Molecular determinants of nuclear receptor–corepressor interaction

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Retinoic acid and thyroid hormone receptors can act alternatively as ligand-independent repressors or ligand-dependent activators, based on an exchange of N-CoR or SMRT-containing corepressor complexes for coactivator complexes in response to ligands. We provide evidence that the molecular basis of N-CoR recruitment is similar to that of coactivator recruitment, involving cooperative binding of two helical interaction motifs within the N-CoR carboxyl terminus to both subunits of a RAR–RXR heterodimer. The N-CoR and SMRT nuclear receptor interaction motifs exhibit a consensus sequence of LXX I/H I XXX I/L, representing an extended helix compared to the coactivator LXXLL helix, which is able to interact with specific residues in the same receptor pocket required for coactivator binding. We propose a model in which discrimination of the different lengths of the coactivator and corepressor interaction helices by the nuclear receptor AF2 motif provides the molecular basis for the exchange of coactivators for corepressors, with ligand-dependent formation of the charge clamp that stabilizes LXXLL binding sterically inhibiting interaction of the extended corepressor helix.

Nuclear receptors (NRs) are a large class of DNA-bound transcription factors, many of which are regulated by the binding of specific ligands and which control numerous critical biological events in development and homeostasis (for review, see Beato et al. 1995; Maegelsdorf et al. 1995). Over the past few years, it has become clear that the transcriptional functions of unliganded and liganded receptors are regulated by coactivators and corepressors that associate with the carboxy-terminal ligand-binding domain (LBD) (for review, see Horwitz et al. 1996; McKenna et al. 1999; C.K. Glass and M.G. Rosenfeld, in prep.). Ligand-dependent transcriptional activation by NRs has been found to depend on a highly conserved motif in LBD, referred to as AF2 (Danielian et al. 1992; Durand et al. 1994; Barettino et al. 1994; Tone et al. 1994). Crystal structures of the LBDs of multiple NRs have revealed that they are folded into a three-layered, anti-parallel, α-helical sandwich. A central core layer of three helices is packed between two additional layers of helices to create a molecular scaffold that establishes a ligand-binding cavity at the narrower end of the domain.

In the unliganded retinoid X receptor (RXR) structure, the AF2 helix extends away from the LBD (Bourguet et al. 1995), whereas in the agonist-bound retinoic acid receptor γ (RARγ), thyroid hormone receptor α (TRα), estrogen receptor (ER), and peroxisome proliferator-activated receptor γ (PPARγ) LBD structures, the AF2 helix is tightly packed against the body of the LBD domain and makes direct contacts with ligand (Renaud et al. 1995; Wagner et al. 1995; Brzozowski et al. 1997; Darimont et al. 1998; Nolte et al. 1998; Shiau et al. 1998). These studies have suggested that ligand-dependent changes in the conformation of the AF2 helix result in the formation of a surface (or surfaces) that facilitates coactivator interactions.

A surprising number of coactivators associate with NRs in a ligand-dependent manner and may combinatorially and/or sequentially be involved in transcriptional activation (for review, see Freedman 1999; McKenna et al. 1999; C.K. Glass and M.G. Rosenfeld, in prep.). A surprising number of these putative coactivators interact based on the presence of helical motifs containing an LXXLL core consensus (Le Douarin et al. 1996; Heery et al. 1997, Torchia et al. 1997a; Ding et al. 1998). Cocrystal structures of PPARγ with a region of steroid receptor

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coactivator-1 (SRC-1) containing two LXXLL motifs, and of liganded ER and thyroid hormone receptor βform (T$_3$R) with a peptide comprising one LXXLL motif of glucocorticoid receptor interacting protein-1 (GRIP-1) (Darimont et al. 1998; Nolte et al. 1998; Shiau et al. 1998), indicate that a critical conserved glutamic acid residue in receptor AF2 helices and a critical conserved lysine residue in helix 3 of the LBD make hydrogen bonds to the backbone amides and carbonyls of leucine 1, and leucine 5, respectively. These contacts form a "charge clamp" that positions and orients the hydrophobic face of the LXXLL helix, allowing the leucine residues to pack into a hydrophobic pocket formed by the surfaces of receptor helices 3, 4, 5 (PPARγ) or 3, 5, 6 (T$_3$R). A critical determinant of coactivator binding is the length of the LXXLL helix, which fits precisely between the conserved glutamate and lysine residues upon closure of the AF-2 in the presence of ligand. Residues outside the core motif appear to provide receptor and ligand-dependent specificity (Darimont et al. 1998; McInerney et al. 1998; Mak et al. 1999). The structure of the PPARγ–SRC-1 cocrystal indicated that two LXXLL motifs from a single SRC-1 molecule interacted with the AF-2 domains of both subunits of the LBD dimer (Nolte et al. 1998).

Intriguingly, the structures of the ER LBD bound to the antagonist raloxifene or dihydroxytamoxifen (OHT) demonstrate a distortion in the position of the AF2 helix (Brzozowski et al. 1997; Shiau et al. 1998). Because of the presence of an additional side chain in these antagonists, the AF2 helix is unable to pack normally and, instead, is translocated to a position that overlaps with the site of coactivator interaction. This conformation prevents coactivator binding and conversely facilitates corepressor binding (for review, see Wurtz et al. 1996; C.K. Glass and M.G. Rosenfeld, in prep.).

A search for proteins that could function as corepressors of the thyroid hormone receptor TR and RAR led to the molecular cloning of cDNAs encoding nuclear receptor corepressor (N-CoR) (Hörlein et al. 1995; Kurokawa et al. 1995; Zamir et al. 1996), a retinoid X receptor (RXR) interacting protein 13 (RIP 13) (Lee et al. 1995), and the highly related factor SMRT [silencing mediator for retinoic acid and thyroid hormone receptors] (Chen and Evans 1995), or T3-associated factor (TRAC2) (Sande and Privalsky 1996). Both N-CoR and SMRT interact with unliganded RARs and TRs via a bipartite nuclear receptor interaction domain in a manner that is enhanced by antagonists or removal of the AF2 domain. Several lines of evidence indicate that NCoR and SMRT are required for the active repression functions of unliganded retinoic acid and thyroid hormone receptors (Chen and Evans 1995; Hörlein et al. 1995; Seol et al. 1996; Zamir et al. 1996; Li et al. 1997; Wong and Privalsky 1998). N-CoR and SMRT are also effective corepressors of Rev–Erb (Zamir et al. 1996), COUP-TF (Shibata et al. 1997), and DAX1 (Crawford et al. 1998). Although unliganded steroid hormone receptors do not appear to interact effectively with N-CoR or SMRT, clear interactions are observed in the presence of antagonists (Vegeto et al. 1992;

Lanz and Rusconi 1994; Xu et al 1996; Jackson et al. 1997; Smith et al. 1997; Lavinsky et al. 1998; Wagner et al. 1998; Zhang et al. 1998a,b), and these interactions appear to be essential for full antagonist activity (Lavinsky et al. 1998; Norris et al. 1999).

In this paper we investigate the molecular mechanisms that determine interactions of corepressors with unliganded TR and RAR and their dissociation by ligand. Our data suggest that the N-CoR/SMRT corepressors interact with unliganded nuclear receptors in a fashion analogous to that utilized by coactivators with liganded receptors but that amino-terminal extension of conserved N-CoR interaction helices, when compared to the LXXLL consensus for coactivator interaction motifs, constitutes a critical distinction in the alternative ligand-independent binding of corepressors and ligand-dependent recruitment of coactivators to nuclear receptors.

Results

Receptor interaction domains in N-CoR

The domain structure of N-CoR is diagrammed in Figure 1A, illustrating the two carboxy-terminal regions involved in NR interactions. N-CoR has been suggested to bind to unliganded receptors in vivo and to be released on ligand binding. This premise is based on the effects of ligands on interactions with DNA-bound receptors in in vitro (Hörlein et al. 1995), and yeast two-hybrid experiments (Chen and Evans 1995), although ligand-dependent release of NCoR is less evident when evaluated on NRs in solution. We therefore first wished to confirm the ligand-dependent release of N-CoR from RARs bound to the βRAR promoter in cells utilizing the chromatin immunoprecipitation (ChIP) assay (Braunstein et al. 1993; Luo et al. 1998). In this experiment we utilized N-CoR-specific anti-IgG, to immunoprecipitate sheared, chromatinized DNA, prepared from cells cultured in the presence or absence of all-*trans* retinoic acid. As shown in Figure 1B, N-CoR could be cross-linked to βRAR promoter in the absence, but not in the presence, of ligand. This observation provides evidence that on endogenous, regulated chromatinized transcription units, N-CoR is physiologically associated with unliganded, but not liganded, DNA-bound RAR. Therefore, exchange of N-CoR occurs on specific promoters in the intact cell, as exemplified by the regulated βRAR promoter.

The carboxy-terminal NR interaction domain (ID-C) previously has been suggested to be localized to a 60-amino-acid region, located between amino acids 2240–2300 (Chen and Evans 1995; Hörlein et al. 1995), although an amino-terminal region spanning from amino acids 2040–2239 was later identified (Zamir et al. 1997; Cohen et al. 1998). To further explore the molecular basis for association of the corepressor complex with unliganded receptors, and to provide an explanation for ligand-dependent dissociation, we systematically mapped the amino-terminal interaction domain (ID-N) of N-CoR. Binding experiments using a series of 50-amino-
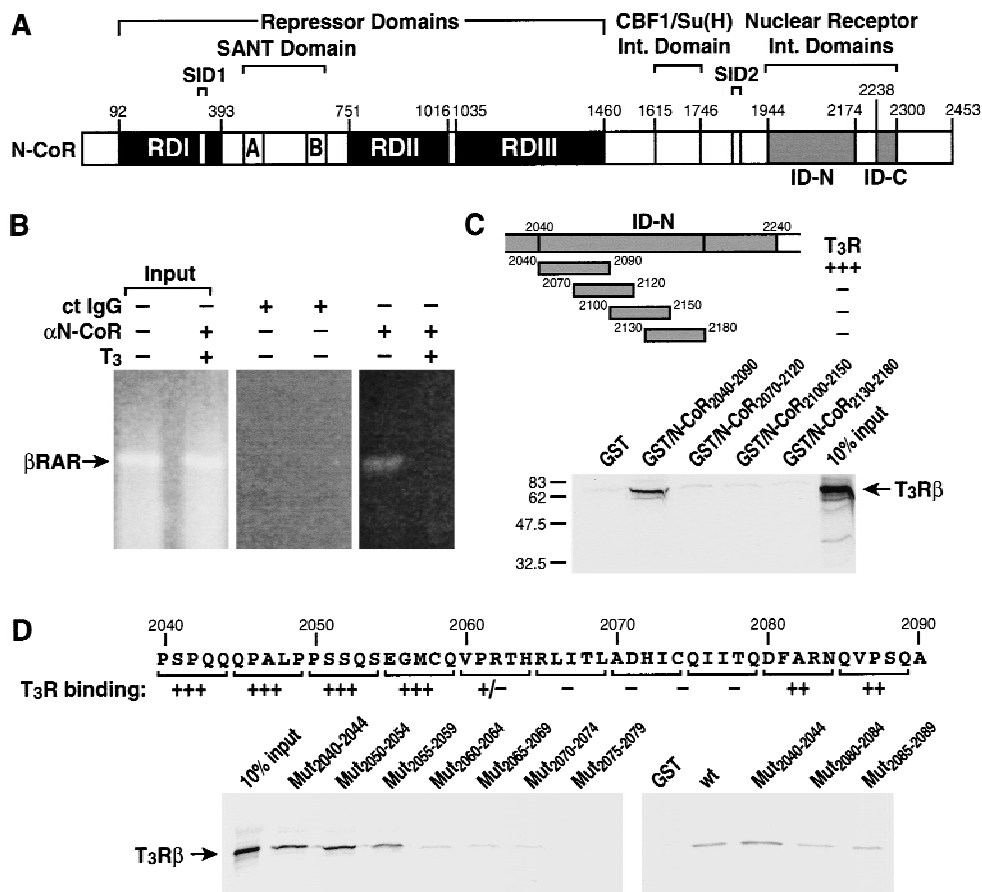
Figure 1. Characterization of the amino-terminal interaction domain of N-CoR. (*A*) Schematic representation of murine N-CoR showing the location of the repressor domains (RDI, RDII, RDIII) and the nuclear receptor interaction domains (ID-N, ID-C). (*B*) ChIP assay of N-CoR binding to the βRAR promoter. The experiments reproducibly revealed the presence of N-CoR on the βRAR promoter in the absence of ligand, but not in the presence of RA; no detectable precipitation of promoter was observed with control preimmune IgG (ct IgG). (*C*) GST pull-down assay testing binding of overlapping fragments of ID-N to T₃Rβ. Sequences spanning amino acids 2040–2090 are required for effective interaction. (*D*) Mapping of the critical residues in the 2040–2090 interaction domain. Cluster mutation of the indicated five adjacent amino acids to alanine residues was performed across the interval. GST pull-down analysis revealed that residues spanning amino acids 2060–2080 were quantitatively the most critical for interaction, with some contribution from amino acids 2080–2090.

acid overlapping fragments from amino acids 2040–2180 of N-CoR, fused to GST, suggested that residues from amino acids 2040–2090 encompassed the most potent interaction region (Fig. 1C). Further mapping revealed that residues 2060–2080 were critical for interaction in vitro (Fig. 1D). Based on previous identification of residues 2268–2298 as the critical region in the ID-C, potential sequence alignments between the two regions were considered. One alignment (Fig. 2A) appeared to be the most likely and was further supported upon consideration of corresponding sequences in both murine and human N-CoR and SMRT. In light of the similarity in this alignment segment with the LXXLL coactivator motif (Heery et al. 1997; Darimont et al. 1998; Ding et al. 1998), structural predictions of these regions were evaluated using several algorithms, including the self-optimized prediction method (Geourjon and Deleage 1994), which predicted an extended helical structure for these putative N-CoR and SMRT recognition domains that is

at least one helical turn longer than the LXXLL helix (Fig. 2A).

Cooperative ID recruitment to DNA-bound receptor heterodimers

Based on this alignment, a series of mutations in ID-C and ID-N were generated to test the potential importance of the predicted leucine and isoleucine residues. Sequences spanning amino acids 2062–2084 or 2268–2289 were each capable of detectable, specific interactions with unliganded TR (data not shown). This was further explored using a mammalian two-hybrid approach involving recruitment of a VP16–RAR fusion protein (Lipkin et al. 1996) to a GAL4/T₃R carboxy-terminal fusion protein. In this assay effective interaction was observed for the ID-C peptide, but the ID-N peptide region could interact only weakly, with either TR or RAR. Therefore,
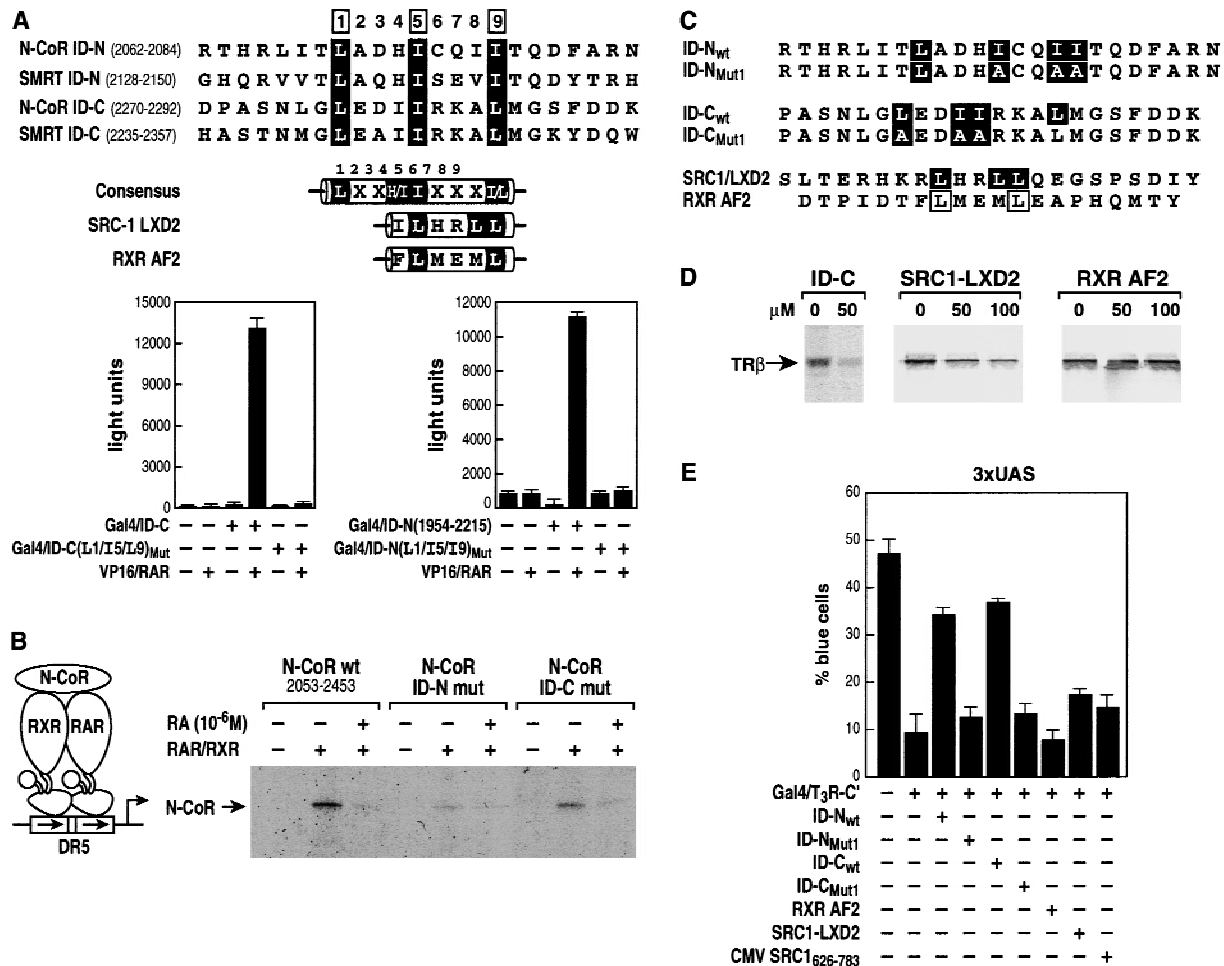
Figure 2. Identification of a putative corepressor extended helix interaction motif. (*A*) Alignment of critical regions of N-CoR and SMRT ID-N and ID-C motifs reveals a conserved LXX I/H IXXX I/L extended helix compared to that of the LXXLL motif of SRC1 or the AF2 domain of RXR. Clustered mutation of these residues in ID-C or in a region (1954–2215) encompassing ID-N resulted in loss of interactions in GST pull-down assays or a mammalian two-hybrid assays, confirming the critical importance of L1, I5, and I9 residues. (*B*) ABCD analysis of N-CoR binding to RXR/RAR heterodimers on a DR+5 element. An N-CoR interaction region spanning amino acids 2053–2453 was bound effectively in the absence, but not in the presence, of RA; mutation of the three conserved L and I residues in either ID-N or in ID-C markedly diminished or abolished N-CoR interaction with the DNA-bound receptor heterodimer. (*C*) Synthetic peptides used for competition studies. (*D*) Peptide competition of NCoR binding by T$_3$R: Addition of ID-C peptide gives clear competition at 50 μM, the RXR AF2 peptide does not compete even at higher concentration; the SRC1–LXD2 peptide gives a detectable slight competition at high concentrations. (*E*) Peptide competition of GAL4/T$_3$R carboxyl terminus fusion protein-dependent inhibition of UAS × 3/*tk*–*lacZ* reporter in single cell nuclear microinjection assays in Rat-1 cells. The nuclear receptor interaction domain (amino acids 570–843 and 626–783) of SRC1 was expressed as a GAL4 fusion protein under control of the CMV promoter.

a region from amino acids 1954-2215 spanning the ID-N domain was utilized in the two-hybrid assay (Fig. 2A). Simultaneous mutation of L1/I5/I9-L9 to alanine residues in either ID-N or ID-C abolished interaction with unliganded NR.

To further examine the possibility of cooperativity between the amino- and carboxy-terminal interaction motifs, we introduced mutations into the L1, I5, and L/I 9 residues of both ID-C and ID-N into an N-CoR carboxy-terminal sequence (amino acids 2053–2453) that encompassed the two interaction domains. Interaction of wild-type and mutant N-CoR with DNA-bound RAR/RXR heterodimers was assessed using the avidin–biotin complex DNA (ABCD) assay (Hörlein et al. 1995; Kurokawa

et al. 1995). Mutations of the L1/I5/L9 residues in the ID-N domain abolished detectable binding, whereas mutations of the comparable residues in the ID-C domain markedly diminished the interaction (Fig. 2B). These data are consistent with a model in which both partners of the DNA-bound heterodimer can bind one of the two N-CoR corepressor interaction motifs, with cooperative recruitment of unliganded receptors (Fig. 2B). Addition of ligand caused the release of N-CoR, consistent with previous ABCD data (Kurokawa et al. 1995; Heinzel et al. 1997; Zamir et al. 1997).

We therefore wished to investigate whether peptides corresponding to the minimal binding regions could inhibit TR binding to N-CoR in vitro or in the context of

the intact cell. We made synthetic peptides of 23 and 22 residues, corresponding to the amino- and carboxy-terminal motifs, respectively. We also evaluated the effects of a 22-amino-acid sequence corresponding to LXD2, the LXXLL domain motif of SRC-1, documented previously to inhibit activation events in the cell (Torchia et al. 1997) as well as a 20-amino-acid peptide corresponding to RXR AF2 motif that was effective for biochemical competition (Westin et al. 1998) on N-CoR interactions with unliganded receptor. As seen in Figure 2D, even the minimal ID-C peptides could inhibit the ability of the N-CoR carboxyl terminus (amino acids 2040–2300) to bind T_3R, whereas the RXR AF2 peptide did not compete. The LXD2, although effective in blocking coactivator function (Torchia et al. 1997) was only minimally effective as a competitor. The ability of a minimal corepressor motif peptide to inhibit N-CoR interaction was studied further using the single cell nuclear microinjection assay, comparing the ability of each peptide to inhibit active repression by a GAL4/T_3R carboxyl terminus fusion protein (Fig. 2E). In this assay a GAL4/T_3R fusion protein inhibited expression of the UAS/tk reporter around fivefold. Both the N-CoR minimal ID-N and ID-C peptides proved capable of effectively inhibiting active repression function, despite the relatively weak interactions of these minimal interaction domains in vitro. In contrast, the RXR AF2 peptide failed to relieve repression function of T_3R-C'carboxy-terminal. Injection of either the SRC-1 LXD2 peptide or overexpression of a transcription unit encoding the SRC-1 nuclear interaction domain (amino acids 626–783) also failed to reverse N-CoR-dependent T_3R repressor function (Fig. 2E).

Residues critical for ID-C and ID-N interactions

Based on these data we further explored the residues that might be required for interactions of either domain with unliganded T_3R by mutation of single or adjacent amino acids in the ID-C (Fig. 3A). Mutations at the extreme amino or carboxyl terminus of the LBD were found to not inhibit binding to the T_3R (Fig. 3B). Clustered mutations of amino acids 2271–2275 or mutation of L1 (amino acid 2277) caused only partial loss of binding; in contrast, mutations of amino acids 2282–2285 or 2286–2290 strongly inhibited binding, as did mutation of L9 (amino acid 2285) (Fig. 3B). The mammalian two-hybrid assay was utilized to confirm that similar requirements for interaction occur in cells: the wild-type, 22-amino-acid ID-C sequence interacted with RAR, but mutation of L1, I5, or L9 abolished this interaction. As in the case of the biochemical assay, point mutation of the extreme amino- or carboxy-terminal residues did not affect interaction (Fig. 3C). However, clustered mutations of the five residues flanking the core motif site at the amino terminus, as well as the carboxyl terminus, abolished interaction, as did mutation of the L1 (amino acid 2285) residue, indicating that for RAR, L1 is a critical residue and that flanking amino acids modulate receptor interactions.
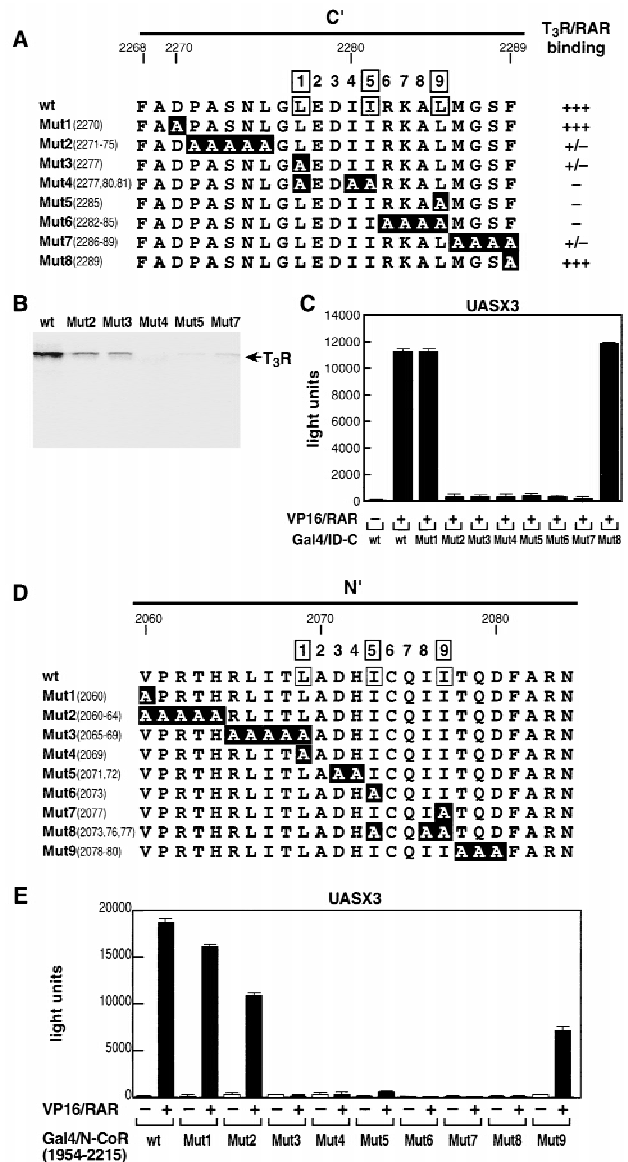

Figure 3. Analysis of ID-C and ID-N amino acid determinants of NR binding to N-CoR. (A) Amino acid mutations introduced in the context of GST or GAL4 fusion proteins with a summary of their effects on receptor interaction. (B) GST pull-down assay of ^{35}S-labeled T_3R by the GST/ID-C(2268–2289) fusion protein. (C) Mammalian two-hybrid assay using GAL4 ID-C fusion proteins in the presence or absence of VP16/RAR with an UAS × 3/p36 luciferase reporter. Results are average of triplicate determinations ±S.E.M., with similar results in three independent assays. (D) Schematic diagram of mutations introduced in the ID-N region in the context of GAL4 N-CoR(1954–2215). (E) Ability of GAL4/NCoR mutants to recruit RAR, thereby activating the UAS × 3/p36 reporter.

In the case of ID-N, the 23-amino-acid (2062–2084) core motif exhibited weak but detectable binding to T_3R, which was enhanced with a 43-amino-acid region (2053–2094) and fully effective interactions were observed with a region extending from amino acid 1954 to 2215. In the context of the 1954–2215 ID-N, L1, I5, and I9 were each

found to be required for NR binding (Fig. 3E). A cluster mutation of the residues 2060–2064 diminished interaction only mildly (Fig. 3E), whereas mutation of residues 2071–2072 (asparitic acid; histidine), intervening in the core motif between the L1 and I5 residues, abolished interactions. In contrast, a mutation of the residues carboxy-terminal to the I9 residue (amino acids 2078–2080), which are not predicted to be an extended helix, caused only a small decrease in the interactions as determined using the mammalian two-hybrid assay. Together, these data suggest that residues within the LXXXIXXXI/L core are critical for corepressor NR interaction, although interactions are clearly strengthened in the context of additional amino- or carboxy-terminal sequences.

Determinants on the T_3R for N-CoR interaction

The similarity of the two N-CoR core nuclear receptor interaction domains with coactivator interaction motifs and the ability of the AF2 helix to inhibit corepressor interaction strongly suggested that coactivators and corepressors utilize an overlapping interaction surface. Inspection of the T_3R crystal structure (Wagner et al. 1995; Feng et al. 1998) indicated that the corepressor interaction surface might involve interactions with either helices 1, 3, 5, 6, or 11. We therefore evaluated the effects of arginine substitutions of specific residues in these helices, based on amino acid positions most likely to represent sites available for interaction. Several mutations in the amino terminus of helix 1 did not significantly affect binding to N-CoR, whereas the previously investigated mutations of conserved residues at the carboxyl terminus (A223, H224, T227 mutated to glycine) fully abolished N-CoR binding (Hörlein et al. 1995; Kurokawa et al. 1998). Here, we show the same effect mutating A223, H224 to glycine or to arginine, and even the single mutation of H224 to alanine diminishes binding (Fig. 4A,B). Mutations of residues in helixes 5 and 6 that are critically involved in coactivator binding (Feng et al. 1998; Nolte et al. 1998) were found to markedly disrupt binding of N-CoR (Fig. 4B). Thus, mutation of V279 and K283, required to position the coactivator helix, impaired or abolished interactions of N-CoR with the T_3R (Fig. 4A,B). Additional residues that disrupt coactivator binding (C293/I297, C304/I307), also disrupted or impaired binding of the corepressor interaction domains. These data were confirmed in the intact cell using a two-hybrid interaction assay with VP16 N-CoR(2053–2453) to detect protein–protein interactions with the T_3R carboxyl terminus (data not shown). Finally, a detailed analysis of helix 11 was performed, introducing mutations throughout the length of the entire helix to test the possibility that the extended corepressor activation helix contacted specific residues in helix 11. This mutational analysis failed to detect any residues that appeared to specifically affect corepressor binding (Fig. 4A,B). As a control, the ability to bind the nuclear receptor interaction domain of SRC-1 was evaluated, finding, as predicted, that mutations of helix 5/6 disrupted coactivator binding (Fig. 4C), and blocked li-

gand-dependent activation in cotransfection assays (data not shown), consistent with previous reports (Feng et al. 1998).

In parallel, each mutant form of the T_3R was evaluated for its ability to function as an N-CoRdependent repressor on a UAS × 3/tk–$lacZ$ reporter. Mutations in the coactivator binding pocket that disrupted corepressor binding in vitro also abolished active repression, whereas mutations in helix 11 that did not affect binding also did not disrupt active repression function (Fig. 4D and data not shown). Together, these data suggest that a series of critical residues in the coactivator binding pocket are essential also for binding and function of corepressor (N-CoR).

These observations raise the intriguing question of how the corepressor recognition helix interacts with the coactivator-binding site without the requirement for the AF2 charge clamp that stabilizes coactivator interactions. The structural prediction of an amino-terminally extended helix in the corepressor interaction motif (Fig. 5A) suggested an essential role of these residues. This possibility was tested introducing single or double amino acid substitutions into the ID-N and converting each residue to glycine to break the putative helical extension (Fig. 5B). Conversion of either or both amino acids to glycine abolished interaction with T_3R by binding assay (data not shown), or in the mammalian two-hybrid assay (Fig. 5C). Therefore, our data are compatible with the suggestion that the corepressor binding motif represents a three amino acid amino-terminal helical extension (LXX) (residues 1–3) beyond a core I/H IXXX I/L (residues 4–9) that permits binding to the same hydrophobic pocket of the receptor occupied by coactivators. To determine whether this extension was sufficient for discrimination of corepressor and coactivator interaction motifs, we tested whether converting the carboxy-terminal protein of the helix IXXXL (residue 5–9) to a consensus LXXLL "coactivator consensus" motif, could also mediate binding. Interestingly, with this modification, no binding was observed (Fig. 5C), consistent with a model in which an extended helix is prevented from binding to the coactivator pocket in the presence of ligand because it is too long to be accommodated by the charge clamp.

Discussion

The ligand-dependent exchange of corepressor for coactivator complexes appears central to regulation of gene expression by NRs. Many of the numerous proposed coactivators of nuclear receptors have proven to share a core recognition domain consisting of a short α helix of consensus sequence LXXLL (Ding et al. 1998; Heery et al. 1997; Torchia et al. 1997; Voegel et al. 1998). This helix appears to be oriented and positioned by a conserved glutamic acid residue in the AF2 helix and a conserved lysine at the end of helix 3. Upon ligand binding, these residues form a charge clamp that makes contacts with the polypeptide backbone at the ends of the LXXLL helix and allows packing of leucine residues into the
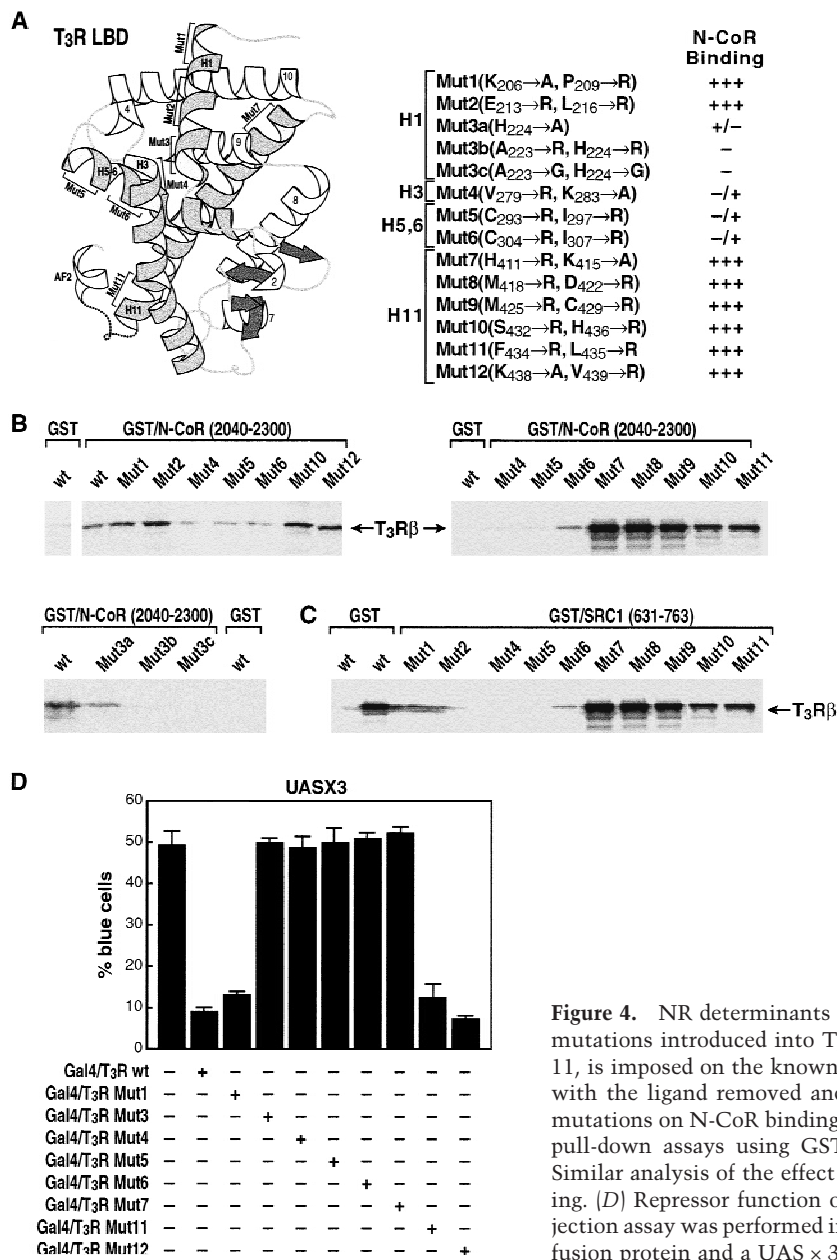
Figure 4. NR determinants of N-CoR binding. (*A*) The position of a series of mutations introduced into $T_3R\beta$, involving residues in helixes 1, 3, 5, 6, and 11, is imposed on the known structure of the $T_3R\alpha$ LBD (Wagner et al. 1995), with the ligand removed and the position of AF2 rotated. The effect of the mutations on N-CoR binding is listed. (*B*) Analysis of these mutations in GST pull-down assays using GST–N-CoR(2040–2300) and ^{35}S-labeled $T_3R\beta$. (*C*) Similar analysis of the effect of $T_3R\beta$ mutations on GST–SRC(631–763) binding. (*D*) Repressor function of mutated T_3R in a single cell nuclear microinjection assay was performed in Rat-1 cells using a GAL4/T_3R carboxy-terminal fusion protein and a UAS × 3/*tk–lacZ* reporter.

Figure 5. Model of corepressor/NR interaction. (*A*) Helical plots of IDN and IDC core motifs. (*B*) Schematic diagram of point mutations designed to disrupt the amino-terminal helical extension (Mut1, Mut2, Mut3) of ID-N or to convert residues 5–9 into an LXXLL consensus (Mut4). (*C*) Mammalian two-hybrid assay of the interaction between wild-type and mutant GAL4 N-CoR(1954–2215) and VP16/RAR using a UAS × 3/p36 luciferase reporter. (*D*) Ribbon diagram of the corepressor extended helix (in red) predicted to contact the hydrophobic (coactivator) binding pocket formed by helices 3, 5, and 6. An idealized helix [sequence (A5)LAAIIAAALRL] was built and transformed it into the coactivator binding site by superimposing the LAAII residues onto the corresponding LXXLL residues of the coactivator peptide using the PPARγ/SRC-1/BRL49653 complex as a model (Nolte et al. 1998). This idealized helix position was then transformed onto $T_3R\beta$ by superimposing PPARγ onto $T_3R\beta$. The carboxy-terminal end of the helix is pointed at helix 1 and the amino-terminal end of the helix is sterically blocked by the AF-2 helix (in yellow) position. The binding of the shorter coactivator helix of GRIP-1 to the same pocket is represented in green. Below is shown an expanded view of AF-2 (yellow), corepressor (red), and coactivator (green) helices. (*E*) Model of the ligand-dependent exchange of corepressor for coactivator. The two related N-CoR interaction helices are suggested to cooperatively be recruited into the helix 3, 5, 6 binding pocket of RXR/RAR or RXR/T_3R heterodimers on DNA, with no requirement for the conserved glutamic acid residues of the AF2 helix. Ligand binding induces exchange for coactivators, which contain the short LXXLL helical motifs, requiring the conserved glutamic acid residue of the AF-2 helix for effective orientation and positioning into the receptor binding pocket.
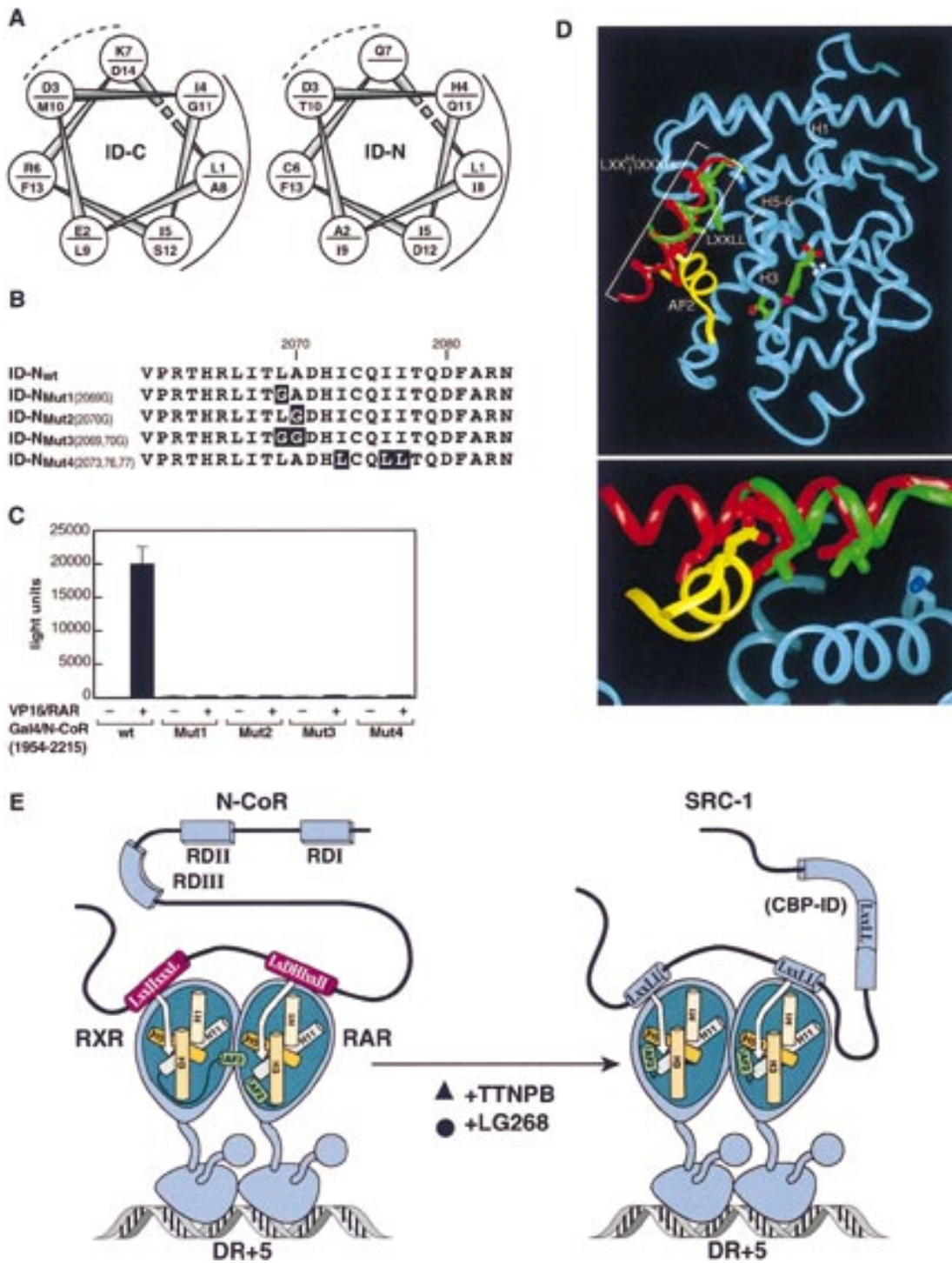
Figure 5. (*See facing page for legend.*)

hydrophobic receptor pocket formed by helices 3, 4, 5, and 6 (Darimont et al. 1998; Nolte et al. 1998; Le Douarin et al. 1996; Shiau et al. 1998). A critical determinant of the specificity of coactivator interaction is that the charge clamp can only accommodate a helix of a particular length. Furthermore, the cocrystal structure of a por-

tion of the SRC-1 nuclear receptor interaction domain containing two LXXLL motifs on a PPARγ LBD dimer (Nolte et al. 1998) supports the suggestion that each member of the receptor homo- or heterodimer pair of DNA-bound NRs can cooperatively recruit one molecule of p160 coactivator. This model has raised intriguing

questions of whether a similar strategy might be utilized in recruitment of the corepressor.

In this paper we have provided evidence that, in an analogous fashion, N-CoR contains two related, but putatively extended helical motifs with amphipathic properties, in which critical spacing of hydrophobic residues constitute a structural determinant of high-affinity interaction with the unliganded RARs and TRs. These motifs appear to share the consensus LXX I/H IXXX I/L, which is predicted to represent an amino-terminally extended helix, when compared to the known LXXLL coactivator helices (Fig. 5B). Based on both biochemical and functional assays, this amino-terminal extension in the N-CoR interaction motif appears to be required for effective binding to unliganded receptor. As would be expected from results with the LXXLL coactivator helical motifs (Darimont et al. 1998; McInerney et al. 1998), residues flanking this motif are of quantitative importance, consistent with additional contacts to stabilize binding.

The critical determinants of corepressor binding appear to reside in the "coactivator" receptor binding pocket formed by the helices 3, 5, and 6. Thus, the corepressor uses, at least in an overlapping fashion, the hydrophobic pocket that is required for coactivator binding. This raises the question why the AF2 helix is fully inhibitory for N-CoR binding to most NRs, whereas it only quantitatively diminishes interactions in the case of the TRs and RARs. Even in the case of RARs and TRs, antagonists that cause distinct placement of the AF2 helix increase binding and function of the corepressor (Lavinsky et al. 1998), indicating that the AF2 helix is inhibitory, and that the conserved glutamic acid residue of AF2 critical for coactivator binding and function is not required in the case of corepressor binding. The amino-terminal extension of the corepressor helix has been modeled on the T_3R carboxyl terminus (Figure 5D). We suggest that the extended helix functions to displace the AF2 helix out of the pocket and to make contact with the receptor coactivator pocket. This is in contrast to coactivator LXXLL motif, which actually requires the AF2 helix to be effectively positioned for packing into the hydrophobic coactivator-binding pocket. As shown in Fig. 5D, I/L9 acts as a fulcrum for motion of the helix and predictions made by energy minimization suggest that the presence of an isoleucine at position 5 is essential to allow L1 to drop deep into the receptor pocket; in this model, I5 gives a better fit than L5 against the sloping wall of the receptor.

Although it was clearly possible that N-CoR contact with helix 11 facilitates binding, our data strongly suggest that helix 11 of the NR is not a component of the corepressor binding contact. Thus, these observations suggest that the extended helix of the corepressor permits binding into the hydrophobic pocket without any requirements for the glutamic acid residue within the AF2 helix, critical for positioning LXXLL coactivator motifs.

Modeling of the corepressor LXX L/I IXXX I/L helix (Fig. 5B) indicates that it cannot make contact with helix

1, although a break in the carboxy-terminal helix could permit contact of more carboxy-terminal residues with helix 1. However, the positions of the H and T residues (Wurtz et al. 1996) are more consistent with the idea that these residues of TRs and RARs interact with and affect the precise placement of other helices sufficiently to facilitate N-CoR binding to unliganded receptors. Modeling also suggests that the AF2 helix, displaced by corepressors, might interact with the carboxyl terminus of helix 1, further facilitating corepressor binding. In receptors such as the estrogen receptor, we propose that the repositioning of the AF2 helix by tamoxifen, as opposed to the unoccupied or agonist-bound receptor, moves the AF2 helix to a position that now permits corepressor binding into the hydrophobic pocket, even without the structural feature of TR and RAR helix 1 (Westin et al. 1998; Le Douarin et al. 1996). Intriguingly, using tamoxifen-bound ERs and an unbiased phage display selection assay, novel related peptides binding to ER were selected (Norris et al. 1999). Several of these peptides tested do not compete with N-CoR for binding to the TR and RAR (V. Perissi et al., unpubl.), suggesting that a distinct surface may be involved for corepressor binding, consistent with the proposal by Norris et al. (1999), that there may be distinct receptor interaction for tamoxifen mediated partial agonist function, perhaps by binding distinct coactivators (Imhof and McDonnell 1996).

Thus, we suggest that a critical evolutionary adaptation of the LBD has been the selection of a LXXLL helix, critical in ligand dependent coactivator binding, and an extended LXX H/I IXXX L/I helix, which has acquired the properties required to permit corepressor binding in the absence of ligand, and that cooperative recruitment on DNA-bound receptor heterodimers occurs in each case (Fig. 5E).

Materials and methods

ChIP assay

ChIP assay for acetyl-histone H4 was conducted as per Upstate Biotechnology protocol ChIP assay Kit (catalog no. 17-229). For N-CoR association with the βRAR promoter 293 cells (2×10^6 cells/10-cm dish) were serum stripped for 24 hr and treated with 10^{-6} M RA for 10 min, protein complexes were cross-linked to DNA with 1% formaldehyde for 30 min at 37°C. Cell pellets, were resuspended in SDS lysis buffer, sonicated, and precleared with salmon sperm DNA/protein A agarose. Samples of supernatants were used for input measurement and the rest was incubated with anti-N-CoR antibody (Santa Cruz, CA) at 4°C overnight. Immune complexes were isolated and cross-linking reversed at 65°C for 4 hr. Samples were subjected to a proteinase K digestion and DNA was extracted and precipitated. Detection of the promoter was determined by PCR amplification with specific primers.

DNA-dependent protein–protein interaction (ABCD) and GST pull-down assays

ABCD assay and GST pull-down assay were performed as described previously (McInerney et al. 1998).

Mammalian two-hybrid assay

Vectors expressing GAL4–DNA-binding domain fusion proteins and VP16–RAR or VP16–T$_3$R were cotransfected in 293 and HeLa cell lines as described previously (Kurokawa et al. 1995). Activation of a UAS × 3/p36 luciferase reporter was then analyzed.

Single cell microinjection assay

Microinjection assays of coactivator function were performed as described previously (Torchia et al. 1997) on Rat-1 fibroblasts. Peptides were generated (Research Genetics) and confirmed by mass spectroscopy.

Mutational analysis

N-CoR and T$_3$R mutations were performed using the Quick Change Mutagenesis kit (Stratagene) and confirmed by sequence. Mutations in the ID-C region were done synthesizing mutated oligonucleotides and cloning them into the GAL4 or GST fusion constructs.

Acknowledgments

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