A Novel Nuclear Receptor Corepressor Complex, N-CoR, Contains Components of the Mammalian SWI/SNF Complex and the Corepressor KAP-1*

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Transcriptional silencing by many transcription factors is mediated by the nuclear receptor corepressor (N-CoR). The mechanism by which N-CoR represses basal transcription involves the direct or indirect recruitment of histone deacetylases (HDACs). We have isolated two multiprotein N-CoR complexes, designated N-CoR-1 and N-CoR-2, which possess histone deacetylase activity that is mediated by distinct HDACs. Based on Western blotting using antibodies against known subunits, the only HDAC found in the N-CoR-1 complex was HDAC3. In contrast, N-CoR-2 contained predominantly HDAC1 and HDAC2 as well as several other subunits that are found in the Sin3A-HDAC complex. Using mass spectrometry and Western blotting, we have identified several novel components of the N-CoR-1 complex including the SWI/SNF-related proteins BRG1, BAF 170, BAF 155, BAF 47/IN11, and the corepressor KAP-1 that is involved in silencing heterochromatin. Indirect immunofluorescence has revealed that both KAP-1 and N-CoR colocalize throughout the nucleus. These results suggest that N-CoR is found in distinct multiprotein complexes, which are involved in multiple pathways of transcriptional repression.

Cellular proliferation and differentiation is critically dependent on the ability of specific DNA-binding transcription factors to activate or repress the transcription of target genes in a coordinated fashion. This is accomplished through transcription factor-mediated recruitment of coactivators and corepressors, which can regulate transcription through the modification of the local chromatin environment and by specifically interacting with components of the core transcriptional machinery (1, 2). The nuclear hormone receptor superfamily provides a unique model to study the mechanism of transcriptional activation and repression and the role of reversible chromatin modification in the control of gene expression. In the absence of ligand, nuclear hormone receptors such as the thyroid hormone (TR)1 and retinoic acid receptors (RAR) repressors by interacting with specific corepressor proteins (3, 4). Ligand binding induces a conformational change in these receptors that results in corepressor release and the recruitment of coactivator proteins (2, 5). The nuclear receptor corepressor (N-CoR), and its related family member silencing mediator for retinoid and thyroid hormone receptor (SMRT), were initially identified by yeast two hybrid screening using unliganded TR or RAR as bait, respectively (3, 4). Several lines of evidence suggest that both N-CoR and SMRT mediate the repressive effects of nuclear hormone receptors. First, both N-CoR and SMRT contain two nuclear receptor interaction domains (IDs) in the carboxyl terminus. Molecular characterization of the IDs reveals the presence of a signature Cor box motif that is necessary and sufficient for receptor interaction and ligand-induced release of N-CoR or SMRT (6–8). More recently, a strong correlation between repression mediated by the TR and recruitment of N-CoR or SMRT have also been demonstrated in Xenopus oocytes (9, 10). Second, microinjection of anti-N-CoR antibodies into living cells blocks T3R- and RAR-mediated repression (11).

N-CoR and SMRT contain three highly conserved repression domains designated RD1, RD2, and RD3 (3, 4, 7, 8, 12). The lack of homology between these domains suggests that their mechanism of action is mediated via distinct pathways. RD1 serves as a major interacting surface for the corepressor Sin3A, which, in turn, can directly interact with the histone deacetylase 1 and 2 (HDAC1 and 2), suggesting that repression by N-CoR and SMRT is linked to the deacetylation of histones (11, 12). Single cell microinjection studies using antibodies against mSin3, or its associated histone deacetylases, inhibits repression by unliganded T3R and RAR (11). Transcriptional repression by nuclear hormone receptors is also blocked by deacetylase inhibitors such as Trichostatin A (TSA) (11). Taken together, these studies suggest that the repressive effects of N-CoR and SMRT are mediated in part by a Sin3-HDAC complex. In contrast, the RD3 of SMRT and N-CoR interacts with the class II histone deacetylases that are structurally related to the yeast Hda1 protein (13–15). The interaction between SMRT and class II HDACs occurs in vivo and in vitro and correlates with the repressor activity of RD3.

Recent studies have also implicated N-CoR and SMRT in transcriptional repression that is mediated by several other families of transcription factors including Notch (16), the homeodomain proteins RXP and Pit-1 (17), p53 (18), and the antagonist-bound estrogen and progesterone receptors (19, 20).

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1The abbreviations used are: T3R, thyroid hormone receptor; RAR, retinoic acid receptor; N-CoR, nuclear receptor corepressor; HDAC, histone deacetylase; SMRT, silencing mediator for retinoid and thyroid hormone receptors; ID, interaction domains; RD, repression domain; TSA, Trichostatin A; PAGE, polyacrylamide gel electrophoresis; MALDI-MS, matrix-assisted laser desorption ionization-mass spectrometry; PSD, postsource decay; BAF, BRG1-associated factor; SR-CAP, SWI/SNF-related CBP activator protein; m, mouse; h, human.
Abnormal recruitment of N-CoR and SMRT has also been linked to pathological disorders such as acute promyelocytic leukemia (PML) that is associated with rearrangements of the gene encoding the RARo receptor and the PML zinc finger protein. This results in the formation of a PML-RAR fusion protein that blocks myeloid differentiation. Treatment with retinoic acid causes the release of N-CoR/SMRT and abolishes the PML-RAR-mediated differentiation block (21, 22).

Biochemical purification of the mammalian Sin3 has identified a Sin3 complex, which consists of 8–10 proteins and includes HDAC1 and 2, Rhap46/48, Sap 18, and Sap 30 (23, 24). However, neither N-CoR nor SMRT were identified as components of the mSin3/HDAC complex, or a related complex known as NURD/Mi2/NRD, which contains both histone deacetylase and nucleosome remodeling activity (25–27). Based on these studies, N-CoR may not be a stable component of a deacetylase complex and may function as an adaptor protein for sequence-specific transcriptional repressors upon activation of specific signaling pathways. Alternatively, it has been suggested that N-CoR may be targeting a different subpopulation of cellular mSin3 or HDACs (28).

To address this issue, we have undertaken a biochemical purification of endogenous N-CoR from HeLa cell nuclear extracts by combining conventional chromatography with immunoaffinity purification using an affinity-purified anti-N-CoR antibody. We have isolated two major N-CoR-containing complexes designated N-CoR-1 and N-CoR-2, which on the basis of gel filtration chromatography are each approximately 2.0 MDA. Both complexes possess intrinsic histone deacetylase activity based on their ability to deacetylate core histones. We have demonstrated that N-CoR-2 contains proteins that are found in the Sin3/HDAC and NURD/Mi2/NRD complexes but are not found in N-CoR-1 suggesting that N-CoR-1 is a novel N-CoR-containing complex. Using mass spectrometry we have identified several components of the N-CoR-1 complex that include several subunits of the SWI/SNF-related chromatin remodeling complexes as well as the corepressor Krab associated protein 1 (KAP-1). Taken together, these findings implicate N-CoR in multiple pathways of transcriptional repression that may involve the recruitment of distinct corepressor complexes.

EXPERIMENTAL PROCEDURES

Western Blotting and Antibody Production—Subcellular fractions of cells were prepared according to standard methods (29). Western blotting was performed as described previously (30). Protein samples were separated by SDS-PAGE, transferred to nitrocellulose, and detected by enhanced chemiluminescence according to the manufacturer’s recommendations (Amersham Pharmacia Biotech). The HDAC2 and Sin3A antibodies were purchased from Santa Cruz Biotechnology. The anti-N-CoR antibody was raised against a His-tagged recombinant N-CoR (amino acids 2323–2453) protein and was purified by protein A-Sepharose chromatography. To further purify the anti-N-CoR antibody, the IgG fraction was passed through an affinity column consisting of the His-tagged recombinant N-CoR protein (amino acids 2323–2453) cross-linked to Sepharose 4B, and the specific antibody was eluted with 100 mM glycine pH 2.8 (31).

Purification of the N-CoR Complexes—Forty liters of HeLa cells, grown to mid-log phase, were obtained from the National Cell Culture Center (Minneapolis, MN). Nuclear extracts were prepared according to standard methods (29). To purify the nuclear N-CoR complexes, the nuclear extract was dialyzed against buffer A (20 mM Tris-HCl, pH 7.9, 0.5 mM EDTA, 0.5 mM EGTA, 10% glycerol, 0.5 mM dithiorthiolutol, 0.2 mM phenylmethylsulfonil fluoride and 5 μg/ml each of leupeptin, aprotonin, and pepstatin A) containing 100 mM KC1 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. At each step, the column was washed with 2 column volumes of buffer containing the corresponding salt concentration to allow UV absorbance to stabilize near baseline prior to collection of the next protein peak. The majority of immunoreactive N-CoR was found in the 0.3- and 0.5-M KCl fractions. The N-CoR-containing fractions were pooled, dialyzed against buffer A containing 100 mM KC1, and then passed through a DEAE-Sepharose column and eluted with an increasing KC1 gradient. All fractions were analyzed by Western blotting using the N-CoR-containing antibody. The fractions containing N-CoR were 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 KC1. The column was washed with buffer A at a flow rate of 0.4 ml/min. Each N-CoR complex eluted as a single large molecular mass peak of approximately 2.0 MDA. The fractions corresponding to each peak were analyzed for N-CoR by Western blotting, pooled, and dialyzed against buffer A containing 100 mM KC1 without diethiothreitol. For affinity purification of the N-CoR complexes, affinity-purified N-CoR antibody was cross-linked to protein A-Sepharose using dimethylpalmilate according to standard procedures (31). 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 five times. The bound proteins were washed with 10 column volumes each of buffer A (without diethiothreitol) containing 0.1% Nonidet P-40 and 0.3 M KCl, 0.1% Nonidet P-40 and 0.5 M KCl, or 0.5% Nonidet P-40 and 0.7 M KCl, and a final wash in buffer A containing 100 mM KC1 no elution with 100 mM glycine (pH 2.8). For mass purification experiments, samples from the gel filtration step were loaded onto protein A-Sepharose alone, or protein A-Sepharose cross-linked to an irrelevant antibody.

Mass Spectrometry—Proteins were separated by 7% SDS-PAGE and then stained with colloidal blue for 1 h followed by destaining in 25% MeOH for an additional 2 h. The protein bands were excised and cut into 1 mm2 pieces. The gel pieces were washed twice in a 50% CH3CN solution for 5 min followed by two washes with a 250-μl solution consisting of 50% CH3CN, 50 mM NH4HCO3 for 30 min. The gel pieces were lyophilized, rehydrated in 10 mM NH4HCO3, pH 8.5 containing 0.1 μg/ml trypsin (Roche Molecular Biochemicals) and incubated overnight at 37°C. The trypptic fragments were extracted by two 30-min washes with a solution containing 60% CH3CN, 10% trifluoroacetic acid. The combined solutions were lyophilized using a Speedvac, resuspended in 20 μl of 0.5% trifluoroacetic acid solution and the peptide suspensions were purified using a ZipTip (Millipore) cartridge according to the manufacturer’s instructions prior to analysis by MALDI-MS. MALDI-MS analyses were carried out by using a Perseptive Biosystems Voyager-DE STR mass spectrometer (Perseptive Biosystems Inc., Framingham, MA), with a pulsed UV nitrogen laser (337 nm, 3-ns pulse) and a dual microchannel plate detector. The spectra were acquired in reflectron-DE mode, acceleration voltage was set to 20 kV, grid voltage at 72% of the acceleration voltage, guide wire voltage at 0.020%, delay time at 140 ns, low mass gate was set at 250 Da, and the mass to charge ratio was calibrated internally with the dimer of α-cyano-4-hydroxycinnamic acid (2M+H+, 390.09 Da) and trypsin autolysis peptide ((M+H)+, 2163.05 Da). Two analysis cycles were combined with mass peak summing (m/z 354–200) and hydroxycinnamic acid and one part of nitrocellulose were dissolved in acetone-isopropl alcohol (4:1) to final concentrations of 20 and 10 mg/ml, respectively. A 0.5-μl volume of this solution was deposited on MALDI target and allowed to dry. 0.5 μl of 1% acetic acid was placed on top of the matrix layer followed by addition of 1 μl of analyte solution. Mass spectra were recorded after evaporation of the solvent and processed using GRAMS software for data collection and analysis.

Peptide sequence analysis was conducted by Post-Source Decay (PSD) technique. The spectra were acquired at DE-reflection mode. The accelerating voltage was set to 20 kV, grid voltage at 75%, guide wire voltage at 0.024%, and delay time at 100 ns. The timed ion selector was preset to the protonated molecular weight of the analyte. The spectra were acquired in 10–13 segments with mirror ratio 1.0 to 0.1542 and finally “stitched” together by the instrument software. The tryptic peptides obtained were used to search for protein candidates in the nonredundant protein sequence database with the program PROWL that is publicly available.

Immunofluorescence—HeLa cells were plated onto Fisher brand microscope slides and maintained in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum at 37°C with 5% CO2. Cells were allowed to adhere for approximately 18 h prior to fixation with 4% paraformaldehyde for 10 min. The cells were then permeabilized with 0.2% Triton X-100 in phosphate-buffered saline and blocked for 30 min in 5% goat serum. After a brief wash, slides were incubated with rabbit anti-N-CoR antibody (1:300 dilution) and anti-KAP-1 mouse monoclonal IgG antibody supernatant (1:10 dilution) in 3% bovine serum albumin at 4°C.
supernatants were pooled and dialyzed extensively against 100 mM buffer (N-CoR-1). N-CoR immunoreactivity was also found in

37 °C for 90 min and stopped by the addition of 0.05 ml of 0.1M HCl, 0.7

was used for liquid scintillation counting.

ethyl acetate. After centrifugation, 0.3 ml of the upper organic phase

containing 0.42M KCl, followed by centrifugation at 10,000

fractions (32). Briefly, 1 liter of HeLa cells were grown to a density of

9% fetal bovine serum. The cells were preincubated with 10 mM

were then extracted twice with 0.2M H2SO4 for 90 min in an ice bath

A similar elution profile was obtained with other members of

that efficient protein separation was achieved with the P11

component of a corepressor complex, we generated a polyclonal

antibody against the carboxyl terminus of N-CoR. The affinity-

purified N-CoR antibody was cross-linked to protein A-Sepharose, and the N-CoR-con-

these complexes further, the affinity purified N-CoR antibody

N-CoR is a 260-kDa protein, this result strongly suggests that N-CoR is found as part of a large multiprotein complex. To purify

eluted from the P11 phosphocellulose column. The salt concentrations

purify the N-CoR complexes.

 results

Identification of N-CoR-containing Complexes—As a first

step in determining whether endogenous N-CoR is a stable

component of a corepressor complex, we generated a polyclonal

antibody against the carboxyl terminus of N-CoR. The affinity-

purified antibody recognized a single protein band of approxi-

mately 2 mg/ml. For each sample, 20 µg of protein were separated by SDS-PAGE, transferred to nitrocellulose, and then probed with the specific antibodies indicated on the left. C, gel filtration chromatography of the N-CoR complexes using a Sephacryl S300 column. The molecular mass standards are indicated at the top of the panel; 660 kDa, thyroglobulin; 440 kDa, ferritin.

FIG. 1. Purification of N-CoR-containing complexes. A, schematic representation of the various chromatographic steps used to purify the N-CoR complexes. B, Western blot analysis of the fractions eluted from the P11 phosphocellulose column. The salt concentrations used to purify the individual fractions are indicated at the top. For each sample, 20 µg of protein were separated by SDS-PAGE, transferred to nitrocellulose, and then probed with the specific antibodies indicated on the left. C, gel filtration chromatography of the N-CoR complexes using a Sephacryl S300 column. The molecular mass standards are indicated at the top of the panel; 660 kDa, thyroglobulin; 440 kDa, ferritin.

purification of N-CoR-containing complexes. Because

the relative abundance of Sin3A was also examined in the

fractions eluted from the phosphocellulose column. Although

Sin3A was detected in all of the N-CoR-containing fractions,

the relative distribution was not consistent with the distribu-

tion of N-CoR. Based on densitometric scanning, only 7% of the total amount of Sin3A was found in the 0.3 M KCl fraction, suggesting that the major N-CoR-containing fraction is not complexed to Sin3A but may function in other contexts.

To characterize these complexes further, we subjected the

P11-purified fractions to purification by DEAE-Sepharose fol-

lowed by gel filtration chromatography using a Sephacryl S-300 column. Immunoblot analysis of the eluates derived from the Sephacryl S300 column demonstrated that N-CoR-1 and N-CoR-2 eluted with the void volume corresponding to a molecular mass of approximately 2.0 MDa (Fig. 1C). Because N-CoR is a 260-kDa protein, this result strongly suggests that it is found as part of a large multiprotein complex. To purify these complexes further, the affinity purified N-CoR antibody was cross-linked to protein A-Sepharose, and the N-CoR-containing fractions isolated from the Sephacryl S300 column were pooled and applied to the immunoaffinity column. After extensive washing with highly stringent buffer containing 0.5% Nonidet P-40 and 0.7 M KCl, the retained proteins were eluted with 100 mM glycine, pH 2.8.
Purification of Corepressor Complexes

**Table I**

| N-CoR-1 complex | Proteins identified |
|-----------------|---------------------|
| p350            | SWI/SNF-related CBP activator protein |
| p300            | Nuclear receptor corepressor (N-CoR) |
| p170            | SWI/SNF-related 170-kDa subunit (BAF 170) |
| p150            | SWI/SNF-related 155-kDa subunit (BAF 155) |
| p130            | Splicing factor 3b SAP190. |
| p120            | Splicing factor 3a120 |
| p110            | Nuclear corepressor KAP-1/TIF1β |
| p48             | hSNF 5/INI 1 (BAF 47) |

This suggests that they are not intrinsic components of the N-CoR-1 complex and are most likely associated with other multiprotein complexes. The only component that was detected by Western blotting was HDAC3, which is consistent with recent observations (9, 10, 34, 35). In addition, we were unable to identify either RhAp48, which has been demonstrated to be a common subunit found in several chromatin-modifying complexes, or Sap 30 a component of the Sin3/HDAC complex (36, 37). Taken together, these observations suggest that the N-CoR-1 complex contains novel components.

The finding that HDAC3 is a component of the N-CoR-1 complex prompted us to examine whether N-CoR-1 complex also possesses deacetylase activity. 3H-labeled core histones were purified from HeLa cells and preincubated with immunopurified N-CoR-1, and the deacetylase activity was monitored by quantifying the release of radiolabeled acetyl-CoA. We observed that N-CoR-1 can deacetylate core histones in this assay, consistent with the presence of HDAC3, and that the deacetylation activity is blocked when the complex was preincubated with the deacetylase inhibitor TSA (Fig. 2D).

**Mass Spectrometric Analysis of the N-CoR-1 Complex**—To identify novel components of the N-CoR-1 complex, SDS-PAGE analysis was performed, and several of the protein bands were excised and subjected to in-gel tryptic digestion. The peptides were then isolated and analyzed by mass spectrometry (Table I, Fig. 3). The majority of the tryptic fragments derived from the 300-kDa protein band corresponded to N-CoR. We also detected several tryptic fragments corresponding to the related family member SMRT, although these were relatively minor components. Interestingly, several of the proteins that consistently copurified with N-CoR have been found as intrinsic components of unrelated, yet biologically important complexes. For example, SAP 130 and SF3a120 are subunits of protein complexes essential for spliceosome assembly (38, 39). However, a function for SAP 130 and SF3a120 in transcriptional regulation has not been previously uncovered.

Four of the proteins identified by mass spectrometry belong to the SWI/SNF family of proteins and include the BRG1-associated factor (BAF) 170, BAF 155, BAF 47/INI1, and the SWI/SNF-related CBP activator protein (SRCAP) (40). BAF 170 and BAF 155 are highly similar homolog of the yeast SWI3 protein, whereas BAF 47/INI1 represents the mammalian homolog of the yeast SNF5 protein. All three proteins have been identified as subunits of several SWI/SNF-related chromatin remodeling complexes including the human BRG1 and hBRM complexes (41, 42). We were unable to obtain definitive evidence for the BRG1 component of the SWI/SNF complex using mass spectrometry. However Western blotting using an anti-BRG1 antibody subsequently demonstrated that the p190 subunit is BRG1, suggesting that the core components of the SWI/SNF complex are found in the N-CoR-1 complex (Fig. 4).

To verify that these components colocalize with N-CoR, Western blotting was performed using polyclonal antibodies...
that recognize either BRG1, BAF 170, BAF 155, or BAF 47. We found that all of these proteins eluted as a high molecular weight complex from the Sephacryl S300 column. Although the overlap with N-CoR was clearly significant, it was not identical suggesting that the association of N-CoR with SWI/SNF is not 100%. (Fig. 4A). Importantly, all of these same proteins are found in the immunoaffinity-purified N-CoR-1 complex (Fig. 4B), consistent with the mass spectrometry data.

**KAP-1 and N-CoR Colocalize in Vivo**—The 110-kDa protein identified by mass spectrometry corresponds to KAP-1, also known as TIF1β (43, 44). KAP-1 is a 97-kDa transcriptional corepressor that interacts with proteins containing the Kruppel-associated box domain (KRAB), a common motif found in many DNA-binding transcriptional repressor proteins. Recent observations have also demonstrated that KAP-1 interacts in vivo and in vitro with members of the heterochromatin protein 1 (HP-1) family, which are important for regulating heterochromatin-mediated gene silencing (45, 46). HP-1 proteins have also been implicated in position effect variegation, a euchromatic silencing mechanism exhibited by genes placed within or adjacent to heterochromatin (47). Interestingly, it has been shown that TSA can interfere with KAP-1/TIF1β-mediated repression (48). This suggests that the mechanism of repression mediated by KAP-1 also involves histone deacetylation and is consistent with the presence of HDAC3 in the N-CoR-1 complex.

To verify that KAP-1 colocalizes with N-CoR, Western blotting was performed using polyclonal antibodies, which recognize either N-CoR or KAP-1. We found that a significant component of KAP-1 also copurified with N-CoR as a high molecular weight complex from the Sephacryl S300 column (Fig. 5A) and was also present in the immunoaffinity-purified N-CoR-1 complex (Fig. 5B). To define a potential in vivo role for KAP-1/N-CoR association, we performed indirect immunofluorescence on asynchronously growing HeLa cells using a monoclonal antibody raised to KAP-1 (kindly provided by Dr. F. Rauscher III) together with affinity-purified anti-N-CoR antibody. Both KAP-1 and N-CoR staining were found throughout the nucleus as an even speckled pattern (Fig. 5C). In some regions, both N-CoR and KAP-1 were concentrated in micropunctate-like structures, which may represent regions of pericentromeric heterochromatin. When KAP-1 and N-CoR staining were directly compared, a significant component of the signals overlap particularly in the granular regions presumed to be pericentromeric heterochromatin. Taken together, these data indicate that a major fraction of KAP-1 and N-CoR are found in the same complex in vivo and may function through a similar mechanism.

**Identification of the N-CoR/Sin3-HDAC Complex**—A similar purification scheme was used to affinity purify the N-CoR-2 complex. However, SDS-PAGE analysis revealed a different protein profile when compared with the N-CoR-1 complex (Fig.
In this case, approximately 12–15 proteins consistently copurified with N-CoR. Surprisingly, we observed that several of the proteins found in the Sin3-HDAC complex are also found in the immunopurified N-CoR-2 complex including Sin3A, HDAC1 and HDAC2, and Sap30 (Fig. 6B). HDAC3 was detected in the N-CoR-2 complex although it does not appear to be a stoichiometric component. We could not detect RbAp48 although it was clearly present in the Sephacryl S300 fraction.

To assess whether N-CoR-2 also possesses histone deacetylase activity, ³H-labeled core histones purified from HeLa cells were preincubated with the immunopurified complexes, and the deacetylase activity was monitored by quantitating the released ³H-labeled acetyl-CoA. The reaction was allowed to proceed for 90 min prior to extraction and quantitation of the released ³H-labeled acetyl-CoA. The results are a representative experiment from at least two independent purifications.

**DISCUSSION**

Functional studies using microinjection of neutralizing antibodies into living cells have provided strong evidence suggesting that the repressive effects of N-CoR and SMRT are mediated, in part, through the recruitment of a Sin3-HDAC complex (11, 12). However, biochemical purification studies have been...
unable to demonstrate the existence of an endogenous N-CoR-Sin3A-HDAC complex (15, 23, 24). Two hypotheses have been proposed to explain these results. First, it is possible that the interactions between N-CoR or SMRT and the Sin3-HDAC complex are transient or require the recruitment of additional factors to become stable components (49). Second, that multiple N-CoR complexes exist, which contain distinct subunits and become associated with different classes of transcription factors. Partial evidence for this hypothesis has come from studies using confocal microscopy, which demonstrated that the distribution of N-CoR is heterogeneous and that only a small fraction of endogenous N-CoR is colocalized with HDAC1(28).

In the present study, we have used phosphocellulose chromatography to separate endogenous N-CoR into two chromographically distinct fractions designated N-CoR-1 and N-CoR-2. We have purified N-CoR-1 and N-CoR-2 to apparent homogeneity and have used both Western blotting and mass spectrometry to demonstrate that each complex contains distinct proteins. For example, although N-CoR-1 and N-CoR-2 both possess HDAC activity, this activity is mediated by different HDACs. HDAC3 represents the major class I deacetylase in the N-CoR-1 complex, whereas HDAC1 and 2 are found only in the N-CoR-2 complex. The presence of different catalytic subunits in each of the N-CoR complexes suggests that they may have unique functional roles in the cell.

Our results are consistent with recent findings demonstrating that HDAC3 is part of a complex, which also includes N-CoR or SMRT (9, 10, 34, 35). It is interesting that the number of proteins which we have identified in the N-CoR complexes is considerably greater than the number of proteins identified in the SMRT core complex. This suggests that there are intrinsic differences between SMRT and N-CoRs ability to interact with other intracellular proteins.

In addition to HDAC3, the N-CoR-1 complex also contains several proteins found in the SWI/SNF complex (50). The SWI/SNF complex is the prototypical chromatin remodeling complex which has been shown to disrupt chromatin structure and facilitate the binding of transcriptional activators to nucleosomal sites (51–53). The glucocorticoid receptor (GR) can target the SWI/SNF complex to chromatinized templates containing GR binding sites in yeast and in mammalian cells resulting in disruption of local nucleosomal structure (54, 55). Multiple mammalian SWI/SNF complexes have also been identified that have diverse subunit compositions although BRG1, BAF 170, BAF 155, and BAF 47 represent the core components (41, 42). BRG1 is homologous to the yeast SWI2/SNF2 and possesses DNA-dependent ATPase activity, a necessary function carried out by all chromatin remodeling complexes identified to date. Reconstitution studies have shown that BRG1 alone can remodel nucleosomes, although this remodeling activity is enhanced when all four core components are present (56). Thus, one possibility is that the core BAFs may be involved in targeting or stabilizing nucleosomes in a particular conformation that is conducive to remodeling activity by BRG1.

The presence of BRG1 strongly suggests that the N-CoR-1 complex possesses chromatin remodeling activity. This hypothesis is consistent with recent studies demonstrating that chromatin remodeling is required for transcriptional repression as well as activation. Genome-wide expression analysis using DNA microarrays have shown that mRNA levels for many genes are elevated in yeast mutants carrying mutations for SWI/SNF (57, 50). In mammalian cells, retinoblastoma protein forms a complex with hSWI/SNF to inhibit transcription of cyclin E and A, resulting in growth arrest (58, 59). Finally, several laboratories have identified multiprotein chromatin remodeling complexes that are believed to be directly involved in transcriptional repression. One such complex, known as NURD, Mi2, or NRD, possesses both histone deacetylase and nucleosome remodeling activity, suggesting that chromatin-modifying enzymes can be coupled to regulate transcription in vivo (25–27). Thus, chromatin remodeling may be necessary step to facilitate the binding of N-CoR by specific factors associated with nucleosomal DNA. Interestingly, the gene encoding BAF 47/INI 1 has been found mutated or deleted in various rhabdoid sarcoma tumor cell lines and in primary rhabdomyosarcoma (60). This suggests that the repressive effects mediated by the N-CoR-1 complex represents a critical mechanism for the regulation of specific genes important for muscle cell proliferation.

We have also established that KAP-1 is an intrinsic component of the N-CoR-1 complex. KAP-1 functions as a bona fide corepressor involved in mediating the repression of a large family of Kruppel-like zinc finger proteins, which contain the KRAB repression domain (KRAB-ZFP) (43, 44). KAP-1 binds to multiple KRAB-containing zinc finger proteins in vivo and in vitro, and mutations that abolish repression also abolish interaction with the KRAB domain. A mechanistic link between KAP-1 and histone modification has also been established with the demonstration that TSA can interfere with KAP-1-mediated repression (46). It has recently been demonstrated that KAP-1 associates with members of the heterochromatin protein 1 (HP1) family in vivo and in vitro (43, 45). HP1 proteins are conserved nonhistone chromosomal proteins associated primarily with pericentric heterochromatin where it is believed they function as regulators of heterochromatin silencing. The finding that KAP-1 and N-CoR colocalize in vitro and in vivo suggests that their mechanism of action is similar. Importantly, N-CoR, through KAP-1-directed recruitment of HP-1 proteins, may be involved in the assembly and/or maintenance of heterochromatin at specific sites within the genome suggesting an entirely novel function for N-CoR.

Based on Western blotting using selected antibodies, N-CoR-2 contains several proteins found in the Sin3A-HDAC complex. These results are in contrast to studies that have failed to demonstrate the presence of such a complex (9, 10, 34, 35). There may be several possible explanations for these differences. First, purification strategies, which rely on the use of specific antibodies, could disrupt specific protein-protein interactions. Second, the presence of a pre-existing complex may be cell-cycle dependent, or its composition can be regulated through post-translational modifications. Consequently, variations in growth conditions might determine the relative abundance of a specific multisubunit complex. Finally, variability in the composition of the initial starting material may be important for determining the existence of specific complexes.

In conclusion, we have provided biochemical evidence for the existence of multiple endogenous N-CoR complexes. The finding that these complexes possess distinct factors suggests that they may perform gene-specific functions by utilizing multiple mechanisms of transcriptional regulation. The continued analysis of these megadalton N-CoR complexes is critical to deciphering common and unique mechanisms of gene repression that are necessary for the homeostatic control of differentiated cell function.

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A Novel Nuclear Receptor Corepressor Complex, N-CoR, Contains Components of the Mammalian SWI/SNF Complex and the Corepressor KAP-1
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