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The N-CoR-HDAC3 Nuclear Receptor Corepressor Complex Inhibits the JNK Pathway through the Integral Subunit GPS2

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Summary

The corepressors N-CoR and SMRT partner with histone deacetylases (HDACs) in diverse repression pathways. We report here that GPS2, a protein involved in intracellular signaling, is an integral subunit of the N-CoR-HDAC3 complex. We have determined structural motifs that direct the formation of a highly stable and active deacetylase complex. GPS2 and TBL1, another component of the N-CoR-HDAC3 complex, interact cooperatively with repression domain 1 of N-CoR to form a heterotrimeric structure and are indirectly linked to HDAC3 via an extended N-CoR SANT domain that also activates latent HDAC3 activity. More importantly, we show here that the N-CoR-HDAC3 complex inhibits JNK activation through the associated GPS2 subunit and thus could potentially provide an alternative mechanism for hormone-mediated antagonism of AP-1 function.

Introduction

N-CoR (nuclear receptor corepressor) and SMRT (silencing mediator for retinoid and thyroid receptors) are related corepressors that mediate transcriptional repression by unliganded nuclear receptors and other classes of transcriptional repressors (reviewed in Glass and Rosenfeld, 2000). Repression mediated by N-CoR and SMRT is thought to involve modification of chromatin structure by associated histone deacetylases (HDACs). Thus, whereas histone acetylation following recruitment of histone acetyltransferases (HATs) by promoter-bound activators facilitates transcription, histone deacetylation following recruitment of HDACs by promoter-bound repressors and corepressors is thought to maintain a condensed chromatin state that inhibits transcription (see review above).

HDACs are a growing family of structurally conserved proteins (Hassig et al., 1998). Mammalian class I HDACs (HDACs 1–3 and 8) are highly related to yeast Rpd3, whereas class II HDACs (HDACs 4–7) are more similar to yeast HDA1. Earlier studies identified multiple HDAC-containing complexes (Hassig et al., 1998; Huang et al., 2000; Humphrey et al., 2001; Zhang et al., 1999), suggesting that they may function in distinct pathways. Biochemical studies have defined a large SMRT complex that contains HDAC3 and the WD40-repeat protein TBL1 (Guenther et al., 2000; Li et al., 2000). Like SMRT, the related N-CoR also associates with HDAC3 (Li et al., 2000; Wen et al., 2000). However, knockout studies have revealed N-CoR-specific repressive functions in vivo, as well as a paradoxical role in transactivation through certain retinoic acid response elements (Jepsen et al., 2000).

Human *GPS2* (G-protein pathway suppressor 2) was isolated via its ability to suppress lethal G protein subunit-activating mutations in the yeast pheromone response pathway (Spain et al., 1996). Pheromone binding to its cognate receptor triggers a $G_{\beta,\gamma}$-mediated kinase cascade (Ste20, Ste11, Ste7, and Fus3/Kss1) that shares striking structural similarities with mammalian MAPK (e.g., Raf-MEK-ERK and MEKK-SEK-JNK) pathways. Consistent with its role in conserved signaling pathways, overexpression of GPS2 in mammalian cells potently suppresses a RAS/MAPK-mediated signal and interferes with JNK1 activation by serum factors or TNFα (Jin et al., 1997; Spain et al., 1996). However, the mechanism by which GPS2 affects signal transduction is not known. Independent studies have shown that GPS2, also known as AMF-1 (Breiding et al., 1997), interacts with the human T cell lymphotrophic virus type I (HTLV-I) Tax oncoprotein, suppressing its ability to activate JNK1 (Jin et al., 1997), and with bovine papillomavirus E2 and tumor suppressor p53 proteins, stimulating their transactivation activities (Breiding et al., 1997; Peng et al., 2000, 2001). These results suggest a role for GPS2, a nuclear protein (Breiding et al., 1997) with ubiquitous expression (Jin et al., 1997), in transcriptional regulation.

To better understand the mechanisms underlying N-CoR and HDAC functions, we have affinity purified and characterized N-CoR- and HDAC3-containing complexes. Our analyses reveal protein-protein interactions that affect the assembly and activity of these complexes, as well as a component (GPS2) that links them to membrane receptor-related intracellular JNK signaling pathways.

Results

GPS2 Is a Stoichiometric Subunit of the N-CoR-HDAC3 Complex

To facilitate structural and functional studies, we have created HeLa S-derived cell lines that stably express FLAG-tagged N-CoR or FLAG-tagged HDACs (HDAC1, 3, or 5). Complexes containing each FLAG-tagged subunit were affinity purified from nuclear extracts of tagged cell lines. Protein subunits were identified by mass spectrometry following resolution by SDS-PAGE.

The polypeptide compositions of the FLAG-N-CoR and FLAG-HDAC3 complexes are shown in Figure 1A. Two members of the heterogenous 55 kDa band were identified as TBL1, which was previously identified in a SMRT complex (Guenther et al., 2000; Li et al., 2000), and a TBL1-related protein (TBLR1) whose complete sequence was revealed by cDNA cloning (Figures 1A and 1B). Peptide mass fingerprints indicated that TBL1

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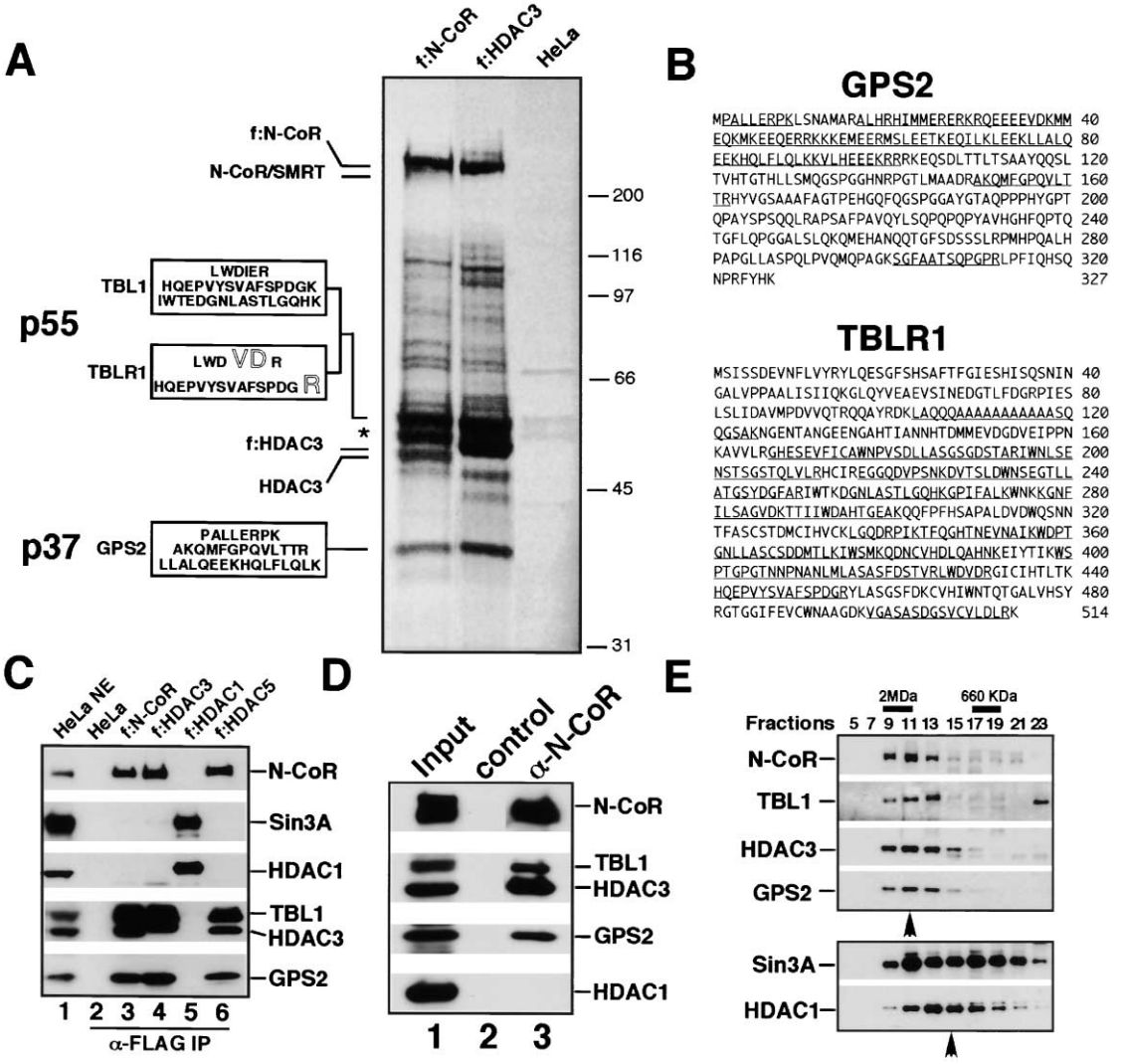

Figure 1. Identification of GPS2 and TBLR1 as Stoichiometric Subunits of the N-CoR-HDAC3 Complex

(A) Polypeptide composition of FLAG-N-CoR (f:N-CoR) and FLAG-HDAC3 (f:HDAC3) complexes. Complexes were affinity purified on M2-agarose from cell lines stably expressing cognate FLAG-tagged polypeptides and analyzed by SDS-PAGE and zinc stain (Bio-Rad kit). HeLa indicates nonspecific polypeptides from control HeLa nuclear extract that were bound to and eluted from M2-agarose beads. Identities of protein bands were determined by mass spectrometric analysis. Peptide sequences for p55 and p37 obtained by MS/MS are indicated. The asterisk denotes a nonspecific band identified by MS.

(B) TBLR1 and GPS2 sequences. Peptide sequences identified by MS or by both MS and MS/MS are underlined.

(C) Presence of GPS2 in N-CoR-, HDAC3-, and HDAC5-containing complexes but not in the HDAC1-containing complex. Complexes (lanes 3–6) were affinity purified on M2-agarose beads (α-FLAG IP) from cell lines stably expressing the corresponding FLAG-tagged proteins and analyzed by Western blot with antibodies to proteins indicated on the right. Lane 1, unfractionated HeLa nuclear extract; lane 2, M2-agarose bound polypeptides from control HeLa nuclear extract.

(D) Western blot analysis of an endogenous N-CoR complex affinity purified on protein G-agarose from HeLa nuclear extract with a specific anti-N-CoR antibody. The N-CoR complex (lane 3) was eluted with a buffer containing the immunizing peptide. Lane 1, 2.5% of total input; lane 2, nonspecific polypeptides from HeLa nuclear extract bound to and eluted from protein G-agarose beads.

(E) Cofractionation of GPS2 with N-CoR, TBL1, and HDAC3 as a large protein complex (~2 MDa mass). HeLa nuclear extract was analyzed by Superose 6 gel filtration (SMART system, Pharmacia) in BC300/0.05% NP-40, and fractions were analyzed by Western blot with antibodies against polypeptides indicated on the left. Identical elution profiles for GPS2, TBL1, N-CoR, and HDAC3 were obtained with the purified FLAG-HDAC3-containing complex (data not shown).

and TBLR1 are equally represented in both N-CoR and HDAC3 complexes (data not shown).

A 37 kDa component (p37) present in stoichiometric amounts in both N-CoR and HDAC3 complexes (Figure 1A) was identified as the G protein pathway suppressor 2 (GPS2) protein (Figures 1A and 1B) (Jin et al., 1997; Spain et al., 1996). Specific association of GSP2 with N-CoR and HDAC3 was further confirmed by Western blot analysis. GPS2 was detected in N-CoR-, HDAC3-, and HDAC5-containing complexes but not in the HDAC1 complex (Figure 1C). In contrast, the accessory corepressor Sin3A was specifically associated with the HDAC1

complex. In agreement with our current results, HDAC5 was shown previously to interact directly with N-CoR and SMRT (Huang et al., 2000).

To further substantiate the physiological relevance of these interactions, we next documented similar interactions between endogenous proteins. An affinity-purified antibody directed against the C terminus of N-CoR was used to isolate an endogenous N-CoR complex from HeLa cell nuclear extract. Consistent with the analyses of the FLAG-N-CoR and FLAG-HDAC3 complexes, endogenous GPS2, TBL1, and HDAC3 specifically associated with endogenous N-CoR (Figure 1D). In a further analysis of endogenous factors, HeLa nuclear extract was fractionated on a Superose 6 gel filtration column under conditions (300 mM KCl, 0.05% NP-40) favoring the dissociation of potential protein aggregates. Consistent with our affinity purification data (Figures 1A, 1C, and 1D) and the notion that they can be present in a single large complex, GPS2, HDAC3, N-CoR, and TBL1 copurified in fractions corresponding to a mass of about 2 MDa (Figure 1E, top). Similar results were obtained upon fractionation with the purified FLAG-HDAC3-containing complex (data not shown). In contrast and consistent with our interaction data (Figures 1C and 1D), distinct fractionation profiles for Sin3A and HDAC1 were observed (Figure 1E, bottom). The cofractionation of GPS2 with other known subunits both from nuclear extract and from affinity-purified complexes strongly suggests that they can indeed be components of a single large complex that, based on compositional (Figure 1A) and size (Figure 1E) analyses, must also contain other as yet unidentified components.

N-CoR, TBL1, and HDAC3 Are Major GPS2-Associated Proteins

An anti-GPS2 antiserum (Ab1) specifically precipitated N-CoR from HeLa nuclear extract (Figure 2A, lane 3), further confirming an endogenous GPS2-N-CoR association. Coimmunoprecipitation was also observed with a peptide-generated anti-GPS2 antiserum (Ab2) (lane 4) and was specifically suppressed in the presence of the immunizing peptide (cf. lanes 4–6). Confirming the endogenous GPS2-HDAC3 association, anti-GPS2 immunoprecipitates contained a strong Trichostatin A (TSA)-sensitive HDAC activity that was comparable in magnitude to that in the anti-N-CoR immunoprecipitates and specifically blocked in the presence of the immunizing peptide (Figure 2B, lanes 1–6).

To establish a stoichiometric association, a stable HeLa S-derived cell line (termed HS4) expressing FLAG-GPS2 was generated, and the GPS2-containing complex was affinity purified from derived nuclear extract. Coomassie blue staining (Figure 2C, lane 2), silver staining (Figure 2C, lane 5), immunoblot analysis (Figure 2D, lanes 1–3), and MS/MS mass spectrometric analysis following SDS-PAGE indicated that N-CoR, TBL1, and HDAC3 are major GPS2-associated proteins. MS/MS analysis also identified significant levels of SMRT and TBLR1 in the GPS2-containing complex(es). In contrast, no association of GPS2 with either Sin3A or HDAC1 was detected (Figure 2D, lanes 1–3). A comparison of complexes affinity purified via different FLAG-tagged subunits (HDAC3, N-CoR, and GPS2) confirmed that they share most major and minor polypeptides (Figure 2C, lanes 3–5).

The FLAG-GPS2 complex displayed both a strong HDAC activity (data not shown) and remarkable stability, being resistant to 1 M KCl and high concentrations of either nonionic (NP-40) or ionic (deoxycholate) detergents and to moderate concentrations of sodium dodecyl sulfate (Figure 2D, lanes 3–10).

GPS2 and TBL1 Interact with Distinct Conserved Regions of the N-CoR RD1 Domain

To determine the N-CoR domain(s) responsible for interactions with TBL1, GPS2, and HDAC3, FLAG-tagged N-CoR derivatives were expressed in 293T cells and immunoprecipitated. While full-length N-CoR associated with the other three subunits, deletion of RD1 (N-terminal 312 amino acids), generating N-CoRΔRD1, abrogated the interactions with GPS2 and TBL1 but was without effect on the interaction with HDAC3 (Figure 3A, cf. lanes 1 and 2). Deletion of a C-terminal region (residues 1446–2453) that interacts with nuclear hormone receptors, generating N-CoR(1–1445), had no effect on interactions with the other three subunits (Figure 3B, lane 3). Another N-CoR derivative (RD1) containing only residues 1–312 failed to interact with HDAC3 but retained the ability to interact with both GPS2 and TBL1 (Figure 3B, lane 2). These results indicate that the N-CoR RD1 domain mediates interactions with GPS2 and TBL1, whereas a region between amino acids 313 and 1445 mediates interaction with HDAC3. Thus, HDAC3 is indirectly associated with GPS2 in the N-CoR-containing complex.

We then tested truncated RD1 mutants (Figure 3C, left) for GPS2 and TBL1 interactions (Figure 3C, middle and right). Truncation at amino acid 225 (mutant A) abolished the TBL1 interaction but had no effect on the GPS2 interaction. Further truncation at amino acid 160 (mutant C) abolished the interaction with GPS2. An internal deletion of amino acids 161 to 225 (mutant B) similarly abrogated the GPS2 interaction but had no effect on the TBL1 interaction. These results suggest that the central subdomain (amino acids 161 to 225) mediates the GPS2 interaction, whereas the C-terminal subdomain (amino acids 226 to 312) mediates the TBL1 interaction. A mammalian two-hybrid assay confirmed that a region within amino acids 226 to 312 is sufficient to mediate a strong intracellular interaction with TBL1 (data not shown).

Both of the RD1 subdomains are highly conserved between N-CoR and SMRT. Structural analysis predicts that a region within amino acids 161 to 225 forms a strong coiled-coil structure (Figure 3D). The corresponding region in the *Drosophila* corepressor SMRTER and a region of GPS2 (amino acids 12 to 103) are also predicted to form similar coiled-coil structures. Coiled-coil interactions mediated by these regions might underlie the structural basis for highly specific N-CoR-GPS2 interaction.

TBL1 Interacts via a Conserved Domain with both GPS2 and RD1 and Enhances the GPS2-RD1 Interaction

The in vivo association between N-CoR(1–225) (mutant A in Figure 3C) and GPS2 was recapitulated in vitro in a GST pull-down assay with a GST-GPS2 fusion protein and in vitro-translated N-CoR(1–225) (Figure 4A). Interestingly, in vitro-translated TBL1 also displayed a strong

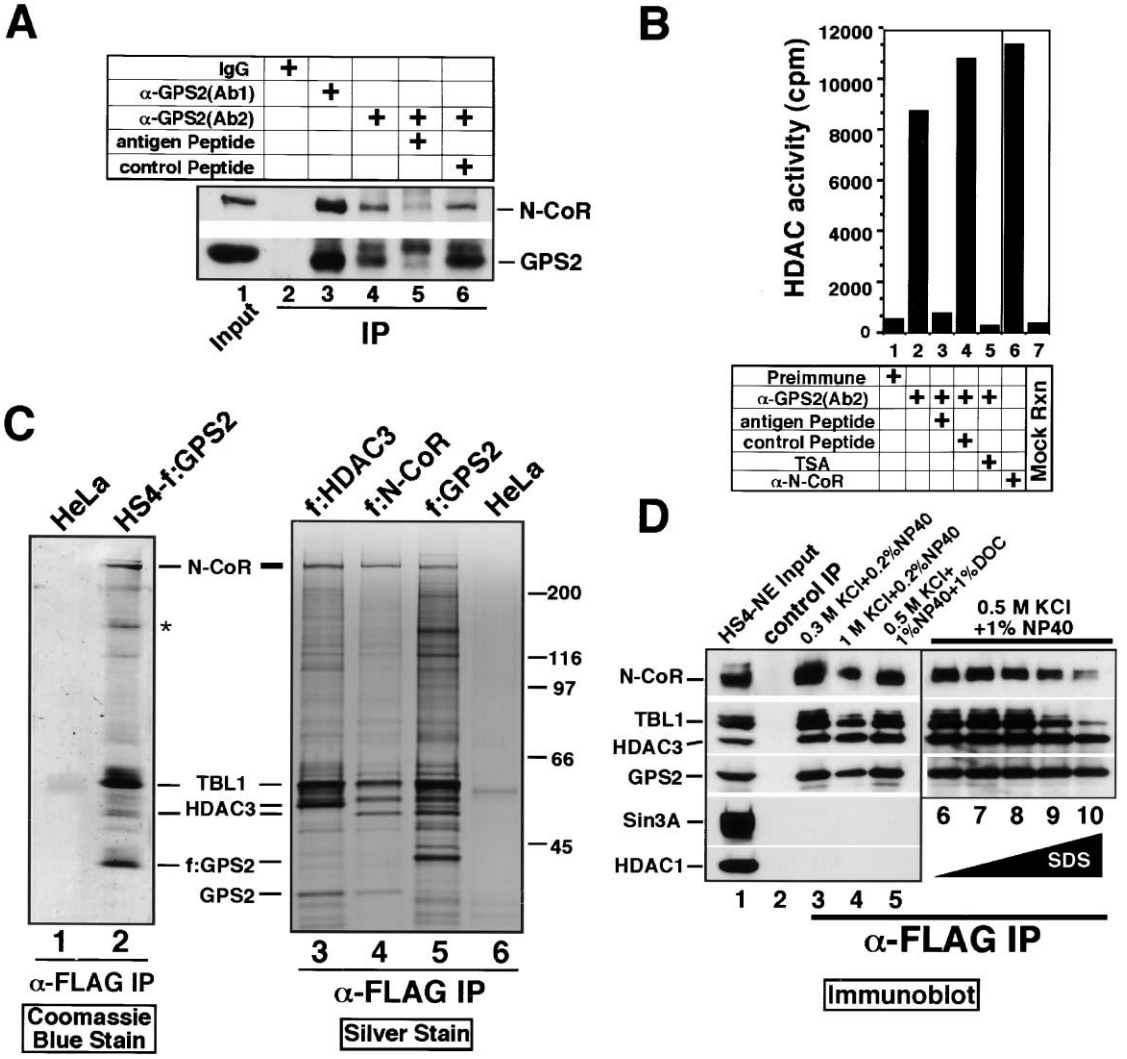

Figure 2. N-CoR, TBL1, and HDAC3 Are Major GPS2-Associated Proteins

(A) Western blot analysis of anti-GPS2 (lanes 3–6) and control rabbit IgG (lane 2) immunoprecipitates (IP) from HeLa nuclear extract with antibodies against proteins indicated on the right. Lane 1, 5% of total input.

(B) HDAC activities of anti-GPS2 (lanes 2–5), anti-N-CoR (lane 6), and control preimmune serum (lane 1) immunoprecipitates from HeLa nuclear extract. Other additions were as indicated.

(C) Coomassie blue stain (lane 2) and silver stain (lane 5) of an SDS-PAGE-resolved FLAG-GPS2 complex affinity purified on M2-agarose from HS4 cell (a stable FLAG-GPS2-expressing cell line) nuclear extract. Identities of protein bands were confirmed by both MS/MS and immunoblot analyses. Affinity-purified FLAG-HDAC3 (lane 3) and FLAG-N-CoR complexes (lane 4) were included for comparison. Lanes 1 and 6, M2-agarose bound nonspecific polypeptides from control HeLa nuclear extract. The asterisk denotes an uncharacterized band that appears to be reproducibly enriched in GPS2-containing complexes purified from HS4 cell nuclear extracts.

(D) Western blot analysis of FLAG-GPS2 complexes affinity purified on M2-agarose from HS4 cell nuclear extracts at various buffer conditions indicated at the top (lanes 3–10). Lane 1, 3.7% of total input; lane 2, nonspecific polypeptides bound to protein G-agarose beads. SDS concentrations (lanes 6–10): 0%, 0.1%, 0.2%, 0.3%, and 0.5%.

interaction with GPS2 (Figure 4A). TBL1 (Figure 4B) contains carboxyl WD40-repeats (TBL1-WD, amino acids 212 to 577) and a unique N-terminal region (TBL1-N, amino acids 1 to 211) that mediates interactions with N-CoR and SMRT (Guenther et al., 2000 and data not shown). We further determined that TBL1-N but not TBL1-WD could similarly interact with GPS2 (Figure 4A).

TBL1 is highly related to TBLR1 and *Drosophila* TBL1/Ebi. Similar to TBL1, TBLR1 was found to interact with GPS2 and N-CoR-RD1 in vitro and with HDAC3 in vivo (data not shown). Alignment of the N-terminal regions of TBL1, TBLR1, and Ebi reveals a highly conserved 91 amino acid region that includes amino acids 52 to 142 of TBL1 and amino acids 1 to 91 of TBLR1 (Figure 4B, top). In an in vitro binding assay, TBL1(1–142) but not TBL1(143–211) displayed strong interactions with both GPS2 and RD1 (Figure 4B, bottom). Similar interactions with GPS2 and RD1 were observed with TBLR1(1–74) (data not shown). Thus, this conserved region of TBL1 functions as both a corepressor (N-CoR) and a GPS2-

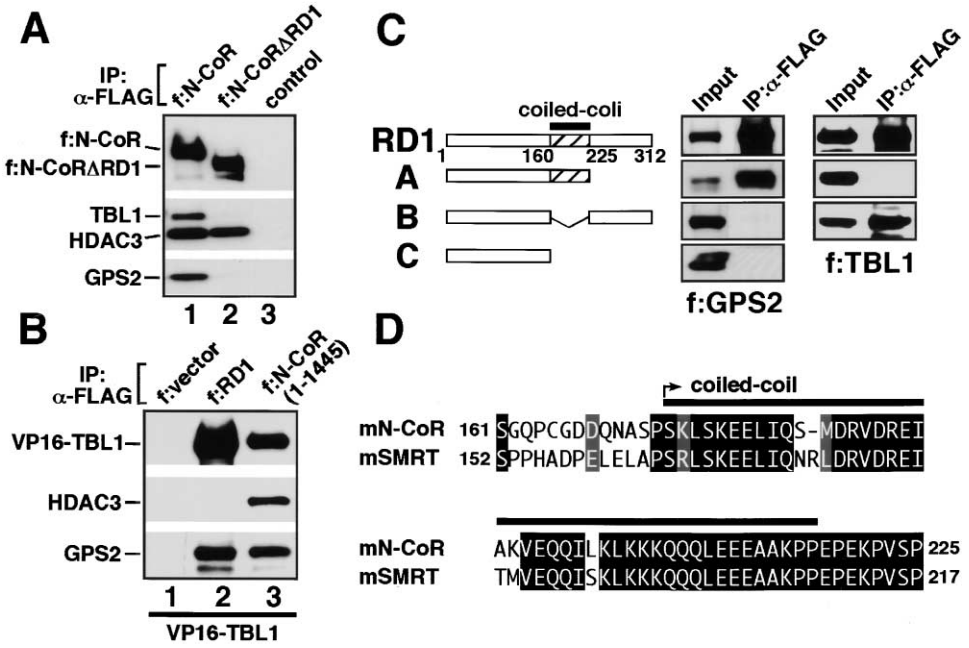

Figure 3. GPS2 and TBL1 Interact with Distinct Conserved Regions of the N-CoR RD1 Domain

(A) Western blot analysis of anti-FLAG immunoprecipitates from 293T cells transfected with FLAG-N-CoR (lane 1) or FLAG-N-CoRΔRD1 (lane 2) or from untransfected 293T cells (lane 3).

(B) Western blot analysis of anti-FLAG immunoprecipitates from 293T cells that were cotransfected with vectors expressing VP16-TBL1 and either the indicated FLAG-tagged N-CoR derivative (lanes 2 and 3) or FLAG peptide alone (lane 1). VP16-TBL1 was probed with an anti-VP16 antibody (Santa Cruz).

(C) Mapping of RD1 subdomains for GPS2 and TBL1 interactions. Anti-FLAG immunoprecipitates from 293T cells that were cotransfected with vectors expressing RD1 or RD1 truncations fused to Gal4-DBD (left panel) and either FLAG-GPS2 (middle panel) or FLAG-TBL1 (right panel) were probed with an anti-Gal4 antibody (Santa Cruz).

(D) Alignment of N-CoR and SMRT sequences showing a highly conserved coiled-coil region within the putative GPS2-interacting domain (amino acids 161 to 225) of N-CoR. Structural prediction was carried out with the DNASTAR PROTEAN program.

interacting domain, which we termed the CGID domain (Figure 4B, top).

No coiled-coil regions are predicted for TBL1. This suggests that it may interact with a GPS2 site distinct from the region (possibly a coiled coil, see above) that interacts with RD1. Consistent with its ability to interact with both GPS2 and RD1, ectopic expression of TBL1 or a VP16-TBL1 fusion protein in 293T cells markedly enhanced FLAG-RD1 interaction with endogenous GPS2 (Figure 4C). This cooperativity further suggests a role for TBL1 in the assembly of the complete N-CoR-TBL1-GPS2-HDAC3 complex. In support of this idea, ectopic FLAG-TBL1 alone but not an ectopic FLAG-HDAC1 control strongly associated with endogenous GPS2, N-CoR, and HDAC3 (Figure 4D). It is likely that TBL1 augments the RD1-GPS2 interaction by stabilizing a GPS2-TBL1-N-CoR heterotrimeric structure, which indirectly associates with HDAC3 through an N-CoR region beyond RD1 and within the N-CoR repression domain (N-CoR-RD) (Figure 4E).

An Extended N-CoR SANT Domain Interacts with and Activates the Latent Activity of HDAC3

Unlike ectopic TBL1, ectopic FLAG-HDAC3 only associates with a low level of other endogenous subunits (N-CoR, TBL1, and GPS2) in transiently transfected 293T cells (data not shown). Immunoprecipitates of FLAG-HDAC3 from these cells also displayed minimal histone deacetylase activity (vector lane versus mock lane in Figure 5A, top), suggesting that HDAC3 itself is virtually inactive. Remarkably, cotransfection of the entire N-CoR repression domain (amino acids 1–1445), which was shown to interact with HDAC3 (Figure 3B), rescued the latent activity of the immunoprecipitated HDAC3 (Figure 5A, top), indicating a critical role of N-CoR in regulating HDAC3 function. To define the responsible N-CoR region(s), a series of N-CoR repression domain mutants were tested for their ability to interact with FLAG-HDAC3 and to rescue its activity in a cotransfection assay. Representative experiments are shown in Figure 5A, and the results are summarized in Figure 5B. The ability to physically interact was found to be necessary and sufficient for activating the HDAC3 activity. Mapping of the N-terminal boundary revealed an essential 8 amino acid region (amino acids 420 to 427).

The N terminus of N-CoR also contains two SANT (SWI3/ADA2/NCoR/TFIIIB) domains (residues 440 to 488 and 627 to 674), which are found in components of a variety of chromatin-associated complexes that include the HDAC1-containing NURD complex, the SWI/SNF complex, and the ISWI complex (Humphrey et al., 2001 and references therein). Mapping of the C-terminal

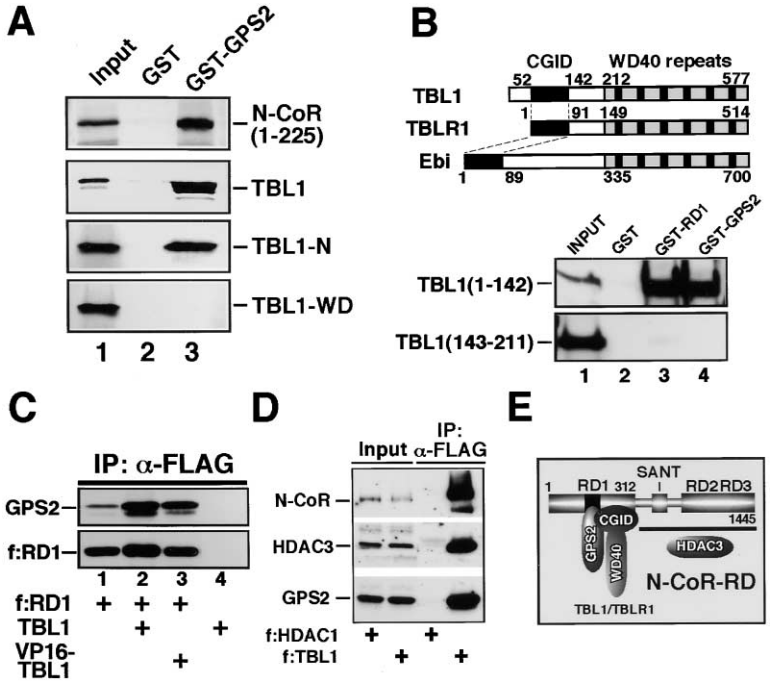

Figure 4. TBL1 Interacts via a Conserved N-Terminal Domain with both GPS2 and RD1 and Enhances GPS2-RD1 Interactions

(A) In vitro interactions of immobilized GPS2 with ^{35}S-labeled (in vitro-translated) N-CoR (1–225), full-length TBL1, TBL1 N terminus (TBL1-N), and TBL1 C terminus with WD40 repeats (TBL1-WD). TBL1-N contains residues 1 to 211 and TBL1-WD contains residues 212 to 577.

(B) A TBL1 N-terminal domain mediates interactions with both N-CoR and GPS2. (Top) Schematic representation of WD40-repeat-containing proteins TBL1, TBLR1, and Ebi. The black box (CGID domain, defined in the text) indicates a highly conserved region among three proteins. (Bottom) In vitro binding assays with TBL1(1–142) and TBL1(143–211) derivatives analyzed as in (A).

(C) TBL1 augments GPS2-RD1 interactions in vivo. Western blot analysis of anti-FLAG immunoprecipitates from 293T cells transfected with the expression vectors indicated at the bottom. FLAG-RD1 was probed with an anti-FLAG antibody.

(D) Ectopic FLAG-TBL1 strongly associates with endogenous GPS2, N-CoR, and HDAC3 in vivo. Western blot analysis of anti-FLAG immunoprecipitates from 293T cells transfected with FLAG-HDAC1 or FLAG-TBL1 vectors. Input lanes show 2% of total.

(E) Schematic representation of the N-CoR repression domain (RD) showing an RD1-GPS2-CGID heterotrimeric structure. A region between amino acids 313 and 1445 is proposed to mediate the N-CoR-HDAC3 interaction. RDs represent three N-CoR repression domains. SANT represents two SANT domains of N-CoR. The black box in RD1 represents the conserved coiled-coil region proposed to mediate the GPS2 interaction.

boundary revealed that while the second SANT domain (SANT2) is dispensable, the first SANT domain (SANT1) is essential for HDAC3 interaction. The minimal deacetylase-interacting domain (DID) thus includes two required regions with 69 amino acids (Figures 5A and 5B). An alignment of N-CoR, SMRT, and the *Drosophila* corepressor SMRTER indicates that both regions are highly conserved, while the middle sequence displays less similarity (Figure 5C). It has been proposed that SANT domain proteins play an important role in the assembly of the HDAC1-containing complexes (Humphrey et al., 2001). Our results suggest that, while required, a SANT domain fold per se is not sufficient for HDAC3 interaction. It is likely that while SANT domains might recognize a general feature among various proteins, such as HDAC1 and HDAC3, high-affinity interactions are conferred by specific flanking sequences.

Thus far, we have determined distinct and highly conserved structural elements that mediate specific, direct, and cooperative interactions. Accordingly, while conserved regions of RD1 and TBL1 direct the formation of a heterotrimeric structure with GPS2, the DID domain of N-CoR interacts with and activates HDAC3 (Figure 5D). Thus, specific interactions are integrated cooperatively and/or allosterically to achieve a highly stable and enzymatically active deacetylase complex.

Promoter-Bound GPS2 Is Capable of Silencing Transcription through the Function of an Associated N-CoR Complex

Earlier studies indicated that GPS2 could function as a cofactor for certain DNA binding transcription factors (see Introduction). Given our determination that GPS2 is a stable, integral subunit of the N-CoR/HDAC3 deacetylase complex, as well as the known "corepressor" function of the N-CoR subunit through interactions with DNA-bound nuclear receptors, we tested whether promoter-bound GPS2 might play a similar corepressor role by directly fusing it to a heterologous Gal4 DNA binding domain. In an in vivo transcription assay with a Gal4 binding site-containing reporter, a Gal4-GPS2 fusion protein showed a potent dose-dependent repression comparable in magnitude to that observed with a Gal4 fusion protein containing the N-CoR repression domain (Figure 6A). These results suggest that GPS2, when brought to a promoter region through interactions with a DNA-bound transcriptional regulatory protein, is capable of mediating transcriptional silencing.

To establish a requirement for N-CoR association for GPS2-mediated transcriptional repression, we mapped the region in GPS2 that mediates the N-CoR interaction. In agreement with earlier results (Figure 3) suggesting that an N-terminal coiled-coil region of GPS2 forms a potential binding surface for N-CoR, coimmunoprecipitation assays showed that full-length GPS2 and GPS2 derivatives containing this coiled-coil region (mutants A and B; Figure 6B, top) but not those without this region (mutants C and D), stably associate with N-CoR in vivo (Figure 6B, bottom). Consistent with a requirement for the N-CoR complex in GPS2-mediated transcriptional repression, further analyses of these GPS2 derivatives showed that their ability to physically interact with N-CoR strictly correlates with their ability to repress transcription (Figure 6C). Surprisingly, Gal4 fusions containing the C-terminal GPS2 fragments (mutants C and D) that failed either to interact with N-CoR or to repress tran-

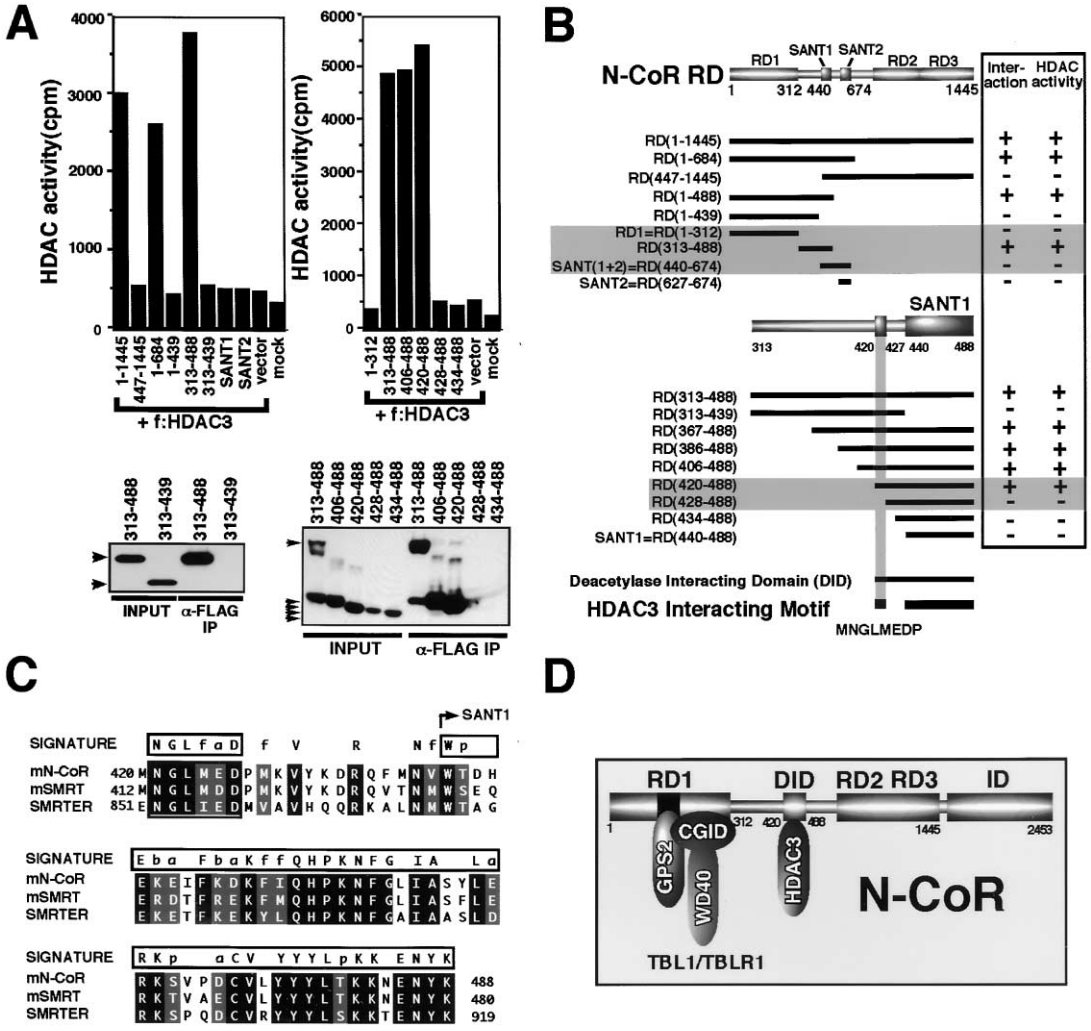

Figure 5. An Extended SANT Domain Interacts with and Activates the Latent Activity of HDAC3

(A) Histone deacetylase (HDAC) assays (top) and Western blot analyses with an anti-Gal4-DBD antibody (bottom) of anti-FLAG immunoprecipitates from 293T cells cotransfected with vectors expressing FLAG-HDAC3 and the indicated N-CoR derivatives fused to Gal4-DBD.

(B) Schematic representation of the N-CoR repression domain and truncated derivatives and a summary of the coimmunoprecipitation results from the experiments in (A) and others not shown. SANT1 and SANT2 represent the two SANT domains of N-CoR.

(C) Alignment of DID domains from N-CoR, SMRT, and SMRTER showing highly conserved regions required for HDAC3 interaction. F, hydrophobic; b, basic; a, acidic; p, polar residues.

(D) Schematic representation of the N-CoR corepressor showing a newly defined DID domain (see text) that contains SANT1 and its N-terminal flanking sequence and mediates N-CoR-HDAC3 interaction. ID represents the nuclear receptor interaction domains.

scription exhibited modest transactivation activities in that they enhanced the reporter activity relative to that observed with Gal4-DBD alone (shown as reduced fold repression in Figure 6C). Interestingly, a sequence alignment reveals a characteristic of the RNA Pol II CTD domain in the GPS2 C terminus (data not shown and Breiding et al., 1997). We speculate that the transactivation property associated with the GPS2 C terminus may be related to this feature or, alternatively, may result from recruitment of a cellular factor such as the previously reported p300 (Peng et al., 2000).

The observation of cooperative interactions between GPS2, TBL1, and N-CoR-RD1 in stabilizing the corresponding heterotrimeric complex (Figures 3 and 4) suggests a role for GPS2 in facilitating the assembly of the complete GPS2-TBL1-N-CoR-HDAC3 complex and, hence, N-CoR-mediated function. In support of this idea, ectopic expression of GPS2(1–155) resulted in a significant increase of N-CoR-mediated repression function (Figure 6D), similar in magnitude to what was observed with ectopic HDAC3 or TBL1 expression (data not shown).

The GPS2 Subunit Links the N-CoR-HDAC3 Corepressor Complex to Intracellular JNK Signaling Pathways

Given the identification of GPS2 as an integral subunit of the N-CoR-HDAC3 complex, as well as indications that N-CoR interactions facilitate GPS2-mediated transcriptional repression (Figure 6), we hypothesized that the observed inhibition of JNK1 activity by ectopically

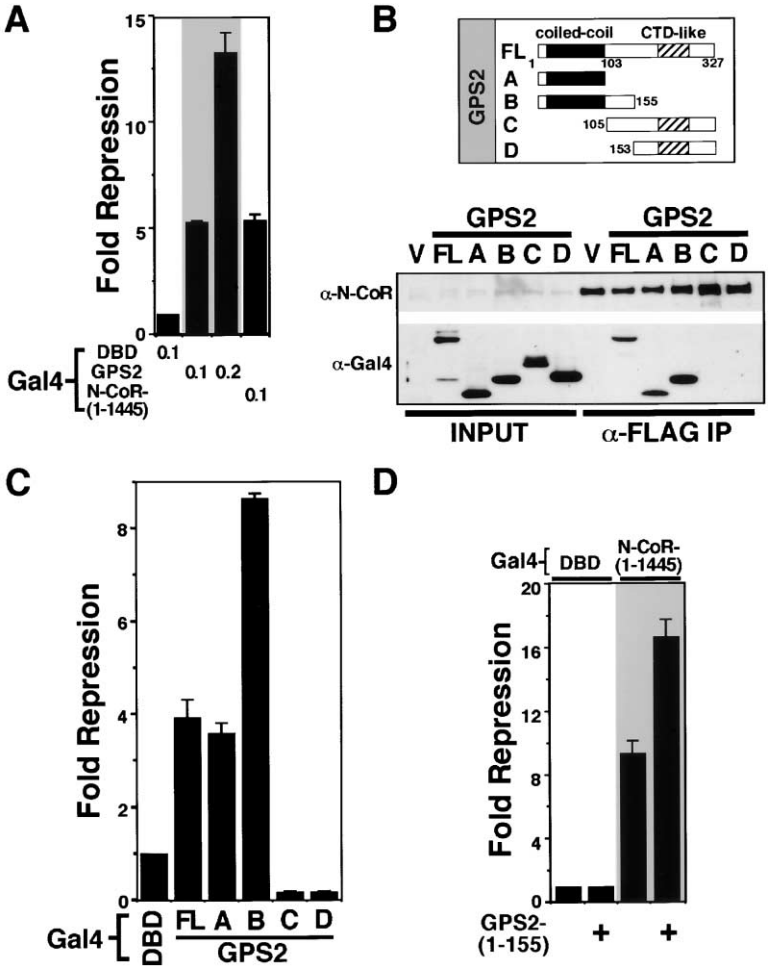

Figure 6. Promoter-Bound GPS2 Is Capable of Silencing Transcription through the Function of an Associated N-CoR Complex

(A) Gal4-GPS2 is a potent transcriptional repressor. 293T cells grown in 12-well plates were transfected with vectors expressing Gal4 fusion proteins with amounts (μg) indicated at the bottom and a Gal4-UAS luciferase reporter (0.1 μg).

(B) (Top) Schematic representation of full-length (FL) GPS2 and its derivatives (A–D). Shown also are an N-terminal coiled-coil region and a C-terminal CTD-like region. (Bottom) GPS2 associates with N-CoR via its N-terminal coiled-coil region. Anti-FLAG immunoprecipitates obtained from 293T cells cotransfected with FLAG-N-CoR and indicated Gal4-DBD fusions or Gal4-DBD alone (V) were subjected to Western blot analysis with anti-N-CoR and anti-Gal4 antibodies.

(C) N-CoR interaction is indispensable for GPS2-medaited repression. Cells were transfected with expression vectors (0.05 μg) for the various Gal4 fusions.

(D) Ectopic expression of a GPS2 subdomain GPS2(1–155) that stably associates with N-CoR significantly potentiates N-CoR-mediated transcriptional repression. Cells were transfected with Gal4-DBD and Gal4-N-CoR(1–1445) expression vectors (0.05 μg) and a GPS2(1–155) expression vector (0.5 μg) as indicated.

expressed GPS2 (see Introduction) may be mediated through an associated N-CoR-dependent corepressor function. An initial analysis showed that transiently expressed GPS2, like endogenous GPS2 (Figure 2), specifically associates with an active endogenous N-CoR-TBL1-HDAC3 deacetylase complex (Figure 7A) in vivo and colocalizes with HDAC3 in the nucleus (confocal immunofluorescence analysis, data not shown). Consistent with previous observations in HeLa and NIH3T3 cells (Jin et al., 1997; Spain et al., 1996), ectopic GPS2 was shown to inhibit JNK1 activity in 293T cells treated with TNFα (Figure 7B, lane 1 versus 2).

To test our hypothesis, cells were cotransfected with GPS2 and N-CoR derivatives that would be expected to block interactions of GPS2 with endogenous N-CoR and, hence, to behave as dominant negatives. Remarkably, overexpression of an N-CoR derivative, N-CoR(1–225), that contains the GPS2-interacting domain within RD1 (Figure 3C) resulted not only in a complete relief of GPS2-mediated inhibition of JNK1 activity but also in a significant enhancement of JNK1 activity (Figure 7B, lanes 1–3 and lanes 4–8). N-CoR(1–312), which contains the entire RD1 domain (Figure 3C), showed a similar dominant-negative effect (Figure 7B, lane 6 versus 5). In contrast, overexpression of N-CoR(1–160), which does not interact with GPS2 (Figure 3C), had no effect on GPS2-mediated inhibition of JNK1 activity (Figure 7B, lane 7 versus 8 and lane 4 versus 5). In a further analysis, ectopic N-CoR(1–684), which lacks the RD2 and RD3 domains (Figure 5B), also behaved as a dominant negative and relieved the GPS2-mediated inhibition of JNK1 activity (Figure 7B, lane 9 versus 7). In contrast and remarkably, ectopic N-CoR(1–1445), which contains the entire repression domain (Figures 5B and 5D) defined by Gal4 fusion protein repression assays, failed to show any dominant-negative activity and even slightly potentiated the GPS2-mediated inhibition of JNK1 activity (Figure 7B, lane 10 versus 7). These results indicate that the entire repression domain (RD1 through RD3) of N-CoR is essential, and perhaps nearly sufficient, for its repression function through GPS2. Finally, ectopic full-length N-CoR strongly potentiated GPS2-mediated JNK1 inhibition (lane 11 versus 7), thus providing direct support for the conclusion that transcriptional repression mediated by a GPS2-N-CoR corepressor complex underlies GPS2-mediated inhibition of JNK1 activation, and also suggesting contributions to repression by regions of N-CoR C-terminal to RD3.

Having established an essential role of N-CoR in GPS2-mediated JNK1 inhibition, we next used the HDAC-specific inhibitor TSA to test an HDAC involvement in GPS2-meditated JNK1 inhibition. Since TSA

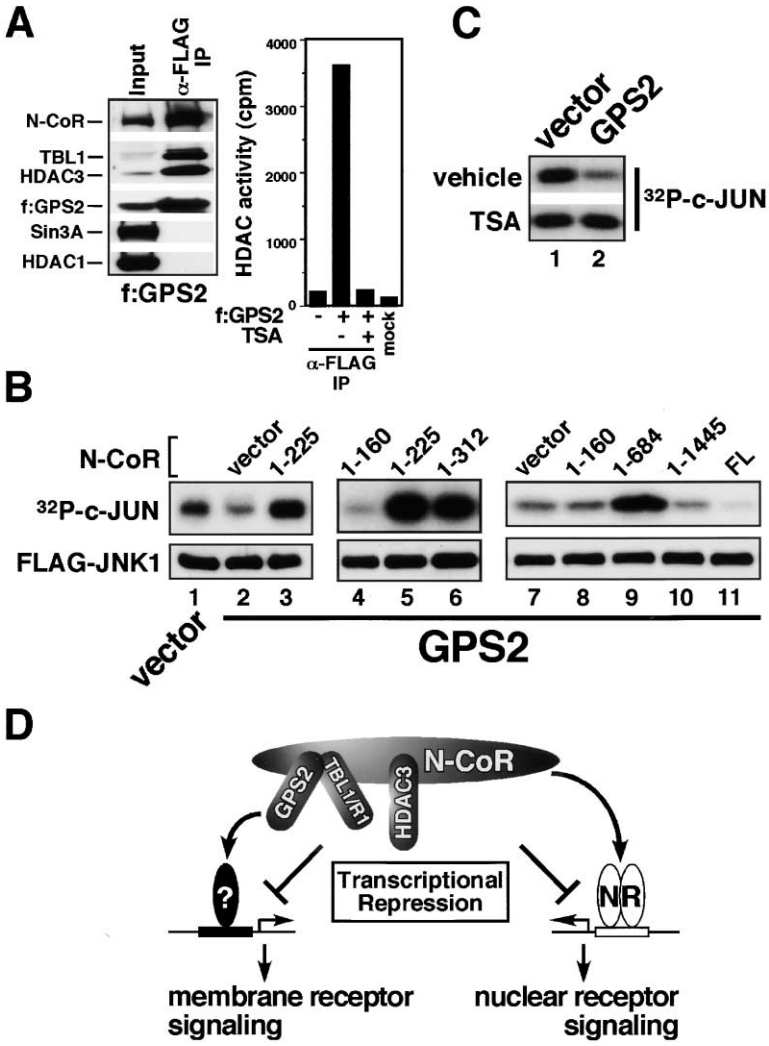

Figure 7. The GPS2 Subunit Links the N-CoR-HDAC3 Corepressor Complex to Intracellular JNK Signaling Pathways

(A) Ectopic GPS2 specifically associates with a functional endogenous N-CoR-TBL1-HDAC3 complex in vivo on the basis of coimmunoprecipitation (left) and HDAC (right) assays with 293T cells transfected with a FLAG-GPS2 expression vector.

(B) N-CoR is essential for GPS2-mediated inhibition of JNK1 activity. 293T cells cotransfected with FLAG-JNK1 (0.15 μg) and expression vectors for GPS2 (0.45 μg) and N-CoR derivatives (0.9 μg) or equivalent empty vectors were subjected to an anti-FLAG immunocomplex kinase assay (upper panel). N-CoR derivatives were expressed as Gal4-DBD(1–147) fusions which showed similar expression levels, except for N-CoR(1–160), whose expression was relatively higher (data not shown).

(C) TSA abrogates JNK1 inhibition by GPS2 in HeLa cells. TSA (250 nM) was added to cells 16 hr posttransfection. Amounts of transfected DNAs were 0.25 μg for FLAG-JNK1 and 0.75 μg for either GPS2 or empty vector.

(D) A model indicating that multiple cellular pathways are linked to the GPS2-TBL1/TBLR1-N-CoR-HDAC3 deacetylase complex through interactions of corresponding factors with either GPS2 or N-CoR. While the N-CoR C terminus mediates a ligand-independent nuclear hormone receptor recognition and signaling, GPS2 interactions with (as yet unidentified) DNA binding transcriptional factor(s) facilitate recruitment of the GPS2-TBL1/TBLR1-N-CoR-HDAC3 complex to target promoter(s) to repress transcription of gene(s) encoding factor(s) important for JNK signaling, and thus result in an inhibition of membrane receptor-mediated JNK signaling pathway (see Discussion).

treatment significantly affects the growth properties of highly proliferative 293T cells (J.Z. and R.D.R., unpublished data), we used HeLa cells whose growth is minimally affected by TSA treatment. GPS2 was found to inhibit JNK activity only in the absence and not in the presence of TSA (Figure 7C). FLAG-JNK1 proteins were recovered in equal amounts from control and GPS2-transfected cells (data not shown). In agreement with the observation of a stable association of GPS2 with HDAC3 and with a potent HDAC activity (Figure 7A), these results thus provide indirect evidence for an important role of HDAC3 in GPS2-mediated JNK inhibition.

Discussion

This study reports the isolation of a deacetylase complex containing apparently stoichiometric amounts of HDAC3, N-CoR, TBL1/TBLR1, and a newly described component, GPS2, that links this complex to membrane receptor-mediated signal transduction pathways. The existence of this complex, and especially the presence of GPS2, has been confirmed by coimmunoprecipitation both of endogenous proteins and of transfected components, by affinity purification of the complex via distinct subunits and by the stability of the complex through distinct chromatographic steps and under highly stringent buffer conditions. In addition, we have elucidated a network of protein interactions, mediated by phylogenetically conserved motifs, in which GPS2, TBL1, N-CoR, and HDAC3 cooperate to form a stable and enzymatically active histone deacetylase complex. Despite the structural similarity between HDAC1 and HDAC3, both class I HDACs, we failed to detect any common subunits in corresponding HDAC complexes (data not shown). This suggests distinct functions for the GPS2-TBL1-N-CoR-HDAC3 complex, including those involved in intracellular signaling (via GPS2) and nuclear receptor function (via a specific N-CoR domain).

Role of GPS2 and TBL1 in Assembly and Function of an N-CoR-HDAC3 Complex

Several observations suggest that GPS2 is an integral component of an active HDAC3-containing corepressor complex: the copurification of GPS2 with N-CoR, TBL1, and HDAC3; the high HDAC activity associated with endogenous GPS2; and the ability of a promoter-bound

Gal4-GPS2 fusion protein to repress transcription. Besides this, our studies have shown direct interactions of GPS2 and TBL1 with each other and with the RD1 domain of N-CoR. Additional cooperative interactions that are shown to stabilize this heterotrimeric subcomplex point to structural roles for GPS2, TBL1, and the N-CoR RD1 domain in the assembly and stabilization of the complete GPS2-TBL1-N-CoR-HDAC3 complex. In support of this conclusion, a GPS2 N-terminal region that stably associates with N-CoR in vivo significantly enhanced N-CoR-mediated repression (Figure 6D). Interestingly, overexpressed full-length GPS2 was found to weakly inhibit N-CoR-mediated repression (data not shown), possibly due to an activation function associated with the GPS2 C terminus (Figure 6C) that could be related to a reported "coactivator" activity of N-CoR on certain response elements (Jepsen et al., 2000; Tagami et al., 1997). Further, our results indicate that the heterotrimeric subcomplex is indirectly linked to HDAC3 through a newly defined N-CoR domain (DID domain, see below) adjacent to the RD1 domain. In support of this model, HDAC3 fails to associate with an RD1-GPS2-TBL1 subcomplex in vivo (Figure 3).

In addition to GPS2, we have identified another integral component, TBLR1, of the N-CoR-HDAC3 complex. Our results indicate that TBLR1 and the previously described TBL1 represent alternative subunits of the N-CoR-HDAC3 complex (data not shown). Interestingly, *TBLR1* was independently isolated as a differentially expressed gene during hematopoietic differentiation (Zhang et al., 2000), suggesting a role of TBLR1, presumably through the N-CoR-, GPS2-, and HDAC3-containing corepressor complex, in controlling cellular differentiation. Further, the high degree of similarity between TBLR1 and TBL1 suggests that TBLR1 could complement normal functions of TBL1 in patients with deletion of the *TBL1* gene (Bassi et al., 1999). Thus, the restricted TBL1 null phenotype (deafness) may fail to reflect a broader array of TBL1/TBLR1 biological functions.

A SANT-HDAC Functional Unit

We show that the HDAC3 activity is markedly stimulated in vivo via interactions with an extended N-CoR SANT domain (DID domain). Interestingly, MTA2, another SANT domain protein, was previously shown to be indispensable for the formation of an enzymatically active HDAC1 complex (Zhang et al., 1999). Thus, the functional dependence of HDACs on a SANT domain-containing subunit is likely to be of general significance. While this paper was under review, similar results were reported independently by another group (Guenther et al., 2001). However, our results also indicate that the SANT domain interaction with HDAC3 is highly specific and critically dependent upon N-terminal flanking sequences that could provide functional specificity for distinct HDAC-containing complexes via specific SANT-HDAC recognition.

GPS2 May Link Viral Regulatory Proteins to a Histone Deacetylase Complex

GPS2 has been shown to interact directly with several viral transcriptional factors (Breiding et al., 1997; Jin et al., 1997), and our preliminary results indicate that it can mediate HTLV-I Tax association with the N-CoR-HDAC3 complex. The Tax oncoprotein is crucial for viral transformation activity through promoter interactions that either activate or repress various promoters (Yoshida, 2001). In addition, Tax was shown to repress nuclear receptor signaling (Doucas and Evans, 1999). Thus, an association of Tax with the GPS2-TBL1-N-CoR-HDAC3 complex could provide additional mechanisms for the pleiotropic effects of Tax in transcription. GPS2 can also be inactivated or sequestered by viral proteins (Degenhardt and Silverstein, 2001), as a potential means to promote viral transformation by affecting both GPS2- and N-CoR-mediated pathways.

GPS2-TBL1-N-CoR-HDAC3 Corepressor Complex Serves as a Platform for a Crosstalk between Membrane Receptor and Nuclear Receptor Signaling Pathways

The identity of GPS2 as a component both of a signal transduction pathway and of an N-CoR-TBL1-HDAC3 complex thus reveals a new and direct link between them. Consistent with this, *Drosophila* TBL1/Ebi was shown both to regulate epidermal growth factor receptor-mediated Ras-MAPK signaling pathways and to be necessary for R7 eye development (Dong et al., 1999). Further supporting a link between signaling pathways and corepressor complexes is the observation that N-CoR-deficient mouse embryos display a specific block in TCR-mediated signaling (Jepsen et al., 2000).

Our studies indicate that promoter-bound GPS2 is capable of repressing transcription through the function of an associated N-CoR complex and, further, that a GPS2-containing N-CoR-HDAC3 complex can actively suppress intracellular JNK activation. In view of other indications that GPS2 can interact with DNA-bound viral regulatory proteins (above), we thus propose that GPS2, like the C terminus of N-CoR, may interact directly with and serve as a corepressor(s) for a DNA binding cellular transcriptional factor(s) whose target gene(s) is directly or indirectly involved in intracellular JNK signaling. Our results suggest that GPS2-N-CoR-mediated JNK inhibition is independent of JNK expression and may occur through modulation of JNK phosphorylation by affecting intracellular levels/activities of JNK-specific MAPK kinases and/or phosphatases (MKPs) (Lee et al., 1999).

Certain hormones, including retinoic acid (RA) and thyroid hormone (T3), not only directly activate their target genes through cognate nuclear receptors but also potently repress an AP-1 (oncoproteins Jun/Fos)-mediated transactivation (Desbois et al., 1991). Among many other proposed models (Karin and Chang, 2001), data described here could potentially lead to an alternative mechanism for some forms of this phenomenon. Thus, in the absence of hormone, limiting N-CoR/SMRT corepressor complexes may associate with (and be sequestered by) unliganded nuclear receptors. Hormone binding would result in release of N-CoR/SMRT corepressor complexes that in turn could act through the newly described GPS2 subunit to inhibit JNK activation and suppress AP-1 transactivation. Earlier observations appear to provide some support for this view. Of note, v-erbA, a viral oncoprotein that constitutively associates with N-CoR/SMRT corepressors, fails to repress the AP-1

activity and, further, was found to abrogate the inactivation of AP-1 by RA or T3 (Desbois et al., 1991). In addition, RA treatment of cells expressing functional retinoid receptors causes a sustained inhibition of JNK activity that requires nascent protein synthesis (Lee et al., 1999). Independent studies show similar hormone- and nuclear receptor-mediated JNK inhibition (Caelles et al., 1997). Furthermore, mutational analyses suggest that RA- or T3-mediated AP-1 antagonism appears to correlate with a hormone-triggered corepressor release from apo-receptors (Desbois et al., 1991; Saatcioglu et al., 1997; Zhang et al., 1997; DiSepio et al., 1999 and references therein). Nonetheless, it should be noted that the hormone-AP-1 antagonism may involve multiple mechanisms to account for complex cell-, receptor- and promoter-specific patterns.

Taken together, our results support the idea of multiple functions of the GPS2-TBL1-N-CoR-HDAC3 complex in distinct cellular pathways. Accordingly, while the C terminus of N-CoR is involved in nuclear receptor recognition and signaling, GPS2 interactions with the N terminus of N-CoR (and the associated TBL1) link complex function, including possible recruitment to target promoters, to membrane receptor signaling (Figure 7D). The N-CoR corepressor complex may thus serve as a platform for a cross-talk between membrane receptor- and nuclear receptor-mediated signaling pathways. The copurification of SMRT with GPS2, TBL1, and HDAC3 (Figures 1 and 2, MS/MS and immunoblot data not shown) and the high sequence similarity between N-CoR and SMRT strongly suggest a similar role for the GPS2-SMRT corepressor complex.

Functional dysregulation of N-CoR/SMRT corepressor complexes, which here are shown to contain GPS2, is involved in pathogenesis and may represent a general mechanism of leukemogenesis. Since AP-1 activity is associated with cell growth and proliferation, whose dysregulation is closely linked to oncogenesis, our model thus suggests that GPS2-N-CoR/SMRT corepressor complexes may function in leukemogenesis, at least in part, through interference with the JNK pathway and AP-1 function. Consistent with this notion, v-ErbA transformation results in a fully active AP-1 complex and abrogates the growth-inhibitory response to retinoic acid treatment (Desbois et al., 1991). A further understanding of the mechanisms by which GPS2-TBL1-N-CoR/SMRT-HDAC3 complexes, as well as hormones, function in intracellular (JNK) signaling pathways could lead to the identification of therapeutic targets for these diseases.

Experimental Procedures

Plasmids and Antibodies

cDNAs for HDAC3 and TBL1 were PCR amplified from corresponding EST clones. cDNAs for N-CoR, HDAC1, and HDAC5 have been previously described (Hassig et al., 1998; Horlein et al., 1995; Huang et al., 2000). Mammalian and bacterial GPS2 expression vectors and a FLAG-JNK1 expression vector were kindly provided by John Colicelli, Dong-Yan Jin, and Roger Davis, respectively. For stable transfection, full-length cDNAs (except for HDAC5, which encoded only the catalytic domain) with FLAG-epitope sequences were subcloned into pCIN4 vector (same as pIRESneo from CLONTECH). All PCR products and subcloning products were verified by DNA sequencing. Rabbit anti-TBL1 antiserum was raised against a GST-TBL1(1–211) fusion protein. Rabbit anti-GPS2 antiserum (Ab2) was raised against a C-terminal peptide (residues 307–327) conjugated to KLH. Rabbit anti-GPS2/AMF1 antiserum (Ab1) was kindly provided by E.J. Androphy. Affinity-purified rabbit anti-HDAC1, anti-HDAC3, anti-N-CoR, and anti-SMRT antibodies were from Affinity BioReagents.

cDNA Cloning

An EST clone containing a partial coding region and 3′ untranslated region of human *TBLR1* was identified based on MS/MS-generated peptide sequences. A full-length human TBLR1 cDNA was isolated through 5′ RACE from a Marathon-Ready HeLa cDNA library (CLONTECH). The sequence was confirmed from multiple independent clones and has been deposited in GenBank (AF314544).

Cell Line Establishment and Affinity Purification of Protein Complexes

HeLa S cells grown in DMEM with 10% FBS were transfected with pCIN4 constructs encoding FLAG-tagged proteins. Selection medium contained 500 μg/ml of G418. Positive clones were confirmed by Western blot analysis. For affinity purification, nuclear extracts prepared from tagged HeLa cells were adjusted to 0.2% NP-40 and incubated with M2-agarose beads (Sigma) at 4°C for 6 hr. After extensive washing with BC300/0.2% NP-40 (20 mM HEPES [pH 7.9], 300 mM KCl, 0.2 mM EDTA, 0.5 mM DTT, 20% glycerol, and 0.2% NP-40), associated complexes were eluted from beads by incubating at 4°C for 60 min with BC100/0.2% NP-40 containing 0.5 mg/ml FLAG peptide. For isolation of endogenous N-CoR- and GPS2-associated complexes with specific antibodies, protein G-agarose beads were used.

Mass Spectrometric Protein Identification

Mass spectrometric measurements were performed on in-house modified MALDI-QqTOF and MALDI-ion trap instruments (Krutchinsky et al., 2001 and references therein) using ZipTip purification of gel-eluted tryptic peptides and 2,5-dihydroxy benzoic acid as matrix. For protein identification, two strategies were employed: (1) High accuracy MS spectra (<15 ppm) were used for peptide mass fingerprint matching with PROWL/ProFound software (Proteometrics, LLC, New York, NY). Proteins identified by this method were confirmed by MS/MS analysis of selected ion peaks using the MALDI-QqTOF instrument. (2) Alternatively, peptide sequence matching alone was performed with MS/MS data obtained on an in-house constructed MALDI-ion trap mass spectrometer, using the Sonar MS/MS search engine (Proteometrics, LLC, New York, NY).

Coimmunoprecipitation Assay

293T cells were transfected with FuGENE 6 reagent (Boehringer Mannheim) according to manufacturer's instructions. Whole-cell extracts in NEHN buffer (300 mM NaCl, 1 mM EDTA, 20 mM HEPES [pH 7.9], 0.5% NP-40, and 20% glycerol) containing protease inhibitor cocktail (Boehringer Mannheim) were incubated with M2-agarose beads (Sigma) for 6 hr at 4°C. Bound proteins (immunoprecipitates) were eluted with BC100/0.2% NP-40 containing 0.5 mg/ml FLAG peptide and subjected to Western blot analysis. Input lane shows 2% of total.

Cell Culture and Transfection

293T cells were maintained in DMEM medium with 10% fetal bovine serum. The Gal4 UAS × 5-SV40-luciferase reporter contains five copies of the Gal4 17-mer binding site. For reporter assays, equal total amounts of plasmids were transfected for each well by adjusting empty vectors. The luciferase light units were normalized to β-gal activity, which served as an internal control for transfection efficiency. Fold repression values are relative to the Gal4-DBD. Figures show the mean and standard error of duplicate samples in representative experiments.

Immunocomplex Kinase Assay

Immunocomplex kinase assays were performed largely as previously described (Yao et al., 1999) but with some modifications, and details are available upon request. In brief, subconfluent 293T or HeLa cells were transfected with FLAG-JNK1 and other expression

vectors. Cells were treated with TNFα (100 ng/ml) (Sigma) for 10 min 48 hr posttransfection. Anti-FLAG immunoprecipitaed FLAG-JNK1 protein was subjected to a JNK kinase assay, and the kinase activity was revealed by autoradiography. Amounts of immunoprecipitated FLAG-JNK1 protein were revealed by anti-JNK1 Western blot analysis (Santa Cruz).

Histone Deacetylase Assay

HDAC assay was performed with a histone deacetylase assay kit (Upstate) according to manufacturer's instructions. Mock reactions contained only ^{3}H-labeled substrate.

In Vitro Binding Assay

In vitro binding was carried out with in vitro-translated ^{35}S-labeled proteins (TNT kit, Promega) and bacterially expressed GST fusions immobilized on Glutathione-Sepharose beads (Amersham Pharmacia). Following incubation and washing with BC300/0.1% NP-40, bound polypeptides were resolved on SDS-PAGE and visualized by autoradiography. Input lanes show 10% of total.

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Accession Numbers

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