Multiple N-CoR Complexes Contain Distinct Histone Deacetylases*

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N-CoR (nuclear receptor corepressor) is a corepressor for multiple transcription factors including unliganded thyroid hormone receptors (TRs). In vitro, N-CoR can interact with the Sin3 corepressor, which in turn binds to the histone deacetylase Rpd3 (HDAC1), predicting the existence of a corepressor complex containing N-CoR, Sin3, and histone deacetylase. However, previous biochemical studies of endogenous Sin3 complexes have failed to find an N-CoR association. Xenopus laevis eggs and oocytes contain all of the necessary components for transcriptional repression by unliganded TRs. In this study, we report the biochemical fractionation of three novel macromolecular complexes containing N-CoR, two of which possess histone deacetylase activity, from Xenopus egg extract. One complex contains Sin3, Rpd3, and RbAp48; the second complex contains a Sin3-independent histone deacetylase; and the third complex lacks histone deacetylase activity. This study describes the first biochemical isolation of endogenous N-CoR-containing HDAC complexes and illustrates that N-CoR associates with distinct histone deacetylases that are both dependent and independent of Sin3. Immunoprecipitation studies show that N-CoR binds to unliganded TR expressed in the frog oocyte, confirming that N-CoR complexes are involved in repression by unliganded TR. These results suggest that N-CoR targets transcriptional repression of specific promoters through at least two distinct histone deacetylase pathways.

Transcriptional regulation by many diverse groups of transcription factors, including nuclear hormone receptors, involves coactivator and corepressor complexes (reviewed in Refs. 1–3). N-CoR1 and SMRT (silencing mediator for retinoid and thyroid receptors) were initially characterized as highly homologous corepressors for unliganded TR (4, 5). Subsequently, N-CoR was identified as a Sin3 corepressor-binding protein (6, 7). This work suggested that N-CoR-mediated repression occurs through Sin3 recruitment of the Sin3-associated histone deacetylase Rpd3 (HDAC1), though an endogenous Sin3-containing N-CoR complex has never been described to date. However, recent work provides evidence that N-CoR can associate directly with HDAC4, HDAC5, and HDAC3, whereas SMRT can form a complex with HDAC5 and HDAC7, or with HDAC3 (8–12). These data suggest that multiple N-CoR-containing histone deacetylase complexes may exist to mediate transcriptional repression. We have previously demonstrated that the frog oocyte is capable of mediating both transcriptional activation by liganded TR and transcriptional repression by unliganded TR, indicating that the oocyte contains the necessary coactivator and corepressor complexes for TR (13–15). To characterize the corepressor complexes present in eggs and oocytes, we biochemically fractionated Xenopus egg extract and purified three distinct complexes. Our data also suggest that one or more of the N-CoR complexes participate in the repression by unliganded TR.

MATERIALS AND METHODS

Chromatography—Eggs were collected from mature female Xenopus laevis, and the high-speed egg extract was prepared exactly as described (16). All chromatography was carried out at 4 °C in Buffer A (20 mM HEPES, pH 7.6, 1.5 mM MgCl2, 1 mM EDTA, 10% glycerol, 10 mM β-glycerophosphate, 0.5 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 2 μg/ml pepstatin A, 1 μg/ml aprotinin, and 1 μg/ml leupetin). Egg extract was initially fractionated using BioReX70 resin (Bio-Rad) in Buffer A. The flow-through fraction was fractionated over DEAE-Sepharose (Amersham Pharmacia Biotech) with a step elution in Buffer A (to 350 mM NaCl). The DEAE (in 350 mM NaCl) step was further fractionated by linear salt gradient elution from Mono Q HR10/10 (Amersham Pharmacia Biotech). The peak N-CoR fractions were pooled and loaded directly onto a 110-ml Superose 6 (Amersham Pharmacia Biotech) gel filtration column in Buffer A (in 150 mM NaCl) with 0.04% Triton X-100. The peak N-CoR fractions were fractionated by linear salt gradient elution from Mono S HR5/5 (Amersham Pharmacia Biotech). The peak N-CoR fractions were pooled and loaded directly onto a 110-ml Superose 6 (Amersham Pharmacia Biotech) gel filtration column in Buffer A (in 150 mM NaCl) with 0.04% Triton X-100. The peak N-CoR fractions were fractionated by linear salt gradient elution from Mono S HR5/5 (Amersham Pharmacia Biotech) column followed by gel filtration through Superose 6 as above. The SP-Sepharose flow-through fraction was fractionated over Mono Q HR10/10 followed by gel filtration as above. The peak N-CoR fractions were further fractionated over Mono Q 5/5 as above.

Coimmunoprecipitations and Antibodies—Either fractionated egg extract (100 μl fraction) or injected oocytes (40 oocytes/sample) were used as the protein sources. Antisera were conjugated to protein A-Sepharose (Amersham Pharmacia Biotech) with dimethyl pimelimidate (DMP) exactly as described (17). Coimmunoprecipitations were carried out in Buffer A (100 mM NaCl + 0.05% Nonidet P-40) at 4 °C for 60 min followed by a series of five washes in Buffer A (150 mM NaCl + 0.05% Nonidet P-40). Proteins were eluted with 100 mM glycine, pH 2.3, or used in the histone deacetylase assay described below. All coimmunoprecipitations were repeated at least three times. Rabbit polyclonal antibodies specific for Xenopus N-CoR were raised against a bacterially expressed N-terminal polyepptide (amino acids 34–272) derived from Xenopus N-CoR cDNA.2 Rabbit polyclonal antibodies raised against Xenopus TRβ3,Sin3,Rpd3, and RbAp48 have been described (13,34).

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1 The abbreviations used are: N-CoR, nuclear receptor corepressor; HDAC, histone deacetylase; T3, triiodothyronine; TR, thyroid hormone receptor.
the BioRad protein assay and bovine serum albumin as a standard.

Protein concentrations estimated from silver-stained gels compared to standards.

**RESULTS**

We used *X. laevis* high speed egg extracts as the source for soluble endogenous proteins to biochemically purify endogenous complexes containing the N-CoR transcriptional co-repressor. The extract was initially fractionated over BioRex70 resin, which bound over 90% of the Sin3 co-repressor protein present in eggs (data not shown). Surprisingly, all of the detectable N-CoR was contained in the flow-through fraction (data not shown). Size fractionation (Sephacryl S-300) of the BioRex70 flow-through pool demonstrated that all of the N-CoR existed in large complexes (>669 kDa) with no detectable free N-CoR (270 kDa, Fig. 1A). Further fractionation of the BioRex70 flow-through protein pool over DEAE-Sepharose separated the N-CoR into two distinct pools. Roughly 50% of the N-CoR bound to the DEAE (complex 1) and 50% remained in the flow-through fraction (Fig. 1B). Fractionation of the DEAE flow-through pool over SP-Sepharose again separated the N-CoR into a bound pool (complex 2) and a flow-through pool (complex 3, Fig. 1B). Extensive purification of all three of the N-CoR protein pools was achieved through a series of ion exchange and gel filtration chromatographic steps as outlined in Fig. 1D. Size fractionation of the purified complexes through Superose 6 showed that all three N-CoR complexes continued to migrate in the MDa range (data not shown), supporting the observation that the three N-CoR protein pools are in fact distinct macromolecular complexes and not dissociation products. A summary of the typical purification is presented in Table 1.

The polypeptide profiles of the three N-CoR complexes were determined by silver-staining SDS-PAGE separations of the final step of fractionation. These results showed that each complex was largely pure and consisted of multiple distinct polypeptides (Fig. 2). Complex 1 contains eight polypeptides, whereas complex 2 and complex 3 each contain four polypeptides. Because N-CoR can interact with Sin3, and Sin3 can adapt to the N-CoR surface for its presence.

**Histone Deacetylase Assay**—Histone deacetylase assays were performed as described (16). Chicken erythrocyte core histones enzymatically acetylated ([H(acytely-CoA) with recombinant yeast HAT1p were used as the substrate (20). Reactions were carried using 25 μl of each fraction in 25 mM Tris, pH 8.0, 50 mM NaCl, 1 mM EDTA, 10% glycerol, and 2 μg of acetylated histones (400-μl final volume). The reactions were incubated at 30 °C for 60 min and terminated with 100 μl of stop solution (0.1 N HCl and 0.16 N acetic acid). Released acetate was extracted with ethyl acetate (800 μl), and 75% of the organic phase was counted by liquid scintillation. Experiments were repeated at least three times.

**Microinjection of Oocytes**—Oocyte preparation and microinjections were carried out as described (18). Oocytes were injected with 3 ng in vitro transcribed capped RNA (Ambion Inc.) encoding *Xenopus* TRβ1 and or RXα. Injected oocytes were incubated for 16 h at 18 °C with or without thyroid hormone (50 mM T3) prior to homogenization in Buffer A (100 mM NaCl).

**TABLE I**

| Complex 1 | Fraction | Total Proteina | Complex 2 | Fraction | Total Proteina | Complex 3 | Fraction | Total Proteina |
|-----------|----------|----------------|-----------|----------|----------------|-----------|----------|----------------|
| Egg Extract | 690 mg | BioRex 70 | 408 mg | DEAE | 82.6 mg | Mono Q | 5.38 mg | Superose 6 | 0.79 mg | Mono S | 25 μgab |
| SP | - | Mono Q | 0.55 mg | Superose 6 | -25 μgab |
| Protein recovery data for the purification of N-CoR complexes |

*Protein concentrations for the fractions were determined using the BioRad protein assay and bovine serum albumin as a standard. Protein concentrations estimated from silver-stained gels compared to standards.*

*HDAC5 antibody was a generous gift from S. Khochbin (19). HDAC3 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).*

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**RESULTS**

We used *X. laevis* high speed egg extracts as the source for soluble endogenous proteins to biochemically purify endogenous complexes containing the N-CoR transcriptional corepressor. The extract was initially fractionated over BioRex70 resin, which bound over 90% of the Sin3 corepressor protein present in eggs (data not shown). Surprisingly, all of the detectable N-CoR was contained in the flow-through fraction (data not shown). Size fractionation (Sephacryl S-300) of the BioRex70 flow-through pool demonstrated that all of the N-CoR existed in large complexes (>669 kDa) with no detectable free N-CoR (270 kDa, Fig. 1A). Further fractionation of the BioRex70 flow-through protein pool over DEAE-Sepharose separated the N-CoR into two distinct pools. Roughly 50% of the N-CoR bound to the DEAE (complex 1) and 50% remained in the flow-through fraction (Fig. 1B). Fractionation of the DEAE flow-through pool over SP-Sepharose again separated the N-CoR into a bound pool (complex 2) and a flow-through pool (complex 3, Fig. 1B). Extensive purification of all three of the N-CoR protein pools was achieved through a series of ion exchange and gel filtration chromatographic steps as outlined in Fig. 1D. Size fractionation of the purified complexes through Superose 6 showed that all three N-CoR complexes continued to migrate in the MDa range (data not shown), supporting the observation that the three N-CoR protein pools are in fact distinct macromolecular complexes and not dissociation products. A summary of the typical purification is presented in Table 1. The polypeptide profiles of the three N-CoR complexes were determined by silver-staining SDS-PAGE separations of the final step of fractionation. These results showed that each complex was largely pure and consisted of multiple distinct polypeptides (Fig. 2). Complex 1 contains eight polypeptides, whereas complex 2 and complex 3 each contain four polypeptides. Because N-CoR can interact with Sin3, and Sin3 can...
interact with Rpd3, we assayed the presence of the Sin3 and Rpd3 proteins as well as histone deacetylase activity in the three N-CoR complexes. Western blot analyses of the final steps of the purifications identified complex 1 as containing Sin3 (p150) and Rpd3 (p58) whereas neither complex 2 nor complex 3 contained either Sin3 or Rpd3 (Fig. 2, B and C). Interestingly, histone deacetylase activity was found to precisely cofractionate with the N-CoR protein for both complexes 1 and 2 (Fig. 3, A and B). These data indicate that complex 2 possesses a Sin3-independent histone deacetylase. Complex 3 contained no detectable histone deacetylase activity (Fig. 3C).

Because N-CoR complexes 1 and 2 each contained histone deacetylase activity, we tested for the presence of RbAp48, a protein that binds to the retinoblastoma A tumor suppressor and has been found in several histone deacetylase complexes (Refs. 21–25). We found that RbAp48 was present in complex 1 but absent in complexes 2 and 3 (Fig. 2). Thus, complex 1 contains the predicted N-CoR complex components, mainly N-CoR, Rpd3, Sin3, and RbAp48, whereas complexes 2 and 3 are novel and do not appear to share any subunits with complex 1.

To confirm the physical associations between N-CoR and the known components of the complexes, we performed coimmunoprecipitation experiments (Fig. 4). Immunoprecipitation reactions were carried out using antiserum against N-CoR, Sin3, or Rpd3 as the precipitating antibodies. Xenopus egg extract fractionated through the DEAE step, which separates complex 1 from complexes 2 and 3 (Fig. 1B), was used as the protein source. The precipitation products were assayed by either Western blot analysis using the N-CoR antibody (Fig. 4A) or by histone deacetylase assay (Fig. 4B). Reactions using either the Sin3, N-CoR, or Rpd3 antibodies as the precipitating antibody all immunoprecipitated N-CoR protein when the DEAE-0.35 M step elution (containing complex 1; Fig. 4A, top panel) was used as the protein source (Fig. 4A, lanes 2–4). However, when the DEAE flow-through fraction (containing complexes 2 and 3; Fig. 4A, lower panel) was used as the protein source, only the N-CoR antibody immunoprecipitated N-CoR protein, whereas the Sin3 and Rpd3 antibodies failed to immunoprecipitate any detectable N-CoR protein (Fig. 4A, lanes 7–8). These data confirm the association of N-CoR with Sin3 and Rpd3 in complex 1 as well as the lack of association between N-CoR and Sin3 or Rpd3 in complexes 2 and 3. For both the DEAE elution and flow-through fractions, an irrelevant antibody (Irr) failed to immunoprecipitate N-CoR protein (Fig. 4A, lanes 5 and 10). In addition, antibodies against N-CoR specifically immunoprecipitated histone deacetylase activity from both protein pools (Fig. 4B). These data support the existence of at least two independent multisubunit N-CoR/histone deacetylase complexes. In addition, these results show a physical association of N-CoR with Sin3, Rpd3, and HDAC activity in complex 1 (but not in complexes 2 or 3) and N-CoR with HDAC activity in complex 2.

We have shown previously that unliganded TRβ represses target promoters in frog oocytes and that this repression can be reversed by blocking HDAC activity (15). To investigate if any of the N-CoR complexes participate in this repression, we overexpressed TRβ and its heterodimer partner RXRα (9-cis retinoic acid receptor) by microinjecting in vitro transcribed mRNA into frog oocytes and analyzing associations with N-CoR. Immunoprecipitation experiments using antibodies against TRβ (Fig. 5A) indicate that TRβ/RXRA heterodimers (lanes 2 and 3), as well as TRβ homodimers (lanes 4 and 5) interact with endogenous N-CoR in a thyroid hormone (T3)-dependent manner. Immunoprecipitation experiments using precipitating antibodies against N-CoR (Fig. 5B) confirm a ligand-dependent interaction between N-CoR and TRβ (lanes 2–5).

**DISCUSSION**

N-CoR was initially characterized as a corepressor for unliganded TRα; however, it has been shown to participate in transcriptional repression through other transcription factors (reviewed in Refs. 2 and 26). Mice lacking N-CoR have multiple developmental defects and show that N-CoR is required for the repressive effects of several classes of DNA-binding transcriptional repressors (27). How N-CoR is differentially targeted to
The precipitation products were assayed by antibody (precipitate products obtained with the N-CoR antibody or an irrelevant negative control containing no starting protein. From the experimental sample divided by the released cpm from a native histone deacetylase. Activity precisely copurified with N-CoR complexes participates in the repression process. This conclusion is supported by the fact that N-CoR interacts with multiple HDACs including both HDAC1 and HDAC3 whereas class II contains HDAC2, and HDAC3 whereas class II contains HDAC4–7 (9, 18, 31–33). Data from other systems indicate that the three N-CoR complexes do not appear to have a 1:1 stoichiometry among all of the associated polypeptides in relation to N-CoR (Fig. 2). In complex 1, six of the eight polypeptides appear to be in a 1:1 ratio with N-CoR, whereas Rpd3 (2:1) and RbAp48 (4:1) are clearly more abundant. The resulting mass of complex 1 is predicted to be slightly more than 1 MDa. The multiple copies of Rpd3 and RbAp48 in the complex may suggest their functions as the catalytic subunit and a histone-interacting subunit, respectively. The four subunits of complex 2 (N-CoR, p115, p60, and p52), appear to have a stoichiometry of 1:1:2:4 respectively. It is possible that the complex exists as a multimer in vivo as the mass of the complex is greater than 1 MDa when assayed by gel filtration chromatography even though the sum of the polypeptides is only 700 kDa. Complex 3 also consists of four polypeptides (N-CoR, p120, p65, and p29), with a stoichiometry of 2:3:1:1, respectively, predicting a mass of 1 MDa. The role of the multiple subunits may indicate that the complexes must interact with multiple targeting proteins or additional corepressor complexes in vivo. Clearly, further studies on the exact stoichiometry of these complexes and their biochemical and molecular properties are required to determine their biological functions and mechanisms.

The ability of unliganded TRs to repress gene transcription in the frog oocyte in a histone deacetylase-dependent manner together with our isolation of two N-CoR-containing histone deacetylase complexes argues that one or more of these complexes participates in the repression process. This conclusion is supported by the fact that N-CoR interacts with multiple HDACs including both HDAC1 and HDAC3 whereas class II contains HDAC2, and HDAC3 whereas class II contains HDAC4–7 (9, 18, 31–33). Data from other systems indicate that the three N-CoR complexes do not appear to have a 1:1 stoichiometry among all of the associated polypeptides in relation to N-CoR (Fig. 2). In complex 1, six of the eight polypeptides appear to be in a 1:1 ratio with N-CoR, whereas Rpd3 (2:1) and RbAp48 (4:1) are clearly more abundant. The resulting mass of complex 1 is predicted to be slightly more than 1 MDa. The multiple copies of Rpd3 and RbAp48 in the complex may suggest their functions as the catalytic subunit and a histone-interacting subunit, respectively. The four subunits of complex 2 (N-CoR, p115, p60, and p52), appear to have a stoichiometry of 1:1:2:4 respectively. It is possible that the complex exists as a multimer in vivo as the mass of the complex is greater than 1 MDa when assayed by gel filtration chromatography even though the sum of the polypeptides is only 700 kDa. Complex 3 also consists of four polypeptides (N-CoR, p120, p65, and p29), with a stoichiometry of 2:3:1:1, respectively, predicting a mass of 1 MDa. The role of the multiple subunits may indicate that the complexes must interact with multiple targeting proteins or additional corepressor complexes in vivo. Clearly, further studies on the exact stoichiometry of these complexes and their biochemical and molecular properties are required to determine their biological functions and mechanisms.

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supported by the interaction of overexpressed TRβ with N-CoR in the frog oocytes, where all of the N-CoR is present in large complexes. Whereas the functional differences among these complexes remain to be investigated, our findings provide an explanation for some inconsistent earlier observations. First, N-CoR was initially shown to interact with Rpd3 (HDAC1) through mSin3s, but N-CoR/mSin3/Rpd3 complexes have eluded biochemical purification in vivo. Second, N-CoR has been shown to be interact with both class I and class II histone deacetylases in vitro and form complexes with them in vivo (8, 9, 11, 12). The nature of such complexes is yet unknown; however, all are independent of mSin3A. Our results here demonstrate that these possibilities coexist in vivo. The failure of other studies to detect the multiple complexes is likely because of the choice of the systems and the purification procedures, which we found to be crucial for the separation and isolation of the three complexes. It is also worth noting that our procedure allowed us to successfully purify the first complex that contains Sin3, Rpd3 (HDAC1), and RbAp48 together. Extensive studies including coimmunoprecipitations have demonstrated pairwise associations between mSin3A, Rpd3 (HDAC1), and RbAp48, suggesting that the three proteins may exist together in a complex. However, until our study here, such a complex had eluded purification.

Our data here provide a potential mechanism whereby N-CoR complexes are differentially recruited by specific transcriptional regulators to repress target genes. An additional level of regulation may come from changes in the ratio among the various N-CoR complexes or from alterations in subunit concentrations. These findings provide an opportunity to investigate these possible mechanisms in the near future.

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