Defining Requirements for Heterodimerization Between the Retinoid X Receptor and the Orphan Nuclear Receptor Nurr1

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Running title: Heterodimerization between RXR and Nurr1
SUMMARY

Nurr1, an orphan nuclear receptor mainly expressed in the central nervous system, is essential for the development of the midbrain dopaminergic neurons. Nurr1 binds DNA as a monomer and exhibits constitutive transcriptional activity. Nurr1 can also regulate transcription as a heterodimer with the retinoid X receptor (RXR) and activate transcription in response to RXR ligands. However, the specific physiological roles of Nurr1 monomers and RXR-Nurr1 heterodimers remain to be elucidated. The aim of this study was to define structural requirements for RXR-Nurr1 heterodimerization. Several amino acid substitutions were introduced in both Nurr1 and RXR in the I-box, a region previously shown to be important for nuclear receptor dimerization. Single amino acid substitutions introduced in either Nurr1 or RXR abolished heterodimerization. Importantly, heterodimerization-deficient Nurr1 mutants exhibited normal activities as monomers. Thus, by introducing specific amino acid substitutions in Nurr1, monomeric and heterodimeric properties of Nurr1 can be distinguished. Interestingly, substitutions in the RXR I-box differentially affected heterodimerization with Nurr1, retinoic acid receptor, thyroid hormone receptor, and constitutive androstane receptor demonstrating that the dimerization interfaces in these different heterodimers are functionally unique. Furthermore, heterodimerization between RXR and Nurr1 had a profound influence on the constitutive activity of Nurr1 which was diminished as a result of RXR interaction. In conclusion, our data show unique structural and functional properties of RXR-Nurr1 heterodimers and also demonstrate that specific mutations in Nurr1 can abolish heterodimerization without affecting other essential functions.
INTRODUCTION

Nuclear receptors (NRs)\(^1\) constitute a large family of ligand-regulated transcription factors including receptors for a variety of small lipophilic ligands such as steroid hormones, retinoids, thyroid hormone, and vitamin D. In addition, several evolutionary conserved proteins with unknown ligands resemble ligand-activated NRs and are referred to as orphan nuclear receptors (1, 2). Nurr1 (NR4A2), which belongs to the category of orphan receptors, is mainly expressed in the central nervous system (CNS) where it plays a critical role in the development of the midbrain dopamine (DA) cells as shown from analyses of gene targeted mice (3-8). Nurr1 continues to be expressed in mature DA cells. Since these cells degenerate in patients with Parkinson’s disease the identification of natural or synthetic ligands modulating Nurr1 activity may be of importance for the treatment of Parkinson’s disease and/or other disorders associated with defective dopamine neurotransmission (9). Two closely related orphan receptors, NGFI-B (NR4A1) and Nor1 (NR4A3), are also expressed in the central nervous system as well as in several peripheral tissues in both overlapping and distinct regions and cell types. Thus, these three orphan receptors are likely to exert both unique and redundant functions \textit{in vivo}.

Nurr1 binds DNA as a monomer and can recognize a NR binding site referred to as NGFI-B response element (NBRE). As a monomer, Nurr1 promotes constitutive transcriptional activation that is dependent on two distinct activation functions (AF1 and AF2) localized in the amino- and carboxyl-terminal regions of Nurr1, respectively (10, 11). In addition, Nurr1 can form heterodimers with the 9-\textit{cis} retinoic acid receptor RXR. These heterodimers recognize a DNA-binding site composed of two consensus NR binding motifs organized as direct repeats separated by five nucleotides (DR5). RXR cannot be efficiently activated by its cognate ligands in complex with most NR partners, e.g. retinoic acid receptor (RAR) and thyroid hormone receptor (TR). In contrast, RXR is efficiently activated by its ligands in RXR-Nurr1 heterodimers (12, 13). Nurr1 may thus be important for signaling in response to natural RXR ligands such as 9-\textit{cis} retinoic acid and docosahexaenoic acid (14). Importantly, the closely
related receptors NGFI-B and Nurr1 are equally efficient in promoting RXR activation whereas Nor1 is unable to form heterodimers with RXR (15).

Heterodimerization of RXR with e.g. RAR, TR or the vitamin D receptor (VDR) depends on two dimerization interfaces localized in the DNA-binding and in the ligand-binding domains (LBD), respectively. The strongest interaction occurs via the dimerization interface in the LBD and has been mapped to a region in the carboxyl-terminal part of the domain, corresponding to helices 9 and 10 in the canonical nuclear receptor LBD structure (I-box; 16, 17). This interface, which has been verified as critical for dimerization in NR crystal structures, is important for dimer formation in both homodimers and RXR heterodimers (18-23).

The highly efficient ligand activation of RXR when interacting with Nurr1 emphasizes the importance of understanding the structural requirements for RXR-Nurr1 heterodimerization. The purpose of this study has been to identify residues in both Nurr1 and RXR that are important for heterodimerization. Our experiments have resulted in the generation of Nurr1 mutants that can be used to discriminate between monomeric and heterodimeric functions.
EXPERIMENTAL PROCEDURES

Site-Directed Mutagenesis and Plasmid constructions

The Nurr1 I-box mutants were generated using the GeneEditor™ in vitro Site-Directed Mutagenesis System (Promega) according to the manufacturer’s instructions. In short, pCMX-Nurr1 expression vector encoding the full-length mouse Nurr1 was used as the template (12). Oligonucleotides with 21-40 nucleotides containing the desired mutation were hybridized to the denaturated template, extended with T4 DNA polymerase and ligated with T4 DNA ligase. The oligonucleotides used were for KLL (554-556) AAA 5’-CCCAACTACCTGTCTGAGCGGCGG-3’; for GKL (557-559) AAA 5’-CTGTCTAAACTGGGCGGCGG-3’; for PEL (560-562) AAA 5’-CTGGAGGAGCTGAGCGACCG-3’; for K558A 5’-AAACTGGGCGCTGGGACAGCACC-3’; for P560A 5’-TTGGGAGCTGGAGGCGACCG-3’; for E561A 5’-AAGCTGCCAGCAGGACC-3’; and L562A 5’-AAGCTGCCAGCAGGACC-3’. The bases coding for the mutated residues are underlined. The bacterial colonies obtained after transformation were screened by direct sequencing. RXR L294R mutant was created using the oligonucleotide 5’-GAGCTGCCCCGGACGACCAG-3’. Other mutations in the human RXRα have been described previously (21, 24). To create the B42-Nurr1 construct, the Nurr1 LBD was amplified by polymerase chain reaction and cloned to EcoRI and XhoI restriction sites of the pJG4-5 vector. LexA fusions of the wild type RXR LBD and its point mutants as well as the B42-fusion of CAR have been previously described (21). Gal4-fusions of the Nurr1 mutants were obtained by cloning the LBD of Nurr1 (residues 354-598) in frame with the yeast Gal4 DNA-binding domain of pCMX-Gal4 vector (12). VP16-RXR fusions were created by cloning the LBDs (residues 222-462) of the mutated hRXRα derivatives in frame with the VP16-activation domain of pCMX-VP16 (25). The (NBRE)3tk-LUC, (βRE)3tk-LUC, and MH100tk-LUC reporter...
constructs and pCMX-Gal4-Nor1 have been described previously (12, 15). To monitor transfection efficiency, pCMX-βgal was used as an internal control.

**Cell culture and Transfections**

Human embryonic kidney 293 cells and human chorion carcinoma JEG3 cells were obtained from American Type Culture Collection. The cells were maintained, transfected with the calcium phosphate precipitation method, and harvested as described (11). Briefly, the cells were maintained in Dulbecco’s modified Eagles medium and minimum essential medium, respectively. The media were supplemented with 10% fetal calf serum. Transfections were performed in 24-well plates using the calcium phosphate precipitation method. The cells were plated 24 h prior to the transfection. Each well was transfected with 200ng of the expression vector for the Nurr1 variants, 100ng of a reporter plasmid, and 200ng of βgalactoside plasmid that was used as an internal control for transfection efficiency. The ability of RXR-Nurr1 heterodimer to activate transcription was examined using a luciferase reporter driven by three copies of the retinoid acid response element (RARE) of the human retinoid acid receptorβ2 (hRARβ2) gene promoter (βRE) upstream of a thymidine kinase promoter. When studying the monomeric Nurr1 activity, a reporter regulated by three copies of the NGFI-B response elements (NBREs) was used. The transcriptional activities of the Gal4-fused mutated ligand-binding domains were assessed on a reporter gene driven by four copies of the Gal4-binding sites. After transfection, the cells received fresh medium supplemented with 10% charcoal-stripped fetal calf serum and RXR ligand (SR11237; 1µM) as indicated. The cells were harvested 24 hours later, lysed, and assayed for luciferase and βgalactosidase activities. All the transfection experiments were performed in quadruplicate dishes and each experiment has been repeated at least twice with identical results.
Yeast two-hybrid assay

The yeast two-hybrid experiments were carried out essentially as described in 21 and 26. For each experiment, three to six independently derived colonies expressing the chimeric proteins were tested.

Mammalian two-hybrid assay

The interaction between the Nurr1 and RXR variants was examined using the mammalian two-hybrid assay. JEG3 and 293 cells were cotransfected with pCMX-Gal4-Nurr1-LBD and pCMX-VP16-RXR-LBD derivatives along with a reporter gene driven by four copies of the Gal4-binding sites. The cells were subsequently harvested and analyzed as described (12).

In vitro DNA binding assay

The DNA-binding experiments were carried out as described in 11. Briefly, Nurr1, RAR, and RXR proteins were produced by coupled in vitro transcription and translation in reticulocyte lysates according to the manufacturer’s instructions (TNT Quick Coupled Transcription/Translation System™; Promega). The proteins were incubated in a binding buffer containing 10mM Tris (pH 8.0), 40mM KCl, 0.05% NP-40, 6% glycerol, 1mM DTT, 0.2mg poly (dI-dC), and protease inhibitors. βRE-probe (agcttaaggGGTTCACCGAAAGTTCActcgcat) was labeled with 32P by fill-in reaction using Klenow fragment. After addition of the probe, the reactions were incubated on ice for 20 min. Protein-DNA complexes were resolved by electrophoresis on 4% non-denaturing polyacrylamide gel in 0.25 x TBE. After electrophoresis, the gels were dried for autoradiography.
RESULTS

Dimerization between RXR and Nurr1 depends on specific residues in the Nurr1 LBD

The interface important for heterodimerization between the LBDs of RXR and its partner nuclear receptors has been mapped to an eleven-residue region referred to as the I-box (16, 17). To assess the importance of Nurr1 I-box residues for heterodimerization with RXR, three-amino acid alanine substitutions were introduced into this region [KLL(554-556)AAA, GKL(557-559)AAA, and PEL(560-562)AAA; Fig. 1]. The ability of these mutants to activate transcription as a heterodimer with RXR was examined by transfection in human chorion carcinoma JEG-3 cells. In these experiments expression vectors encoding wild-type and mutated Nurr1 derivatives were co-transfected with a luciferase reporter gene containing three copies of the retinoic acid receptor response element from the human RARβ2 gene (βRE), a DNA binding site previously shown to be efficiently activated by RXR-Nurr1 in response to RXR ligands (12). In contrast to the wild-type Nurr1, all three Nurr1 I-box mutants, as well as the non-dimerizing receptor Nor1, failed to promote efficient reporter gene activation in transfected cells treated with the synthetic RXR-specific ligand SR11237 (Fig. 2A). Thus, the results suggest that the Nurr1 I-box is required for RXR-Nurr1 heterodimer-mediated transactivation.

Comparison of the amino acid composition of I-box regions from dimerizing (Nurr1, RXR, RAR, and TR) and non-dimerizing (Nor1 and SF-1) nuclear receptors revealed that sequences of helices 9 and 10 are quite closely conserved among all compared nuclear receptors (see also Fig. 1). However, dimerizing receptors have a positively charged residue at the position corresponding to K558 in Nurr1 (Fig. 1). In contrast, non-dimerizing Nor1 and SF-1 have non-polar residues at this position. In addition, dimerizing receptors contain a proline or threonine at the position corresponding to P560 of Nurr1. Therefore, it was of interest to examine if these two residues in Nurr1 are important for
heterodimer formation by using site-directed mutagenesis to substitute these residues for alanines. Mutating Nurr1 lysine 558 to alanine (Nurr1 K558A) influenced dimer function only moderately (Fig. 2B). In contrast, Nurr1 P560A was totally inactive in response to RXR ligand SR11237 when assayed in JEG-3 or 293 cells cotransfected with the βRE luciferase reporter (Fig. 2B, and data not shown). Transactivation induced by Nurr1 L562A was reduced in similar experiments while Nurr1 E561A was fully efficient in RXR dimer-mediated transactivation. Thus, the results identify residues P560 and L562 in Nurr1 as critical for heterodimer function.

A mammalian two-hybrid system was used to ensure that mutations affected dimerization rather than the ability of the heterodimers to activate transcription in response to RXR ligands. In these experiments, wild-type or mutated Nurr1 LBDs were fused to the yeast Gal4 DNA-binding domain and coexpressed in JEG-3 and 293 cells with VP16-RXR, a derivative in which the LBD of RXR is fused to the VP16 activation domain of Herpes simplex virus. The results demonstrate that the Gal4-Nurr1 I-box mutants containing three amino acid substitutions [KLL(554-556)AAA, GKL(557-559)AAA, and PEL(560-562)AAA; Fig. 1] failed to interact with VP16-RXR (Fig. 3A). Moreover, the single residue mutant that was completely inactive in response to the RXR ligand in the reporter gene assay (Nurr1 P560A) abolished heterodimerization with RXR. In contrast, mutants that promoted RXR-mediated transactivation efficiently on the βRE reporter (Nurr1 K558A and E561A) also retained the ability to heterodimerize with RXR when examined by the mammalian two-hybrid assay (Fig. 3A). Gal4-Nor1 was used as a negative control and was unable to interact with VP16-RXR as predicted from previous studies (15).

Electrophoretic mobility shift assay was used to analyze heterodimerization in vitro. Nurr1 mutant derivatives and wild-type RXR were made by in vitro transcription and translation and tested for their ability to heterodimerize on a 32P-labeled βRE oligonucleotide probe. Nurr1 bound βRE as a
monomer and upon addition of RXR, a more slowly migrating heterodimer complex was detected. Consistent with the transfection experiments, Nurr1 E561A could dimerize with RXR and Nurr1 K558A showed somewhat reduced heterodimerization. Those mutants that failed to interact with RXR in cells were entirely unable to form heterodimers with RXR also in vitro (Fig. 3B). Importantly, all tested Nurr1 derivatives were able to interact with DNA as monomers also when the monomeric binding element NBRE was used as the probe (data not shown).

Mutations in the Nurr1 I-box do not influence the monomeric activity

The I-box localizes to the LBD, which contains a region important for transcriptional activation by Nurr1 monomer. Therefore, the ability of Nurr1 I-box mutants to promote transcriptional activation as monomers was examined. As shown in Figure 4A, all tested mutants activated a luciferase reporter driven by three copies of the monomer binding site (NBRE) in transfected 293 cells. Interestingly, the KLL(554-556)AAA variant was even more efficient than the wild-type Nurr1 in activating the reporter gene as a monomer. Moreover, examining the transcriptional activities of the mutated LBDs in the context of Gal4-derivatives confirmed that I-box mutations did not seem to alter the overall integrity of the domain, since all Gal4-Nurr1 LBD mutants promoted strong transactivation (Fig. 4B).

Identification of RXR I-box mutations influencing heterodimerization with Nurr1

The RXR I-box is important for heterodimerization with other NRs such as RAR, TR, peroxisome proliferator activated receptor (PPAR), and constitutive androstane receptor (CAR; 17, 21). To examine how various RXR I-box mutations influence heterodimerization with Nurr1, mutated RXR LBDs were expressed in yeast as LexA-fusions and their ability to interact with B42-fused Nurr1 LBD was examined. Wild-type RXR interacted with Nurr1 in the yeast two-hybrid assay (Fig. 5A). Addition of 9-cis retinoic acid (9-cis-RA) did not have any significant effect on dimerization. Several RXR
amino acid substitutions were tested for their influence on heterodimerization with Nurr1. RXR A416D, RXR A416K, and RXR R421P were unable to heterodimerize with Nurr1 while retaining the ability to interact with CAR. Notably, previous studies have demonstrated that the latter mutated RXR derivative heterodimerizes with both RAR and TR establishing unique structural requirements for different NR heterodimers (21).

Results in yeast were verified in mammalian cells using the two-hybrid system. As in the yeast experiments, VP16-fusion proteins with the LBDs of RXR A416K and RXR R421P were unable to heterodimerize with Gal4-Nurr1 (Fig. 5B). Substitution of aspartic acid for RXR A416 diminished interaction with Nurr1 but did not totally abolish heterodimerization. Mutation of RXR R421 to alanine had no effect on Nurr1-dimerization. Finally, the ability of the RXR mutants to heterodimerize with Nurr1 in vitro was examined using electrophoretic mobility shift assay. Under the conditions used, none of the mutants formed heterodimeric complexes with Nurr1 whereas RXR A416D and R421A heterodimerized efficiently with RAR as reported previously in yeast and mammalian cells (Fig. 5C; 21). The difference in the results on RXR-Nurr1 dimerization in cells and in vitro probably reflects more stringent heterodimerization requirements in vitro. This conclusion is also suggested by the differential dimerization efficiency of Nurr1 K558A with RXR under in vitro conditions and in mammalian cells (Fig. 3A and B). In conclusion, these data demonstrate that the RXR I-box is crucial for dimerization with Nurr1 and that individual RXR I-box residues are differentially involved in heterodimerization with Nurr1, RAR, TR, and CAR.

Behavior of RXR-Nurr1 heterodimers on monomeric Nurr1 response elements

As Nurr1 heterodimerizes with RXR and efficiently promotes RXR-mediated transactivation on a DR5 response element, we next asked whether Nurr1 interacts with RXR also on the monomeric response element NBRE. As seen in Figure 6A, cotransfected RXR repressed the transcriptional activity of the
NBRE bound Nurr1 monomer in a dose-dependent manner. Treatment with the RXR ligand SR11237 stimulated NBRE-reporter gene activity by about three-fold and abolished the suppressive effect of apo-RXR. The repressive effect of RXR on Nurr1 could be due to squelching of transcriptional coactivator proteins or recruitment of corepressors by RXR. To address these possibilities, an RXR AF2 mutant unable to interact with coactivators [RXR ML(454,455)AA] and a mutant lacking the ability to bind corepressors (RXR L294R; data not shown and 27) were used in co-transfection experiments. However, both mutants retained the ability to repress Nurr1 efficiently (Fig. 6B). In contrast, no repression of Nurr1-mediated activation was observed when heterodimerization functions in either RXR or Nurr1 were abolished [RXRA416K and Nurr1 KLL(554-556)AAA; Fig. 6B and data not shown]. The repressive effect of RXR did not seem to be due to interference with Nurr1 DNA binding since RXR inhibited also activation by Gal4-fused Nurr1 LBD on a reporter gene driven by Gal4-binding sites. Moreover, RXR had no effect on the ability of Nurr1 to interact with NBRE in vitro (data not shown).
DISCUSSION

The ability of Nurr1, and its close relative NGFI-B, to activate transcription both as a monomer and heterodimer with RXR is a unique property within the nuclear receptor family. As a consequence, the physiological phenomena regulated by Nurr1 are probably modulated not only by signals affecting the activity of Nurr1, but also by ligands binding to the dimerization partner RXR. Characterization of the structural requirements for dimerization presented in this study has generated mutants that can distinguish between monomeric versus heterodimeric activities. We envision that this will prove useful in attempts to understand the physiological roles of these two forms of Nurr1. Ultimately, analyses of mice in which the wild-type allele of Nurr1 is replaced by one of the dimerization deficient derivatives of Nurr1 will allow definitive elucidation of the in vivo significance of RXR-Nurr1 heterodimers.

The results firmly identified the I-box of Nurr1 as critical for heterodimerization with RXR. Importantly, all analyzed Nurr1 I-box mutants functioned as fully active monomers demonstrating that the overall protein conformation was not disrupted by these mutations. This conclusion was supported by the finding that neither DNA-binding to the NBRE nor βRE as monomers was affected (Fig. 3B and data not shown). Notably, the Nurr1 mutant KLL(554-556)AAA was even more efficient in activating transcription as a monomer than the wild-type Nurr1 for reasons that remain to be examined.

We cannot exclude that additional residues outside of the I-box modulate dimerization with RXR. Indeed, this seems likely based on comparison with the closely related receptor Nor1. Nor1, which is unable to heterodimerize with RXR, differs slightly in the I-box region from Nurr1 and other heterodimerizing receptors. However, substituting Nor1 I-box amino acids for the corresponding Nurr1 residues did not allow heterodimerization with RXR demonstrating that additional differences in amino acid composition outside of the I-box are important for Nurr1’s ability to interact with RXR.
Several amino acids are conserved in RXR heterodimerizing nuclear receptors. However, an important finding in this study was the demonstration that different RXR heterodimers have structurally unique dimerization interfaces. Thus, several mutations abolishing heterodimerization with Nurr1 did not disrupt dimerization with RAR, TR, and CAR. These unique properties may reflect functional differences in the behavior of different heterodimers. For example, ligand binding has been reported to regulate heterodimerization of RXR with both RAR and TR (28-31). In contrast, 9-cis-RA did not significantly affect RXR-Nurr1 heterodimerization (Fig. 5A).

RXR-Nurr1 heterodimers have the capacity to bind both DR5 and NBRE response elements (Fig. 2A, 6A; 12, 13). Heterodimerization on both response elements requires intact I-box regions in both receptors. As shown here, unliganded RXR can suppress the constitutive activity of Nurr1 bound to the NBRE. It is also interesting to note that constitutive activation is not observed when Nurr1 is binding to the βRE (Fig. 2; 12). This is presumably due to the more efficacious heterodimerization with RXR on βRE elements and is consistent with the suppressive activity of apo-RXR. RXR does not repress Nurr1 by squelching of common coactivator proteins since the RXR AF2 mutant unable to interact with coactivators was fully capable of repressing Nurr1. Moreover, suppression does not seem to depend on the interaction between RXR and transcriptional corepressor proteins as mutations abolishing RXR corepressor interactions did not influence to ability to suppress Nurr1. Furthermore, heterodimerization with RXR does not prevent Nurr1 DNA binding. Thus, it seems most likely that RXR heterodimerization hinders recruitment of Nurr1 coactivators e.g. by a mechanism involving steric hindrance. Alternatively, heterodimerization can induce a conformation of Nurr1 that is non-permissive for coactivator interactions.

Due to its crucial role in the midbrain dopaminergic neurons, Nurr1 is a highly interesting target for the development of pharmacological compounds aiming at the treatment of disorders involving
dopamine transmission such as Parkinson’s disease. Currently, neither endogenous nor pharmacological Nurr1 ligands have been identified. In theory, either Nurr1 monomers, RXR-Nurr1 heterodimers, or both may be useful as drug targets. The mutants identified in this study should prove valuable in ligand screening in distinguishing between ligands activating either Nurr1 alone or RXR-Nurr1 heterodimers.

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REFERENCES

1. Truss M and Beato M (1993) Endocr Rev 14: 459-479
2. Mangelsdorf DJ, Thummel C, Beato M, Herrlich P, Schütz G, Umesono K, Blumberg B, Kastner P, Mark M, Chambon P, and Evans RM (1995) Cell 83: 835-839
3. Law SW, Conneely OM, DeMayo FJ, and O'Malley BW (1992) Mol Endocrinol 6: 2129-2135
4. Ohkura N, Hijikuro M, Yamamoto A, and Miki K (1994) Biochem Biophys Res Commun 205: 1959-1965
5. Zetterström RH, Solomin L, Jansson L, Hoffer BJ, Olson L, and Perlmann T (1997) Science 276: 248-250
6. Saucedo-Cardenas O, Quintana-Hau JD, Le WD, Schmidt MP, Cox JJ, Mayo FD, Burbach JPH, and Conneely OM (1998) Proc Natl Acad Sci USA 95: 4013-4018
7. Castillo SO, Baffi JS, Palkovits M, Goldstein DS, Kopin IJ, Witta J, Magnuson MA, and Nikodem VM (1998) Mol Cell Neurosci 11: 36-46.
8. Wallén Å. Zetterström RH, Solomin L, Arvidsson M, Olson L, and Perlmann T (1999) Exp Cell Res 253: 737-746
9. Buervenich S, Carmine A, Arvidsson M, Xiang F, Zhang Z, Sydow O, Jonsson EG, Sedvell G, Leonard S, Freedman R, Chowdari K, Nimogaonkar VL, Perlmann T, Anvert M, and Olson L (2000) Am J Med Genet 96: 808-813
10. Wilson TE, Fahrner TJ, Johnston M, and Milbrandt J (1991) Science 252: 1296-1300
11. Castro DS, Arvidsson M, Bondesson Bolin M, and Perlmann T (1999) J Biol Chem 274: 37483-37490
12. Perlmann T and Jansson L (1995) Genes Dev 9: 769-782
13. Forman BM, Umesono K, Chen J, and Evans RM (1995) Cell 81: 541-550
14. Mata de Urguiza A, Liu S, Sjöberg M, Zetterström RH, Griffiths W, Sjövall J, and Perlmann T (2000) *Science* 290: 2140-2144

15. Zetterström RH, Solomin L, Mitsiadis T, Olson L, and Perlmann T (1996) *Mol Endocrinol* 10: 1656-1666

16. Perlmann T, Umesono K, Rangarajan PN, Forman BM, and Evans RM (1996) *Mol Endocrinol* 10: 958-966

17. Lee S-K, Na S-Y, Kim H-J, Soh J, Choi H-S, and Lee JW (1998) *Mol Endocrinol* 12: 325-332

18. Bourguet W, Ruff M, Chambon P, Gronemeyer H, and Moras D (1995) *Nature* 375: 377-382

19. Bourguet W, Vivat V, Wurtz J-M, Chambon P, Gronemeyer H, and Moras D (2000) *Mol Cell* 5: 289-298

20. Gampe RT, Montana VG, Lambert MH, Miller AB, Bledsoe RK, Milburn MV, Kliwer SA, Wilson TM, and Xu HE (2000) *Mol Cell* 5: 545-555

21. Lee S-K, Lee B, and Lee JW (2000) *J Biol Chem* 275: 33522-33526

22. Gorla-Bajszczak A, Juge-Aubry C, Pernin A, Burger AG, and Meier CA (1999) *Mol Cell Endocrinol* 147: 37-47

23. Chen S, Costa CHRM, Nakamura K, Ribeiro RCJ, and Gardner DG (1999) *J Biol Chem* 274: 11260-11266

24. Botling J, Casto DS, Öberg F, Nilsson K, and Perlmann T (1997) *J Biol Chem* 272: 9443-9449

25. Perlmann T, Rangarajan PN, Umesono K, and Evans RM (1993) *Genes Dev* 7: 1411-1422

26. Lee JW, Choi HS, Gyuris J, Brent R, and Moore DD (1995) *Mol Endocrinol* 9: 243-254

27. Hu X and Lazar MA (1999) *Nature* 402: 93-96
28. Collingwood TN, Butler A, Tone Y, Clifton-Blight RJ, Parker MG, and Chatterjee KK (1997) *J Biol Chem* 272: 13060-13065

29. Kakizawa T, Miyamoto T, Kaneko A, Yajima H, Ichikawa K, and Hashizume K (1997) *J Biol Chem* 272: 23799-23804

30. Liu Y-Y, Nguyen C, and Peleg S (2000) *Mol Endocrinol* 14: 1776-1787

31. Depoix C, Delmotte M-H, Formstecher P, and Lefebre P (2001) *J Biol Chem* 276: 9452-9459

**FOOTNOTES**

1 Abbreviations used: NR, nuclear receptor; CNS, central nervous system; DA, dopamine; NBRE, NGFI-B response element; RXR, retinoid X receptor; RAR, retinoic acid receptor; TR, thyroid hormone receptor; VDR, vitamin D receptor; LBD, ligand-binding domain; βRE, retinoic acid receptor response element from the human RARβ2 gene.
FIGURE LEGENDS

Fig. 1. **Identification of Nurr1 and RXR residues critical for heterodimerization.**
Alignment of the I-box residues of Nurr1, RXRα, RARγ, TRβ and the non-dimerizing Nor1 and SF1. The *underlined* residues in the Nurr1 sequence were mutated to alanines [KLL(554-556)AAA, GKL(557-559)AAA, and PEL(560-562)AAA, respectively]. The lysine, proline, glutamic acid, and leucine in *bold* were individually converted to alanines (K558A, P560A, E561A, and L562A, respectively). In RXR sequence, the two residues in *bold* (A416 and R421) were mutated.

Fig. 2. **Mutations in the Nurr1 I-box influence RXR-Nurr1-mediated transactivation.**
A) JEG 3 cells were cotransfected with the (βRE)₃tk-LUC reporter, pCMX-βgal internal control plasmid, and with expression vectors for wild-type Nurr1, Nor1, or with mutated Nurr1 derivatives as indicated. The cell cultures were treated with or without the synthetic RXR ligand SR11237 as depicted. After a 24h incubation, the cells were harvested, and the cell extracts assayed for luciferase and β-galactosidase activities.

B) JEG 3 cells were transfected with the expression vectors for Nurr1, Nor1, and the Nurr1 single amino acid substitution mutants and treated with SR11237 as indicated.

Fig. 3. **Mutations in the Nurr1 I-box abolish heterodimerization with RXR.**
A) The effects of Nurr1 I-box mutations on heterodimerization with RXR were examined in JEG 3 cells. The cells were cotransfected with the wild-type or mutated Gal4-Nurr1 derivatives along with VP16-RXR and a luciferase reporter gene driven by four copies of Gal4-binding sites.

B) The effect of I-box mutations on RXR-Nurr1 heterodimerization on DNA *in vitro*. The ability of the Nurr1 derivatives to bind DNA as a heterodimer with RXR was assessed using a gel-shift assay.
Radioactively labeled hRARβ2 promoter RARE (βRE) was used as probe. The complexes corresponding to Nurr1 monomer and RXR-Nurr1 heterodimer are indicated.

Fig. 4. **I-box mutations have no effect on the monomeric activity of Nurr1.**

A) The monomeric activities of the Nurr1 mutants were examined in 293 cells. The cells were cotransfected with (NBRE)_3tk-LUC reporter, pCMX-βgal, and with expression vectors for the Nurr1 variants.

B) To study the activities of the mutated Nurr1 LBDs, expression vectors for Gal4-Nurr1 LBD derivatives were cotransfected to 293 cells with a reporter gene regulated by four copies of Gal4-binding sites.

Fig. 5. **Specific mutations in the RXR I-box impair heterodimerization with Nurr1.**

A) The ability of Nurr1 and CAR LBDs to dimerize with mutated RXR LBDs was examined using the yeast two-hybrid method in the presence (solid bars) and absence (open bars) of 9-cis-RA. The data has been reproduced with three to six independently derived colonies with identical results.

B) The ability of the VP16-fused RXR variants to interact with Gal4-Nurr1 derivatives was examined in 293 cells.

C) The ability of RXR mutants to bind DNA as heterodimers with Nurr1 and RAR *in vitro* was assessed by the gel-shift assay using radioactively labeled βRE as the probe. The Nurr1 monomer as well as RXR-Nurr1 and RXR-RAR heterodimer complexes are indicated.
Fig. 6. **RXR modulates Nurr1 signaling on NBREs**

A) 293 cells were cotransfected with (NBRE)_3tk-LUC reporter along with 100ng of pCMX-Nurr1 and 50ng, 100ng, or 200ng of pCMX-RXR as indicated. After transfection, the cell cultures were treated with or without the synthetic RXR ligand SR11237 as depicted.

B) The ability of RXR mutants to repress Nurr1 activity was examined in 293 cells by cotransfecting the cells with (NBRE)_3tk-LUC reporter plasmid, 100ng of pCMX-Nurr1, and 100ng of pCMX-RXR variants as indicated.
Aarnisalo et al. Fig. 1

Nurr1  553  S  K  L  L  G  K  L  P  E  L  R  563
RXRα   416  A  K  L  L  L  R  L  P  A  L  R  426
RARγ   377  P  R  M  L  M  K  I  T  D  L  R  387
TRβ    419  P  K  L  L  M  K  V  T  D  L  R  429
Nor1   583  P  K  V  L  R  A  L  V  E  L  R  593
SF-1   417  Q  Q  L  L  L  C  L  V  E  V  R  427

I-box

NT  DBD  LBD
Aarnisalo et al. Fig. 2

**A**

![Graph A](image)

**B**

![Graph B](image)
A

![Graph showing RLU values for different conditions](image)

B

![Western blot image with RXR-Nurr1 heterodimer and Nurr1 monomer](image)

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Aarnisalo et al., Fig. 3

RXR

K558A

P560A

E561A

Nor1

RLU

WT

KLL(554-556)AAA

GKL(557-559)AAA

PEL(560-562)AAA

Norr1

200

400

600

800

1000
Aarnisalo et al. Fig. 4

A

FOLD INDUCTION

I  WT  KLL(554-556)AAA  GKL(557-559)AAA  PEL(560-562)AAA  K558A  P560A  E561A  L562A

B

700

KLL(554-556)AAA  GKL(557-559)AAA  PEL(560-562)AAA
Aarnisalo et al. Fig. 5

**A**

RELATIVE β-GALACTOSIDASE ACTIVITY

|          | RXRwt | A416D | A416K | A416P | R421A | R421P |
|----------|-------|-------|-------|-------|-------|-------|
| B42/-    |       |       |       |       |       |       |
| B42/Nurr1-LBD |   |       |       |       |       |       |
| B42/CAR  |       |       |       |       |       |       |

-9cis-RA +9cis-RA

**B**

RLU

|          | RXR wt | A416D | A416K | R421A | R421P |
|----------|--------|-------|-------|-------|-------|
|          |        |       |       |       |       |

**C**

heterodimer →
monomer →

|          | RXR/RAR | heterodimer | Nurr1 |
|----------|----------|-------------|-------|
|          | Wt       | A416D | A416K | R421A | R421P |
|          |          |       |       |       |       |

RAR
Figure 6

A

Relative repression of Nurr1 by SR11237 in cells transfected with increasing concentrations of RXR

B

Relative repression of RXR mutants by SR11237

RXR wt
RXR ML(454,455)AA
RXR A416K
RXR L294R
