Direct Association between the CREB-Binding Protein (CBP) and Nuclear Receptor Corepressor (N-CoR)†

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ABSTRACT: The binding of ligand to nuclear hormone receptors induces a conformational change that results in corepressor release and the recruitment of coactivator proteins that contain or recruit histone acetyltransferase (HAT) activity. As such, the coactivator and corepressor complexes and their associated HAT and histone deacytlase (HDAC) activities are often believed to be segregated into distinct complexes. However, there have been several reports that suggest that coactivators and corepressors may not be strictly segregated and in some cases even interact directly. In the present study, we have utilized a biochemical approach to assess whether the nuclear receptor corepressor (N-CoR) is capable of associating with the HAT coactivator CREB-binding protein (CBP). We demonstrate, using both immunoaffinity purification and conventional chromatography, that a subset of the N-CoR−HDAC3 complex copurifies with CBP in HeLa cells. In addition, indirect immunofluorescence also indicates an association between N-CoR and CBP in intact MCF-7 cells. This association may be direct as *in vitro* pulldown assays using recombinant purified proteins indicated that the amino terminus of N-CoR interacts directly with CBP. Interestingly, we also demonstrate that increasing concentrations of N-CoR are capable of attenuating CBP HAT activity *in vitro*, suggesting that N-CoR may have a functional role in modulating HAT activity. This is the first report of a direct interaction between N-CoR and CBP, and suggests that the role of N-CoR in mediating transcriptional events may be more complex than previously anticipated.

Gene expression is critically dependent upon the ability of sequence-specific DNA-binding proteins to regulate the transcription of target genes in a coordinated fashion. This is accomplished, in part, through transcription-factor-mediated recruitment of coactivators and corepressors that regulate transcription via several mechanisms. These mechanisms include the modification of the local chromatin environment via post-translational modifications such as acetylation, methylation, and phosphorylation, as well as through direct interactions with the core transcriptional machinery (*1*). The nuclear hormone receptor superfamily provides a unique model to study the mechanism of transcriptional activation/repression and the role of reversible chromatin modification in the control of gene expression. In the absence of ligand, nuclear hormone receptors function as repressors by interacting with corepressor proteins such as the nuclear receptor corepressor (N-CoR)[1] or the closely related silencing mediator for retinoid and thyroid hormone receptors (SMRT) (*2*). The binding of ligand induces a conformational change in the receptor that results in corepressor release and the recruitment of coactivator proteins that possess or recruit various enzyme activities, such as histone acetyltransferase (HAT) activity. N-CoR and SMRT are 260 kDa proteins that were identified on the basis of their direct interaction with unliganded thyroid hormone receptor (TR) or retinoic acid receptor (RAR), respectively (*3*, *4*). This interaction is mediated by two or three receptor interaction domains (RIDs) located within the carboxy terminus of N-CoR and SMRT (*3*, *5*−*8*). In addition to TR and RAR, N-CoR and SMRT can serve as corepressors for several other members of the NR superfamily including the vitamin D receptor, PPARα, and the antagonist-bound estrogen (ER) and progesterone receptors (PR) (*9*−*11*). N-CoR has also been implicated in transcriptional repression by several other classes of transcription factors, such as Notch (*12*) and the homeodomain proteins RPX and Pit-1 (*13*).

Transcriptional repression by N-CoR is mediated through four repression domains (RD) designated RD1−RD4, that function as docking surfaces for the recruitment of additional components of the corepressor complex (*14*). Biochemical purification of endogenous N-CoR using HeLa cell nuclear extracts has identified several "core proteins" that appear to

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[1] Abbreviations: N-CoR, nuclear receptor corepressor; CBP, CREB-binding protein; HDAC3, histone deacetylase 3; HDAC, histone deacetylase; HAT, histone acetyltransferase; SMRT, silencing mediator of retinoid and thyroid receptors; TBL1, transducin-like protein 1; TBLR1, TBL-1-related protein; GPS2, G protein suppressor 2; TSA, trichostatin A.

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associate directly and stochiometrically with N-CoR (*14*−*17*). One of these constituents is histone deacetylase 3 (HDAC3) that catalyzes the deacetylation of conserved lysines found within the histone tails. The direct association of HDAC3 with N-CoR is required for both its recruitment to the promoter and activation of its enzymatic activity (*18*). Transducin-like protein 1 (TBL-1) and TBL-1-related protein (TBLR1) are two additional core proteins that have been identified. TBL1 and TBLR1 are highly homologous proteins that appear to be essential for mediating repression by unliganded TR. Interestingly, TBL and TBLRI serve as exchange factors for components of the proteasome and are also required for some nuclear receptor activation events (*19*). Recent studies have also identified G protein suppressor 2 (GPS2) as a N-CoR-associated protein that may couple signal transduction pathways originating at the cell surface to transcriptional repression (*14*−*17*). The methyl CpG-binding protein Kaiso has also been identified as a constituent of a N-CoR complex that also contains HDAC3, TBL1, and TBLR1 and is recruited to the MTA-2 gene in a methylation-dependent manner (*20*). These findings suggest that N-CoR is also involved in mediating transcriptional repression associated with DNA methylation.

Many additional proteins have been identified that interact or copurify with N-CoR or SMRT, in what appears to be a more transient fashion, although the functional significance of these associations remains to be defined. These proteins include SKI (*21*), SNO (*22*), KAP-1 (*23*), SHARP (*24*), the SWI/SNF chromatin remodeling complex (*23*), the PRP4 kinase (*25*), the class-II HDACs (*26*), and mSin3 (mammalian switch-independent 3) (*27*).

Recent models of gene regulation have suggested that transcription is a highly dynamic process that involves the rapid exchange of coregulators in a combinatorial fashion (*2*). A dominant feature of this mechanism has been the notion that acetyltransferases and deacetylases are generally found in separate complexes. However, several studies have suggested that the activities of HATs and HDACs are not entirely segregated and that their activities may in some cases be linked. For example, co-immunoprecipitation and FRET analysis have demonstrated that the class-I HDACs are associated with the HAT PCAF in HeLa cells (*28*). The transcription factor YY1 interacts with both HATs and HDACs, allowing it to function as an activator or repressor (*29*). Other studies have shown that the TSHβ promoter contains "negative hormone response elements" and that TSHβ is activated in the absence of thyroid hormone (*30*, *31*). The ligand-independent activation of TSH has been shown to involve the recruitment of N-CoR and CREB-binding protein (CBP). Overexpression of N-CoR enhanced expression of these genes and was associated with increases in the acetylation status of a transient reporter containing the TSH gene promoter. More recently, it was shown that the targeted disruption of N-CoR in mice blocked the ability of the retinoic acid to activate a reporter gene containing a DR-1 response element (*32*). In addition, the N-CoR-dependent activation of the DR-1 required the function of HDAC3 and CBP, supporting a requirement for both deacetylase and acetylase function in specific types of transcriptional activation events.

In the present study, we have utilized a biochemical approach to demonstrate that a subset of endogenous N-CoR is capable of associating with CBP both *in vitro* and in intact cells. In addition, we have shown that the amino terminus of N-CoR can interact directly with full-length CBP, suggesting that, in some cases, the association between N-CoR and CBP may be direct. Importantly, we have found that increasing concentrations of N-CoR attenuates CBP HAT activity when using free histones as substrates. Our data suggest several novel roles of N-CoR in regulating transcription and broaden our present understanding of N-CoR function.

EXPERIMENTAL PROCEDURES

Western Blotting. Protein samples were separated by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) and transferred to nitrocellulose, and Western blotting was performed using the indicated antibodies. Signals were detected by enhanced chemiluminescence according to the recommendations of the manufacturer (ECL plus, Amersham Pharmacia Biotech). CBP antibodies (A-22 and C-1) were purchased from Santa Cruz Biotechnology; the HDAC3 antibody was purchased from Affinity Bioreagents; the M5 FLAG antibody was purchased from Sigma-Aldrich; the p300 (RW128) antibody was purchased from Upstate; the SMRT antibody (1212) was purchased from Santa Cruz; and the N-CoR polyclonal antibody has been described previously (*23*).

Affinity Purification of the N-CoR. A total of 40 L of HeLa cells, grown to mid-log phase, was obtained from the National Cell Culture Center (Minneapolis, MN). The affinity purification of N-CoR has been previously described (*23*). Briefly, the nuclear extract was dialyzed against buffer A [20 mM Tris-HCl at pH 7.9, 0.5 mM ethylenediaminetetraacetic acid (EDTA), 0.5 mM ethylene glycol bis(2-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), 10% glycerol, 0.5 mM dithiothreitol (DTT), 0.2 mM phenylmethylsulfonyl fluoride (PMSF), and 5 μg/mL each of leupeptin, aprotinin, and pepstatin A] containing 100 mM KCl and was loaded onto an 80 mL P11 phosphocellulose column pre-equilibrated with the same buffer. The flow through was collected, and the column was washed sequentially in a stepwise fashion using buffer A containing 0.3, 0.5, and 1.0 M KCl. The 1.0 M KCl N-CoR-containing fractions were pooled, dialyzed against buffer A containing 100 mM KCl, then passed through a DEAE-Sepharose column, and washed with an increasing KCl gradient. All fractions were analyzed by Western blotting using anti-N-CoR antibody. The N-CoR-containing fractions were again pooled, concentrated by precipitating with 20−60% ammonium sulfate, and then applied to a Sephacryl S300 column pre-equilibrated with buffer A containing 100 mM KCl. The column was washed with buffer A at a flow rate of 0.4 mL/min. The fractions were analyzed for N-CoR by Western blotting, pooled, and dialyzed against buffer A containing 100 mM KCl without DTT. For affinity purification of N-CoR, affinity-purified N-CoR antibody was cross-linked to protein A-Sepharose using dimethylpalmilidate according to standard procedures (*33*). The pooled fractions from the gel-filtration step were precleared using protein A-Sepharose cross-linked to rabbit IgG. The precleared sample was then loaded onto the affinity column at a flow rate of 0.4 mL/min, and the flow through was collected and reloaded onto the affinity column 5 times. The bound proteins were washed with 10 column volumes each of buffer A (without DTT) containing 0.1% Nonidet

P-40 (NP-40) and 0.3 M KCl, 0.1% NP-40 and 0.5 M KCl, or 0.5% NP-40 and 0.7 M KCl and a final wash in buffer A containing 100 mM KCl prior to elution with 100 mM glycine (pH 2.8). For mock purification experiments, samples from the gel-filtration step were loaded onto a protein A-Sepharose cross-linked to the IgG column, reloaded 5 times, and eluted following the same procedure as for the N-CoR affinity purification (see above).

Chromatographic Purification of N-CoR. The 1.0 M KCl fraction eluted from the phosphocellulose column was dialyzed against buffer A containing 100 mM KCl, then passed through a DEAE-Sepharose column, and washed with an increasing KCl gradient. The fractions containing N-CoR and CBP were pooled and dialyzed against buffer A containing 1 M ammonium sulfate and then applied to a phenyl Sepharose column, and proteins were eluted by decreasing the ammonium sulfate concentration. The fractions corresponding to the major protein peaks were analyzed for N-CoR or CBP by Western blotting, pooled, and dialyzed against buffer A containing 100 mM KCl. The dialyzed sample was then applied to a Source Q column, and the proteins were eluted off of the column with an increasing KCl gradient. The major protein peak was assayed for N-CoR and CBP by Western blotting, and fractions containing both N-CoR and CBP were pooled, concentrated using a Biomax 10K NMWL centrifugal filter, and purified through a Superose 6 gel-filtration column. Individual fractions were concentrated using a 0.5 mL Biomax 10K NMWL centrifugal filter and Western blotted for N-CoR, CBP, and HDAC3.

Measurement of Acetyltransferase Activity. HAT activity was measured using a previously described protocol (*34*). Typically, approximately 100 ng of purified proteins bound to protein-G agarose was suspended in 30 μL of IPH buffer (50 mM Tris-HCl at pH 8.0, 100 mM NaCl, 5 mM EDTA, 0.1% NP-40, and 0.1 mM PMSF) containing 10 mM of the HDAC inhibitor sodium butyrate, 1 μL of [^{3}H]acetyl-CoA (1.85 mBq, 10 mCi/mmol, Amersham), and 1−1.5 μg of free histones and incubated for 45 min at 30 °C. The reactions were stopped by spotting on p81 filter paper and allowed to air-dry for 10 min. The p81 filters were then soaked in $NaCO_3$ buffer (50 mM $NaHCO_3$ and 50 mM Na_2CO_3 at pH adjusted to 9.2 with HCl) for 30 min at 37 °C and then washed 3 times (10, 5, and 5 min) in a 1:1:1 mixture of MeOH/$CHCl_3$/acetone. The washed p81 filters are then allowed to air-dry for 10 min before quantification by scintillation counting. To assess the effects of N-CoR on CBP HAT activity, 50 ng of full-length CBP was preincubated with increasing amounts of N-CoR (1−844 amino acids) and free histones prior to the addition of [^{3}H]acetyl-CoA and HAT activity was assessed as described above. The amount of specific histone acetylation was determined by subtracting the CPMs obtained from reactions containing only CBP to account for CBP autoacetylation.

Measurement of Deacetylase Activity. Histone deacetylase activity was monitored as described (*35*). Aliquots of the affinity-purified N-CoR were retained on 25 μL of protein A-Sepharose-affinity resin containing cross-linked anti-N-CoR antibody. The beads were washed extensively with buffer A containing 500 mM KCl and 0.5% NP40 and then resuspended in buffer consisting of 20 mM Tris-HCl (pH 7.6), 100 mM KCl, 0.1 mM EDTA, 0. 2 mM DTT, and 0.2 mM PMSF and containing [^{3}H]histones (20 000 cpm/rxn) to a final volume of 200 μL. The reactions were allowed to proceed at 37 °C for 90 min and stopped by the addition of 0.05 mL of a 0.1 M HCl/0.7 M acetic acid solution. Released [^{3}H]acetate was extracted with 0.6 mL of ethyl acetate. After centrifugation, 0.3 mL of the upper organic phase was used for liquid scintillation counting.

Expression and Purification of Recombinant Proteins. FLAG-tagged CBP, HA-tagged N-CoR (1−844 amino acids) cDNAs were subcloned into the pFastbac vector (Invitrogen), and recombinant proteins were expressed using the Bac-to-Bac baculovirus expression system according to the instructions of the manufacturer. Epitope-tagged proteins were prepared by the infection of SF9 cells with the appropriate recombinant baculovirus, followed by immunoaffinity chromatography with anti-FLAG M2 or anti-HA-affinity resin. Proteins were eluted with 20 mM Tris buffer at pH 7.9, 100 mM KCl, 10% glycerol, 0.5 mM EDTA, and 0.2 mg/mL of the appropriate peptide competitor, followed by dialysis to remove the peptide competitor, and frozen at −80 °C. N-CoR deletion mutants were generated by amplifying specific regions of N-CoR using polymerase chain reaction (PCR) and subcloning the amplified cDNAs into the pGEX bacterial expression vector in-frame with the glutathione-*S*-transferase (GST) moiety. The resulting plasmid was transformed into the *Escherichia coli* strain BL-21, and protein expression was induced with 1 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG) for 3 h at 30 °C. The bacterial cells were then disrupted, and the expressed proteins were affinity-purified using GST-Sepharose beads. Bound proteins were eluted with buffer A containing 10 mM reduced glutathione. Proteins were then dialyzed against a buffer consisting of 20 mM Tris at pH 7.9, 100 mM KCl, 10% glycerol, 0.5 mM EDTA, and 0.5 mM DTT and stored at −80 °C.

Immunoprecipitations and in vitro Interaction Assays. Immunoprecipitations were carried out on 100 μL (3 mg/mL) of HeLa nuclear extract diluted in buffer A without DTT and containing 300 mM KCl, 0.1% NP-40, and 1 mg/mL bovine serum albumin (BSA). The nuclear extract was precleared with protein-G agarose for 1 h at 4 °C. Immunoprecipitations were done with approximately 2 μg of the indicated antibodies or normal IgG for 1 h at 4 °C and then pulled-down with 20 μL of protein-G agarose for 1 h or overnight. The immunoprecipitates were washed at least 4 times with wash buffer (20 mM Tris-HCl at pH 7.9, 300 mM KCl, 0.1% NP-40, 0.5 mM EDTA, 0.5 mM EGTA, and 10% glycerol) and then eluted with SDS-sample buffer and analyzed by Western blotting. For the copurification of CBP with N-CoR, HeLa nuclear extract (1 mL) was diluted with 4 mL of buffer A without DTT and containing 300 mM KCl and 0.1% NP-40 and loaded onto the control affinity column consisting of protein A-Sepharose cross-linked to rabbit IgG (100 μL packed volume). The flow through was collected and reloaded onto the control column 5 times. The precleared sample was then loaded onto the affinity anti-N-CoR column (100 μL packed volume), and the flow through was collected and reloaded onto the affinity column 5 times. The control and anti-N-CoR columns were washed with 10 column volumes each of buffer A (without DTT) containing 0.1% NP-40 and 0.3 M KCl, 0.1% NP-40 and 0.5 M KCl, or 0.5% NP-40 and 0.7 M KCl and a final wash in buffer A, containing 100 mM KCl prior to elution with elution buffer

[100 mM glycine (pH 3.0) and 100 mM KCl], and analyzed by Western blotting. For the *in vitro* interaction assay, approximately 500 ng of recombinant HA-N-CoR (1−844 amino acids) was bound to anti-HA agarose and washed extensively with buffer A containing 300 mM KCl and 0.1% NP-40. To this immobilized HA-N-CoR (1−844 amino acids) agarose or anti-HA agarose as a control, approximately 500 ng of purified recombinant FLAG-CBP was incubated in 200 μL of buffer A containing 300 mM KCl, 0.1% NP-40, and 1 mg/mL BSA overnight at 4 °C with end-over-end rotation. The bound proteins were then washed extensively, eluted with SDS-sample buffer, and analyzed by Western blotting. For the GST interaction assays, 0.5 μg of either GST alone, GST-N-CoR, or HA-N-CoR (1−844 amino acids) were incubated with 0.5 μg of FLAG-CBP in buffer A containing 300 mM KCl, 0.1% NP-40, and 1 mg/mL BSA at 4 °C for approximately 16 h. Protein complexes were then captured using either GST- or anti-HA-Sepharose, for 1 h at 4 °C. The protein complexes were then washed extensively with buffer A containing 300 mM KCl and 0.1% NP-40 and then analyzed by SDS−PAGE and Western blotting using anti-CBP rabbit polyclonal antibody.

Co-infection of N-CoR and CBP. Sf9 cells were infected with FLAG-CBP baculovirus alone or co-infected with FLAG-CBP and HA-N-CoR (1−844 amino acids) baculoviruses. A total of 48 h postinfection, the cells were harvested and resuspended in buffer A containing 300 mM KCl and 0.1% NP-40 and disrupted by 10 strokes in a dounce homogenizer and the cell lysate was clarified by centrifugation. To each of the cell extracts, anti-HA agarose was added, incubated for 2 h at 4 °C, and washed with lysis buffer and a final wash with buffer A containing 100 mM KCl and 0.1% NP-40. The bound proteins were eluted with SDS-sample buffer and analyzed by Western blotting.

Immunofluorescence. MCF-7 cells were plated onto microscope slides and maintained in αMEM with 10% fetal bovine serum (FBS) at 37 °C with 5% CO_2. Cells were allowed to adhere for approximately 18 h prior to fixation with 4% paraformaldehyde for 10 min, then permeabilized with 0.1% Triton X-100 in phosphate-buffered saline (PBS), and washed 4 times in PBS over 5 min. The cells were then preincubated in blocking solution (3% BSA and 1.5% FBS) for 1 h, and CBP was labeled using an mouse monoclonal anti-CBP antibody (1:20 dilution, Santa Cruz, sc-7300). N-CoR was labeled using an affinity-purified rabbit polyclonal anti-N-CoR (1:20 dilution) or a Santa Cruz goat polyclonal antibody (1:50 dilution, Sc-1609). Acetylated histones were labeled with rabbit polyclonal acetylated-histone-H4 antibody (1:50 dilution, Cell Signaling Technology) for 1 h at room temperature. The cells were then washed 4 times with PBS before labeling with anti-rabbit IgG conjugated to TRIT-C (1:100 dilution, Sigma), anti-mouse IgG conjugated to fluorescein (1:100 dilution, Santa Cruz), or anti-sheep IgG conjugated to Alexa 488 (1:1000 dilution, Molecular Probes) for 1 h at room temperature. Images were captured at 100× (oil immersion) using a Z-stack on a Leica microscope, and the 3D stack was digitally deconvolved using Velocity and analyzed using Openlab 3.0 and Adobe Photoshop (parts A and B of Figure 5). Images were also captured at 100× (oil immersion) using an Olympus AX70 microscope and overlaid using Adobe Photoshop (parts C and D of Figure 5).

RESULTS

To assess whether N-CoR and CBP associate in intact cells, preliminary experiments were performed by fractionating HeLa cell nuclear extracts using a Superose 6 size-exclusion column. Western blots of fractions eluted from this column indicated that N-CoR was found in a large molecular-weight complex (Figure 1A), consistent with previous observations (*23*). CBP also eluted in similar fractions, although the elution profile was much broader, suggesting that CBP is also found in complexes not containing N-CoR (Figure 1A). Immunoprecipitation experiments of endogenous N-CoR and CBP were also conducted using specific antibodies. Western blotting of proteins that copurified with an anti-N-CoR antibody indicated that CBP could also be co-immunoprecipitated (Figure 1B). Conversely, immunoprecipitations using a specific anti-CBP antibody followed by Western blotting demonstrated that N-CoR is associated with CBP, suggesting that both N-CoR and CBP may be found in the same complex (Figure 1B). Further co-immunoprecipitation experiments demonstrate that N-CoR also co-immunoprecipitates with the CBP-related protein p300 but not with actin, which was used as a negative control (Figure 1B). To address whether the related protein SMRT copurifies with CBP or p300, additional co-immunoprecipitation experiments were performed and demonstrate that SMRT does not copurifiy with CBP or p300 (Figure 1C). In addition, we performed *in vitro* HAT assays using proteins immunoprecipitated with either N-CoR or CBP antibodies. These results indicate that immunopurified CBP and N-CoR possess acetyltransferase activity that is not seen with the control IgG immunoprecipitation (Figure 1D).

These preliminary observations were followed up by a more detailed biochemical analysis. First, a large-scale purification of N-CoR was performed using the scheme shown in Figure 2A, which combined conventional purification techniques with a final immunoaffinity purification. The initial step utilized a P11 phosphocellulose column with elution by increasing salt concentrations. The results of this preliminary chromatographic separation indicated that the majority of N-CoR eluted at salt concentrations corresponding to 0.3 and 0.5 M KCl as described previously (*23*). A relatively minor component of the total N-CoR was found in a fraction requiring 1.0 M KCl for elution (data not shown). Further purification of this fraction using gel-filtration chromatography indicated that both N-CoR and CBP were found in similar fractions and that the size of the immunoreactive peak corresponded to approximately 1 MDa (Figure 2B). We also tested the individual fractions for HAT activity, which again indicated a direct overlap between HAT activity and copurification of both N-CoR and CBP (Figure 2B). The N-CoR-containing fractions were pooled and then passed through an anti-N-CoR immunoaffinity column. After extensive washing, the bound proteins were eluted and assayed by Western blotting, which indicated that both N-CoR and CBP were present (Figure 3A). In contrast, neither protein was detected when the partially purified fractions were passed through a control immunoaffinity column (Figure 3A). Silver staining of the affinity-purified N-CoR complex identified approximately 10 proteins, ranging in size from approximately 46 to 300 kDa, that copurify with N-CoR and were not found in the control immunoaf-

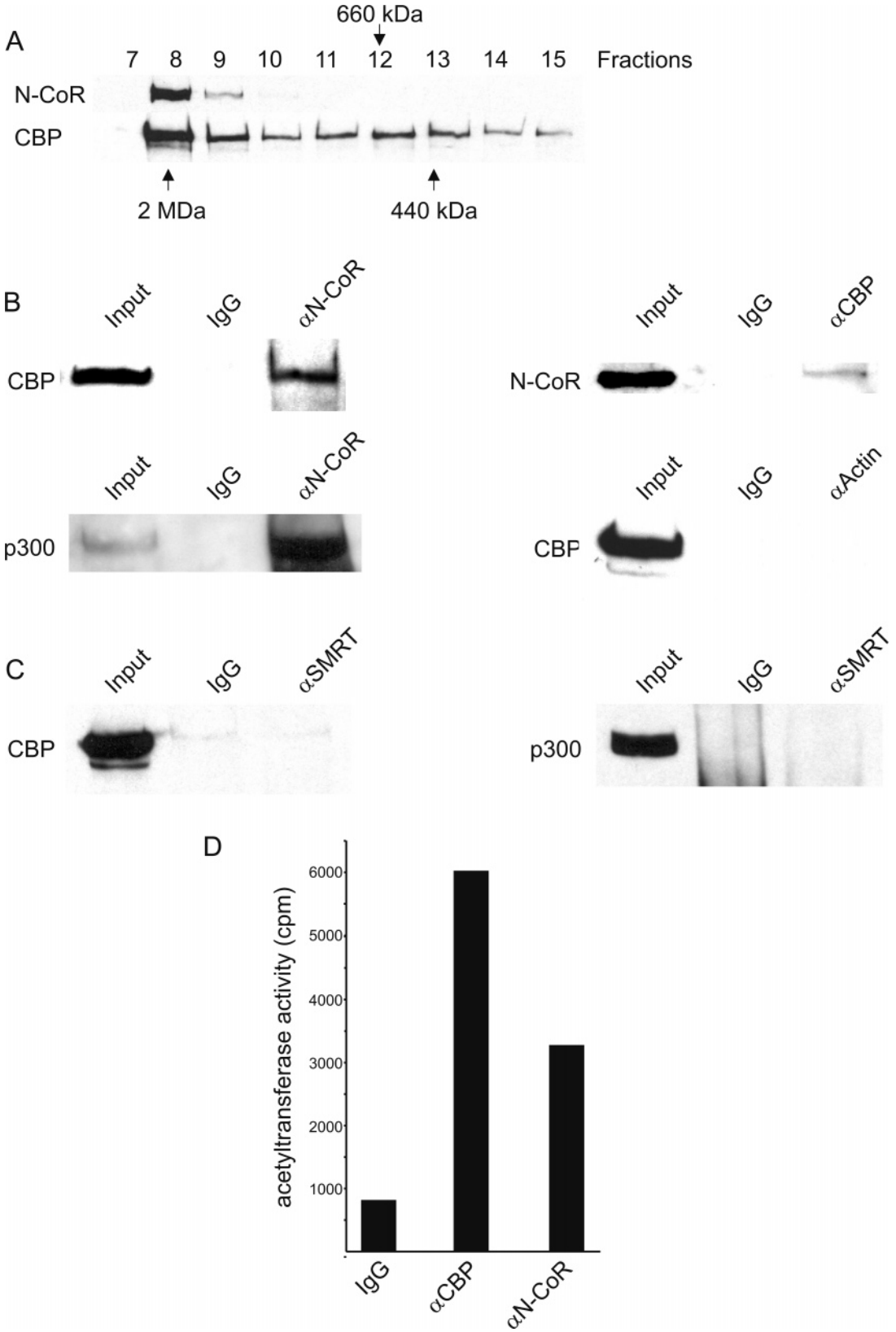

FIGURE 1: N-CoR copurifies with CBP and HAT activity. (A) N-CoR and CBP copurify by gel-filtration chromatography. HeLa cell nuclear extracts were fractionated using a Sephacryl S300 column, and the presence of N-CoR and CBP in the collected fractions was determined by Western blotting. (B) N-CoR co-immunoprecipitates with CBP and p300 from HeLa cell nuclear extracts. Co-immunoprecipitation experiments from HeLa nuclear extracts conducted using specific antibodies for N-CoR, CBP, p300, or actin. The resulting immunopurified complexes were separated by SDS−PAGE, and the presence of CBP, N-CoR, and p300 was analyzed by Western blotting. (C) Co-immunoprecipitation experiments from HeLa nuclear extracts using a SMRT-specific antibody. The resulting immunoprecipitated complexes were separated by SDS−PAGE, and the presence of CBP or p300 was analyzed by Western blotting. (D) Immunoprecipitated N-CoR possesses HAT activity. Immunoprecipitation of HAT activity using anti-CBP or anti-N-CoR antibodies, or normal rabbit IgG as a control, from HeLa nuclear extract. The resulting immunoprecipitated complexes were extensively washed and assayed for HAT activity *in vitro* using free histones as substrates.

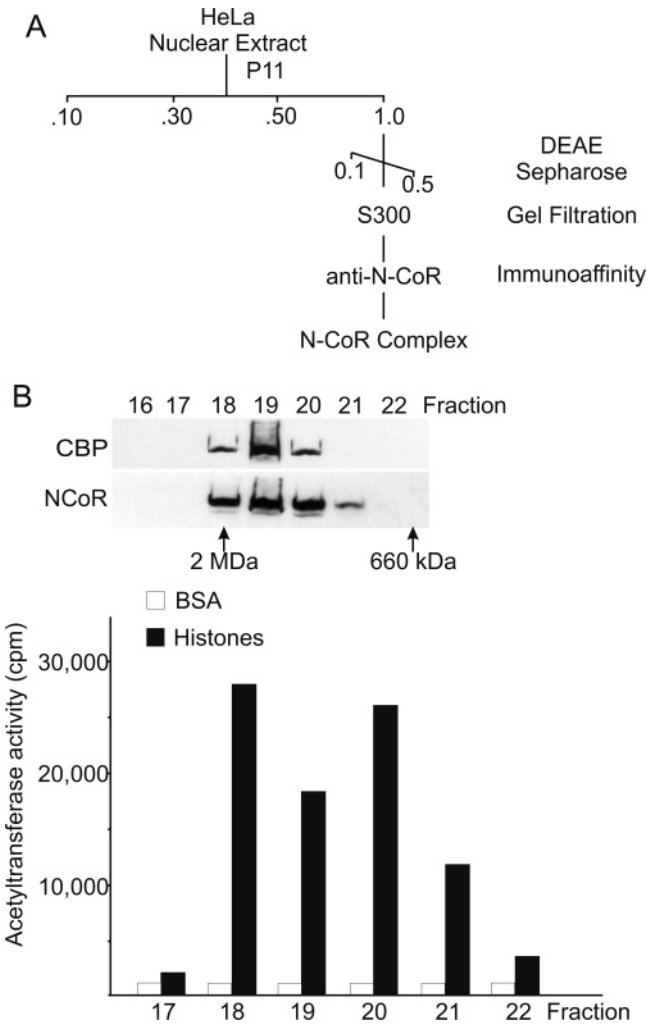

FIGURE 2: Purification of a N-CoR complex containing CBP. (A) Schematic representation of the various chromatographic steps used to purify the N-CoR complex. (B) N-CoR copurifies with CBP and HAT activity. (Upper panel) Western blot analysis of the fractions eluted from the Sephacryl S300 column. A total of 20 μg of protein from the corresponding fractions was separated by SDS−PAGE, transferred to nitrocellulose, and probed with the specific antibodies indicated on the left. The molecular-mass standards are indicated at the top of the panel: 2 MDa, void; 660 kDa, thyroglobulin. (Lower panel) Fractions corresponding to the gel-filtration chromatographic separation were also assessed for HAT activity using free histones or BSA as substrates.

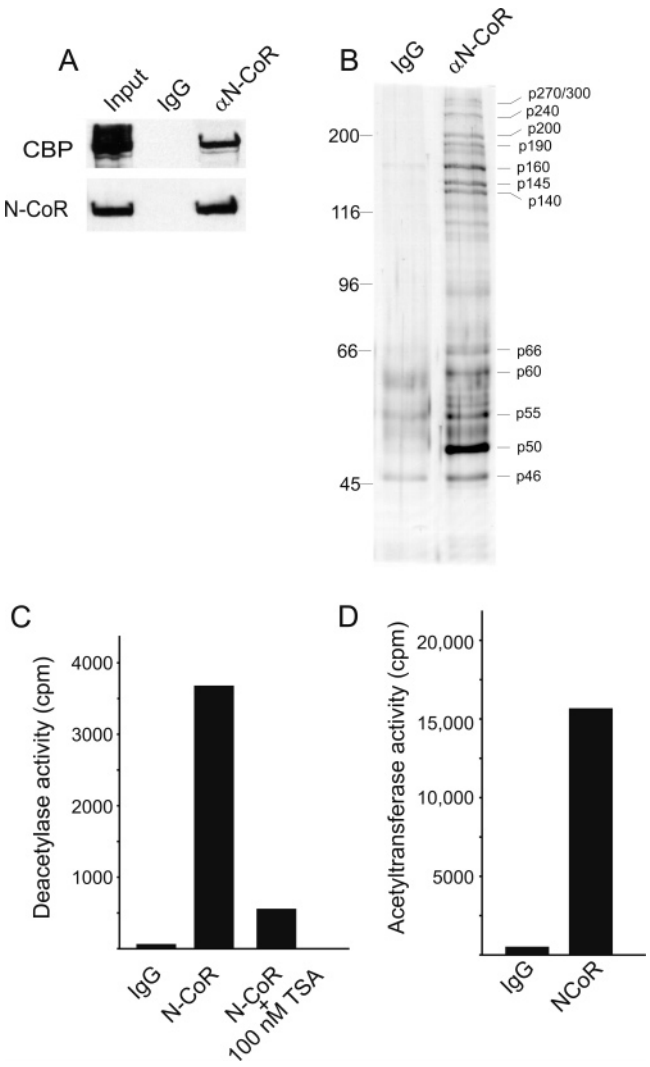

FIGURE 3: Immunopurified N-CoR copurifies with both HDAC and HAT activity. (A) Western blot analysis of the immunopurified N-CoR complex. N-CoR eluted from the Sephacryl S300 fraction was immunopurified using an anti-N-CoR affinity column or control column. The purified proteins were separated by SDS−PAGE and immunoblotted using anti-N-CoR or anti-CBP antibodies. (B) Silver stain of the immunopurified N-CoR complex. The Sephacryl S300 fractions containing N-CoR were pooled and purified by affinity chromatography using an anti-N-CoR affinity column or IgG control column as indicated in the Experimental Procedures. Bound proteins were eluted from the affinity column using 100 mM glycine at pH 2.8 and separated by SDS−PAGE. Approximate molecular masses of the isolated polypeptides are indicated on the right. Molecular-mass standards are indicated on the left. (C) Deacetylase activity of the immunopurified N-CoR complex. Immunopurified N-CoR or control IgG elutions were preincubated either alone or with 100 nM TSA and ^{3}H-labeled core histones. The reaction was allowed to proceed for 90 min prior to extraction and quantification of the released ^{3}H-labeled acetyl-CoA. The results are a representative experiment from at least two independent purifications. (D) HAT activity of the immunopurified N-CoR complex. Immunopurified N-CoR or IgG-purified proteins were assessed for HAT activity using free histones as substrates. The results are representative of at least two independent experiments.

finity purification (Figure 3B). We also tested the immunopurified fractions for both acetyltransferase and deacetylase activities. Surprisingly, both activities were detected when using free histones as substrates (parts C and D of Figure 3), further suggesting that N-CoR is capable of interacting with both HDACs (Figure 3C) and HATs (Figure 3D).

Second, to assess the stability of the association between N-CoR and CBP, we attempted to purify N-CoR by passing the 1.0 M KCl fraction eluted from the P11 phosphocellulose column through a series of additional chromatographic columns (Figure 4A). At each chromatographic step, the fractions containing both N-CoR and CBP were pooled and passed through a subsequent column. As shown in Figure 4B, overlapping peaks of immunoreactivity could be detected for both CBP and N-CoR through every column that was employed. The final step included gel-filtration chromatography, which again indicated that both CBP and N-CoR, as well as HDAC3, copurified (Figure 4B). Silver staining of the pooled fractions from each step of the chromatographic purification indicates that approximately 10 proteins copurify with N-CoR using this alternate purification scheme. Furthermore, the association of N-CoR with CBP could not be prevented in cells pretreated with the HDAC inhibitor TSA (data not shown). Collectively, these results support the

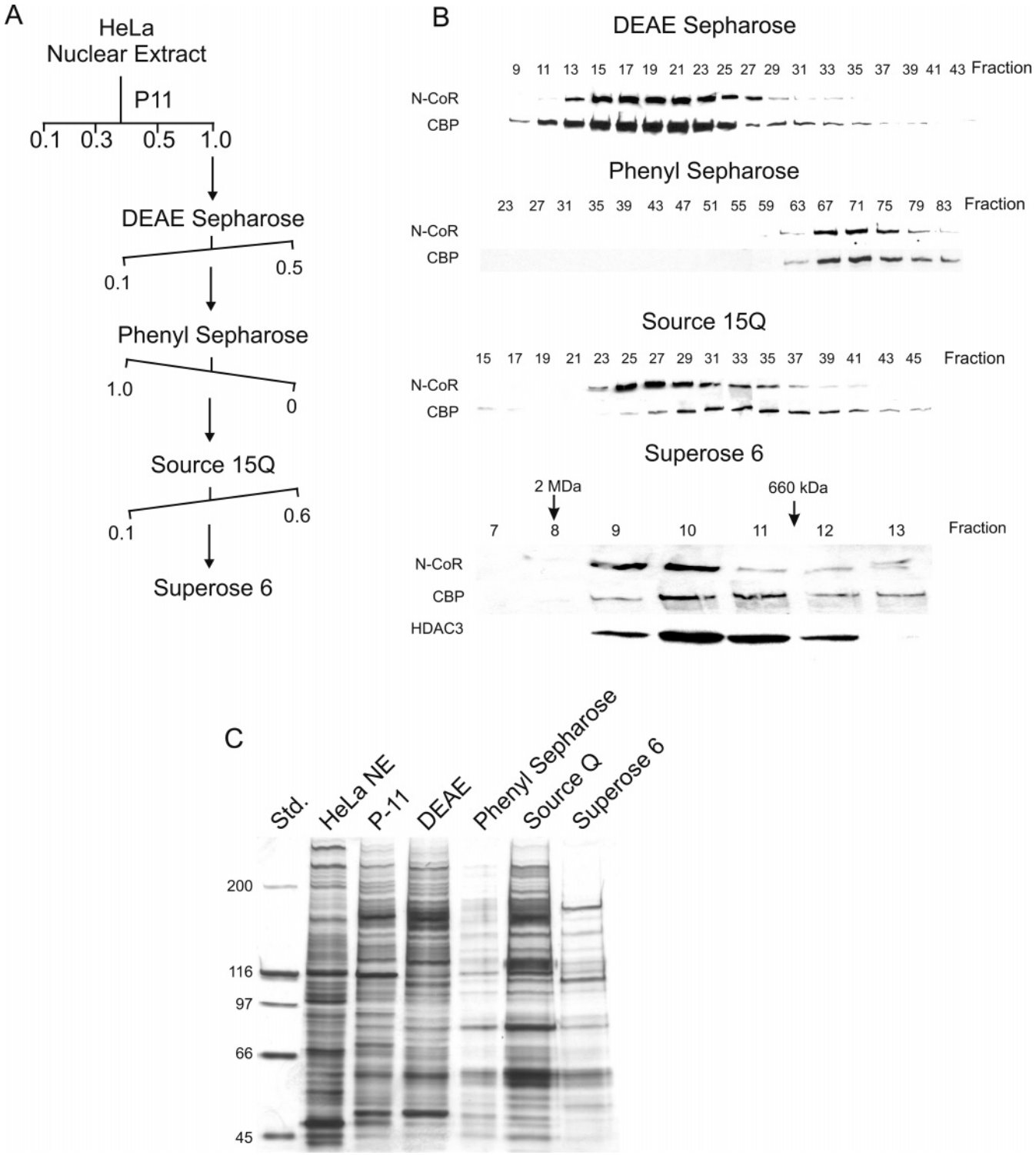

FIGURE 4: Chromatographic copurification of N-CoR and CBP. (A) Schematic representation of the conventional chromatographic steps used to purify N-CoR and CBP. (B) N-CoR copurifies with CBP and HDAC3 through multiple conventional chromatography fractionations. Western blot analysis of the fractions eluted from each of the chromatographic columns utilized. For each sample, approximately 20 μL were separated by SDS−PAGE, transferred to nitrocellulose, and then probed with the antibodies indicated on the left. (C) Silver-stain SDS−PAGE gel of aliquots obtained from the conventional chromatography purification. Fractions after each step of conventional chromatography were pooled, and an aliquot of the pooled fractions was separated by SDS−PAGE and silver-stained. Molecular-mass standards are indicated on the left.

notion that a fraction of CBP does indeed copurify as a component of the N-CoR complex, and this association does not appear to be dependent upon deacetylase activity.

To assess the colocalization of N-CoR and CBP in intact cells, we performed indirect immunofluorescence on asynchronously growing MCF-7 cells using a mouse monoclonal antibody raised against CBP together with affinity-purified anti-N-CoR antibody. Both N-CoR (red staining) and CBP (green staining) were found throughout the nucleus (DAPI, blue staining) as an even speckled pattern excluded from the nucleolus (Figure 5A). Upon merging the images obtained for N-CoR and CBP within the same optical section,

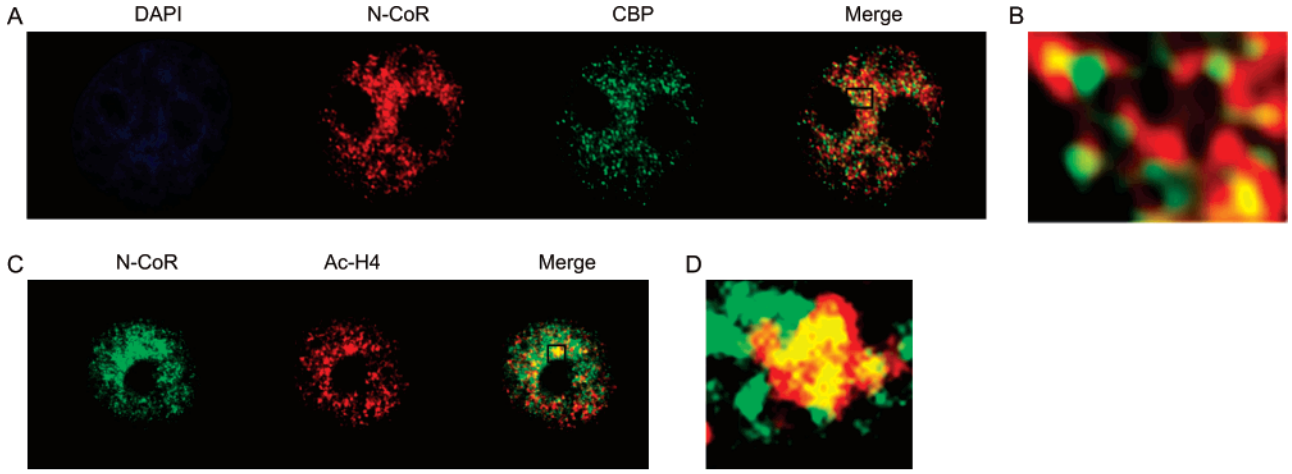

FIGURE 5: N-CoR colocalizes with CBP and acetylated histones within the nucleus in intact cells. (A) N-CoR colocalizes with CBP within the nucleus. Indirect immunofluorescence of asynchronous MCF-7 cells using a mouse monoclonal anti-CBP antibody together with affinity-purified anti-N-CoR antibody. Both CBP and N-CoR staining were found as an even nuclear speckled pattern excluded from the nucleolus. When CBP and N-CoR staining were directly compared, a fraction of both proteins colocalize as demonstrated by the yellow-staining pattern. (B) Enlargement of the area indicated by the black box in the merged image shown in A. (C) N-CoR colocalizes with acetylated histones. Indirect immunofluorescence of asynchronously growing MCF-7 cells using a goat polyclonal antibody anti-N-CoR and a rabbit polyclonal antibody anti-Ac-H4. Both N-CoR and Ac-H4 staining were found as an even nuclear speckled pattern excluded from the nucleolus. When the two images are merged, the resulting yellow color represents the colocalization of N-CoR with acetylated histone H4. (D) Enlargement of the area indicated by the black box in the merged image shown in C.

some colocalization of the two antibodies was observed, as evidenced by the yellow-staining pattern (Figure 5B). However, a significant fraction of the staining for the two proteins was found not to colocalize, suggesting that a substantial fraction of N-CoR and CBP also reside in separate compartments within the nucleus (Figure 5B). To assess if N-CoR could be found in regions of the nucleus containing acetylated histones, we performed indirect immunofluorescence on asynchronously growing MCF-7 cells. N-CoR was stained using a goat polyclonal antibody, and acetylated histones were labeled with rabbit polyclonal acetylated-histone-H4 antibody. Both N-CoR (green staining) and acetylated-histone-H4 (red staining) were found throughout the nucleus as an even speckled pattern excluded from the nucleolus (Figure 5C). Upon merging the images obtained for N-CoR and acetylated-histone-H4 within the same optical section, some colocalization of the two antibodies was observed (yellow-staining pattern, Figure 5D). However, as was the case for the colocalization of N-CoR and CBP, a significant fraction of the staining for the two proteins was found not to colocalize (Figure 5D). Collectively, these data indicate that a fraction of CBP and N-CoR are found in the same complex *in vivo*.

Direct Interaction between N-CoR and CBP. To assess whether N-CoR is capable of directly interacting with CBP, experiments were performed using insect cells overexpressing N-CoR (1−844 amino acids) containing an HA tag at its amino terminus and full-length FLAG-tagged CBP (Figure 6A). A total of 48 h after co-infection, cells were disrupted, extracts were affinity-purified using anti-FLAG Sepharose, and purified proteins were analyzed by Western blotting using an anti-HA antibody. These results indicate that CBP associates with the amino terminus of N-CoR following overexpression in insect cells (Figure 6B). To determine if the association of N-CoR and CBP is the result of a direct interaction, *in vitro* pulldown assays were performed using purified recombinant N-CoR (1−844 amino acids) and purified full-length CBP. The results of these experiments indicated that full-length CBP is capable of interacting directly with the amino terminus of N-CoR (Figure 6C).

The association between N-CoR and CBP may have a number of functional implications. In addition to recruiting other proteins, it has been demonstrated that N-CoR and SMRT have the ability to activate the deacetylase activity of HDAC3. This deacetylase activation function is dependent upon a direct interaction between N-CoR and HDAC3 (*18*). To test if N-CoR has a regulatory role in modulating CBP acetyltransferase activity, we tested N-CoR (1−844 amino acids) for its ability to regulate CBP HAT activity *in vitro*. Surprisingly, increasing concentrations of N-CoR resulted in a dose-dependent inhibition of HAT activity, with a maximal inhibition occurring at approximately 400 nM (Figure 6D). Furthermore, the presence of the HDAC inhibitor sodium butyrate, to inhibit any copurifying deacetylase activity, suggests that the inhibition of HAT activity is not due to the recruitment of HDAC activity but is mediated through an alternate mechanism.

To map the region of N-CoR that interacts directly with CBP and is capable of inhibiting CBP HAT activity, we generated N-CoR deletion mutants fused to GST spanning 1−312 amino acids, which consists of repressor domain 1 (RD1), N-CoR (403−488 amino acids), which contains the first SANT domain and the deacetylase activation domain (DAD), and N-CoR (516−685 amino acids), which contains the second SANT domain and a putative histone interaction domain (HID) (*41*) (Figure 7A). These deletion mutants were tested for *in vitro* binding to full-length recombinant CBP. The results of these experiments demonstrate that the minimal region of N-CoR that is capable of interacting with CBP is found at amino acids 1−312, although a consistently stronger interaction was detected with N-CoR (1−844 amino acids) (Figure 7B). These same deletion mutants were also tested for inhibition of CBP HAT activity *in vitro.* Surprisingly, the region of N-CoR capable of inhibiting CBP HAT activity was found at amino acids 516−685 (Figure 7C) and did not include amino acids 1−312. Interestingly, N-CoR

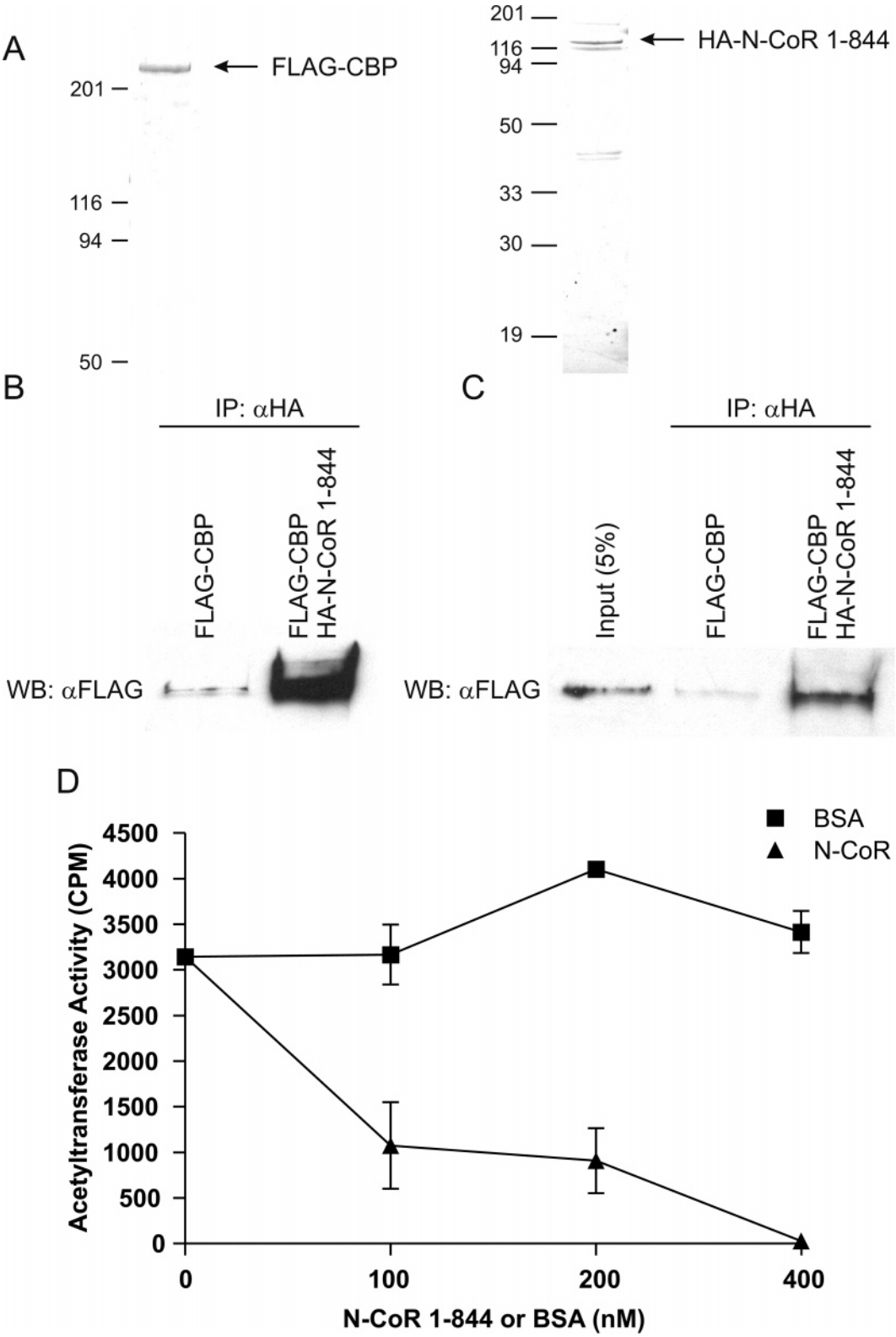

FIGURE 6: N-CoR interacts directly with CBP and inhibits CBP HAT activity. (A) Coomassie stain of recombinant tagged N-CoR and CBP proteins. Sf9 insect cells were infected with baculovirus FLAG-tagged CBP or HA-tagged N-CoR (1−844 amino acids) baculoviruses. A total of 48 h postinfection, the insect cells were lysed and proteins were immunopurified with anti-FLAG or anti-HA agarose, washed extensively, and eluted using FLAG or HA peptide, respectively. The immunoprecipitated proteins were resolved by SDS−PAGE and stained with Coomassie blue. (B) Amino terminus of N-CoR copurifies with CBP. Sf9 insect cells were infected with baculovirus FLAG-tagged CBP alone or in combination with HA-tagged N-CoR (1−844 amino acids) baculoviruses. A total of 48 h postinfection, the insect cells were lysed and proteins were immunopurified with anti-HA agarose. The immunoprecipitated proteins were resolved by SDS−PAGE and probed with anti-FLAG antibody. (C) Amino terminus of N-CoR interacts directly with CBP. Purified recombinant FLAG-CBP was incubated with recombinant HA-N-CoR (1−844 amino acids) immobilized onto anti-HA agarose and then washed extensively. The resulting immunoprecipitated proteins were resolved by SDS−PAGE and probed with anti-FLAG antibody. (D) Amino terminus of N-CoR attenuates CBP HAT activity. Purified recombinant FLAG-CBP was preincubated with increasing amounts of HA-N-CoR (1−844 amino acids) or BSA prior to the measurement of HAT activity *in vitro*.

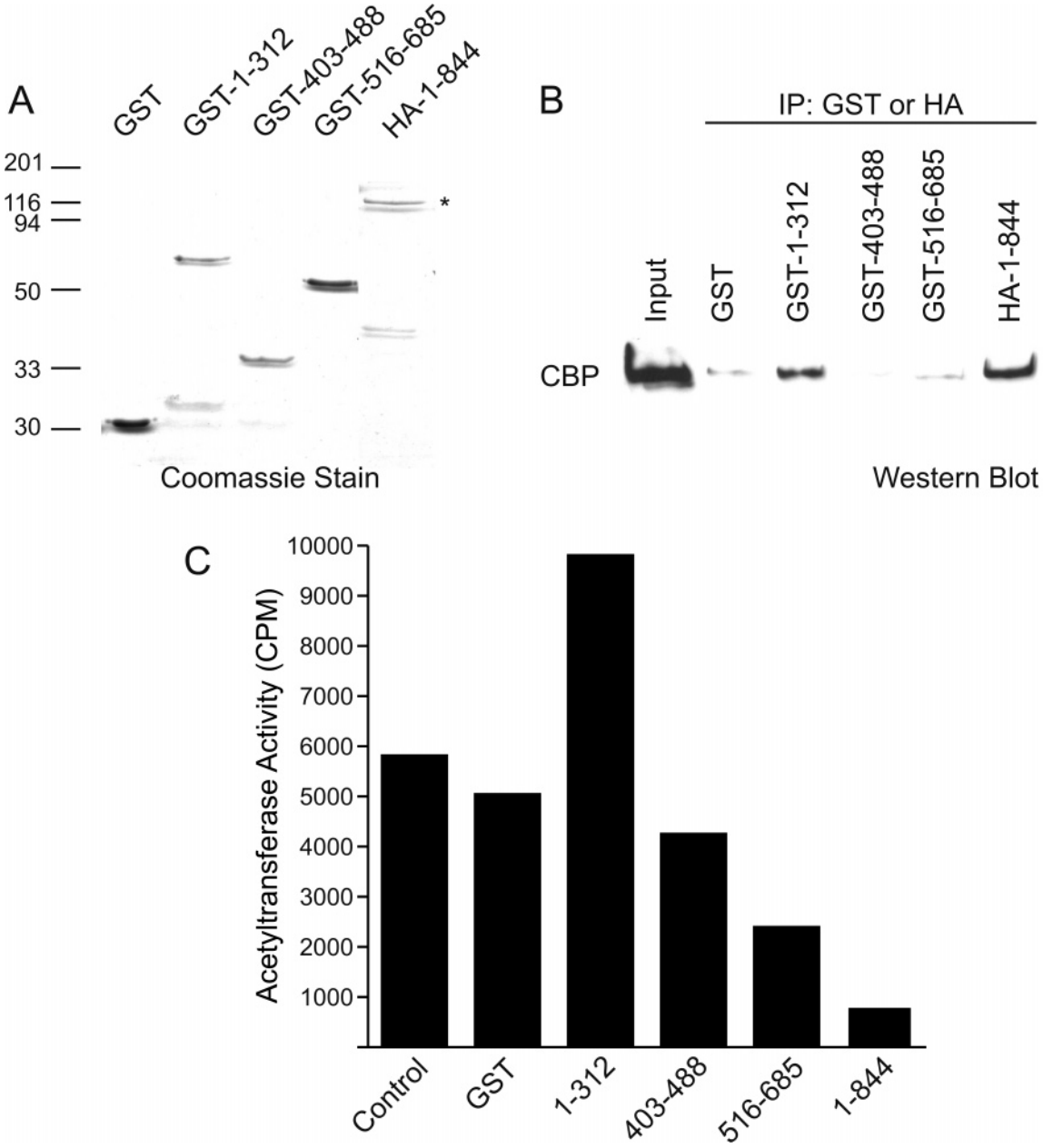

FIGURE 7: Mapping of the CBP interaction and HAT inhibitory domains. (A) Coomassie stain of purified recombinant N-CoR amino-terminal deletion mutants. N-CoR deletion were expressed and purified as described under the Experimental Procedures. Approximately 0.5 μg of the immunopurified proteins were resolved by SDS−PAGE and stained with Coomassie blue. The asterisk represents the correct HA-N-CoR (1−844 amino acids) band. (B) Mapping of the N-terminal N-CoR/CBP interaction domain. Approximately 0.5 μg of recombinant full-length FLAG-tagged CBP was incubated with 0.5 μg of N-CoR deletion mutants and affinity-purified, and the immunoprecipitated complexes were resolved by SDS−PAGE and probed with anti-CBP antibody. The results shown are representative of at least two independent experiments. (C) Mapping of the N-terminal N-CoR CBP HAT inhibitory domain. A total of 50 ng of recombinant purified FLAG-tagged CBP was incubated alone or preincubated with 100 nM purified recombinant N-CoR deletion mutants prior to the measurement of HAT activity *in vitro* using 1 μg of free histones as substrates. The results shown are representative of at least two independent experiments.

(1−312 amino acids) was found to stimulate HAT activity *in vitro*, although as observed with the *in vitro* interaction data, N-CoR (1−844 amino acids) is a more effective inhibitor of acetyltransferase activity. Collectively, these data suggest that the amino terminus of N-CoR is capable of both directly interacting with CBP and inhibiting HAT activity.

DISCUSSION

The purpose of this study was to determine if the protein acetyltransferase CBP could stably associate with N-CoR. We have provided several lines of evidence to demonstrate that a component of endogenous N-CoR does indeed associate with CBP. First, biochemical purification studies using both immunoaffinity and conventional chromatography show that CBP copurifies with N-CoR. We also demonstrate that N-CoR is capable of copurifying with the related protein p300; however, it appears that these interactions are not conserved with the related corepressor SMRT. In addition, HAT activity based on the acteylation of free histones copurifies with the N-CoR complex, which is consistent with the presence of CBP. We further demonstrate that the amino terminus of N-CoR is capable of interacting directly with full-length CBP *in vitro*, suggesting that the association may be direct. Second, immunofluorescence studies clearly

demonstrate that a component of both N-CoR and CBP colocalize in the nucleus. However, the colocalization is not complete, and a significant fraction of these proteins reside in separate compartments.

Recent studies have suggested that transcriptional activation and repression are highly dynamic processes involving a large repertoire of coactivator and corepressor proteins that assemble at specific promoters in a sequential and combinatorial fashion (*36*, *37*). Although it has often been observed that coactivators and corepressors are functionally separated, this separation is not always absolute. For example, ChIP studies indicate that HDAC1 and HDAC7, which are normally viewed as corepressors, are integral components of the transcriptional activation cycle and play a role in transcriptional activation (*36*). Genome-wide expression analysis using DNA microarrays has shown that mRNA levels for many genes are elevated in yeast mutants carrying mutations for SWI/SNF (*38*, *39*). These studies also demonstrate that gene regulation by SWI/SNF occurs at the level of individual promoters rather than over chromosomal domains, providing further evidence that SWI/SNF is targeted to specific genes to repress transcription. Studies investigating the genome-wide function of histone deacetylase in yeast have provided further evidence for a direct role of HDACs in the transcriptional activation of a subset of genes. It has also been shown that, within chromatin regions of high transcriptional activity, HATs and HDACs can coexist within the same nuclear compartment. Thus, it is possible that the association between N-CoR and CBP may occur in such a compartment and provide a mechanism for the efficient regulation of hormone-dependent gene expression.

Our work demonstrates that the region of N-CoR (516−685 amino acids) containing a HID is responsible for inhibiting CBP HAT activity *in vitro* and is consistent with a previous report demonstrating that the corepressor SMRT (1−891 amino acids) was also capable of attenuating HAT activity (*41*). The major CBP interaction domain was found at N-CoR (1−312 amino acids), a region that contains RD1 and is distinct from the HAT inhibitory domain. On the basis of these results, we propose that N-CoR contains two distinct domains that function to regulate CBP activity. The first domain (N-CoR 1−312 amino acids) functions to sequester CBP through direct binding, while the second domain (N-CoR 516−685 amino acids) silences CBP HAT activity through a mechanism that may involve direct binding of N-CoR to histones. Accordingly, such a mechanism may function to position a coactivator at a promoter in a transcriptionally inactive state so that it is poised to respond to specific signals that activate transcription. Indeed, such a concept has been previously proposed to explain the direct interaction observed between N-CoR and ACTR (*40*).

This does not, however, exclude the possibility that the direct association between N-CoR and CBP may have other functional consequences. The N-CoR/CBP association may represent a distinct complex specifically involved in transcriptional activation events that are dependent upon the promoter context. For example, studies using embryonic fibroblasts isolated from N-CoR knockout mice indicate that disruption of N-CoR results in inhibition of retinoic-acid-dependent transcription activation from a DR1-containing reporter plasmid (*32*). Interestingly, this activation could also be attenuated by disruption of CBP and HDAC3, suggesting that N-CoR recruitment is a necessary requirement for activation of the DR1-containing reporter and that this activation is dependent upon receptor conformation, which may be dictated by the transcription factor binding site (*32*). Also of note, a recent paper using a purified *in vitro* transcription system has demonstrated a transcriptional repression activity associated with the CBP-related coactivator protein p300 (*42*). Indeed, there are several published reports of CBP and p300 participating in transcriptional repression (*43*−*45*).

Another possibility is that the HAT activity of CBP may modulate transcriptional repression by directly acetylating N-CoR or additional N-CoR-associated proteins during transcriptional repression. Although acetylation is most commonly associated with the modification of histones, numerous studies have shown that HATs also play a role in modifying nonhistone proteins. The HAT activity of p300 can both promote and inhibit transcription by modifying nonhistone proteins. For example, direct acetylation of p53 by p300 contributes to its activation by facilitating DNA-binding activity and recruitment of coactivators (*46*). In contrast, acetylation of the PLZF DNA-binding domain by p300 promotes repression by p300 (*47*). Alternatively, acetylation could regulate protein−protein interactions necessary for the recruitment of additional proteins by N-CoR. The nuclear receptor coactivator ACTR is acetylated *in vivo* by CBP, and this acetylation is required to terminate the ligand-dependent interaction between the retinoic acid receptor and ACTR (*48*). Interestingly, while this paper was in review, it was demonstrated that p300 co-immunoprecipitates with the glucocorticoid receptor (GR) and HDAC1 and that acetylation of HDAC1 by p300 reduces HDAC1 deacetylase activity (*49*). Thus, it is conceivable that N-CoR or one of its core complex components may be directly acetylated by CBP, and that this acetylation may modulate the function of the complexes.

Finally, HATs and HDACs may both be required for mediating the transcriptional repression function of N-CoR by covalently modifying histones in a coordinated fashion. Therefore, the switch from transcriptional repression to activation may not be an all or none event only involving an exchange of complexes. Rather, it may be the balance between acetylation and deacetylation that defines the overall activation or repression potential. This would imply that the combination of acetylation and deacetylation is hierarchical, such that the covalent modification at one site results in allosteric changes that serve as a prerequisite for covalent modification at a subsequent site within a histone or nonhistone protein. This hypothesis is consistent with the theory of a histone code, where distinct histone amino-terminal modifications such as acetylation and/or methylation can generate synergistic or antagonistic interaction affinities for chromatin-associated proteins, which in turn dictate dynamic transitions between transcriptional activation/repression.

In conclusion, we have identified a novel association between N-CoR and CBP and suggest several novel roles for N-CoR in transcription. In addition to recruitment and activation of HDAC3, N-CoR may mediate its transcriptional effects by inhibiting CBP HAT activity. Although the full ramifications of the presence of coactivators and corepressors

in a single coregulatory complex is not yet fully understood, our work provides additional evidence that coactivators and corepressors interact directly, and that their simultaneous activity may in some cases be required to regulate transcription.

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