Phosphorylation of Thyroid Hormone Receptor-associated Nuclear Receptor Corepressor Holocomplex by the DNA-dependent Protein Kinase Enhances Its Histone Deacetylase Activity

M. Jeyakumar, Xue-feng Liu, Hediye Erdjument-Bromage, Paul Tempst, and Milan K. Bagchi

Received for publication, September 22, 2006, and in revised form, January 22, 2007 Published, JBC Papers in Press, January 22, 2007 DOI 10.1074/jbc.M609009200

From the Department of Molecular and Integrative Physiology, University of Illinois, Urbana, Illinois 61801 and Memorial Sloan-Kettering Cancer Center, New York, New York 10021

It is well documented that unliganded thyroid hormone receptor (TR) functions as a transcriptional repressor of specific cellular target genes by acting in concert with a corepressor complex harboring histone deacetylase (HDAC) activity. To fully explore the cofactors that interact with the transcriptionally repressive form of TR, we biochemically isolated a multiprotein complex that assembles on a TR-retinoid X receptor (RXR) heterodimer in HeLa nuclear extracts and identified its polypeptide components by mass spectrometry. A subset of TR-RXR-associated polypeptides included NCoR, SMRT, TBL1, and HDAC3, which represent the core components of a previously described NCoR/SMRT corepressor complex. We also identified several polypeptides that constitute a DNA-dependent protein kinase (DNA-PK) enzyme complex, a regulator of DNA repair, recombination, and transcription. These polypeptides included the catalytic subunit DNA-PKcs, the regulatory subunits Ku70 and Ku86, and the poly(ADP-ribose) polymerase 1.

Density gradient fractionation and immunoprecipitation analyses provided evidence for the existence of a high molecular weight TR-RXR-corepressor holocomplex containing both NCoR/SMRT and DNA-PK complexes. Chromatin immunoprecipitation studies confirmed that unliganded TR-RXR recruits both complexes to the triiodothyronine-responsive region of growth hormone gene in vivo. Interestingly, DNA-PKcs, a member of the phosphatidylinositol 3-kinase family, was found to phosphorylate HDAC3 when the purified TR-RXR-corepressor holocomplex was incubated with ATP. This phosphorylation was accompanied by a significant enhancement of the HDAC activity of this complex. Collectively, our results indicated that DNA-PK promotes the establishment of a repressive chromatin at a TR target promoter by enhancing the HDAC activity of the receptor-bound NCoR/SMRT corepressor complex.

The thyroid hormone receptors (TRs), α and β, are hormone-inducible transcription factors. They mediate the physiological actions of thyroid hormone, which regulates the growth, development, and metabolism of a wide variety of tissues in higher organisms (1). At the target gene promoter, TR α or β interacts with a distinct DNA sequence, termed thyroid hormone response element (TRE), either as a homodimer or a heterodimer with retinoid X receptor (RXR) (2, 3). The DNA-bound receptor then functions either as a transcriptional activator or a repressor, depending on its hormonal status, the host cell, and the promoter context (3, 4). In the absence of thyroid hormone, TR generally functions as a silencer of basal level transcription from the target promoter (5–7). Ligand binding to TR releases transcriptional silencing and leads to the activation of target gene expression. Investigations into the molecular basis of this ligand-dependent switch have led to the identification of distinct cellular coregulatory factors, corepressors and coactivators, which mediate the transcriptional response by the receptor (8–10). In the unliganded state, the receptor exists in a conformation that allows it to interact with a corepressor, which mediates transcriptional repression (8–12). Ligand binding triggers a dramatic change in receptor conformation, resulting in the displacement of the corepressor and recruitment of a coactivator, which mediates transcriptional activation.

Several laboratories have characterized the corepressors that interact with TR (8, 13). Two distinct but structurally related corepressors, nuclear receptor corepressor (NCoR) and silencing mediator of retinoid and thyroid receptors (SMRT), have been identified (11, 12). The NCoR and SMRT polypeptides harbor multiple independent repression domains, which contribute to the overall repression function of these corepressors (11, 12). NCoR or SMRT uses its carboxyl-terminal receptor interaction domain to interact directly with the ligand-binding domain of unliganded TR but does not interact with the ligand-occupied receptor. Within the receptor interaction domain of the corepressor, two corepressor-nuclear receptor boxes con-
Biochemical characterization of cellular NCORS and SMRT revealed that these proteins exist in large multicomponent complexes (16–18). The core complexes of NCORS and SMRT are essentially the same and composed of NCORS/SMRT, histone deacetylase 3 (HDAC3), a WD40-repeat protein TBL1, and the G protein suppressor 2. Interestingly, there are also reports that NCORS or SMRT associates with a complex containing Sin3, HDAC1, and HDAC2 (19, 20). Despite significant progress in the isolation and functional characterization of NCORS/SMRT complexes, the identities of the coregulatory proteins that interact with unliganded TR-RXR have not been fully investigated. It is unclear whether TR-RXR needs to interact with regulatory factors in addition to the NCoR/SMRT corepressor complex, the other one is a DNA-dependent protein complex. Although one of these complexes represents the previously described NCoR/SMRT corepressor 2. Interestingly, there are also reports that NCoR or SMRT interacts with regulatory factors in addition to the NCoR/SMRT corepressor complex containing the ligand-binding domain (14). It has been demonstrated that the loss of interaction with NCORS or SMRT impairs transcriptional silencing by TR, indicating that these factors are bona fide corepressors (15).

In this report, we describe the biochemical isolation of an SMRT corepressor to function as a transcriptional repressor. The repressive form of TR-RXR interacts with regulatory factors in addition to the NCoR/SMRT core complex, the other one is a DNA-dependent protein complex that contributes to TR-mediated transcriptional repression by increasing histone deacetylation and thereby influencing chromatin structure and function.

EXPERIMENTAL PROCEDURES

Reagents—Glutathione-Sepharose-4B and reduced glutathione were purchased from Amersham Biosciences. Antibodies against DNA-PKcs, TBL-1, TRα, TRβ1, TRβ2, RXRα, RXRβ, RXRγ, and GST were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against HDAC1 and HDAC3 were purchased from Cell Signaling Technology (Beverly, CA). Rabbit anti-NCOR antibody was raised against carboxyl-terminal amino acids 2057–2453 of murine NCOR fused to GST. Wortmannin, phosphatase inhibitor mixture I and II, and trichostatin-A were obtained from Sigma. NU7026 was obtained from Calbiochem. Acetyl-[3H]CoA (200.00 mCi/mmol) and [γ-32P]ATP (6000 mCi/mmol) were purchased from PerkinElmer Life Sciences.

Isolation of TR-RXR-interacting Polypeptides from HeLa Nuclear Extracts Using Receptor Affinity Chromatography—Nuclear extracts (NE) of HeLa cells were prepared as described previously (21). The bacterial expression constructs encoding GST-hTRβ and His6-tagged hRXRα (full-length) have been described previously (21). Briefly, GST-TR (0.5 μg) was incubated with glutathione (GSH)-Sepharose beads (50 μl, 1:1 slurry) for 2 h at 4°C. The beads were washed four times with a wash buffer containing 20 mM Tris-HCl, pH 7.9, 100 mM NaCl, 15% glycerol, and 0.02% IGEPEAL CA-630 (Sigma). The immobilized TR was incubated with purified His6-hRXRα (2 μg) for 1 h at room temperature followed by repeated washing with the wash buffer. The bead-bound TR-RXR heterodimer was then incubated with HeLa cell NE (2.5 mg, 2.5 μg/μl) for 2 h at 4°C on an end-to-end shaker. The beads were subsequently washed four times with the wash buffer. For identification of polypeptides by mass spectrometry, the bound proteins were dissociated under denaturing condition with 0.2% sodium N-lauroyl sarcosinate. For analyses of the TR-RXR holocomplex by gel electrophoresis, the bound-complex is eluted under native conditions using 20 mM GSH.

Protein Identification by Mass Spectrometry—Gel-resolved proteins were digested with trypsin and batch-purified on a reversed-phase micro-tip, and resulting peptide pools were individually analyzed by matrix-assisted laser desorption/ionization reflectron time-of-flight mass spectrometry for peptide mass fingerprinting, as described (22, 23). Selected peptide ions (m/z) were taken to search a “non-redundant” protein data base (NCBI, Bethesda, MD) utilizing the Peptide Search algorithm (Applied Biosystems, Foster City, CA).

Glycerol Density Gradient Centrifugation—Protein complexes bound to TR-RXR heterodimer were eluted using reduced GSH and fractionated on a 15–60% glycerol gradient based on a previously published procedure (24).

Immunoprecipitation—A rabbit polyclonal antibody that recognized both NCORS and SMRT (10 μg) was covalently coupled to protein-A-Sepharose, using the chemical cross-linking agent dimethyl pimelimidate, as described previously (25). The immobilized antibody was incubated with purified TR-RXR holocomplex for 3 h at 4°C. Following repeated washings with wash buffer, the bound proteins were eluted with SDS sample buffer and analyzed by Western blotting using antibodies against NCORS/SMRT and DNA-PK.

Phosphorylation of TR-RXR Holocomplex and HDAC3—The TR-RXR heterodimer-bound protein complexes were isolated from HeLa nuclear extract as detailed above, except that phosphatase inhibitor cocktails (I and II) were used during receptor immobilization and incubation with nuclear extract. After the final wash, the beads were suspended in 50 μl of kinase buffer (20 mM HEPES, pH 7.9, 10 mM MgCl2, 1 mM EDTA, 10% glycerol, 100 mM KCl, 0.02% IGEPEAL, and 1 mM dithiothreitol). The purified complex was then phosphorylated using a mixture of γ32P-labeled ATP (3.6 fmol of 3000 Ci/mmol) and unlabeled ATP (50 μM) in the presence or absence of the PI3K inhibitor wortmannin (1 μM) as previously described (26).

For immunoprecipitation of phosphorylated proteins, 32P-labeled TR-RXR holocomplex was suspended in radioimmunoprecipitation assay buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 1.0% IGEPEAL, 0.5% deoxycholate, 0.1% SDS) and incubated with protein-A-Sepharose beads bound to an antibody against GST or NCORS or DNA-PKcs or HDAC3 or control IgG (10 μg) for 2 h at 4°C. Following extensive washing with radioimmunoprecipitation assay buffer, the bound proteins were eluted by

Role of DNA-PK in a TR-RXR-Corepressor Holocomplex

MARCH 30, 2007•VOLUME 282•NUMBER 13
JOURNAL OF BIOLOGICAL CHEMISTRY 9313
Role of DNA-PK in a TR-RXR-Corepressor Holocomplex

boiling with SDS sample buffer and analyzed by SDS-PAGE and autoradiography.

For the expression of FLAG epitope-tagged human HDAC3, cDNA encoding the full-length HDAC3 was cloned into the NdeI and BamH1 sites of pET-15b vector (Novagen) downstream of a 3×FLAG sequence (inserted at the NCo1 site). The 3×FLAG-HDAC3 fusion protein was expressed in bacteria as described previously (21). FLAG-HDAC3 or GST (0.5 μg) immobilized on either M2 beads or GSH beads was subjected to a phosphorylation reaction using 5.0 units of DNA-PK enzyme (Promega, Madison, WI). The proteins were denatured in SDS-sample buffer, resolved on an SDS-PAGE, stained with Coomassie Blue, and visualized by autoradiography.

Histone Deacetylase Assay—Histone deacetylase assay was carried out using a HDAC assay kit as per manufacturer’s instruction (Upstate Biotechnology). Briefly, an H4 synthetic peptide (residues 2–24) was acetylated in the presence of [3H]acetyl-CoA and p300/CBP-associated factor, a histone acetyltransferase. HDAC activity was assayed by incubating streptavidin-bound radiolabeled H4 peptide with bead-bound purified TR/RXR holocomplexes. Wortmannin (1 μM) and trichostatin A (1 μM) were used where indicated.

ChIP Assay—The ChIP assays were carried out as described previously (27).

Cell Culture and Transfection—HeLa cells were grown in Dulbecco’s modified Eagle’s medium with 5% fetal calf serum containing penicillin and streptomycin. 48 h prior to transfection, medium was replaced with DMEM containing 5% T3-stripped serum (28). Cells were transfected with an expression plasmid pCI-hTR (0.025 or 0.05 μg) or GST-TR (0.5 μg) and tri-thymine kinase vector (Promega, Madison, WI). The bound proteins were eluted with 0.2% sodium deoxycholate, resolved in an 8% SDS-PAGE and visualized by silver staining. The polypeptides that were found to interact specifically with TR-RXR holocomplexes were excised bands containing individual polypeptides from SDS-PAGE, and subjected these bands to in-gel tryptic digestion as described under “Experimental Procedures.” The bound proteins were eluted with 0.2% sodium deoxycholate, resolved in an 8% SDS-PAGE and visualized by silver staining.

RESULTS

Unliganded TR-RXR Heterodimer Interacts with the Components of NCoR/SMRT and DNA-PK Complexes—To identify the polypeptides that interact with TR-RXR heterodimer, we employed protein affinity chromatography, using the preformed heterodimers as bait. Human TRb and RXRα were expressed in Escherichia coli as recombinant proteins fused to GST and polyhistidine tags, respectively. Recombinant GST-TR was first immobilized on GSH beads and then incubated with purified His-RXR to form the TR-RXR heterodimers. As shown in Fig. 1, the immobilized TR-RXR heterodimers contained stoichiometrically equivalent amounts of each receptor partner (lane 4). In addition, multiple proteolytic degradation products of each receptor were also retained on the beads. The GSH beads bound to either GST alone (control) or hormone-free TR-RXR (test) were then incubated with or without HeLa nuclear extracts. The beads were washed extensively, and the bound proteins were eluted with a buffer containing 0.2% N-lauroyl sarcosine. The fractions eluted from the control and test beads were analyzed by SDS-PAGE, and the polypeptide components were visualized by silver staining. We noted that the control GST beads non-specifically retained a number of proteins (NS1–5) from HeLa nuclear extracts (lane 2). The same polypeptides were also retained by the TR-RXR beads (lane 4). Strikingly, about fifteen additional polypeptides with relative molecular masses ranging from 50 to 400 kDa were detected in the fraction eluted from the GST-TR-RXR column but were absent in the fraction eluted from the control beads (lane 4). These polypeptides, therefore, interacted specifically with unliganded TR-RXR heterodimers and represented potential constituents of one or more corepressor complex.

To determine the identities of the TR-RXR-associated polypeptides, we performed large scale purification using TR-RXR affinity beads, excised bands containing individual polypeptides from SDS-PAGE, and subjected these bands to in-gel tryptic digestion followed by mass spectrometry analysis as described under “Experimental Procedures.” Peptide sequences were obtained from several of these polypeptides, and their identities were established (Table 1). Consistent with previously published reports, several signature components of the NCoR/SMRT complex such as nuclear receptor corepressor 1 (NCoR), nuclear receptor corepressor 2 (SMRT), transducin β-like 1 (TBL1), G-protein pathway suppressor 2, and histone deacetylase 3 (HDAC3) were found to be associated with TR-RXR. Interestingly, we also identified four known components of the DNA-PK complex, namely, DNA-dependent protein kinase catalytic subunit (DNA-PKcs), PARP1, ATP-dependent DNA helicase II ( Ku86), and thyroid autoantigen...
(Ku70), as proteins that were retained specifically by TR-RXR. The mass spectrometric analysis also identified specific association of three other proteins, SMC1, DNA-ligase III, and HSP70, with the TR-RXR heterodimer (Table 1). Additionally, we obtained seven mass spectrometric identifications that represented minor polypeptides in the SDS-PAGE. These polypeptides are nucleolin (NCBI JH0148), KIAA1380 protein (BAA92618), RAD50 (XP_034865), zinc finger CW type with coiled-coil domain 2 (NP_078933), thyroid receptor interacting protein 8 (Q15652), eukaryotic translation elongation factor 1 (NP_078933), and thyroid receptor interacting protein 8 (Q15652).

The TR-RXR-associated polypeptides, therefore, included the components of at least two distinct complexes, the NCoR/SMRT corepressor complex, and the DNA-PK enzyme complex. It is notable that the approach used here did not result in the isolation of any known coactivator, thereby indicating the highly specific nature of our affinity chromatography. The identification of known corepressors such as NCoR and SMRT also gave us confidence that the polypeptides retained by the TR-RXR beads may represent molecules with relevant functions in the receptor-mediated repression pathway.

**Binding of T3 to TR Inhibits the Interaction of Both NCoR/SMRT and DNA-PK Complexes with TR-RXR Heterodimer—**

Binding of T3 to TR is reported to trigger the dissociation of NCoR/SMRT. The receptor-bound polypeptides were then eluted as described above in Fig. 1 and analyzed by Western blotting using antibodies against NCoR/SMRT and DNA-PKCs. As shown in Fig. 2A, addition of T3 markedly reduced the association of both NCoR/SMRT and DNA-PKCs with TR-RXR. Binding of T3 to TR-RXR, however, had a significantly greater impact on the retention of NCoR/SMRT than that of DNA-PKCs. Whereas treatment with T3 resulted in >90% reduction in the binding of NCoR/SMRT to TR-RXR, the binding of DNA-PKCs was reduced to ~40%.

In a parallel experiment, we investigated whether T3 is able to dissociate NCoR/SMRT and DNA-PK complexes bound to TR-RXR. In this experiment, immobilized TR-RXR heterodimers

### TABLE 1

**Identification of TR-RXR-associated polypeptides by mass spectrometry**

For protein mass spectrometric analysis, a large-scale isolation of TR-RXR-associated polypeptides was performed, and the polypeptides were resolved in an 8% SDS-PAGE and stained with Coomassie R250. Proteins were designated according to their apparent molecular sizes. The specific polypeptide bands from lane 4 were excised, and the band identities are shown. The number of actual peptides identified for each of the 13 polypeptides listed ranged from 4 to 14. Here we have listed only the sequences representing the top two peptides that displayed the tallest peak heights in the mass spectrum.

| Band | Protein identity | NCBI no. | Peptide sequence |
|------|------------------|----------|-----------------|
| p400 | DNA-PKcs         | WPVAGQIR, LSPFVYPPR |
| p470 | NcoR             | EREREREK, YRSDVSQSPVLR |
| p270 | SMRT             | SHPLAFDTPSSIR, YPHYLSVYFQIAR |
| p143 | SMC1             | VVQHIYKSEELEK, RAVISGGAADLK |
| p113 | PARP1            | AAS174554, IEQAEVEVAPP |
| p86  | Ku86             | NQQLQVLF, ILKPVLPYX |
| p70  | HSP70            | VITMFQQR, LTIGSNLSIR |
| p70  | TBL1             | ILELDQFK, KPGGFDISLFYR |
| p56  | TBLR1            | DGNLASTLGQHK, MSISDEVNFLYR |
| p55  | HDAC3            | TFQGHTEVNAIK, DNLASTLQHFK |
| p55  | GPS2             | DNLASTLQHFK, TFQGHTEVNAIK |

**Notes:** For protein mass spectrometric analysis, a large-scale isolation of TR-RXR-associated polypeptides was performed, and the polypeptides were resolved in an 8% SDS-PAGE and stained with Coomassie R250. Proteins were designated according to their apparent molecular sizes. The specific polypeptide bands from lane 4 were excised, and the band identities are shown. The number of actual peptides identified for each of the 13 polypeptides listed ranged from 4 to 14. Here we have listed only the sequences representing the top two peptides that displayed the tallest peak heights in the mass spectrum.
Role of DNA-PK in a TR-RXR-Corepressor Holocomplex

Figure 3. Glycerol gradient sedimentation analysis of the TR-RXR-corepressor holocomplex. A, TR-RXR:corepressor holocomplex was first assembled from HeLa NE as described in legends to Fig. 1. The bead-bound proteins were eluted under native condition with reduced GSH (15 mM). The purified proteins were loaded onto a 15–60% discontinuous glycerol gradient (GG), resolved by centrifugation, and subjected to fractionation. Proteins in each fraction were precipitated by 20% trichloroacetic acid and analyzed by Western blotting using indicated antibodies. The "bound" represents 10% of the total TR-RXR-associated proteins before density gradient analysis. C, fractions 9–11 containing the high molecular weight TR-RXR complex(es) were pooled and concentrated by Centricon filters (10,000 molecular weight cut off, Amicon Bioseparations) and assayed for HDAC activity using a synthetic [3H]histone-4 peptide (residues 2–24) as substrate. The HDAC activity in lane 1 corresponds to the total activity that was loaded onto the gradient, and the activity in lane 3 corresponds to the HDAC activity that was recovered in the high molecular weight TR-RXR fractions. Where indicated, HDAC inhibitor TSA (1 μM) was used. The HDAC activities are representative of the results of three independent GG analyses.
To analyze the molecular composition of complex II, we pooled the fractions 9–11 and performed Western blot analysis using antibodies specific for individual components of both NCoR and DNA-PK complexes. We confirmed that the signature components of the core NCoR/SMRT corepressor complex, namely NCoR, HDAC3 and TBL1, are present in complex II (Fig. 3B). The signature components of the DNA-PK complex, namely, DNA-PKcs and PARP1, were also detected in this high molecular size complex (Fig. 3B). As much as 40% of the TSA-sensitive HDAC activity that was loaded on the density gradient was recovered in the fractions containing complex II, indicating that it represents a bona fide TR-RXR-corepressor complex (Fig. 3C). These results are consistent with our hypothesis that the NCoR/SMRT and DNA-PK complexes coexist in a large holocomplex that interacts with TR-RXR.

To further verify the simultaneous association of the NCoR and DNA-PK complexes with TR-RXR to form a holocomplex, we performed immunoprecipitation experiments. The receptor corepressor complex was first assembled by incubating TR-RXR with HeLa nuclear extracts as described in Fig. 1. TR-RXR and the associated polypeptides were then eluted from the GSH beads under native condition (GSH elution). The eluted proteins were immunoprecipitated using an NCoR/SMRT antibody or preimmune serum. The immunoprecipitates were analyzed by Western blotting using antibodies specific for individual components of both NCoR and DNA-PK complexes. As expected, immunoblotting confirmed the presence of TR and RXR, NCoR/SMRT, and DNA-PK antibodies. As expected, immunoblotting confirmed the absence of TR and RXR (data not shown) and NCoR/SMRT in the immunoprecipitate (Fig. 4, upper panel). Probing with DNA-PK antibody revealed that DNA-PK is also present in the immunoprecipitate (Fig. 4, lower panel). Coimmunoprecipitation of NCoR/SMRT and DNA-PK with TR-RXR heterodimer strengthened our view that the components of the NCoR/SMRT and DNA-PK complexes coexist in a large multisubunit holocomplex assembled on unliganded TR-RXR.

NCoR/SMRT and DNA-PK Complexes Simultaneously Occupy a TRE in Vivo—We next performed ChIP to examine whether the components of NCoR and DNA-PK complexes are recruited at the regulatory regions of an endogenous TR-regulated gene. The rat growth hormone (GH) promoter contains a well characterized thyroid hormone response element (TRE) located between −189 and −162 with respect to the transcriptional start site (Fig. 5A). In GH3 pituitary tumor cells, the GH gene is actively silenced in the absence of T3 and activated in the presence of T3. These cells are known to express three TR isoforms, TRα, TRβ1, and TRβ2, and three RXR isoforms, RXRα, RXRβ, and RXRγ (29, 30). At first, we determined the identity of the TR and RXR isoforms that occupy the TRE of the GH promoter in the absence or presence of T3. As shown in Fig. 5B,
Role of DNA-PK in a TR-RXR-Corepressor Holocomplex

TRα is the predominant TR isoform that occupies the GH TRE in GH3 cells. We also detected occupancy of this TRE by TRβ1, although to a lesser extent than TRα. No significant binding of TRβ2 was seen at this TRE. We noted occupancy of the GH TRE by both RXRα and RXRβ. Very little RXRγ was seen at this site. Neither TR nor RXR showed any interaction with the distal promoter regions of GH promoter (data not shown).

We observed that the TR and RXR isoforms remained bound to the GH promoter irrespective of thyroid hormone (Fig. 5B). As expected, NCoR/SMRT, HDAC3, and TBL1, the components of the NCoR/SMRT corepressor complex, were bound to the GH TRE in the absence of T₃. No significant recruitment of HDAC1 was also recruited at the GH TRE region in the absence of T₃. Like HDAC3, respectively, we carried out immunoprecipitation of the NCoR/SMRT complex, DNA-PKcs was also released from the promoter site upon T₃ treatment. Similar T₃-sensitive GH promoter occupancy by Ku70, another component of the DNA-PK complex, was also noted (data not shown). The results of our in vivo ChIP experiments are, therefore, generally consistent with the results of our in vitro biochemical studies. These results also provided strong support to our hypothesis that a TR-RXR-corepressor holocomplex containing the components of both DNA-PK and NCoR/SMRT complexes binds to cellular target genes in vivo and mediates transcriptional repression. Hormone binding to TR is accompanied by the dissociation of NCoR and DNA-PK complexes from TRE-bound heterodimers, thereby releasing the repression.

DNA-PK Phosphorylates HDAC3 in a TR-RXR-Corepressor Holocomplex—The presence of DNA-PKcs in the TR-RXR-corepressor holocomplex raised the possibility that this kinase may phosphorylate one or more components of the holocomplex to modulate the corepressor function. To test this possibility, we first assembled a TR-RXR-corepressor holocomplex containing the components of both DNA-PK and NCoR/SMRT complexes binds to cellular target genes in vivo and mediates transcriptional repression. Hormone binding to TR is accompanied by the dissociation of NCoR and DNA-PK complexes from TRE-bound heterodimers, thereby releasing the repression.

A, WM

B, DNA-PK

DHAC3

GST

FIS

NS

50 KD

WM

+ + + +

+ + + +

+ + + +

DNA-PK

HDAC3

GST

NCoR

NS

50 KD

Fig. 6. DNA-PK-dependent phosphorylation of HDAC3 in TR-RXR holocomplex. A, TR-RXR holocomplex was formed upon incubation with HeLa NE as described in Fig. 1. The bead-bound proteins were suspended in a kinase buffer and phosphorylated in the presence of γ-32P-labeled ATP as described under “Experimental Procedures.” After extensive washings, the protein complexes were dissociated by boiling with SDS sample buffer and resolved in an 8% SDS-PAGE. The radiolabeled protein bands were detected by autoradiography. Left panel represents the total TR-RXR-associated polypeptides that were phosphorylated in the absence (lane 1) and presence (lane 2) of 1 μM wortmannin (WM), a DNA-PK inhibitor. Right panel: after phosphorylation reaction, 32P-labeled TR-RXR-associated protein complexes were dissociated from the GSHagarose beads using 15 mM reduced GSH, suspended in radioimmune precipitation assay buffer and immunoprecipitated with antibodies against GST (for GST-TR), NCoR, DNA-PK, HDAC3 and control IgG as described under “Experimental Procedures.” The immunoprecipitates were resolved in a SDS-PAGE, and the radiolabeled proteins were detected by autoradiography. NS denotes a nonspecific band. 50 KD denotes the HDAC3 protein. B, a purified preparation of DNA-PK specifically phosphorylates HDAC3. FLAG-HDAC3 immobilized on beads linked to anti-FLAG antibody, and GST bound to GSH beads (0.5 mg each) were subjected to a phosphorylation reaction using 5 units of a purified preparation of DNA-PK (Promega) and γ-32P-labeled ATP as described under “Experimental Procedures.” Following extensive washings, the bead-bound proteins were dissociated by boiling with SDS sample buffer, resolved in an 8% SDS-PAGE and stained by Coomassie Blue (upper panel). The gel was then dried and subjected to autoradiography (lower panel). 1 μM wortmannin was used where indicated. The results are representative of two independent sets of experiments.

400-kDa polypeptide, confirming that it indeed represented DNA-PKcs (data not shown). Most importantly, as shown in Fig. 6A, in vitro phosphorylation of HDAC3 antibody precipitated the 32P-labeled 50-kDa polypeptide, indicating that it represented HDAC3. These results provided strong evidence that the DNA-PKcs present in the TR-RXR corepressor complex targets the HDAC3 subunit of the complex for phosphorylation.

To demonstrate directly that HDAC3 is a target of phosphorylation by DNA-PKcs, we expressed HDAC3 as a FLAG-tagged polypeptide in bacteria and immobilized the recombinant protein on beads linked to anti-FLAG antibody. The FLAG-HDAC3 or control GST protein was then incubated with a purified preparation of catalytically active DNA-PKcs (commercially available from Promega) in the presence of γ-32P-labeled ATP. We found that HDAC3, but not GST, was effi-
Role of DNA-PK in a TR-RXR-Corepressor Holocomplex

The present study is aimed at the biochemical identification and functional characterization of transcriptional coregulatory proteins recruited by the repressive form of the TR-RXR heterodimer. We provide several lines of evidence in support of the concept that unliganded TR-RXR interacts simultaneously with two distinct protein complexes, the NCoR/SMRT corepressor complex and the DNA-PK enzyme complex, to assemble a single, large holocomplex. First, mass spectrometry analysis of polypeptides associated with TR-RXR identified signature components of both DNA-PK (DNA-PKcs, Ku86, Ku70, and PARP1) and NCoR/SMRT (NCoR/SMRT, TBL1, and HDAC3) complexes. Second, components of both complexes were simultaneously released from the TR-RXR heterodimer upon treatment with T₃, indicating a possible direct association between them. Third, a TR-RXR complex of high molecular size comigrated with the components of both NCoR/SMRT and DNA-PK complexes during density gradient fractionation. Fourth, immunoprecipitation of a TR-RXR-corepressor complex using a NCoR/SMRT antibody coprecipitated DNA-PK. Previous studies reported that the DNA-PK complex binds to single-stranded DNA ends (31, 32). In our experimental conditions, however, purified TR-RXR heterodimers bound to the DNA-PK complex in the absence of any

DISCUSSION

The present study is aimed at the biochemical identification and functional characterization of transcriptional coregulatory proteins recruited by the repressive form of the TR-RXR heterodimer. We provide several lines of evidence in support of the concept that unliganded TR-RXR interacts simultaneously with two distinct protein complexes, the NCoR/SMRT corepressor complex and the DNA-PK enzyme complex, to assemble a single, large holocomplex. First, mass spectrometry analysis of polypeptides associated with TR-RXR identified signature components of both DNA-PK (DNA-PKcs, Ku86, Ku70, and PARP1) and NCoR/SMRT (NCoR/SMRT, TBL1, and HDAC3) complexes. Second, components of both complexes were simultaneously released from the TR-RXR heterodimer upon treatment with T₃, indicating a possible direct association between them. Third, a TR-RXR complex of high molecular size comigrated with the components of both NCoR/SMRT and DNA-PK complexes during density gradient fractionation. Fourth, immunoprecipitation of a TR-RXR-corepressor complex using a NCoR/SMRT antibody coprecipitated DNA-PK. Previous studies reported that the DNA-PK complex binds to single-stranded DNA ends (31, 32). In our experimental conditions, however, purified TR-RXR heterodimers bound to the DNA-PK complex in the absence of any

...
Role of DNA-PK in a TR-RXR-Corepressor Holocomplex

A. TR 0.025 μg TR 0.050 μg

B. TR 0.05 μg TR 0.10 μg

FIGURE 8. Transcriptional repression by TR is impaired in the presence of DNA-PK inhibitors. A, HeLa cells were grown in T₃-free medium with or without 0.5 μM wortmannin (WM). The cells were transiently transfected with a reporter vector, TRE-luciferase (0.5 μg), a TR expression vector pCI-hTRβ (0.025 and 0.05 μg), a RXR expression vector pCI-hRXRα (0.025 μg), and an internal control vector expressing Renilla luciferase (0.01 μg). 48 h after transfection, cells were harvested and assayed for luciferase activity as described previously (59). The basal reporter activity in the absence of transfected pCI-hTRβ was taken as 100%, and the relative values for test samples were obtained. B, transient transfections were carried out in HeLa cells as described above with the following modifications: 1 μM NU7026 was used instead of wortmannin, and slightly different amounts of pCI-hTRβ (0.05 and 0.1 μg) were used. Three independent sets of each experiment were carried out, and the results are represented as mean value ± S.E.

added DNA. Moreover, we did not see any significant reduction in the retention of DNA-PK on TR-RXR even after extensive treatment of HeLa nuclear extracts and purified TR-RXR complexes with DNase I (data not shown), ruling out any contribution of DNA-dependent interactions to the binding of these proteins by TR-RXR. Collectively, these biochemical experiments are consistent with the concept that in the unliganded state TR-RXR recruits both NCoR/SMRT and DNA-PK complexes, which are held together via protein-protein interactions between certain of their components.

The results of these in vitro experiments found support from our in vivo analysis of T₃-regulated growth hormone gene expression in GH₃ pituitary tumor cells using chromatin immunoprecipitation. Both TR and RXR occupied the growth hormone promoter region containing a well characterized TRE, irrespective of thyroid hormone status of these cells. In the absence of T₃, the signature components of both NCoR/SMRT and DNA-PK complexes were found to bind to this region, consistent with the findings of our in vitro experiments that these complexes are associated with the transcriptionally repressive form of TR-RXR. Binding of T₃ to TR, which triggers the transition of this receptor from the repressive to an active form, was accompanied by the dissociation of the components of the NCoR/SMRT complex from the TRE-bound heterodimer. Interestingly, hormone binding to TR also released the DNA-PK complex from the TRE site. These results provided strong evidence not only for the recruitment of a DNA-PK complex by unliganded TR-RXR at a natural target gene but also hinted at its close interaction with the components of the NCoR/SMRT complex.

The DNA-PK core complex is composed of four subunits: DNA-PKcs, PARP1, Ku86, and Ku70 (32, 33). The DNA-PKcs, a serine/threonine protein kinase, belongs to a family of PI3K-related protein kinases that also includes ATM (ataxia-telangiectasia, mutated), ATR (ATM and Rad3-related), mammalian target of rapamycin, and transformation/transcription domain-associated protein. The members of this family are reported to be involved in double-stranded DNA break repair and V(D)J recombination (32, 33). Autophosphorylation of DNA-PKcs activates the enzyme and is critical for its DNA repair function (34–36). The DNA-PKcs is also known to have a role in transcriptional regulation due to its ability to phosphorylate and regulate the activity of sequence-specific transcription factors and their cofactors. It was observed that DNA-PK and other PI3Ks such as ATM and ATR target the tumor suppressor p53 and its negative regulator mdm2 to modulate the p53-mediated transcriptional pathway (37, 38). Recently Ko and Chin (39) reported that TRBP (thyroid hormone receptor-binding protein), a nuclear receptor coactivator, exhibited reduced coactivation function for GR in DNA-PK-deficient cells in response to dexamethasone, indicating a role in this kinase in regulating coactivator function.

Direct association of a DNA-PK complex with a nuclear receptor was reported by Sartorius et al. (40). They observed that the DNA-binding domain of progesterone receptor is able to interact with an intact DNA-PK complex containing DNA-PKcs, PARP1, Ku86, and Ku70 present in HeLa nuclear extracts (40). Weigel et al. previously reported that transcriptionally active progesterone receptor is phosphorylated by DNA-PK (41). Studies by Giffin et al. (42) indicated that DNA-PK interacts with glucocorticoid receptor and phosphorylates it at the Ser-527 residue situated between the DNA-binding domain and ligand-binding domain. Although these studies collectively suggested that DNA-PK is able to interact directly with certain nuclear receptors to modify their transcriptional activities, the precise mechanism by which the phosphorylation by this kinase affects receptor function remained unclear.

An important finding of this report is that the DNA-PK modulates the activity of HDAC3, a key component of the NCoR/SMRT corepressor complex, to regulate chromatin function and transcription. We did not detect any significant phosphorylation of either TR or RXR when the purified TR-RXR-corepressor holocomplex was incubated with radiolabeled ATP (data not shown). We, however, observed that the HDAC3 subunit of the NCoR/SMRT complex is phosphorylated under these conditions, and this phosphorylation is sensitive to wortmannin, an
Role of DNA-PK in a TR-RXR-Corepressor Holocomplex

inhibitor of DNA-PK and other PI3Ks. Because mass spectrometry analysis identified DNA-PKcs as the only PI3K present in the purified TR-RXR holocomplex, we propose that DNA-PK is the kinase that phosphorylates HDAC3. This conclusion is further supported by the observation that purified, catalytically active DNA-PK is able to phosphorylate HDAC3.

The PI3K-mediated phosphorylation of HDAC3 represents a novel post-translational modification of the class I HDAC enzymes. Previous studies documented that both HDAC1 and HDAC2 exist as phosphoproteins in the nuclear extracts. It was also determined that these phosphorylation events were mediated by casein kinase II (43, 44). HDAC3 is also phosphorylated upon incubation with casein kinase II in vitro (45). It is conceivable that the close contacts between the components of the DNA-PK and NCoR/SMRT complexes in the holocomplex allow the DNA-PKcs to target HDAC3 for phosphorylation. It is of interest to note that sequence analysis of human HDAC3 protein by MotifScan (scansite.mit.edu/cgi-bin/motifscan_seq), a phosphorylation site prediction program, pointed to amino acids Thr-390 and Ser-405 as potential phosphorylation sites for DNA-PK. It, however, remains to be seen whether these amino acids represent actual sites of phosphorylation by DNA-PK.

Previous studies indicated that increased phosphorylation of the class I HDACs promotes their histone deacetylase activities (43–45). Consistent with these reports, we observed that DNA-PK-mediated phosphorylation of the HDAC3 component of the TR-RXR-corepressor holocomplex significantly enhanced the HDAC enzyme activity of the purified complex (Fig. 7). It is well established that the NCoR/SMRT corepressor complex containing HDAC3 is recruited by promoter-bound unliganded TR within the class I HDACs promotes their histone deacetylase activities (43–45). Consistent with these reports, we observed that DNA-PK-mediated phosphorylation of the HDAC3 component of the TR-RXR-corepressor holocomplex significantly enhanced the HDAC enzyme activity of the purified complex (Fig. 7). It is well established that the NCoR/SMRT corepressor complex containing HDAC3 is recruited by promoter-bound unliganded TR-RXR and that it promotes transcriptional silencing of target genes via local deacetylation of histones. The deacetylation function of HDAC3 removes the acetyl groups from critical lysine and arginine groups of histones to create a compact chromatin structure that is conducive to efficient transcriptional repression. We propose that PI3K signaling via DNA-PK plays a critical role in this process by phosphorylating and thereby enhancing the catalytic activity of HDAC3. If the DNA-PK activity were critical for maintaining a robust deacetylase function of the NCoR/SMRT corepressor complex, then one would predict that its blockade would lead to a suppression of TR-mediated transcriptional silencing in vivo. Indeed, we noted that the ability of TR to function as a transcriptional repressor in a cell-based transfection assay was markedly reduced in the presence of wortmannin, an inhibitor of DNA-PK.

The factors that regulate the activity DNA-PK within the TR-RXR-corepressor holocomplex are unknown. Earlier reports indicated that the DNA-PKcs must be physically associated with DNA to be fully active (31). Although binding to DNA ends is a well known mode of activation of this kinase, other mechanisms, including regulation by distinct kinases and protein-protein interaction, have been shown to promote DNA-PK activity (46–48). The study by Ko and Chin has shown that the coactivator TRBP can stimulate the activity of the DNA-PK complex in the absence of any added DNA (39). Our studies with the purified TR-RXR holocomplex suggest that the DNA-PK activity may derive from the protein-protein interactions of the DNA-PKcs with other polypeptides within this complex.

The functional roles, if any, of the Ku subunits and PARP1 in TR-mediated silencing are presently unclear. PARP-1, the best characterized member of a family of enzymes that catalyzes the transfer of ADP-ribose from NAD+ to target proteins, is involved in multiple DNA repair pathways (49). ADP ribosylation of histones by PARP1 promotes their dissociation to cause a local DNA relaxation that allows the assembly of repair proteins and the initiation of the repair process (50). Cross covalent modifications, i.e. phosphorylation of PARP1 by DNA-PK and ADP-ribosylation of DNA-PK by PARP-1, have been found to be critical for the function of the DNA repair machinery (51, 52). There is now increasing evidence that PARP1 plays a critical role in transcriptional regulation. Miyamoto et al. (53) reported that overexpression of PARP1 strongly inhibits T3-dependent transactivation by TR in transient transfection experiments. Ju et al. (54) have shown that PARP1 is a stable and integral component of the TLE corepressor complex, which is involved in the repression of MASH1 gene by transcription factor HES1 during neural cell differentiation. A recent study indicated that a complex containing DNA topoisomerase IIα and PARP1 is recruited to target promoters during hormone-mediated transactivation by the nuclear receptors (55). Topoisomerase IIα induces transient, site-specific double-stranded DNA breaks and PARP1 mediates nucleosome-specific modifications in histone H1, leading to local changes in chromatin architecture. Although our study did not address the recruitment of topoisomerase IIα during TR-mediated transcriptional repression, we did note that DNA ligase III and structural maintenance of chromosome 1 are associated with the repressive form of TR-RXR (Table 1). These proteins are known to play critical functions in DNA break and repair events (56–58). Further studies are clearly necessary to understand how the molecules involved in DNA break and repair pathways contribute to TR-mediated transcriptional activation and repression mechanisms.

In summary, our study provides compelling evidence for the existence of a TR-RXR-corepressor holocomplex endowed with multiple enzymatic activities such as HDAC3 (histone deacetylase), DNA-PK (kinase), and PARP1 (ADP-ribosylase). Coexistence of several modifying activities in a single large complex offers the potential of regulation by multiple signaling mechanisms. It is possible that DNA-PKcs can target additional components of the holocomplex besides HDAC3 for phosphorylation. Similarly, PARP1 may modify components of the holocomplex via ADP ribosylation. Additionally, these activities may also alter chromatin structure and function by modifying histones. When recruited to a target gene promoter, the corepressor holocomplex will thus provide a highly versatile and efficient enzymatic machinery to locally alter chromatin structure that modulates the transcriptional function of TR. Future studies will analyze the nature of these chemical modifications and determine their functional roles in TR-mediated gene repression.

Acknowledgments—We thank David Savitsky, Ronald Baker, and J. R. Paige for expert technical help. We thank Arpi Nazarian for help with mass spectrometric analysis. We also thank Michael Schall for identifying several TR-RXR-associated polypeptides at the Rockefeller University Mass Spectrometry Facility.
Role of DNA-PK in a TR-RXR-Corepressor Holocomplex

REFERENCES

1. Yen, P. M., Ando, S., Feng, X., Liu, Y., Maruvada, P., and Xia, X. M. (2006) J. Biol. Chem. 281, 6091–6101
2. Tsai, M. J., and Omalley, B. W. (1994) Annu. Rev. Biochem. 63, 451–486
3. Suen, C. S., Yen, P. M., and Chin, W. W. (1994) J. Biol. Chem. 269, 1314–1322
4. Lees-Miller, S. P. (1996) Biochem. Cell Biol. 74, 503–512
5. Meek, K., Gupta, S., Ramsden, D. A., and Lees-Miller, S. P. (2004) Immuno.
6. Collis, S. J., DeWeese, T. L., Jeggo, P. A., and Parker, A. R. (2005) Oncogene
7. Douglass, P., Sakpota, G. P., Morrice, N., Yu, Y. P., Goodarzi, A. A., Merkle, D.,
8. Li, J. W., Wang, J., Wang, J. X., Nawaz, Z., Liu, J. M., Qin, J., and Wong,
9. Rachez, C., Suldan, Z., Ward, J., Chang, C. P. B., Burakov, D., Erdjument-
10. Casanova, J., Helmer, E., Selmiruby, S., Qi, J. S., Aufliegner, M., Desaiya-
11. Chen, J. D., and Evans, R. M. (1995) Mol. Endocrinol. 19, 2644–2649
12. Erdjument-Bromage, H., Lui, M., Lacomis, L., Grevel, A., Annan, R. S.,
13. Koeppen, B. R., and Omalley, B. W. (1994) Mol. Cell. Biol. 14, 5756–5765
14. Privalsky, M. L. (2004) Annu. Rev. Physiol. 66, 315–360
15. Glass, C. K., and Rosenfeld, M. G. (2000) Genes Dev. 14, 121–141
16. Smith, C. L., and O’Malley, B. W. (2004) Endocr. Rev. 25, 45–71
17. Chen, J. D., and Evans, R. M. (1995) Nature 377, 454–457
18. Horlein, A. J., Naar, A. M., Heinzel, T., Torchoia, J., Gloss, B., Kurokawa, R.,
19. Hu, X., Li, Y., and Lazar, M. A. (2001) Mol. Cell. Biol. 21, 1747–1758
20. Ishizuka, T., and Lazar, M. A. (2003) Mol. Cell. Biol. 23, 5122–5131
21. Yoon, H. G., Chan, D. W., Huang, Z. Q., Li, J. W., Fondell, J. D., Qin, J., and
22. Zhang, J. S., Kulkarni, M., Chait, B. T., and Roeder, R. G. (2002) Mol. Cell 9,
23. Guntert, M. G., Barak, O., and Lazar, M. A. (2001) Mol. Cell. Biol. 21, 6091–6101
24. Heinzel, T., Lavinsky, R. M., Mullen, T. M., Soderstrom, M., Laherty, C. D.,
25. Heiden, J. J., and Lazar, M. A. (2001) Mol. Cell. Biol. 21, 5836–5848
26. Winkler, G. S., Lacomis, L., Philip, J., Erdjument-Bromage, H., Svejstrup,
27. Winkler, G. S., Lacomis, L., Philip, J., Erdjument-Bromage, H., Svejstrup,
28. Rachez, C., Sudan, Z., Ward, J., Chang, C. P. B., Burakov, D., Erdjument-
29. Li, J. W., Wang, J., Wang, J. X., Nawaz, Z., Liu, J. M., Qin, J., and Wong,
30. Carter, T., Vancurova, I., Sun, I., Lou, W., and Deleon, S. (1990) Mol. Cell.
31. Liu, X. F., and Bagchi, M. K. (2004) J. Biol. Chem. 279, 15050–15058
32. Samuels, H. H., Forman, B. M., Horowitz, Z. D., and Ye, Z. S. (1989) Annu.
33. Samuels, H. H., Forman, B. M., Horowitz, Z. D., and Ye, Z. S. (1989) Annu.
34. Liu, X. F., and Bagchi, M. K. (2004) J. Biol. Chem. 279, 15050–15058
35. Samuels, H. H., Stanley, F., and Casanova, J. (1979) Endocrinology 105, 80–85
36. Samuels, H. H., Forman, B. M., Horowitz, Z. D., and Ye, Z. S. (1989) Annu.
37. Liu, X. F., and Bagchi, M. K. (2004) J. Biol. Chem. 279, 15050–15058
38. Samuels, H. H., Stanley, F., and Casanova, J. (1979) Endocrinology 105, 80–85
39. Samuels, H. H., Forman, B. M., Horowitz, Z. D., and Ye, Z. S. (1989) Annu.
40. Liu, X. F., and Bagchi, M. K. (2004) J. Biol. Chem. 279, 15050–15058
41. Samuels, H. H., Forman, B. M., Horowitz, Z. D., and Ye, Z. S. (1989) Annu.
42. Liu, X. F., and Bagchi, M. K. (2004) J. Biol. Chem. 279, 15050–15058