of nuclear receptors that do not have a defined regulatory ligand [12]. They have similar functional domains as nuclear receptors, however the mechanism of regulation is unknown [9]. COUP-TFs belong to a subfamily of orphan nuclear receptors that function as transcriptional repressors in transient transfection experiments [13]. However, in the context of certain promoters, COUP-TFs can activate target genes [14].

The COUP-TF subfamily is composed of three members: COUP-TFI, ARP1/COUP-TFII, and Ear2/COUP-TFIII. While COUP-TFI and ARP1 share a high degree of sequence similarity, Ear2 has less similarity to COUP-TFI and ARP1 [15], [16]. Each COUP-TF orphan receptor can function to repress ligand

dependent transcriptional activation of target genes mediated by several nuclear receptors such as retinoic acid [17], thyroid hormone [18], and estrogen receptor [19]. The mechanism of repression mediated by COUP-TFs is largely unknown, however there are several possible mechanisms that could mediate its signaling pathway. COUP-TF proteins are known to promiscuously bind to a wide variety of response elements and thus competition for response element binding with other nuclear receptors would be possible [20]. COUP-TFs could also titrate general transcription factors or coactivators, or could recruit corepressor complexes to the promoter region.

COUP-TF family members are highly expressed in brain and embryo tissues. Deletion of the COUP-TFI gene in mice results in defective neuronal axonal guidance [21]. COUP-TFI null animals exhibit perinatal death associated with defects in the glossopharyngeal ganglion and the VIth cranial nerve [21]. ARP1/COUP-TFII is highly expressed in mesenchymal cells during organogenesis [22]. ARP1 null animals die at embryonal day 10, possibly due to defects in angiogenesis and embryonic heart development [23]. Ear2/COUP-TFIII is ubiquitously expressed during embryonic development and in the adult organism [15] but the function of this protein is unknown. Ear2 heterodimerizes with COUP-TFI and ARPI [16], which suggests that Ear2 may be involved in both signaling pathways.

CTIPs--Novel Proteins Mediating Transcriptional Repression of COUP-TFs

Avram, et. al. (2000) have identified a family of novel proteins that may be involved in COUP-TF mediated transcriptional repression. CTIP1 and CTIP2 interact directly and specifically with COUP-TF family members. CTIP1 is expressed in high levels in the brain and less in the embryo, lung, heart, and liver, while CTIP2 is expressed in the brain and lung but not the liver or heart. This indicates that expression patterns of the CTIP genes are only partially overlapping [1] (fig. 2).

CTIP1 and CTIP2 are members of a novel family of Cysteine 2, Histidine 2 (C_2H_2) zinc finger proteins [13]. The zinc finger motif is defined by an anti-parallel β sheet and an α helix that fold around a central zinc ion and the α helix binds the major groove of DNA [24]. C_2H_2 zinc finger proteins contain multiple zinc finger motifs with the conserved sequence $Y/FXCX_{(2-5)}CX_3F/YX_5LX_2HX_{(3-5)}H$, where X is any amino acid [24] (fig. 3). Zinc finger containing proteins are the most abundant transcription factors eukaryotes [25]. C_2H_2 zinc finger proteins usually bind DNA as monomers or assist other DNA binding proteins [24]. C_2H_2 zinc fingers are also implicated in protein-protein interactions such as the C_2H_2 zinc finger protein from FOG, which interacts with the amino terminal C_2H_2 zinc finger domain of GATA-1 protein [26].

CTIP1 proteins were isolated in a yeast two-hybrid screening of mouse brain cDNA using the hinge and LBD region of ARP1 [20] as bait. Several positive clones of CTIP1 were isolated from the mouse library and then

overlapping clones of CTIP1 were used to generate a cDNA encoding full-length CTIP1, a protein of 776 amino acids [13].

Studies by Avram, et al. (2000) were done to determine the function of CTIP1 in relationship with the repression function of ARP1. CTIP1 interacts with ARP1 *in vitro* and this interaction requires the carboxyl terminus of ARP1, which contains the activation function 2 (AF-2) motif. The AF-2 motif is important for coactivator as well as corepressor binding among nuclear receptors [9]. The CTIP1 interaction with ARP1 is mediated through the CTIP1 carboxyl terminus, and there is an additional interaction site in the CTIP1 core region (amino acids 264-378) [13]. Cotransfection of CTIP1 and ARP1 in cells resulted in enhancement of the repression function of ARP1. CTIP1 mediated transcriptional repression also occurred in the presence of trichostatin A (TSA), an inhibitor of histone deacetylase, which indicates that the repression mechanism probably does not require TSA-sensitive histone deacetylases [13]. CTIP1 bears autonomous transcriptional repression domains when fused to a GAL4 DBD [13]. The amino terminal of CTIP1 (amino acids 1-171) mediated transcriptional repression of a reporter gene driven by a 17-mer reporter when fused to GAL4 DBD. The CTIP1 core region (amino acids 171-434) also exhibited autonomous repression function similar to the CTIP1 amino terminus [13]. Transcriptional repression mediated by CTIP1 alone also occurred in the presence of TSA, indicating that the repression mechanism probably does not require TSA-sensitive histone deacetylases.

ARP1 presents a diffuse pattern of staining while CTIP1 presents a punctate staining pattern in the nuclei of transfected cells. Immunocytochemistry of cotransfected cells revealed that CTIP1 redistributes ARP1 to punctate nuclear substructures which correlate with the repression function in the sense that mutants unable to interact with CTIP1 are unable to be recruited to punctate structures and unable to repress transcription [1]. The punctate staining patterns of CTIP1 are similar to proteins that localize in juxtaposition of heterochromatic regions [27], [28], which may suggest that heterochromatin is involved in the repression mechanism mediated by CTIP1.

Heterochromatin, which contains hypoacetylated histones, may play a role in CTIP1-mediated transcriptional repression through interaction with heterochromatin-associated proteins (HP1s) [27], [28]. HP1 proteins have three distinct domains: the amino terminal chromo domain (chromosome organization modifier), a hinge region in the center of the protein, and a carboxyl terminal chromo shadow domain that is structurally similar to the chromo domain [29]. The HP1 chromo shadow domain seems to be implicated in protein-protein interactions, as HP1 chimeric proteins containing a chromo domain are still recruited to heterochromatin [30], [31]. Smothers et. al. (2000) found that HP1 interacting proteins harbor a consensus hydrophobic pentapeptide sequence and HP1 proteins contain a corresponding hydrophobic groove in the chromo shadow domain that is capable of binding these short peptides [32].

To elucidate a possible novel mechanism of CTIP1 mediated transcriptional repression we generated different CTIP1 deletion mutants and performed protein-protein interaction studies to determine which regions of CTIP1 interacts with full-length HP1 proteins. Here we show that CTIPs interact with HP1-α, β, and γ. We also found that the amino terminal and central core regions of CTIP1, which bear transcriptional repression functions are the domains implicated in interaction with HP1 proteins. This interaction has functional significance as indicated by the fact that CTIP1 and HP1 proteins interact in cells.

Materials and Methods

HP1 Constructs

HP1 full-length proteins cloned into pGEX (Life Technologies, Inc.) were expressed in bacteria to generate GST-HP1 fusion proteins. HP1 constructs were kind gifts from R gine Losson and Pierre Chambon.

CTIP1 Constructs

CITP1 mutants were generated by PCR amplification using specific forward and reverse primers (Life Technologies, Inc.) and CTIP1/pcDNA3 (Invitrogen) [1] (fig. 4B) as a template. Each primer included appropriate restriction enzyme sites for appropriate insertion into digested vectors. Primers

also contain an ATG start site, a Kozak sequence and a stop codon for proper expression.

The following CTIP1 mutants were generated with forward and reverse primers that contained *BamHI* (Promega) and *Xba* (Promega) restriction sites, respectively. These CTIP1 mutant fragments were digested accordingly and cloned into *BamHI/Xba* digested pCDNA3 vectors. The CTIP1 amino truncated mutant (amino acids 71-171, fig. 4K) was generated using the forward oligo 5 - ATGTACCCTTATGATGTGCCAGATTATGCC-3 and reverse oligo 5 - GCAAATTCCTCTAGATGACGTT-3 . CTIP1 amino terminal mutant with amino acids 171-210 (fig. 4J) was generated with forward oligo 5 - ATGTACCCTTATGATGTGCCAGATTATGCC-3 and reverse oligo 5 - CCGCGGGGTCAGGGGACT-3 . CTIP1 amino terminal mutant with amino acids 71-210 (fig. 4I) was generated with forward oligo 5 - ATGTACCCTTATGATGTGCCAGATTATGCC-3 and reverse oligo 5 - CCGCGGGGTCAGGGGACT-3 .

The following CTIP1 mutants were generated with forward and reverse primers that contained *BamHI* and *Xh*o (Promega) restriction sites, respectively. The CTIP1 PCR products were digested accordingly and cloned into *BamHI/Xh*o digested pCDNA3 vectors. CTIP1 amino terminal mutant with amino acids 1-263 (fig. 4F) was constructed with forward oligo 5 - ATGTCTCGCCGCAAGCAAGGC-3 and reverse oligo 5 - CACTTATAGGGCTTCTCACAGT-3 . The CTIP1 mutant containing the amino terminal end up to, but not including, the third zinc finger (amino acids 1-378,

fig. 4E) was made using forward oligo 5'-ATGTCTCGCCGCAAGCAAGG3 and a reverse oligo 5'-TGACTTGGACTTGACCGGGGG-3 .

The following CTIP1 deletion mutants were generated with primers that contain a *BamHI* restriction site in the forward primer and an *Xba* restriction site in the reverse primer. The CTIP1 PCR products were not digested according to their restriction sites and were cloned into a blunt end cutting *Ecl*II (Promega) digested pBluescript (Stratagene) vector. The CTIP1 amino terminal truncated mutant (amino acids 1-171, fig. 4H) was generated with the forward oligo 5 -CGCGGATCCACCATGTCTCGCCGCAA-3 and reverse oligo 5 -CCCAAGCTTGTGTAGCTGCTGGGCTCATCTTT-3 . The CTIP1 core construct containing zinc fingers 2, 3, and 4 (amino acids 171-434, fig. 4C) was generated with forward oligo 5 -TGTACAACTTGCAAACAGCCATTC-3 and reverse oligo 5 -CATGGGGGACGATTTGTGCATG-3 . A naturally occurring splice variant (fig. 4G) was synthesized by Reverse transcriptase PCR amplification using forward oligo 5 -CGCGGATCCACCATGTCTCGCCGCAA-3 and reverse oligo 5 -CCCAAGCTTAACTTAAGGGTTCTTGACCTTCC-3 from rat brain cDNA.

The CTIP1 carboxyl terminal mutant that contained zinc finger motif 4 (amino acids 407-776, fig. 4D) was cloned from the yeast vector pACT2 (CLONTECH, Palo Alto, CA) in yeast 2-hybrid experiments [13]. The pACT2 construct were digested with *Bgl*II and cloned into *Bam*HI-digested pTL1[33].

The PCR reactions used to generate CTIP1 truncations were performed for 25-30 cycles with a 94 °C melting temperature, a primer dependent

annealing temperature that ranged from 56-60 °C, and a 72 °C amplification temperature.

The PCR products were loaded on a 1% agarose gel for electrophoresis and purified from the gel according to the QIAquick gel extraction protocol (Qiagen). Then the PCR products were digested with appropriate restriction enzymes (see above; note that PCR products cloned into pBluescript do not require digestion, as they are cloned into a blunt cut vector). pCDNA3 and pBluescript vectors were digested with appropriate enzymes (see above). The digested PCR products were purified according to the Qiaquick PCR purification protocol. The CTIP1 PCR products were then ligated with digested vectors overnight at 14 °C using 50 ng of PCR product, 10 ng of vector, 0.5μl of T4 DNA ligase (Promega), and 0.5 μl of 10X T4 DNA ligase buffer (Promega). 2μl of each ligated CTIP1 construct was then transformed into 50μl of competent *Escherichia coli (E. coli*) XL1-blue cells (Stratagene) and plated on LB agar with ampicillin (0.1 mg/ml) to select for viable cells containing the construct. Colonies of E. coli were selected and grown in LB and ampicillin. The plasmid DNA was isolated according to plasmid mini prep protocol (Qiagen) and digested with restriction enzymes to check for plasmids containing the CTIP1 insert.

CTIP2 construct

A fragment encoding CTIP2 full-length protein was cloned into *BamHI/Xba* digested pcDNA3 (fig. 4A).

In vitro translation

In vitro synthesis of CTIP1 truncated mutant proteins was performed using the TNT T7 Coupled Reticulocyte Lysate System (Promega). 2μg of CTIP1 mutant DNA was added to a mixture containing rabbit reticulocyte lysate, TNT reaction buffer, T7 (or T3 for CTIP1 mutants in pBluescript) RNA polymerase, a mixture of amino acids minus methionine, ^{35}S-methionine and RNase inhibitor (Rnasin). The mixture was incubated in a 30 °C water bath for 90 min and then stored at -80 °C. 5ul of the reaction and 20ul of sample buffer were run on a SDS polyacrylamide gel electrophoresis (SDS-PAGE). The gel was treated with 7% glacial acetic acid for 15 min, 10% 2, 5-diphenyloxazole for 10 min, and water for 5 min. The gels were vacuum dried for 30 min and exposed to an X-ray film overnight.

GST Fusion Protein Production

HP1 constructs inserted into pGEX plasmid vectors are fused to a glutathione-S-transferase (GST) protein. The GST fusion proteins were produced and crude bacterial lysates prepared as described previously [34]. Briefly, the pGEX plasmids were transformed into competent *E. coli* cells and grown in 0.34 mg/ml chloramphenicol and 0.1 mg/ml ampicillin LB broth to an

optical density of 0.6 at 600 nm. The culture was induced to produce protein with addition of 0.1 mM IPTG (1:1000 dilution) and after two hr, the culture was harvested and freezing and thawing generated crude bacterial lysate. The GST proteins were stored at —80 °C with 10% glycerol.

GST Pulldown Experiments

GST-HP1 fusion proteins were partially purified on glutathione-Sepharose 4B (Pharmacia Biotech Inc.). ^{35}S methionine-labeled CTIP1 mutants were added to the GST fusion protein-bound resin equilibrated in binding buffer (10mM HEPES-NaOH, pH7.5, 1mM EDTA, 1mM dithiothreitol, 150mM NaCl, 10% glycerol, 0.1% Nonidet P-40), and incubated with rotation at 4 °C for 2 hr. Unbound proteins were removed by centrifugation and three washes with binding buffer. The remaining bound proteins were denatured in sample buffer and analyzed by SDS-PAGE and autoradiography.

Cell Culture and Nuclear Extract Preparation

NIH 3T3 cells (American Cell Culture Collection) were cultured in Dulbecco's modification of Eagle's medium (D-MEM) (Cellegro) supplemented with 10% bovine calf serum (Life Technologies, Inc.) and penicillin and streptomycin (Life Technologies, Inc.). Cells were grown to 50% confluence and transiently transfected using Lipofectamine Plus reagent (Life Technologies, Inc.) Briefly, 100mm plates were transfected with 10ug of flag-CTIP1 expression vector (pcDNA3) that has been pre-complexed with 4ul of

plus reagent (Life Technologies, Inc.) diluted with D-MEM without serum or antibiotics. Then D-MEM diluted lipofectamine reagent was added to the diluted DNA and plus reagent and incubated at room temperature for 15 min. The solution was added to cells and incubated at 37 °C with 5% CO_2 for 3-8 hr. D-MEM with 20% serum was added to the cells after 3-8 hr, and 24 hr later, fresh D-MEM with serum and antibiotics was added.

48 hr after transfection, cells were harvested in phosphate buffered solution (PBS) and nuclear extracts were prepared for further experiments. Cells were centrifuged and the pellet was resuspended in low salt buffer NET-N (15mM NaCl, 60mM KCl, 5mM MgCl2, 250mM sucrose, 1mM EDTA, 15mM Tris-HCl, and 0.3% NP-40 with protease inhibitor cocktail) and incubated on ice. The nuclei were pelleted at 2000g and resuspended in DNase nuclear extraction buffer (250mM NaCl, 1mM EDTA, 25mM Tris-HCl, 5mM MgCL2, 0.2% NP-40, 10% glycerol, and protease inhibitors) and incubated for 2 hr at 4 °C. The cell debris was pelleted by centrifugation. The supernatant containing nuclear proteins was mixed with cytoplasmic supernatant to collect nuclear proteins in the cell lysate. The extracts were used immediately for immunoprecipitation experiments [35].

Co-Immunoprecipitiations

Nuclear plus cytoplasmic extracts from CTIP1 transiently transfected NIH3T3 cells were incubated with anti HP1-α, β, and γ antibodies for 30 min and then rabbit anti-mouse antibodies (Southern Biotech, Inc.) were added with

protein A sepharose (Promega) and incubated an additional 30 min on ice. The extracts were then incubated by rotation at 4 °C overnight. The CTIP1 and empty expression vector transfected nuclear extracts were washed 5 times with buffer NET-N and resuspended in sample buffer. The extracts were denatured at 100 °C and proteins were separated by SDS-PAGE and transferred to a nitrocellulose membrane for western blot analysis. The membrane was treated with Flag antibodies (Southern Biotech, Inc.) overnight with 4 °C rotation. The membrane was treated with horseradish peroxidase antibodies (Southern Biotech, Inc.) and exposed to X-ray film for 30 sec to 30 min (depending on the amount of protein expressed) and developed immediately.

Results

CTIP1 constructs generated

In order to study CTIP1 interfaces implicated in interaction with HP1 proteins we generated the following constructs:

I. Seven amino terminal constructs

1. 1-378 Terminates just before zinc finger motif three (fig. 4)
2. 1-263 Terminates just after the second zinc finger motif (fig. 4)
3. Splice Variant Contained the amino terminal end with two zinc fingers and the carboxyl terminal end of the protein (fig. 4)
4. 1-171 Contained only the first zinc finger motif (fig. 4)
5. 71-210 Amino terminus just after zinc finger one , carboxyl terminus just after zinc finger two (fig. 4)

6. 171-210 Short mutant harboring zinc finger motif two (fig 4)
7. 71-171 No zinc finger motif (fig. 4)

II. One carboxyl terminal construct

1. 407-776 Contained zinc finger motif four and contains a complete minimal COUP-TF interaction domain (CID) (fig. 4)

III. One central core construct

1. 171-434 Amino terminus just after the first zinc finger motif and terminated just before the COUP-TF interaction domain (CID) motif (fig. 4)

IV. CTIP2 construct

1. Full-length CTIP2 (fig. 4)

Interaction of CTIPs and HP1 proteins in vitro

GST pull-down experiments using full-length and truncated mutants of CTIP1 were conducted to verify the hypothesis that CITP1 directly interacts with HP1 proteins. ^{35}S-methionine labeled full-length CTIP1 protein interacted directly and specifically with each full-length HP1 α, β, and γ GST fusion protein (fig. 5B). ^{35}S-methionine labeled CTIP2 full-length protein also interacted directly with each HP1 protein (fig. 5A).

CTIP1 Amino Terminal and Core Domains Interact with HP1

As shown in figure 5, the CTIP1 splice variant and the amino terminal mutants 1-378, 1-263, 1-171, 71-210, and 71-171 interacted with HP1α. Amino

terminal mutant 171-210 did not interact with HP1α (fig. 5J), indicating that zinc finger 2 is not necessary for this interaction. Based on this study, we narrowed the HP1 interaction amino terminal domain of CTIP1 to 100 amino acids; 71-171 (fig. 5K), a region that harbors neither zinc fingers nor a motif that resembles the conserved pentapeptide sequence [1] known to be present in other HP1 interacting proteins [37]. Therefore, we believe that the amino terminal domain contains a novel motif implicated in the interaction with HP1 proteins.

The CTIP1 amino terminal and core domains (fig. 5E and 5C) found to interact with HP1 proteins harbor transcriptional repression function as shown previously [1], suggesting a functional significance of this interaction. The carboxyl terminal domain, which does not repress transcription, does not interact with HP1 proteins.

CTIP1 and HP1 interact in cells

Although CTIP1 and HP1 interact *in vitro*, a physiological interaction between these two proteins will only occur in cells in which they are both expressed. NIH3T3 cells that contain endogenous HP1 proteins were transfected with Flag-CTIP1 or empty pCDNA3 expression vector and the nuclear extracts were immunoprecipitated with anti-HP1α, HP1β, and HP1γ antibodies (fig. 6A lanes 3-5 and 8-10). The immunoprecipitated complexes were separated by SDS-PAGE and transferred to nitrocellulose membrane analyzed by western blot. Flag-CTIP1 was detected with anti-Flag antibodies

(fig. 6A, lanes 8-10). Flag-CTIP1 expression was required for co-immunoprecipitation with HP1 proteins (fig. 6A, compare lanes 3-5 and 8-10). The expression of Flag-CTIP1 did not affect the efficiency by which HP1 antibodies recognized endogenous HP1 proteins (fig. 6B). Flag-CTIP1 was not detected in co-immunoprecipitates with the empty expression vector (fig. 6A lanes 3-5).

Discussion

Our results indicate that CTIP1 and CTIP2 proteins directly interact *in vitro*. This interaction has functional significance as indicated by the fact that CTIP1 is present in the complexes immunoprecipitated from cells with antibodies against HP1 proteins. *In vitro* interaction studies show that CTIP1 harbors two domains involved in the interaction with HP1 proteins, the amino terminal and the zinc finger core. These domains were previously shown to harbor transcriptional repression function [1] and therefore we believe that the interaction of CTIP1 through these domains have functional significance and may point toward a mechanism of transcriptional repression mediated by association with HP1 proteins. Such a mechanism was described also for the C_2H_2 zinc finger Kruppel proteins [40]. These proteins associate with the corepressor KAP1, which in turn associates with HP1 proteins and potentially nucleates heterochromatin formation in euchromatic foci where target genes are repressed by Kruppel transcription factors [40]. Interestingly, the CTIP1 amino terminal, as well as the zinc finger core domains does not bear the

conserved hydrophobic pentapeptide motif present in other proteins interacting with HP1. However, the HP1 interaction sequence is also absent from several HP1 interacting proteins such as the inner centromere protein (INCENP), origin of replication protein (ORC), actin-related protein (ARP4), and lamin B receptor [32]. Therefore, CTIP1 may harbor a novel interaction motif with HP1.

As stated previously [13], CTIP1 potentiates COUP-TF mediated transcriptional repression [1]. When CTIP1 is cotransfected with ARP1, it redistributes ARP1 to the punctate structures formed by CTIP1, which is similar to the punctate foci formed in heterochromatic regions in nuclei of cells [36]. We therefore can speculate that CTIP1 may redistribute ARP1 in the nucleus and recruit it to heterochromatin and mediate its repression function through association with HP1 proteins. This would create an environment that is not permissive for transcription and thereby prevent gene expression. Heterochromatin is known to repress transcription as in the case of Position Effect Variegation (PEV), in which euchromatic genes in *Drosophila* are repressed as a result of positioning adjacent to heterochromatin [37]. Mutations that suppress PEV were in proteins associated with heterochromatin [37], such as *Drosophila* heterochromatin-associated protein 1. Transcriptional repression mediated by the COUP-TF family of orphan nuclear receptors could, therefore, rely on the repression mediated by heterochromatin.

Summary

The functional significance of the interaction between CTIP1 and HP1 is an ongoing study in the laboratory of Mark Leid. The conserved hydrophobic pentapeptide of HP1-interacting proteins defined by Smothers, et al. (2000) is not present in CTIP1 [1]. Generating more CTIP1 deletion mutants as well as site directed mutations will determine the motif in CTIP1 required for interaction with HP1. Immunocytochemistry studies will be conducted to define the localization of CTIP1 and HP1 in the nucleus and establish their juxtaposition as suggested by preliminary data. Finally, HP1 domains implicated in the interaction with CTIP1 will be studied.

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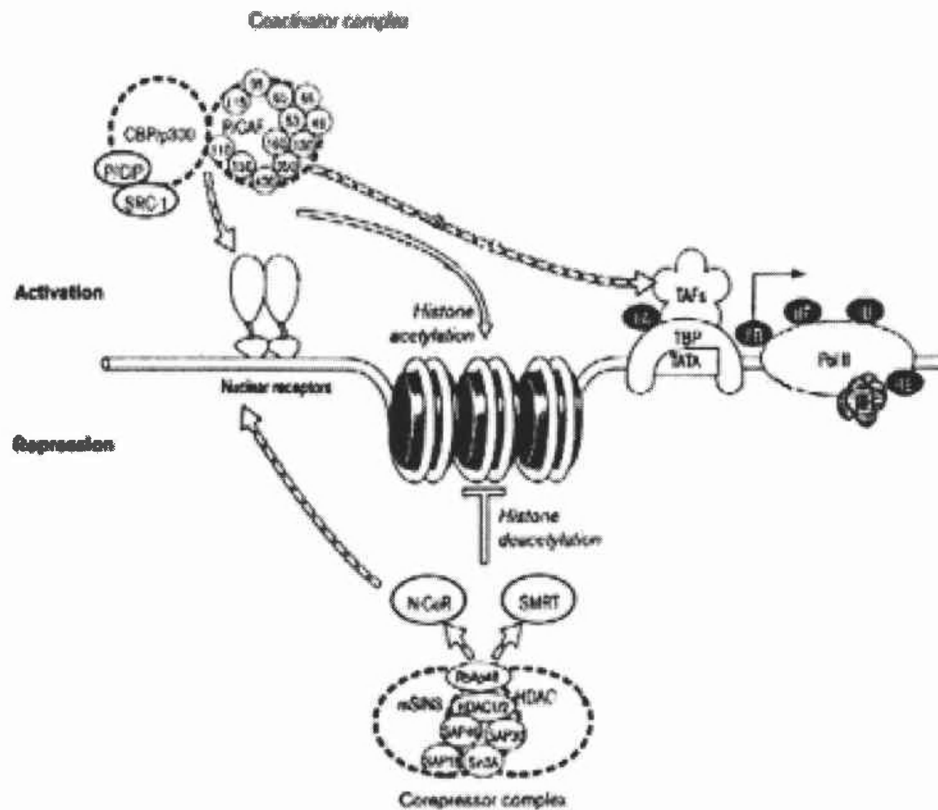

Figure 1. Coactivator and corepressor complexes in transcription. Coactivator complexes include CBP/p300 and p/CAF, which possess histone acetyltransferase activities. These complexes are recruited to the promoter region in response to a ligand bound nuclear receptor. In the absence of ligand, corepressor complexes that minimally include histone deacetylases, are recruited by NcoR or SMRT corepressors. The assembly of TBP, TAFs, general transcription factors, and RNA Polymerase II is dependent on the activity of the coactivator complexes in response to the ligand bound nuclear receptor. (Modified from Xu, 2000).

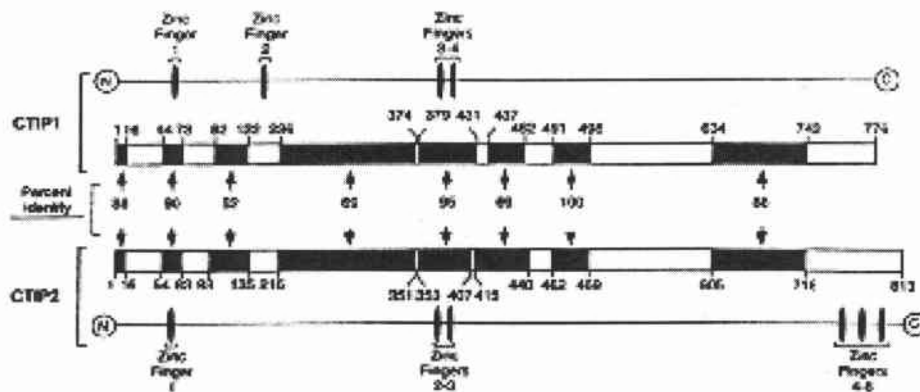

Figure 2. Diagram of CTIP1 and CTIP2 protein homology. The homologous regions are denoted by black boxes and the percentage of homology is indicated. (Modified from Avram, et al., 2000)

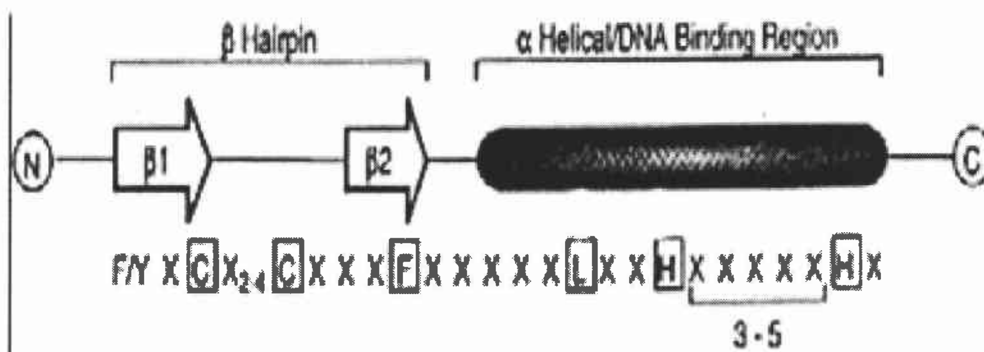

Figure 3. Schematic diagram of the secondary structure of the C2H2 zinc finger motif. Conserved amino acids are boxed and variable amino acids are denoted by an X . The structural features are indicated.

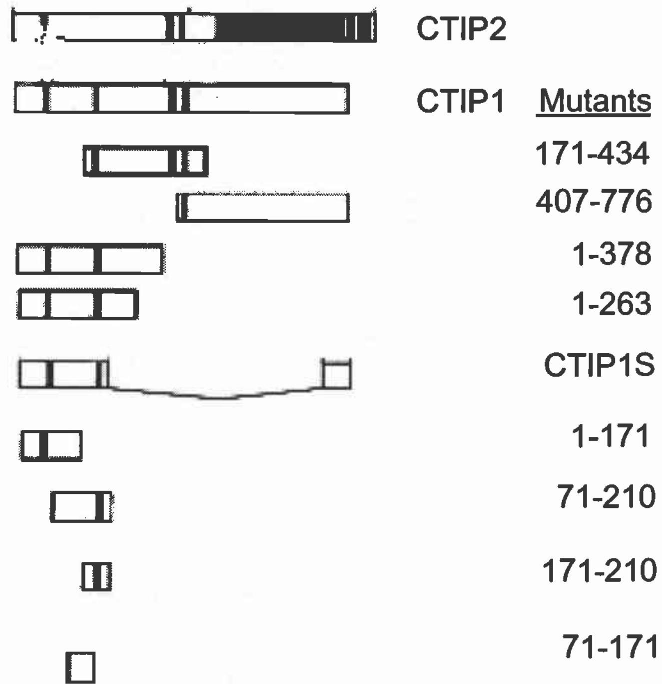

Figure 4. Schematic diagram of CTIP proteins. Schematic representations of CTIP2 (1-813), CTIP1 (1-776), and all CTIP1 mutants are shown. Vertical bars represent zinc finger motifs.

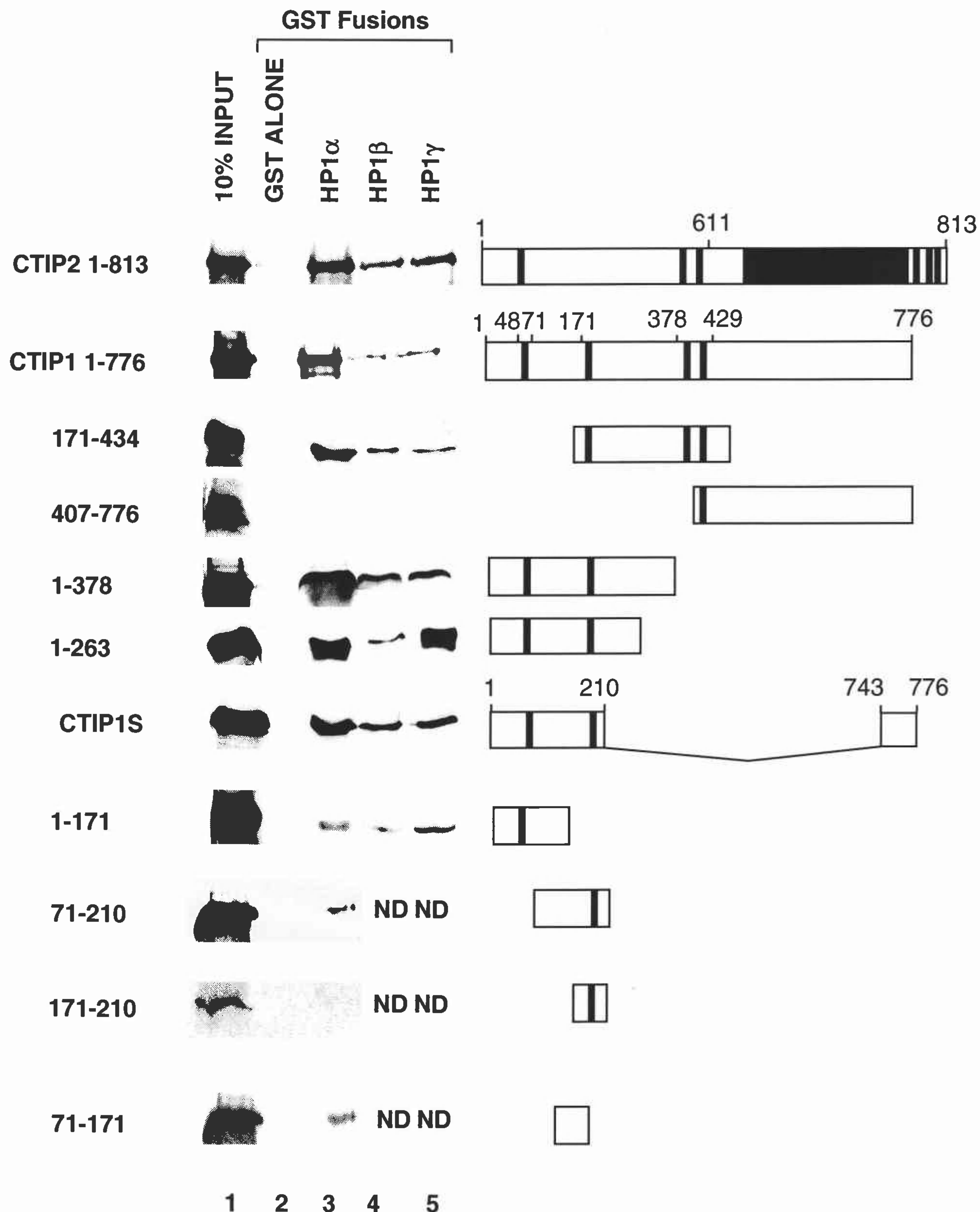

Figure 5. CTIPs interact with heterochromatin associated proteins in vitro. GST pulldown experiments using GST (lane 2 of all panels) or GST fused to HP1a, b, or g (lanes 3-5 of all panels). 10% input of each protein is shown in lane 1 of all panels. Schematic representations of wild-type CTIP1 (1-776) and CTIP2 (1-813) and all CTIP1 mutants are shown on the right side of the figure. Zinc fingers are denoted by vertical bars.

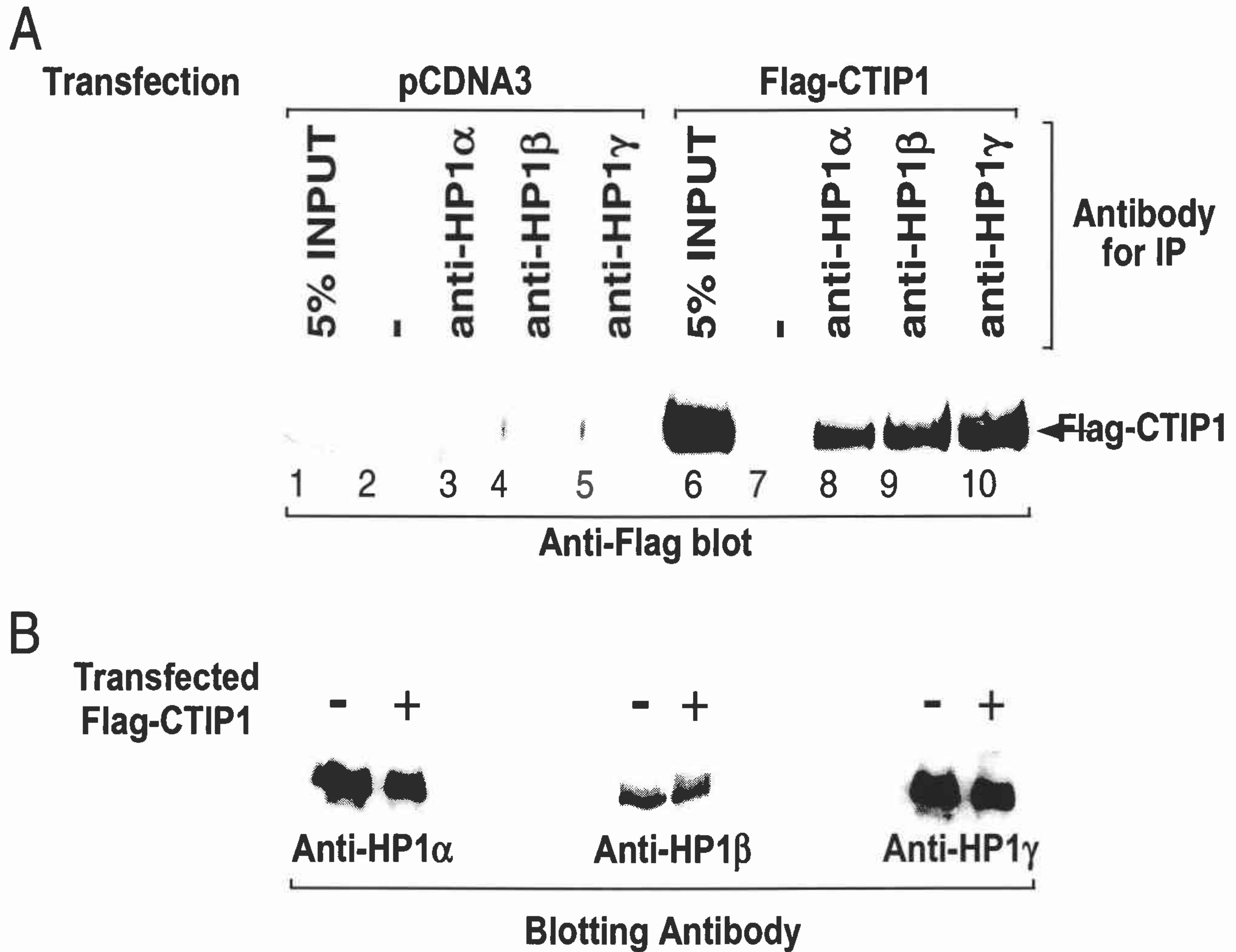

Figure 6. CTIP1 associates with HP1 proteins in mammalian cells. **(A)** Immunoprecipitation experiments using anti-HP1 α, β, and γ antibodies. Immunoprecipitates were subjected to immunoblot analysis using an anti-Flag antibody to reveal Flag-CTIP1 (indicated by arrow). **(B)** Expression of Flag-CTIP1 does not affect the quality of immunoprecipitated HP1 proteins. The immunoprecipitates in A were subjected to immunoblot analysis using HP1 antibodies to verify that equivalent amounts of HP1 proteins were immunoprecipitated in the absence and presence of Flag-CTIP1 expression. The levels of immunoprecipitated HP1 α, β, and γ did not vary as a function of Flag-CTIP1 expression.