Downstream signaling mechanism of the C-terminal activation domain of transcriptional coactivator CoCoA

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ABSTRACT

The coiled-coil coactivator (CoCoA) is a transcriptional coactivator for nuclear receptors and enhances nuclear receptor function by the interaction with the bHLH-PAS domain (AD3) of p160 coactivators. The C-terminal activation domain (AD) of CoCoA possesses strong transactivation activity and is required for the coactivator function of CoCoA with nuclear receptors. To understand how CoCoA AD transmits its activating signal to the transcription machinery, we defined specific subregions, amino acid motifs and protein binding partners involved in the function of CoCoA AD. The minimal transcriptional AD was mapped to approximately 91 C-terminal amino acids and consists of acidic, serine/proline-rich and phenylalanine-rich subdomains. Transcriptional activation by the CoCoA AD was p300-dependent, and p300 interacted physically and functionally with CoCoA AD and was recruited to a promoter by the interaction with CoCoA AD. The FYDVASAF motif in the CoCoA AD was critical for the transcriptional activity of CoCoA AD, the interaction of CoCoA with p300, the coactivator function of CoCoA for estrogen receptor α and GRIP1 and the transcriptional synergy among coactivators GRIP1, CARM1, p300 and CoCoA. Taken together these data extend our understanding of the mechanism of downstream signaling by the essential C-terminal AD of the nuclear receptor coactivator CoCoA; they indicate that p300 is a functionally important interaction partner of CoCoA AD and that their interaction potentiates transcriptional activation by the p160 coactivator complex.

INTRODUCTION

The p160 transcriptional coactivators, GRIP1, SRC-1 and AIB1, bind directly to nuclear receptors (NRs) and many other types of transcription factors and serve as protein scaffolds for the assembly of multicomponent coactivator complexes (1–3). The central NR interaction domain directly binds to NRs (4). The C-terminal activation domains (AD), AD1 (amino acids 1040–1120 of GRIP1) and AD2 (amino acids 1122–1462 of GRIP1), recruit the histone acetyltransferases p300 and CBP and histone methyltransferases such as CARM1 and PRMT1, respectively (5–8). These histone-modifying enzymes act synergistically with p160 coactivators to enhance NR function (7,9,10) and are recruited to the target gene promoters in a hormone-dependent manner (11–13).

The N-terminal AD, AD3 (amino acids 5–479 of GRIP1), recruits the coiled-coil coactivator (CoCoA), GRIP1-associated coactivator 63 (GAC63), Flightless I (Fli-I), ankyrin repeats containing cofactor-1 (ANCO-1), BAF57 and MMS19 (14–19). CoCoA binds to the basic helix–loop–helix/Per-Arnt-Sim (bHLH-PAS) domain (AD3) of p160 coactivators but not directly to NRs, and the coactivator activity of CoCoA for NRs is highly dependent on the presence of a p160 coactivator. CoCoA cooperates synergistically with GRIP1, CARM1 and p300 to enhance transcriptional activation by estrogen receptor (ER) α (15). In addition, CoCoA binds to and cooperates synergistically with β-catenin as a secondary coactivator for AR and TCF/LEF (20). Even though CoCoA was initially identified as a secondary coactivator for NRs, CoCoA binds directly to aryl hydrocarbon receptor (AHR) and AHR nuclear translocator (ARNT) and serves as a primary coactivator for AHR/ARNT (21). CoCoA is a physiologically relevant part of several transcriptional activation processes. Endogenous CoCoA binds to the native promoters of target genes for NR, AHR/ARNT and TCF/LEF transcription factors and is required for efficient ER, GRIP1, AHR/ARNT, TCF/LEF and β-catenin function (15,20,21).

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CoCoA has been dissected into several functional domains that contribute to its coactivator activity (Figure 1A). The central region (amino acids 150–500) of CoCoA contains the coiled-coil domain that interacts with the bHLH-PAS domains of p160 coactivators, AHR and ARNT (15,21). Both the N-terminal (amino acids 1–190) and C-terminal (amino acids 501–691) regions of CoCoA were capable of binding to β-catenin (20). The C-terminal domain of CoCoA possesses strong autonomous transcriptional activation activity when fused to the Gal4 DNA binding domain and is essential for the coactivator function of CoCoA for NRs, GRIP1 and AHR/ARNT (15,21). The AD of CoCoA consists of 20% acidic amino acids, 24% serine and proline (S/P) and 30% hydrophobic amino acids which are interspersed with acidic residues (Figure 1A).

To activate the transcription of a particular gene, transcriptional activators must orchestrate the assembly of transcriptional complexes of numerous proteins. The transcriptional ADs of transcription factors and coactivators interact with distinct coregulators and many general transcription factors to help assemble the basal transcription machinery (22,23). Therefore, a better understanding of the mechanism of gene regulation requires a precise delineation of these interactions. Although a large number of transcriptional ADs are known, these functional domains share little sequence homology and have only loosely been classified by the preponderance of amino acid residues such as glutamine, proline and acidic residues (22,23). However, the most important amino acid residues for transactivation are not necessarily the predominant residues such as glutamines or acidic amino acids. Instead, in many cases, hydrophobic residues that are interspersed with acidic amino acids appear to be important elements for transactivation (24,25). Some ADs assume a specific three-dimensional structure only upon binding to an interaction partner, thus undergoing an induced fit (26–28). This low homology of ADs and their conformational flexibility have made it difficult to characterize these essential domains in transcription factors and coactivators.
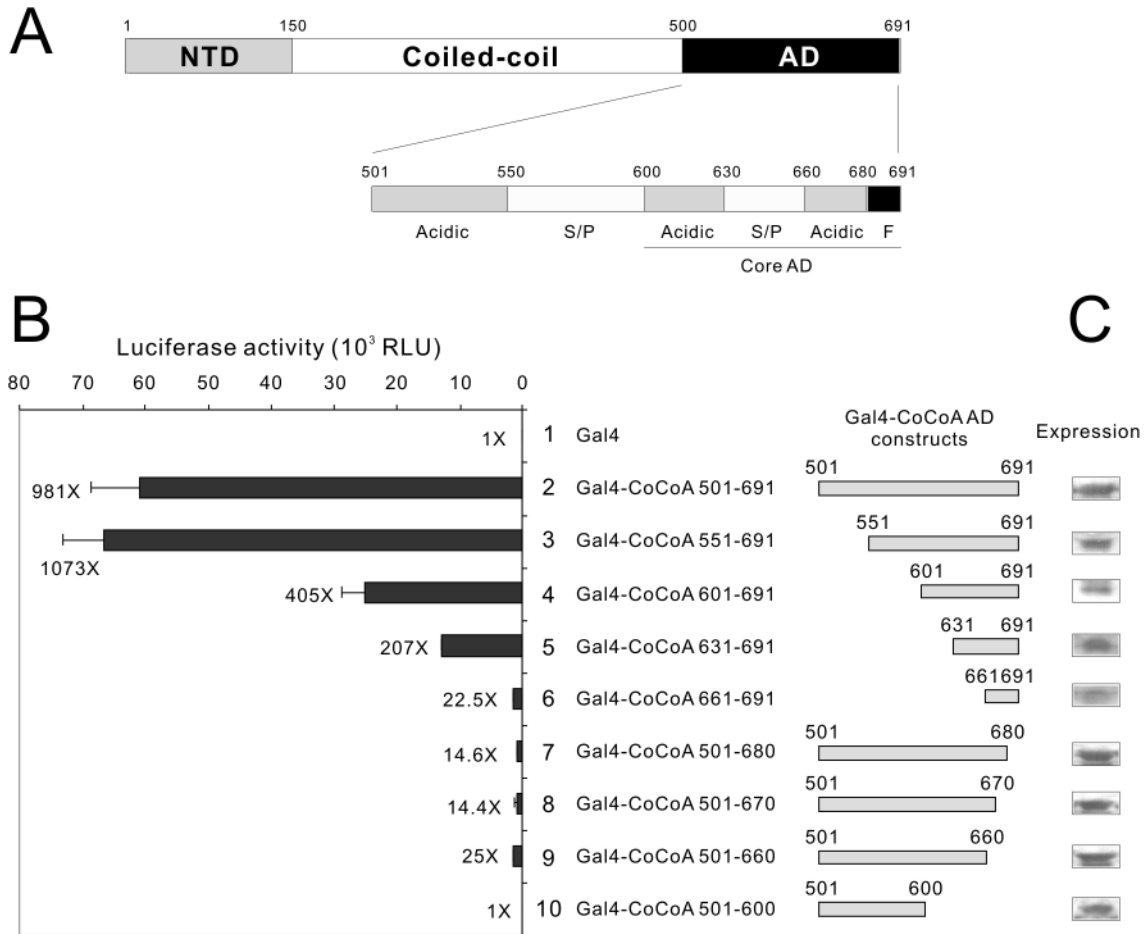

Figure 1. Fine mapping of the transcriptional AD of CoCoA. (**A**) Domain structure of CoCoA. The coiled-coil domain interacts with the bHLH-PAS domains of transcriptional activators and coactivators. The function of the N-terminal domain (NTD) is unknown. The C-terminal 191 amino acids of CoCoA function as a transcriptional AD. The CoCoA AD consists of a phenylalanine (F)-rich subdomain, three acidic subdomains and two serine and proline (S/P)-rich subdomains. In the diagrams, numbers indicate CoCoA amino acid positions. (**B**) CV-1 cells in 12-well plates were transfected with GK1-LUC (200 ng) and a plasmid encoding Gal4 DBD or Gal4 DBD fused to various CoCoA AD fragments (200 ng), as indicated. Luciferase activity was measured 48 h after transfection. Luciferase activity results shown are from a single experiment which is representative of three independent experiments. Fold induction compared with Gal4 DBD is shown to the left of each bar. (**C**) Expression levels of Gal4-CoCoA proteins. COS-7 cells were transfected with pM vectors encoding the indicated CoCoA fragment. Cell extracts were subjected to immunoblot analysis with antibodies against the Gal4 DBD.

Identification of functional sequence motifs that are hidden in ADs would help to dissect the functions of ADs and understand their composite regulations.

To understand the function of CoCoA AD in the p160 coactivator complex and its mechanism of action, we characterized CoCoA AD and identified important subregions and amino acid motifs for its transactivation function. Deletions and mutations of these motifs of CoCoA AD significantly reduced coactivator activity of CoCoA for GRIP1 and ER and impaired synergy among GRIP1, CARM1, p300 and CoCoA. Protein–protein interaction assays and chromatin immunoprecipitation (ChIP) assays demonstrated that CoCoA AD recruits p300. This interaction was essential for transcriptional activation by CoCoA AD. Moreover, CoCoA mediated the interaction between p300 and GRIP1 AD3. Finally we demonstrated that CoCoA and p300 synergistically enhanced the transcriptional activity of GRIP1 AD3 in a CoCoA AD-dependent manner. Our results suggest that recruitment of p300 through CoCoA AD contributes to CoCoA-mediated transactivation.

MATERIALS AND METHODS

Plasmids

pSG5.HA-CoCoA, pSG5.HA-CoCoA 1–500, pSG5.HA-CoCoA 150–500, pSG5.HA-CoCoA 150–691, pSG5.HA-CoCoA 1–190, pSG5.HA-CoCoA 470–691, pSG5.HA-GRIP1N (amino acids 5–479), pSG5.HA-GRIP1, pSG5.HA-CARM1, pcDNA3.1-CoCoA/V5-HIS, pCMV-p300, pM.CoCoA 501–691, pM.CoCoA 470–691, pM.GRIP1N, pHE0 and GK1-LUC were described previously (15). The following plasmids were described previously as indicated: pGEX-p300-KIX, pGEX-p300-CH3, pGEX-p300-Q, pGEX-p300-GBD, pGEX-p300-N, pGEX-p300-C (29); pM.GRIP1 (30). PCR amplification and subcloning into the indicated restriction sites were performed to create plasmids encoding the following CoCoA fragments (with amino acid numbers): CoCoA 501–691, CoCoA 1–149, CoCoA 274–510 into EcoRI and XhoI sites of pSG5.HA (8); CoCoA 501–600, CoCoA 601–691 into EcoRI and XhoI sites of pSG5.HA and EcoRI and SalI sites of pM (Clontech); CoCoA 501–660, CoCoA 501–670, CoCoA 501–680, CoCoA 551–691, CoCoA 631–691, CoCoA 661–691 into EcoRI and SalI sites of pM; CoCoA 1–600 into XhoI and BglII sites of pSG5.HA. Mutations were introduced in the CoCoA cDNA by site-directed mutagenesis using the Quick-Change II Site-Directed Mutagenesis Kit (Stratagene). The sequences of oligonucleotides used for mutagenesis are available upon request. p300 1155–1673 (HAT) was cloned into EcoRI and XhoI sites of pGEX-5X-1 (Amersham). To generate the 2ERE-TATA-LUC reporter plasmid, double stranded oligonucleotides containing two ERE sequences (15) and a minimal TATA sequence (-42 to $+14$) of *Xenopus laevis* vitellogenin B1 promoter were cloned into MluI and XhoI sites and BglII and HindIII sites, respectively, of the pGL3-basic vector (Promega). E1A and E1AΔ2-36 expression plasmids were kind gifts from Fang Liu (State University of New Jersey) (31). pCI-FLAG-p300 and its ΔHAT mutant expression plasmids, pCI-FLAG-p300Δ1472-1522

and pCI-FLAG-p300Δ1603-1653, were kindly provided by Yoshihiro Nakatani (Harvard University) (32). pcDNA3.1-HDAC1.Myc and pcDNA3.1-HDAC4.Myc were kindly provided by Mitchell A. Lazar (University of Pennsylvania) (33).

Cell Culture and Transient Transfection

CV-1, COS-7, HEK 293T and HLR cells were grown in DMEM with 10% fetal bovine serum. For reporter gene assays, CV-1 cells were plated at 5×10^4 or 1×10^5 cells/well in 24-well or 12-well plates, respectively, and transiently transfected by TargeFect F1 reagent (Targeting Systems). Total amount of plasmid DNA added to each well was adjusted to 0.6 or 1.0 μg by adding the necessary amount of pSG5.HA empty vector. For ER experiments, after transfection, cells were incubated in phenol red-free DMEM containing 5% fetal bovine serum treated with dextran-coated charcoal (Gemini Bioproducts), 20 mM Na-HEPES, pH 7.2, penicillin and streptomycin, with or without 100 nM E2. Cell extracts were prepared and assayed for luciferase activity 48 h after transfection, as described previously (15). HEK 293T cells were transiently transfected using Lipofectamine 2000 (Invitrogen) as described previously (21). The results shown are the means and SD of triplicate points. Instead of using internal controls, results shown are representative of multiple independent experiments, as indicated in figure legends.

Histone Acetyltrasferase (HAT) assay

COS-7 cells were transfected with pSG5.HA-CoCoA or pSG.HA-CoCoA 470–691. HA-tagged CoCoA and CoCoA 470–691 expressed in COS-7 cells were immunoprecipitated with 1 μg anti-HA antibody 3F10 (Roche) and 20 μl protein A/G agarose suspension (Santa Cruz Biotechnology). Immunoprecipitates were incubated at 30°C for 1 h with 5 μg calf thymus core histones (type IIA, Sigma) and 0.5 μCi [³H]-acetyl-CoA (Amersham) in buffer containing 50 mM Tris–HCl (pH8.0), 30 mM KCl, 10% glycerol, 10 mM sodium butyrate, 1 mM DTT in a total volume of 30 μl. Reactions were stopped by adding 4× SDS sample buffer and fractionated on 15% SDS–PAGE gels. Gels were fixed with 50% (vol/vol) methanol-10% (vol/vol) acetic acid for 30 min, then soaked with Amplify (Amersham) for 30 min, and vacuum dried. Fluorography was performed overnight.

Coimmunoprecipitation and immunoblotting

COS-7 or HEK 293T cell transfection, coimmunoprecipitation (CoIP) and immunoblotting were performed as described previously (15,21). For CoIP, cell lysate containing 1 mg of protein was incubated with 1 μg of the anti-p300 antibody RW128 (Upstate) or 1 μg of mouse normal IgG (Santa Cruz Biotechnology) and 20 μl of protein A/G agarose suspension or 20 μl of Ni-NTA HIS-Bind agarose (Novagen) overnight at 4°C. Immunoblots were then performed on the precipitated proteins with anti-HA antibody 3F10, anti-p300 antibody RW128 or anti-V5 antibody R960-25 (Invitrogen). Gal4 DBD fusion proteins were detected using an anti-Gal4 DBD antibody (Upstate).

GST pull-down assay

HA or V5 epitope-tagged CoCoA and its fragments were synthesized *in vitro* using TNT-Quick coupled transcription/ translation system (Promega) according to the manufacturer's protocol. GST pull-down assays were performed as described previously (15,21). GST fusion proteins were expressed in *Echerichia coli* BL21 and bound to glutathione-Sepharose-4B beads (Amersham Pharmacia). Bound proteins were analyzed by immunoblot with anti-HA antibody or anti-V5 antibody R960-25.

Chromatin immunoprecipitation and reporter coimmunoprecipitation

HLR cells (Stratagene) with stably integrated Gal4-LUC reporter (pFR-LUC) in 150 mm dishes were transiently transfected with pM or pM.CoCoA 501–691 (8 μg) using Lipofectamine 2000. ChIP assays were performed largely as described previously (15,21). After 48 h transfection, the cross-linked, sheared chromatin solution was used for immunoprecipitation with 1 μg of anti-Gal4 antibody 06–262 (Upstate), 1 μg of anti-p300 antibody RW128, or 1 μg of mouse normal IgG. The immunoprecipitated DNAs were purified by phenol–chloroform extraction, precipitated by ethanol and amplified by PCR using primers flanking the Gal4 responsive elements: 5′-GGTAC-CGAGCTCGAATTCCAGCTT-3′ (forward) and 5′-GCGT-ATCTCTTCATAGCCTTATGC-3′ (reverse). Quantitative real-time PCR (qPCR) was performed with Brilliant SYBR Green QPCR Master Mix according to the manufacturer's instructions (Stratagene). The qPCR conditions were identical to the standard PCR. Reporter Co-IP assays were performed as described previously (15,34). COS-7 cells in 100 mm dishes were transfected with pG5-LUC (Promega) and pGL3-Basic (Promega) reporter plasmids and Gal4 DBD or Gal4-CoCoA 501–691 expression plasmids as indicated in figure legend. Soluble chromatin fraction was prepared and immunoprecipitated, and PCR was performed as described previously (15,34). The following primers spanning the Gal4 responsive elements of pG5-LUC reporter were used for PCR amplification: 5′-CAAGTGCAGGTGCCAGAACA-3′ (forward) and 5′-CCACCTCGATATGTGCATCTGT-3′ (reverse). The amount of each immunoprecipitated DNA sample was titrated to determine the linear range for the PCR reactions, and the results shown are within the linear range.

RESULTS

Fine mapping of CoCoA AD

The transcriptional AD of CoCoA was mapped to amino acids 501–691 in our previous study (Figure 1A) (15). To further define the CoCoA transcriptional AD more precisely, progressive N-terminal or C-terminal deletions were introduced in the C-terminal AD of CoCoA. Various regions in CoCoA AD were fused to the Gal4 DBD and fusion constructs were cotransfected with a reporter plasmid carrying Gal4 responsive elements. The C-terminal AD (amino acids 501–691) of CoCoA showed strong transactivation activity (Figure 1B, assay 2). A mutant with a deletion of 50 amino

acids from the N-terminus of CoCoA AD retained wild-type activity (Gal4-CoCoA 551–691) (assay 3). Further deletion of the N-terminal sequences sharply reduced the transactivating potential of CoCoA AD. However, although Gal4-CoCoA 601–691 and Gal4-CoCoA 631–691 lost ∼60 and 80%, respectively, of the wild-type CoCoA AD activity (assays 4–5), we still observed ∼400-fold and 200-fold stimulation of reporter gene activity. Gal4-CoCoA 661–691 showed a nearly 95% loss of function (assay 6). Interestingly, deletion of 11 amino acids (681–691) from the extreme C-terminus, leaving Gal4-CoCoA 501–680, almost abolished strong transactivation activity, and further deletion up to 660 (leaving Gal4-CoCoA 501–660) had no further effect (assays 7–9). Further deletion of an additional 60 amino acids (Gal4-CoCoA 501–600) virtually abolished the transcriptional potential of CoCoA AD (assay 10). All of the fusion proteins were expressed at similar levels (Figure 1C). These results suggest that the transactivation ability of CoCoA is primarily contained within the residues 601–691 (the minimal or core AD) and that this minimal AD can be divided into two subdomains, amino acids 601–660 (acidic/S/P-rich subdomain) and amino acids 661–691 (acidic/F-rich subdomain) (Figure 1A). The combination (core AD) of these two subdomains can synergistically activate transcription (assays 4, 6 and 9). The residues 501–600 may be a subdomain that can affect the degree of CoCoA transactivation activity. In addition, the results show that the last 11 amino acids (F-rich region) of CoCoA are essential for its strong transcriptional activity.

CoCoA is associated with p300

To investigate the mechanism through which CoCoA activates transcription and identify proteins capable of interacting with CoCoA AD, we tested whether CoCoA is associated with histone-modifying enzymes. We performed histone acetyltransferase (HAT) assays using HA-tagged CoCoA and CoCoA 470–691 fragment immunoprecipitated with anti-HA antibody from COS-7 cell extracts. The anti-HA immunoprecipitates from HA-CoCoA and HA-CoCoA 470–691 transfected cells had higher levels of HAT activity, compared with similar immunoprecipitates from control cells transfected with pSG5.HA empty vector (Figure 2A). This observation suggests that CoCoA physically associates with an HAT activity. The bacterially expressed GST-CoCoA and GST-CoCoA 470–691 did not harbor any intrinsic HAT activity (data not shown), suggesting that CoCoA is not an HAT by itself.

To test whether CoCoA can interact with p300 in cells, COS-7 cells were transfected with CoCoA-His and p300, and cell lysates were pulled down with Ni-bound agarose or agarose beads, followed by immunoblotting analysis with anti-p300 antibody. p300 was identified along with CoCoA among the proteins pelleted by Ni-agarose beads but not by control agarose beads (Figure 2B). Similar data were obtained with p300 and CoCoA-His in HEK 293T cells (data not shown). In a reciprocal experiment, p300 was transfected into COS-7 cells alone or in combination with HA-CoCoA, and CoCoA was coimmunoprecipitated specifically with p300 (Figure 2C). These results indicate that CoCoA can physically interact with p300 *in vivo*.
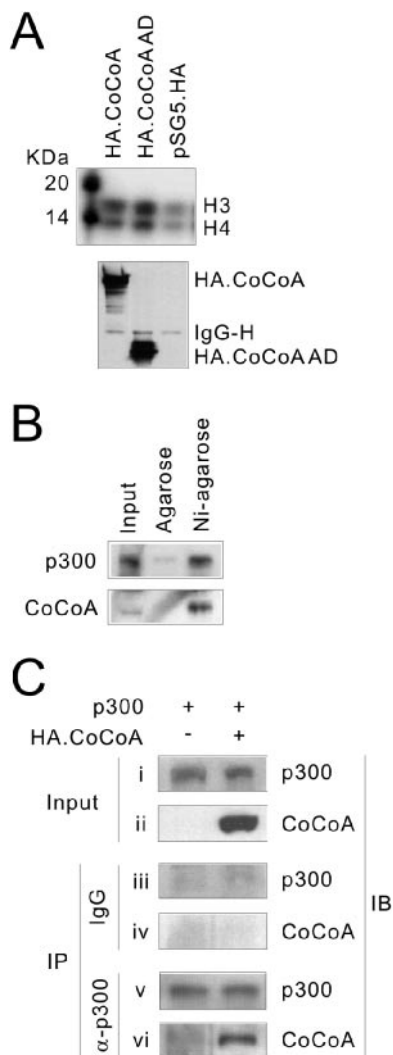
Figure 2. CoCoA is associated with p300. (**A**) CoCoA is associated with HAT activity. COS-7 cells were transfected with pSG5.HA-CoCoA, pSG5.HA-CoCoA 470–691 or pSG5.HA (3 μg) and immunoprecipitated with 1 μg of anti-HA antibody. Immunoprecipitates were assayed for HAT activity using 5 μg of core histones. Of immunoprecipitated samples 50% were also analyzed by western blotting using the anti-HA antibody to detect immunoprecipitated CoCoA proteins. IgG-H, immunoglobulin G heavy chain from the immunoprecipitation. These results were reproducible from three independent experiments. (**B**) Interaction of CoCoA with p300 *in vivo*. COS-7 cells were transfected with pcDNA3.1-CoCoA.V5/HIS (3 μg) and pCMV-p300 (6 μg). Cell extracts were prepared 48 h after transfection and incubated with Ni-NTA HIS-Bind agarose beads or control agarose beads. Bound CoCoA-V5/HIS and coprecipitated p300 proteins were detected by immunoblot analysis with anti-V5 antibody or anti-p300 antibody. A portion of the original cell extract was also examined for CoCoA and p300 expression (5% input). (**C**) COS-7 cells were transfected with pCMV-p300 (6 μg) either alone or together with pSG5-HA.CoCoA (3 μg). Cell lysates were immunoprecipitated (IP) with anti-p300 antibody (v and vi) or control normal mouse IgG (iii and iv). Immunoprecipitated p300 (v) and coimmunoprecipitated HA-CoCoA (vi) were detected by immunoblotting (IB) with anti-p300 or anti-HA antibodies. A portion of the original cell extract was also examined for CoCoA and p300 expression (5% input, i and ii).

CoCoA interacts with KIX and CH3 domains of p300

To identify the part(s) of p300 that can interact with CoCoA, the N-terminal, KIX, HAT and C-terminal domains of p300 (Figure 3A) were expressed as GST fusion proteins in

E.coli, purified, and incubated with *in vitro* translated CoCoA. GST-p300 KIX and GST-p300 C bound efficiently to CoCoA, whereas GST-p300 N and GST-p300 HAT failed to do so (Figure 3B). Next, we scanned the p300 C-terminal fragment for interaction with CoCoA using three GST-p300 fusion proteins encoding CH3, GBD (GRIP1 binding domain) and Q-rich regions (Figure 3A). Although the p300 Q-rich region possessed marginal binding activity, CH3 domain bound with greater efficiency to CoCoA (Figure 3C). These results indicate that p300 contains two main regions, KIX and CH3, which can bind to CoCoA.

Using similar approaches, we investigated the region in CoCoA that is required for the interaction with p300. While CoCoA AD (amino acids 501–691) and the N-terminal domain (amino acids 1–190) were able to bind p300 KIX and CH3 domains, CoCoA coiled-coil domain (amino acids 150–500) failed to interact with p300 KIX and CH3 domains (Figure 3D and E). These results therefore suggest that CoCoA contains at least two autonomous and separable interaction domains for p300. In these assays, CoCoA fragments were capable of interacting with p300 as long as they contained at least the C-terminal AD. Interestingly, the coiled-coil domain (amino acids 150–500) negatively regulated the interaction between the N-terminal domain of CoCoA and p300, while it did not affect CoCoA AD binding to p300 (Figure 3D and E). The core AD (CoCoA 601–691) itself still interacted strongly with p300 (Figure 3F). However, deletion of the core AD drastically reduced p300 KIX interaction relative to the full length (amino acids 501–691) and core (amino acids 601–691) ADs but did not abrogate it (Figure 3D). Thus, the core AD is required and sufficient for the interaction with p300. The control GST alone failed to bind any of the *in vitro* translated proteins. Therefore, p300 KIX and CH3 domains interact with CoCoA AD, and the core AD is important for that interaction. Thus, the major p300 binding domain of CoCoA (defined in Figure 3) co-localizes with the core AD (defined in Figure 1B). Taken together, our data show that CoCoA directly interacts with KIX and CH3 domains of p300 and that CoCoA domains involved in the interaction with p300 are distinct from the GRIP1-binding domain in the coiled-coil region.

Endogenous p300 is required for the transcriptional activity of CoCoA AD

As an initial test of p300 dependence, we tested the effects of p300 expression on the ability of CoCoA AD to activate transcription in a mammalian one-hybrid assay (Figure 4A). A p300 expression plasmid was cotransfected with various Gal4-CoCoA AD expression plasmids into 293T cells. In these assays, p300 activated CoCoA AD-mediated transcriptional activation about 5-fold (assays 6). However, coexpression of the coactivator PCAF had no apparent effect (data not shown). The transcriptional activity mediated by CoCoA 501–680, CoCoA 501–660 or CoCoA 661–691 was also enhanced by p300 about 16-fold, 15-fold and 8-fold, respectively (assays 2, 3 and 5). In contrast, p300 had a minimal effect (~2-fold) on the transcriptional activity of Gal4 alone or Gal4-CoCoA 501–600 lacking core AD (assays 1 and 4). These results suggest functional interaction between
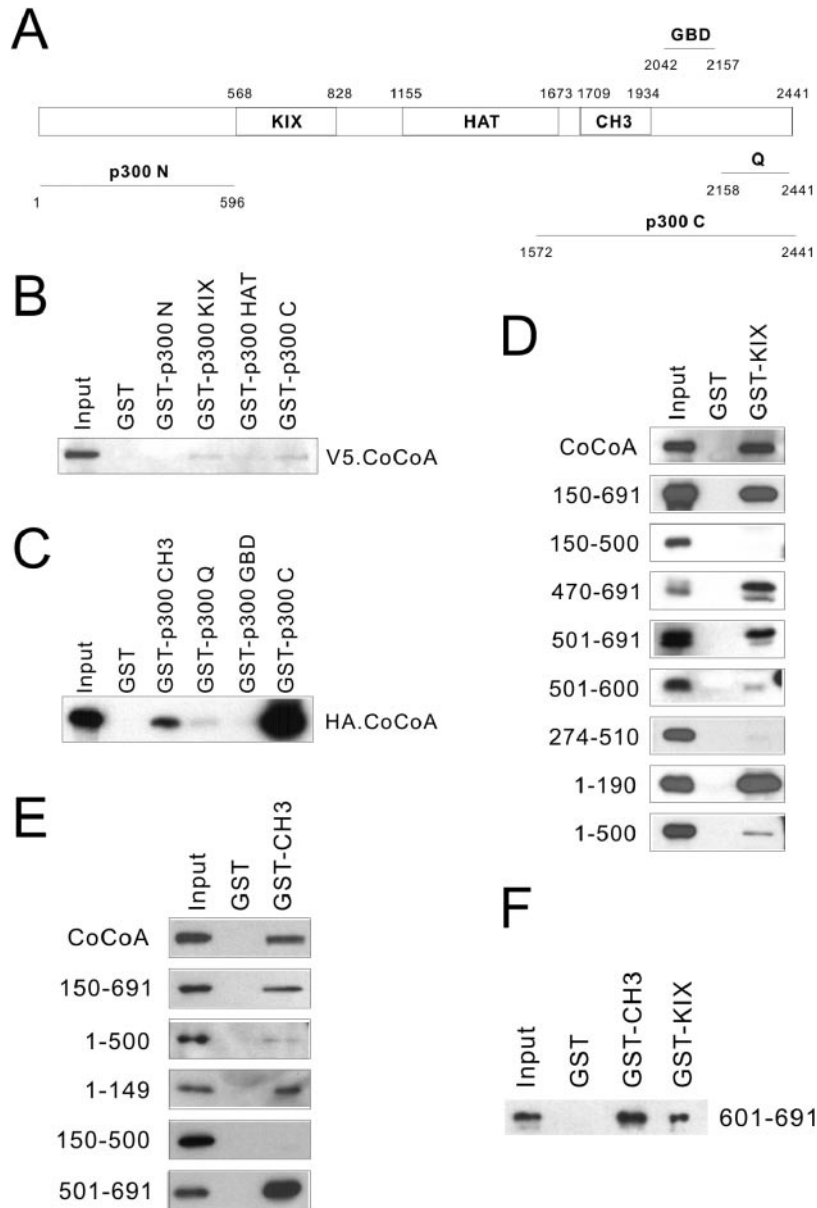
Figure 3. Domain requirements for CoCoA and p300 interaction. (**A**) Schematic representation of p300-derived protein fragments fused to GST. (**B** and **C**) CoCoA interacts with KIX and CH3 regions of p300. Equal amounts of various GST-fusion fragments of p300 were incubated with *in vitro* translated V5- or HA-tagged CoCoA. Bound proteins were analyzed by immunoblot analysis with anti-V5 or anti-HA antibodies. A portion of *in vitro* translated protein was loaded directly (10% input). (**D**) Interaction of CoCoA with the KIX domain of p300 *in vitro*. HA-tagged CoCoA fragments synthesized *in vitro* were incubated with equal amounts of GST or GST-KIX fusion protein. Bound proteins were analyzed by immunoblot with anti-HA antibody. A portion of *in vitro* translated protein was loaded directly (10% input). (**E**) Interaction of CoCoA with the CH3 domain of p300 *in vitro*. GST pull-down assays were performed as describe above using *in vitro* translated CoCoA or its fragments and GST or GST-CH3 bound to beads. (**F**) Core AD of CoCoA interacts with KIX and CH3 regions of p300. GST pull-down assays were performed as described above using *in vitro* translated CoCoA 601–691 and GST, GST-KIX or GST-CH3 bound to beads.

CoCoA AD and p300 and demonstrate that the core AD (amino acids 601–691) of CoCoA AD is essential for the ability of CoCoA AD to be enhanced by p300.

To assess the role of endogenous p300/CBP in transcriptional activation by CoCoA AD, we used the p300/CBP inhibitor E1A. This viral protein inhibits p300/CBP-dependent transactivation by a number of transcription factors that interact with p300/CBP (35,36). To investigate whether E1A interferes with CoCoA AD-mediated transcription, we performed mammalian one-hybrid assays using

Gal4-CoCoA 501–691 and E1A constructs in 293T cells (Figure 4B). The wild-type E1A repressed CoCoA AD activity in a dose-dependent manner (assays 2–5). In contrast, the mutant E1AΔ2-36, which lacks the interaction domain for p300/CBP, had no significant inhibitory effect on the transcriptional activity of CoCoA AD (assays 6–9), while the expression level of the mutant was even higher than that of wild-type E1A (Figure 4D). The inhibitory effect of E1A on CoCoA AD-mediated transcription indicated that endogenous p300 may be involved in this process.
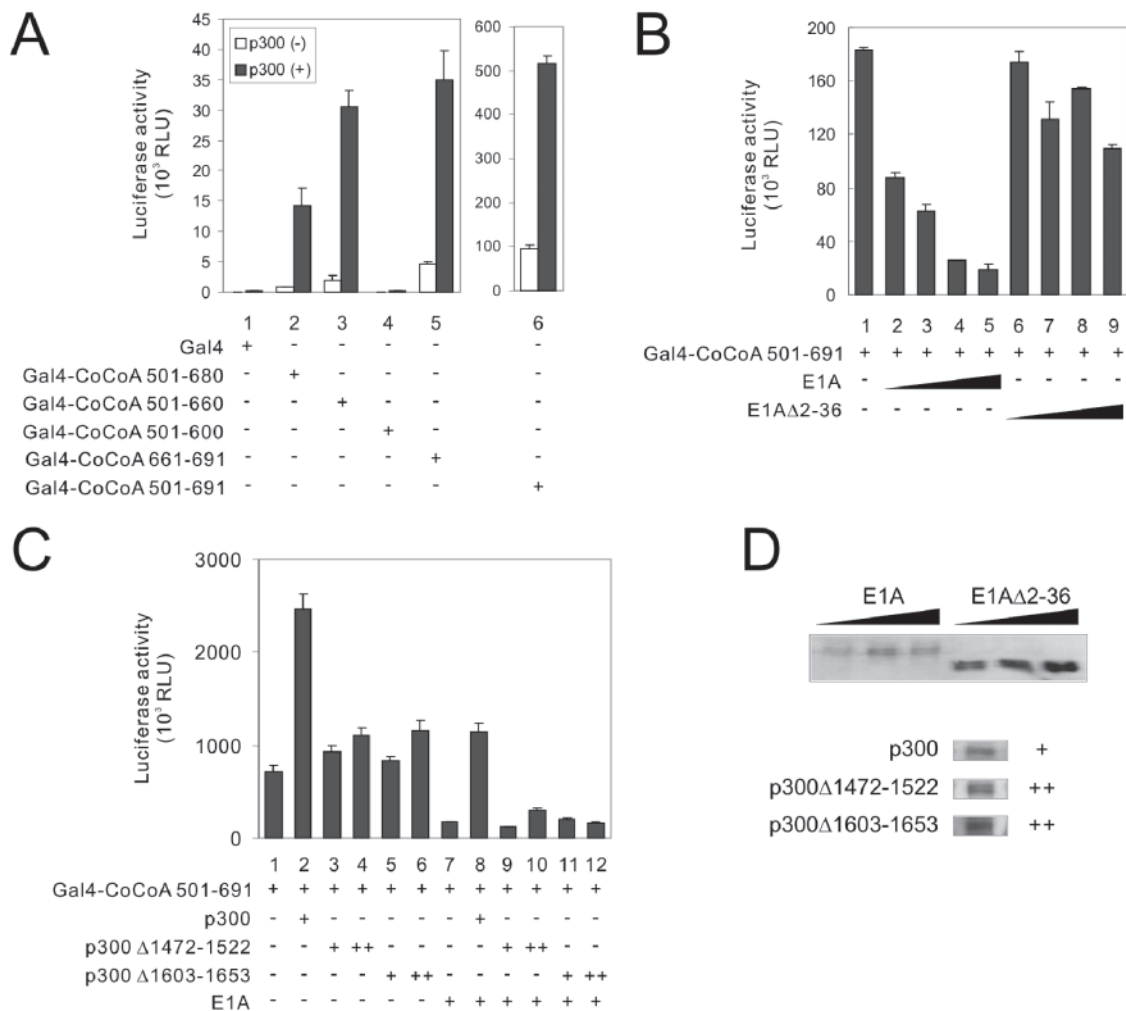
Figure 4. Requirement of p300 for the transactivation activity of CoCoA AD. (**A**) p300 enhances the transactivation activity of CoCoA AD. 293T cells in 24-well plates were transfected with GK1-LUC (100 ng), plasmids encoding Gal4 DBD or Gal4-CoCoA AD fragments (100 ng), and pCMV-p300 (100 ng), as indicated. Luciferase activity was measured 24 h after transfection. Luciferase activity results shown are from a single experiment which is representative of four independent experiments. (**B**) Adenovirus E1A expression inhibits CoCoA AD-mediated transactivation. 293T cells in 24-well plates were transfected with GK1-LUC (100 ng), Gal4-CoCoA 501–691 (100 ng), and variable amounts of E1A expression plasmids (25, 50, 100 and 200 ng), as indicated. Luciferase activity was measured 24 h after transfection. Luciferase activity results shown are from a single experiment which is representative of four independent experiments. (**C**) HAT activity of p300 is required for its function as coactivator of CoCoA AD. Transient transfections using pCI-FLAG-p300 (100 ng) or pCI-FLAG-p300ΔHAT mutants (+, 100 ng and ++, 200 ng) and E1A (50 ng) were performed as in (B). Luciferase activity results shown are from a single experiment which is representative of four independent experiments. (**D**) Expression levels of transfected E1As and p300s. COS-7 cells were transfected with 50, 100 and 200 ng of E1A expression plasmids. The expression levels of E1As were detected with anti-E1A antibody. The expression levels of FLAG-tagged p300s (+, 100 ng and ++, 200 ng) transfected in 293T cells were detected using anti-FLAG antibody.

We also tested whether the HAT activity of p300 is necessary to enhance CoCoA AD activity and whether p300 can rescue E1A-mediated repression (Figure 4C). Again, expression of p300 enhanced the transcriptional activity of CoCoA AD (assay 2). In contrast, no significant enhancement was observed when p300ΔHAT mutants were expressed (assays 3–6), while mutant and wild-type p300s were expressed at similar levels when 100 ng of wild-type p300 and 200 ng of p300 ΔHAT mutants were used in the transfection experiment (Figure 4D). Interestingly, although p300 rescued E1A-mediated repression (assay 8), p300ΔHAT mutants failed to rescue CoCoA AD activity (assays 9–12). Thus, the HAT activity of p300 is essential for its ability to potentiate transcriptional activation mediated by CoCoA AD. Overall, these results demonstrate that the interaction with

enzymatically active p300 is important for and correlates with the transactivation activity of CoCoA AD.

Endogenous p300 is recruited to the promoter by CoCoA AD

A modified ChIP experiment (reporter CoIP) was performed to test whether endogenous p300 participates in the transcriptional activation by CoCoA AD. COS-7 cells were transfected with pG5-LUC reporter plasmid and Gal4 DBD or Gal4-CoCoA AD expression vector. As an internal control, the pGL3-Basic reporter plasmid, which has the same plasmid backbone as pG5-LUC but no Gal4 response elements, was also included in the transfection. The cross-linked and sheared nuclear fractions were subjected to

immunoprecipitation with anti-Gal4 and anti-p300 antibodies, and the precipitated DNA was analyzed by PCR amplification using primers against sequences on the backbone of the pGL3-Basic reporter plasmid. PCR amplification using these primers produces a 406 bp product from the pG5-LUC reporter and a 269 bp product from the pGL3-Basic reporter (Figure 5A). Similar input PCR signals were generated from pG5-LUC and pGL3-Basic reporters, indicating similar amounts of the two reporters in the transfected COS-7 cells. Gal4 DBD only and Gal4-CoCoA AD were preferentially recruited to the pG5-LUC reporter containing the Gal4 responsive elements. However, preferential recruitment of endogenous p300 to the pG5-LUC reporter was observed only in the presence of Gal4-CoCoA AD, compared with the p300 recruitment to the pG5-LUC reporter in the COS-7 cells transfected with the Gal4 DBD expression vector. A no-antibody control showed equal low background levels of both plasmids. Therefore, endogenous p300 is specifically recruited to the Gal4 responsive elements through the interaction with Gal4-CoCoA AD.

To further investigate whether endogenous p300 is recruited to a chromosomally integrated Gal4-regulated promoter through the interaction with CoCoA AD, we used a HeLa cell line (HLR) harboring a stably integrated reporter gene controlled by 5 Gal4 response elements (pFR-LUC). HLR cells transfected with Gal4 DBD or Gal4-CoCoA AD expression vector were analyzed by the ChIP assay. Consistent with our reporter CoIP data, occupancy at Gal4 response elements was similar for Gal4 DBD only and Gal4-CoCoA AD; however, endogenous p300 was specifically recruited to the integrated Gal4 responsive elements in a CoCoA AD-dependent manner (Figure 5B). By quantitative real-time PCR analysis we observed a 5-fold increase in p300 recruitment to Gal4 responsive elements by Gal4-CoCoA AD, compared with Gal4 DBD alone, even though Gal4 DBD and Gal4-CoCoA AD were recruited to the Gal4 responsive elements with equal efficiency (Figure 5C). Thus, endogenous p300 is recruited to promoter-bound CoCoA AD, demonstrating a functional interaction between p300 and CoCoA AD occurring in an *in vivo* setting. Our reporter CoIP and ChIP data indicate that strong transactivation activity of CoCoA AD in mammalian one hybrid assays correlates with the recruitment of endogenous p300 to CoCoA AD.
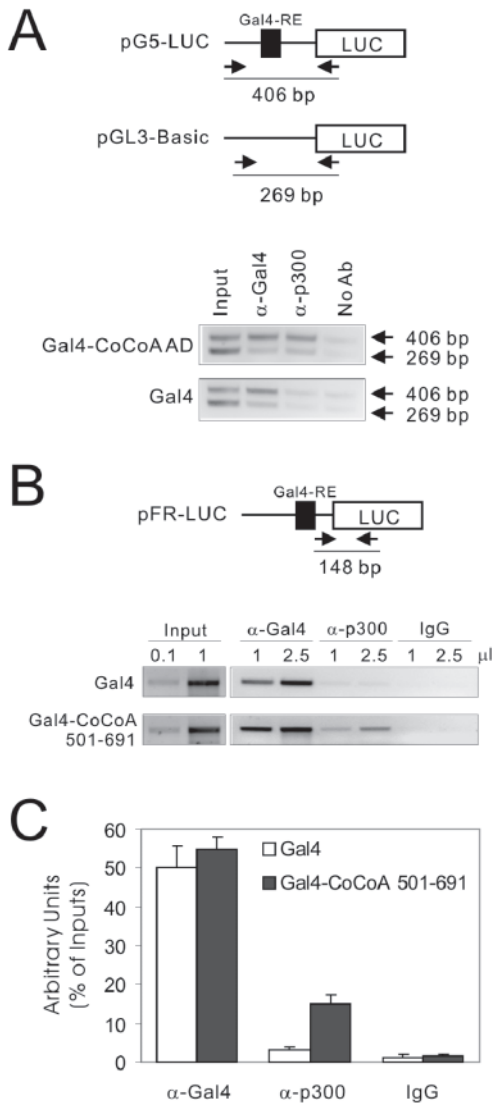
HDACs negatively regulate CoCoA AD-mediated transcription

The acetyltransferase activity of p300 is necessary to potentiate CoCoA AD activity (Figure 4C). We therefore investigated the functional impact of the HDAC inhibitor trichostatin A (TSA) and overexpression of HDACs on the transcriptional activity of CoCoA AD. TSA treatment augmented the transcriptional activity of Gal4-CoCoA AD fragments containing the core AD (Figure 6A, assays 2, 3 and 5). In contrast, the activity of Gal4 DBD only and Gal4-CoCoA 501–600 lacking the Core AD was only marginally

Figure 5. Specific recruitment of endogenous p300 to Gal4 responsive promoters by Gal4-CoCoA AD (501–691). (**A**) Recruitment to the transiently transfected Gal4 responsive promoter. COS-7 cells in 100 mm dishes were transfected with pG5-LUC (3 μg), pGL3-Basic (3 μg) and pM or pM.CoCoA 501–691 (3 μg). After 48 h transfection, crosslinked, sonicated nuclear extracts were immunoprecipitated with the indicated antibodies. PCR analysis was performed with primers that recognize identical backbone sequences in both reporter plasmids but produce different size PCR products from the two reporters, as illustrated in the diagram. The data shown were obtained with 0.1 μl of input DNA and 1 μl of precipitated DNA samples. Results shown are representative of three independent experiments. (**B**) Occupancy of the stably integrated Gal4 responsive promoter. HLR cells, containing a stably integrated Gal4-LUC reporter gene, were transiently transfected in 150 mm dishes with pM or pM.CoCoA 501–691 (8 μg). After 48 h transfection, crosslinked, sonicated nuclear extracts were immunoprecipitated with the indicated antibodies. The coprecipitated DNA was amplified by PCR using primers to amplify the Gal4 responsive promoter. PCR was performed with varying amounts of input (1, 0.1 and 0.01 μl) and precipitated DNA samples (5, 2.5, 1 and 0.2 μl) to determine the linear range for the amplification (data not shown). The data shown were obtained with 0.1 or 1 μl of input DNA and 1 or 2.5 μl of precipitated DNA samples. Position of the PCR primers amplifying a region of the Gal4 responsive promoter is indicated by the opposing pair of arrows in the diagram. The results shown are representative of two independent experiments. (**C**) Real-time PCR analysis was performed with the same primers and samples used in (B). The data shown were obtained with 1 μl of input and 1 μl or 2.5 μl of precipitated DNA samples (1 μl of α-Gal4 samples and 2.5 μl of α-p300 and IgG samples). The results are shown as percentage of input and are the mean and SD from triplicate reactions.
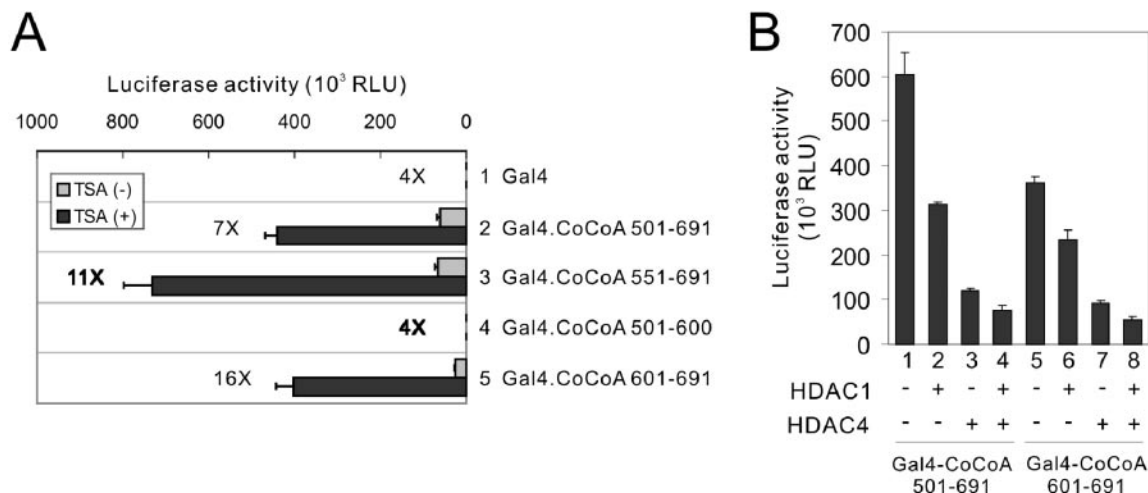
Figure 6. HDACs repress CoCoA AD activity. (**A**) TSA enhances the transactivation activity of CoCoA AD. CV-1 cells in 12-well plates were transfected with GK1-LUC (200 ng) and plasmids encoding Gal4 DBD or Gal4-CoCoA AD fragments (200 ng), as indicated. Cells were treated with 100 nM TSA for 18 h before harvesting. Luciferase activity was measured 48 h after transfection. Luciferase activity results shown are from a single experiment which is representative of three independent experiments. Fold induction by TSA is shown at the left of each pair of bars. (**B**) CV-1 cells were transfected with GK1-LUC (200 ng), plasmids encoding Gal4-CoCoA 501–691 or Gal4–CoCoA 601–691 (200 ng) and HDAC expression plasmids (200 ng), as indicated. Luciferase activity results shown are from a single experiment which is representative of two independent experiments.

affected by TSA (assays 1 and 4). Consistent with these results, overexpression of HDAC1 and HDAC4, either alone or together, led to the inhibition of the transcriptional activity of CoCoA AD (Figure 6B). These data suggest that HDACs can repress the transcriptional activity of CoCoA AD, and again that endogenous HAT activity is required for CoCoA AD activity.

Mutational analysis of FXXΦΦXXΦ motif in the Core AD

The core AD of CoCoA contains a stretch of amino acids FYDVASAF which fits a consensus sequence (F/YXX-ΦΦXXΦ, where X represents any amino acid and Φ represents any hydrophobic amino acid) shown to be important for the transcriptional activation function of several transcription factors and for their interaction with p300 KIX (37). This consensus sequence is highly conserved in CoCoA proteins of various species (Figure 7A). This motif is found in the ADs of p53, VP16, CREB and p65 (26,37–39). The relative importance of the putative KIX binding motif was examined by testing the effects of mutations in the core AD on its transcriptional activity in mammalian one-hybrid assays and its interaction with KIX and CH3 domains of p300 in GST pull-down assays. Secondary structure analysis using the HNN (hierarchial neural network) method (40) predicts that FYDVASAF motif of CoCoA AD has the potential to form an α-helical structure. The projection of residues 628–635 of core AD onto a helical wheel reveals that three hydrophobic residues, Phe-628, Val-631 and Phe-635, lie along one face of the helix (Figure 7B). These three residues were replaced with Ala (F628A, F628A/V631A and F628/635A). To disrupt the α-helix formation in another mutant, Asp-630, which is located on the other side of the helix, was substituted with helix breaking Pro (D630P). To analyze transactivation potential, each CoCoA AD point mutant was fused with Gal4 DBD, and its

expression plasmid was transfected into CV-1 cells with a Gal4-responsive reporter plasmid. All of the fusion proteins were expressed at similar levels (Figure 7D). Mutations F628A, D630P and F628A/V631A resulted in a reproducible reduction (~40–60%) in the level of transcriptional activity in mammalian one-hybrid assays, indicating that the helical structure and hydrophobic residues in the putative KIX binding motif are important for the transactivation activity of CoCoA AD (Figure 7C). Double mutation F628/635A more severely impaired the transactivation activity of CoCoA AD, demonstrating the critical role of these phenylalanine residues in creating a fully functional AD.

To determine whether the reduced transactivation potential of F628/635A mutant was paralleled by a reduction in physical interaction with p300, we performed GST pull-down experiments with p300 CH3 and KIX fragments fused to GST and *in vitro* translated CoCoA proteins. In low salt concentration (200 mM NaCl), the F628/635A mutant still retained its ability to bind p300 (data not shown). However, at a higher salt concentration (300 mM NaCl) wild-type CoCoA still strongly interacted with CH3 and KIX domains of p300, but little or no interaction above background was observed with the F628/635A mutant (Figure 7E). Thus, the two phenylalanine residues in the FYDVASAF motif of CoCoA AD are critical for high affinity interaction with the KIX and CH3 domains of p300. Taken together, these results suggest that the helical FYDVASAF motif of CoCoA AD contributes to the interaction with p300, and that CoCoA-p300 interaction contributes to the transcriptional activity of CoCoA AD.

The C-terminal AD of CoCoA is required for coactivator function of CoCoA and synergistic activation of ER function

We have previously shown that CoCoA functions as a secondary coactivator in transient transfection assays using
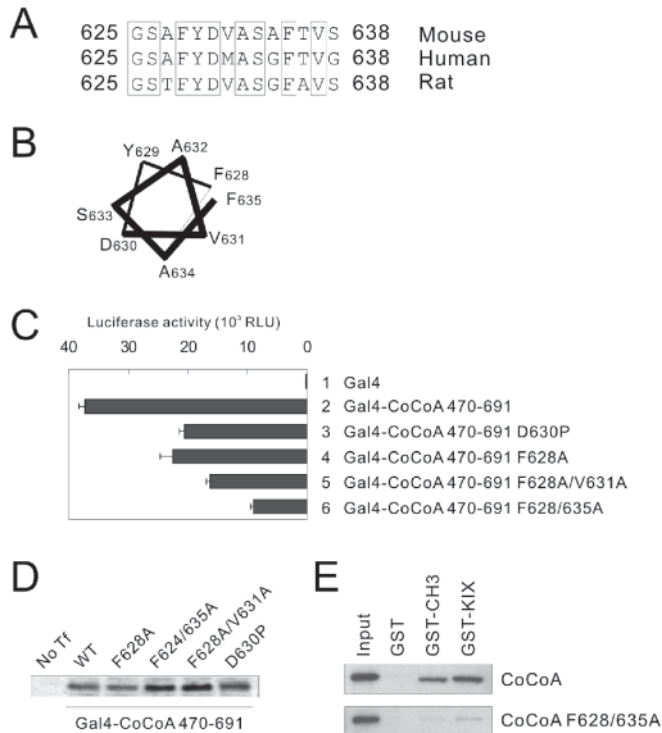
Figure 7. Helical FYDVASAF motif is important for the transcriptional activity of CoCoA AD and its binding to p300. (**A**) Alignment of amino acid sequences of the FXXΦΦXXΦ motif in the C-terminal AD of mouse, human and rat CoCoAs. The well-conserved amino acids are indicated by boxes. (**B**) Helical wheel presentation of residues 628–635 of CoCoA. (**C**) Mutations in the FYDVASAF motif impair the transactivation activity of CoCoA AD. CV-1 cells in 12-well plates were transfected with GK1-LUC (200 ng) and plasmids encoding Gal4 DBD, Gal4-CoCoA 470–691, or its mutants (200 ng), as indicated. Luciferase activity was measured 48 h after transfection. Luciferase activity results shown are from a single experiment which is representative of three independent experiments. (**D**) Expression levels of Gal4-CoCoA proteins. COS-7 cells were transfected with pM vectors encoding the indicated CoCoA fragment. Cell extracts were subjected to immunoblot analysis with antibodies against the Gal4 DBD. (**E**) p300 binding of CoCoA is greatly impaired by mutations at phenylalanine residues in the FYDVASAF motif. GST pull-down assays were performed using *in vitro* translated CoCoA or its mutant CoCoA F628/635A and GST, GST-KIX or GST-CH3 bound to beads as in Figure 3F.

relatively high level ER conditions. Furthermore, at low ER levels which allow synergy among three or more coactivators to be observed (10), GRIP1, CoCoA, p300 and CARM1 enhanced ER function in a synergistic manner (15). In our previous studies the C-terminal AD of CoCoA was required for CoCoA coactivator function to enhance the transcriptional activity of the bHLH-PAS domain of GRIP1 or AHR (15,21). We first examined the effect of the deletion and mutations of the C-terminal AD of CoCoA on its coactivator function at a high level of ER (Figure 8A). Full length CoCoA potentiated ER activity in the presence of GRIP1 (assays 4–5), as previously shown (15). In contrast, the coactivator activity of CoCoA was severely impaired by deletion of the core AD or point mutations in the FYDVASAF motif (assays 6–17). All of the CoCoA proteins were expressed at similar levels (data not shown).

To confirm that the core AD of CoCoA is necessary for its coactivator function, we next tested the effect of CoCoA or its AD deletion and point mutants on the transcriptional

activation function of GRIP1 in a mammalian one-hybrid system (Figure 8B). The modest transcriptional activation function of GRIP1 fused to Gal4 DBD was strongly stimulated by wild-type CoCoA (assay 4). In contrast, deletion of the core AD or mutations in the FYDVASAF motif resulted in significant loss of coactivator activity of CoCoA (assays 5–8). This is consistent with the data observed in ER experiments (Figure 8A). Thus, the core AD of CoCoA and its FYDVASAF motif are required for its coactivator function for GRIP1 and ER.

Previous studies have shown that CoCoA, p300 and CARM1 interact with different domains of p160 coactivators (AD3 for CoCoA, AD1 for p300 and AD2 for CARM1) (5,6,8,15); and four coactivators, GRIP1, CoCoA, p300 and CARM1, synergistically stimulate ER transcriptional activity (15). We tested the activity of CoCoA mutants in transient transfection assays using low-ER conditions, which facilitate multiple coactivator synergy (Figure 8C) (10,15). In agreement with a previous report (15), these four coactivators synergistically enhanced ER function, and this synergy was GRIP1-dependent (assays 4, 5, 12 and 14). Importantly, deletion of CoCoA AD (CoCoA mutants 1–500 and 1–600) severely reduced the synergy with p300, CARM1 and GRIP1 (assays 6, 8 and 15), while a CoCoA mutant (CoCoA 150–691) lacking the N-terminal domain exhibited wild-type synergy (assay 7). Note that all of the CoCoA deletion constructs used in this experiment have an intact coiled-coil domain and are capable of GRIP1 binding. CoCoA F628A and D630P mutants also acted synergistically with GRIP1, p300 and CARM1, but with reduced efficacy (assays 16–17). Interestingly, the double mutation within the FYDVASAF motif, F628/635A, resulted in nearly complete loss of transcriptional activity (assay 18), which was comparable with that of CoCoA 1–600 (assay 15). These results suggest that, within the core AD of CoCoA, two phenylalanines in the FYDVASAF motif are critical for the synergy. In addition, these two phenylalanine residues within the core AD are highly conserved in CoCoA proteins of various species (Figure 7A).

p300 enhances GRIP1 AD3 activity through the functional interaction with CoCoA

GRIP1 is believed to provide appropriate interaction surfaces, AD3 and AD1, for recruitment of CoCoA and p300, respectively, and the finding of direct physical interaction between CoCoA and p300 suggested that CoCoA, p300 and GRIP1 might form a ternary complex. To test the idea that CoCoA mediates the interaction between GRIP1 AD3 and p300 and to investigate the functional consequence of p300 on the activity of CoCoA, we tested the effect of p300 on the CoCoA-dependent transcriptional AD (AD3, bHLH-PAS domain) of GRIP1 in a mammalian one-hybrid system (Figure 9). As expected, CoCoA efficiently enhanced the transcriptional activation function of GRIP1 AD3 in a dose-dependent manner (assays 5 and 7). p300 alone had no effect on GRIP1 AD3 activity (assay 4), but in the presence of CoCoA, p300 synergistically enhanced GRIP1 AD3 function (assays 6 and 8). The dependence of p300 coactivator function for GRIP1 AD3 on CoCoA indicates that p300 associates indirectly with GRIP1 AD3 through CoCoA. To test the importance of the functional interaction between CoCoA
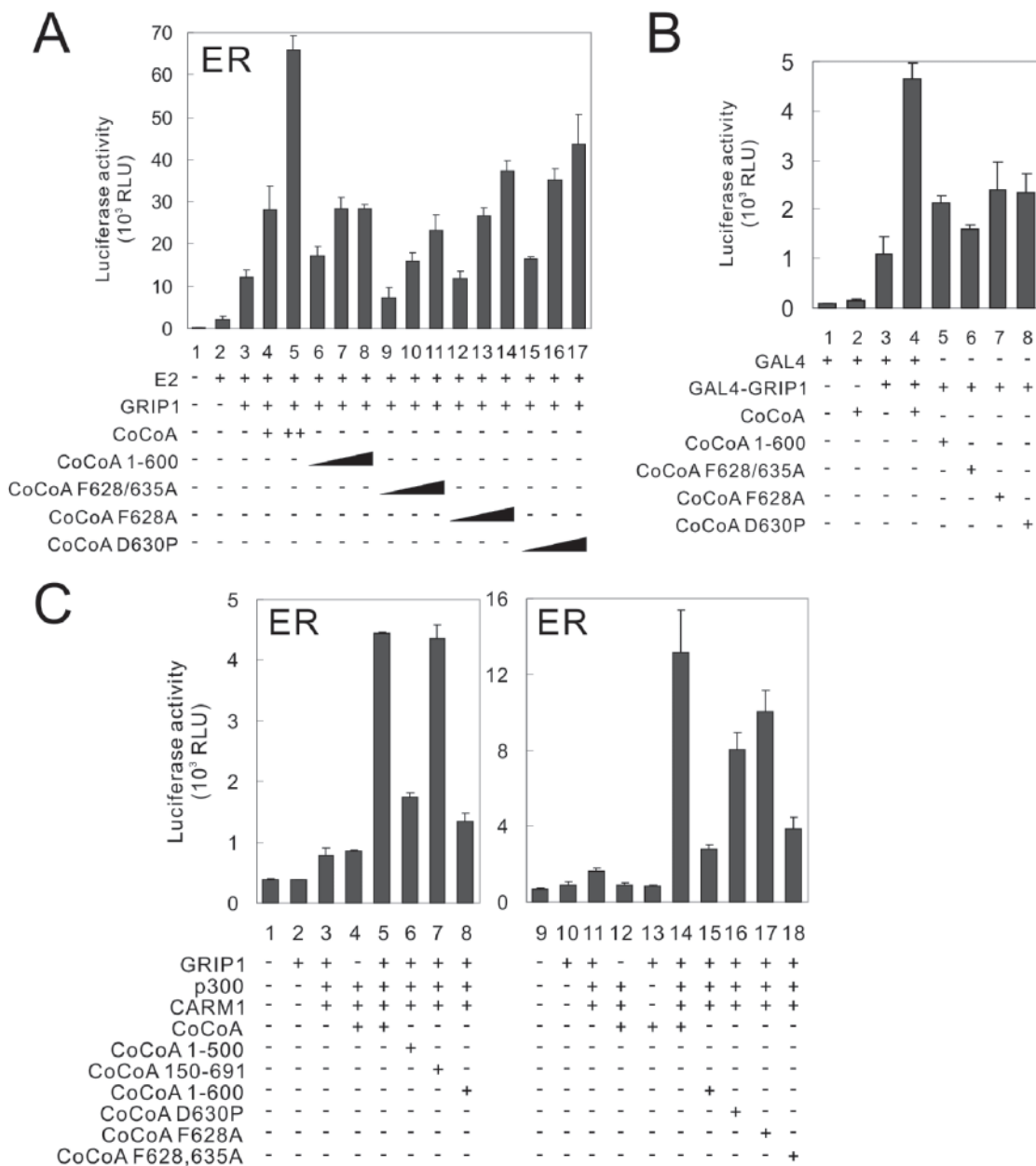
Figure 8. The C-terminal AD is required for the NR coactivator activity of CoCoA. (**A**) CV-1 cells in 24-well plates were transfected with p2ERE-TATA-LUC (100 ng), pHE0 encoding human ERα (1 ng), pSG5.HA-GRIP1 (100 ng) and pSG5.HA-CoCoA (+,100 ng; ++, 200 ng) or its mutants (100, 200 and 400 ng), as indicated. Luciferase activity was measured 48 h after transfection. Luciferase activity results shown are from a single experiment which is representative of three independent experiments. (**B**) CV-1 cells in 24-well plates were transfected with GK1-LUC (100 ng), plasmids encoding Gal4 DBD or Gal4-GRIP1 (100 ng), and pSG5.HA-CoCoA or its mutants (200 ng), as indicated. Luciferase activity was measured 48 h after transfection. Luciferase activity results shown are from a single experiment which is representative of two independent experiments. (**C**) CV-1 cells in 24-well plates were transfected with p2ERE-TATA-LUC (100 ng), pHE0 encoding human ERα (0.01 ng), pSG5.HA-GRIP1 (100 ng), pSG5.HA-CARM1 (100 ng), pCMV-p300 (100 ng), and pSG5.HA-CoCoA (100 ng) or its mutants (100 ng), as indicated. Luciferase activity was measured 48 h after transfection. Luciferase activity results shown are from a single experiment which is representative of three independent experiments.

AD and p300, we also tested the coactivator activity of p300 in cooperation with a CoCoA deletion mutant lacking the core AD. Neither CoCoA 1–600 nor both CoCoA 1–600 and p300 had a significant effect on GRIP1 AD3 activity (assays 9–12). Thus, both the core AD of CoCoA and the functional interaction between CoCoA and p300 are required for their synergistic coactivator function for GRIP1 AD3.

DISCUSSION

Transcriptional activation is a dynamic process regulated by protein–protein interactions that assemble, disassemble and modulate a variety of DNA binding, coactivator, chromatin, basal transcription factor and RNA polymerase complexes. Characterizing these fundamental protein–protein interactions and their regulation will provide a molecular basis for
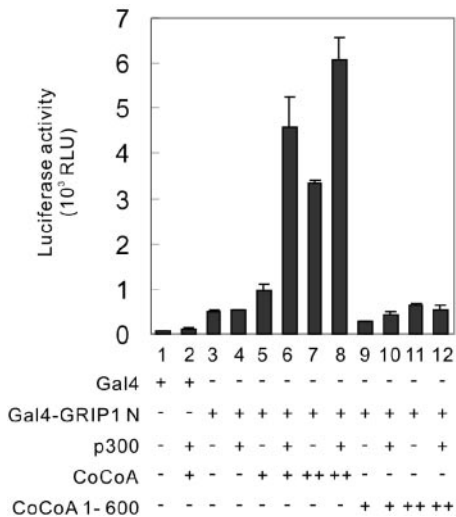
Figure 9. Coactivator function of p300 for GRIP1 AD3 depends on the presence of CoCoA. CV-1 cells in 24-well plates were transfected with GK1-LUC (100 ng), plasmids encoding Gal4 DBD or Gal4-GRIP1N (amino acids 5–479) (100 ng), pCMV-p300 (100 ng), and pSG5.HA-CoCoA or pSG5.HA-CoCoA 1–600 (+,100 ng; ++, 200 ng), as indicated. Luciferase activity was measured 48 h after transfection. Luciferase activity results shown are from a single experiment which is representative of three independent experiments.

understanding how gene expression is regulated by a variety of stimuli. In this study, we have characterized the C-terminal AD of CoCoA and demonstrated that the coactivator CoCoA directly interacts and functionally cooperates with p300 in GRIP1- and ER-mediated transcription.

Transcriptionally important motifs in CoCoA AD

The C-terminal AD of CoCoA can be divided into several subdomains, which appear to function synergistically: acidic (amino acids 501–550, 601–630 and 660–680), S/P-rich (amino acids 550–600 and 630–660) and F-rich (amino acids 680–691) subdomains (Figure 1A). The core AD (amino acids 601–691), which functioned as a minimal autonomous transactivation domain (Figure 1B), could be subdivided into two subdomains, acidic/S/P-rich region (amino acids 601–660) and acidic/F-rich region (amino acids 661–691). Although each subdomain by itself had very little transcriptional activity, the combination of two subdomains was capable of synergistically enhancing transcriptional activation. It is possible that these two subdomains recruit or activate different downstream coactivators to synergistically stimulate transcription.

The acidic ADs of VP16 and p65 contain critical core phenylalanine residues (24,25). We also found that two phenylalanine residues in the FYDVASAF motif and the F-rich region were critical for transcriptional activation by CoCoA AD (Figures 1B and 7B). Mutations in the FYDVASAF motif which resulted in significant loss of transactivation by CoCoA AD and NR coactivator activity of CoCoA also resulted in proportional loss of binding to p300 (Figures 7 and 8). We conclude that the predicted helical motif, FYDVASAF, is an important functional motif of CoCoA and that interaction between this motif and p300 is required for transcriptional activation by CoCoA. However, in addition to p300 it is also possible that this motif may

also interact with some other coactivators. In fact, the conserved stretch of FXXΦΦXXΦ in the ADs of p53, VP16 and p65 can bind directly to TAF9 (a TATA box-binding protein-associated factor previously referred to as TAFII31) (26,38,39).

A CoCoA AD mutant lacking the C-terminal F-rich region (amino acids 681–691) has very weak transactivation activity (Figure 1B). Thus, the extreme C-terminal sequence of CoCoA AD is critical for its transactivation function. This F-rich sequence may be important to maintain the proper tertiary structure of CoCoA AD or it may be important for binding other cellular factors. In view of the fact that mutants lacking this sequence still bind to p300 (Figure 3 and data not shown), and p300 still enhances transcriptional activities of the mutants (Figure 4A), it is more likely that the sequence may be important to recruit other transcriptional activators that are required for its strong transactivation function. In fact, several short fragments (amino acids 601–691, 601–680 and 680–691) of CoCoA AD also behave as potent transactivating elements in yeast cells, which possess neither p300 nor CBP (data not shown). Additional, as yet unidentified, coactivators must act through these small regions of CoCoA AD to enable p300-dependent and independent transactivation. Therefore, an important issue in understanding the function of the F-rich region is to identify specific target molecules for the F-rich region.

Although amino acids 501–600 showed no significant transactivation potential (Figure 1B), this region could interact weakly with p300 (Figure 3D) and was required for the optimal transcriptional activity of CoCoA AD (Figure 1B). Thus, amino acids 501–600 apparently harbor other critical motifs or structures which cannot activate transcription alone but can enhance the activity of the core AD. Analysis of the amino acid sequence of CoCoA AD showed that it is rich in proline residues, much like the transcriptional ADs of other well characterized transcriptional activators including p53 (41,42). A recent study showed that the proline repeat motifs (PXXPXXP or PXPXXP) of the ADs of p53 and Smad4 bind directly to p300 and are required for maximal transcriptional activity (41). Two proline repeat motifs, 564-PPGPREP-570 and 579-PAPIAP-584, actually reside within amino acids 501–600, suggesting that these motifs may function as additional p300 contact sites.

The CoCoA AD is serine-rich. The prediction of phosphorylation sites of CoCoA AD by the NetPhosK 1.0 server (43) indicated that it contains putative phosphorylation sites for multiple kinases, such as mitogen-activated protein kinases (MAPKs), casein kinases (CKs), protein kinase A (PKA) and protein kinase C (PKC). Immunoprecipitation assays with anti-phosphoserine antibodies indicated that this region was phosphorylated (data not shown). The human CoCoA was recently described to be an *in vitro* substrate of p42 MAPK and of CDK2/Cyclin E (44). It remains to be determined whether phosphorylation of the CoCoA AD by the various kinases may differentially influence CoCoA coactivator activity.

p300 functionally interacts with CoCoA AD

Our results indicated that the coactivator p300 and its HAT activity are involved in CoCoA AD-mediated transactivation.

Substantial HAT activity was associated with CoCoA and CoCoA AD (Figure 2A) and CoCoA interacted with p300 *in vitro* and *in vivo* (Figure 2 and Figure 3), but not with PCAF (data not shown). Thus, p300 might be the predominant CoCoA AD-associated HAT. CoCoA AD recruited endogenous p300 to the promoter region (Figure 5). E1A, a p300 inhibitor, strongly repressed CoCoA AD-mediated transcriptional activation and overexpression of p300 rescued E1A-mediated repression of CoCoA AD activity (Figure 4). Moreover, an E1A mutant lacking p300 binding domain failed to repress CoCoA AD activity. The mechanism by which p300 regulates the activity of CoCoA AD is presumably through its acetyltransferase activity since p300 ΔHAT mutants failed to enhance CoCoA AD activity. A growing list of transcriptional activators has been shown to be acetylated by p300 (45). In most cases, acetylation is responsible for transcription stimulation by enhancing DNA-binding activity or regulating protein–protein interaction. We showed in the present study that HDACs strongly repressed CoCoA AD activity, and TSA treatment led to an increase in CoCoA AD activity (Figure 6). We also found that CoCoA AD immunoprecipitated from COS-7 cells was significantly acetylated (J.H. Kim and M.R. Stallcup, unpublished data). However, CoCoA AD was not acetylated by p300 and PCAF *in vitro*. Thus we believe that interaction of CoCoA with p300 is part of a mechanism by which CoCoA alters chromatin structure or the activity of other transcription factors or coactivators at target gene promoters by recruiting p300. What acetyltransferases are responsible for the acetylation of CoCoA AD, whether p300 can also acetylate CoCoA *in vivo* and whether the acetylation of CoCoA AD affects the coactivator activity of CoCoA remain to be determined.

Role of CoCoA in p160 coactivator complex

CoCoA acted synergistically in combination with p300 and CARM1 in low-ER transient transfection assays, and this coactivator synergy is entirely dependent on the presence of GRIP1 (Figure 8C). These results are consistent with the model that GRIP1 as a primary coactivator recruits multiple secondary coactivators which contribute synergistically to ER-mediated transcription. The role of CoCoA in the p160 coactivator complex is most probably multifunctional through multiple protein–protein interactions (Figure 10). The coiled-coil domain interacts with GRIP1, and CoCoA AD interacts with p300/CBP and might help recruit or potentiate the HAT activity of p300/CBP (Figure 5). The formation of a ternary complex among GRIP1, p300 and CoCoA and the ability of CoCoA to bind simultaneously GRIP1 and p300 were strongly supported by mammalian one-hybrid assays using the GRIP1 AD3 fragment fused to Gal4 DBD (Figure 9). Moreover, p300 enhanced synergistically GRIP1 AD3 activity only in the presence of CoCoA, suggesting that p300 stimulated GRIP1 AD3 function by the interaction with CoCoA. Because the deletion of core AD eliminated the coactivator function of CoCoA and p300 for GRIP1 AD3, the contribution of p300 to CoCoA-p300 synergy was mediated by functional interaction between p300 and core AD of CoCoA. The core AD of CoCoA is required for stimulation by p300
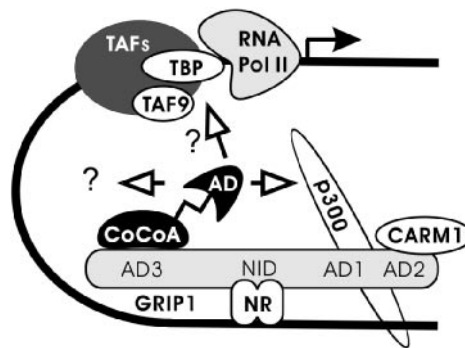

Figure 10. The possible roles of CoCoA in the p160 coactivator complex. DNA-bound NRs recruit GRIP1. The ADs, AD1, AD2 and AD3, of GRIP1 transmit the transactivation signal by recruiting secondary coactivators, p300, CARM1 and CoCoA, respectively. CoCoA may contribute to transcriptional activation for NRs by one or more of multiple mechanisms: facilitating or stabilizing the p160 coactivator complex through the simultaneous interaction with GRIP1 and p300, enhancing the HAT or coactivator activity of p300, bridging between the p160 coactivator complex and basal transcription factors such as TBP and TAF9, and recruiting currently unknown downstream coactivators.

(Figure 4A) and is, by itself, capable of interacting with p300 (Figure 3F). These findings are most consistent with a direct action of p300 on the transactivation activity of CoCoA AD or vice-versa.

Another possible explanation for the role of CoCoA AD in p160 coactivator complex might be to potentiate transcriptional cross talk between the basal transcriptional machinery and p160 coactivator complex (Figure 10). Transcriptional activation by acidic transcriptional ADs has been correlated with their ability to interact with components of the general transcription machinery. The acidic ADs of VP16 and p53, for example, bind to several general transcription factors including TFIIH, TFIIB, TAF9 and TBP (26,38,46–48). As discussed above, the hydrophobic sequence FXXΦΦXXΦ, which is known as a KIX binding motif in the acidic ADs of VP16, p53 and p65, also makes direct contacts with TAF9 (26,38,39). We also observed the interaction between CoCoA and components of TFIID including TBP and TAF9, and the core AD of CoCoA was required for these interactions (data not shown). The exact binding sites and physiological relevance of these interactions remains to be determined.

Residual coactivator activities of CoCoA AD deletion and point mutants in reporter gene assays (Figure 8) suggest that other domain(s) of CoCoA may also contribute to the maximal activation of p160 coactivator complexes. Our recent study showed that the N-terminal domain of CoCoA is essential to activate β-catenin-mediated transcription, although it is dispensable for GRIP1-mediated transcription (20). Furthermore, we showed that p300 directly interacts with the N-terminal domain of CoCoA (Figure 3D and E). These results suggest that there may be an additional transcriptional AD in the N-terminus of CoCoA. It is also possible that the different ADs of CoCoA may be differentially used depending on the interaction partners. The flexibility provided by multiple ADs and by multiple subdomains in the ADs could make CoCoA a versatile transcriptional coactivator.

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Conflict of interest statement. None declared.

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