Activity of the Nurr1 Carboxyl-terminal Domain Depends on Cell Type and Integrity of the Activation Function 2*

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Nurr1, a member of the nuclear hormone receptor superfamily, was recently demonstrated to be of critical importance in the developing central nervous system, where it is required for the generation of midbrain dopamine cells. Nuclear receptors encompass a transcriptional activation function (activation function 2; AF2) within their carboxyl-terminal domains important for ligand-induced transcriptional activation. Since a Nurr1 ligand remains to be identified, the role of the Nurr1 AF2 region in transcriptional activation is unclear. However, here we show that the Nurr1 AF2 contributes to constitutive activation independent of exogenously added ligands in human embryonic kidney 293 cells and in neural cell lines. Extensive mutagenesis indicated a crucial role of the AF2 core region for transcriptional activation but also identified unique features differing from previously characterized receptors. In addition, Nurr1 did not appear to interact with, and was not stimulated by, several previously identified coactivators such as the steroid receptor coactivator 1. In contrast, adenovirus protein E1A, stably expressed in 293 cells, was shown to contribute to AF2-dependent activation. Finally, while the AF2 core of RXR is required for ligand-induced transcriptional activation by Nurr1-RXR heterodimers, the functional integrity of Nurr1 AF2 core is not critical. These results establish that the ligand binding domain of Nurr1 has intrinsic capacity for transcriptional activation depending on cell type and mode of DNA binding. Furthermore, these results are consistent with the possibility that gene expression in the central nervous system can be modulated by an as yet unidentified ligand interacting with the ligand binding domain of Nurr1.

Nuclear receptors (NRs) constituting a large family of ligand-inducible transcription factors including receptors for steroid hormones, retinoids, vitamin D, and thyroid hormone (1–3). In addition, a large number of structurally related proteins, referred to as orphan receptors, belong to the same superfamily of transcription factors but lack identified ligands. NRs bind to DNA sequences termed hormone response elements (HREs) in the vicinity of genes that they regulate. Such HREs are often composed of repeats of a common half-site sequence recognized by receptor dimers. While, for example, steroid hormone receptors bind repeats as homodimers, several other NRs, including receptors for retinoic acid (RAR), thyroid hormone (TR), and vitamin D, bind to DNA as heterodimers with the 9-cis-retinoic acid receptor, RXR (1). Yet an additional category of receptors bind their cognate response elements as monomers, recognizing additional nucleotides upstream of the minimal consensus half-site sequence.

NRs encompass several functional domains including a well conserved DNA binding domain and a somewhat less conserved carboxyl-terminal ligand binding domain (LBD). Two major regions essential for transcriptional activation, situated in the amino-terminal (AF1) and carboxyl-terminal (AF2) regions, respectively, have been identified by using mutated receptor derivatives in transcription assays in vivo and in vitro. The AF2 region plays a key role in the ligand-induced conformational transition of the LBD, which, upon ligand binding, results in activation of transcription. A region close to the carboxyl terminus contains an amphipathic α-helix, which has proven to be of critical importance for ligand-induced transcriptional activation and is referred to as the AF2 core. Recent x-ray crystallographic studies have yielded a relatively clear understanding of the ligand-induced structural transition occurring within NRs. Notably, these analyses have demonstrated that ligand binding results in a conformational change involving repositioning of the AF2 core α-helix (4–6).

The AF2 region interacts with auxiliary proteins termed coactivators, which mediate the transcriptional activation of liganded receptors (7, 8). An important group of coactivators is the thyroid receptor coactivator 1 (SRC-1) and closely related proteins referred to as the p160 family of coactivators. These proteins are components of a multiprotein complex including cAMP-response element-binding protein-binding protein (CBP)/p300 and the histone acetylase p500/CoBP-associated factor (9). Thus, the activities of these proteins result in localized modulation of chromatin structure as a consequence of increased histone acetylation in the vicinity of hormone-regulated target genes. An additional and distinct coactivator multiprotein complex, which apparently lacks histone acetylase activity, was recently identified and found to enhance transcription by liganded thyroid hormone and vitamin D receptors in vitro transcription assays (10, 11). A specific motif composed of the amino acid sequence LXXLY in NR coactivators has been demonstrated to interact directly with the LBDs of liganded receptors (12–14). Reciprocally, mutations introduced in the carboxyl-terminal domain of NRs, in particular in the AF2 core, which abolish interactions with coactivators, have de-
The involvement of unique coactivators and/or Nurr1 ligands in the transcriptional activation depending both on cell type specificity and regulation of Nurr1 in the developing and adult central nervous system plays a role in transcriptional activation, since such information is available on the structural requirements for transcriptional activation. In particular, therefore, only limited information is available on the structural requirements for transcriptional activation. In particular, RXR is permissive to ligand-induced activation, whereas several other heterodimerization partners, including RAR, have been shown to inhibit the inducibility of RXR (31, 32, 34–38).

Target genes regulated by Nurr1 have not been identified, and the role of a putative ligand remains to be investigated. Moreover, only limited information is available on the structural requirements for transcriptional activation. In particular, it is important to understand whether the AF2 region of Nurr1 plays a role in transcriptional activation, since such information could give clues regarding the potential for ligand-induced regulation of Nurr1 in the developing and adult central nervous system. The results presented here provide evidence demonstrating unique properties for the AF2 region in transcriptional activation depending both on cell type specificity and mode of DNA binding. Together, these data suggest the involvement of unique coactivators and/or Nurr1 ligands in the developing and mature central nervous system.

**EXPERIMENTAL PROCEDURES**

**Plasmids**—The luciferase reporters used in transfection experiments contain three copies of the human RARβ2 gene promoter retinoic acid response element (NBRE) and function as constitutively active transcription factors in various cell lines (29, 30). In addition, Nurr1 and NGFI-B, but not Nor1, form heterodimers with RXR that bind to direct repeats of the consensus half-site sequence AGGTCA spaced by 5 nucleotides (31–33). For example, such heterodimers efficiently recognize a retinoic acid response element in the RARβ2 gene promoter (βRE), which is composed of a direct repeat spaced by 5 nucleotides. In these heterodimers, RXR is permissive to ligand-induced activation, whereas several other heterodimerization partners, including RAR, have been shown to inhibit the inducibility of RXR (31, 32, 34–38).

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**RESULTS**

**AF2-dependent Transcriptional Activation by Nurr1**—Members of the NGFI-B/Nurr1/Nor1 subfamily of orphan NRs have been shown to be constitutively active in transcription as monomers. When assessing transcriptional activation, we noted a significant difference in transactivation efficiency between Nurr1, NGFI-B, and Nor1 monomers in different cell lines. In human embryonic kidney 293 cells, Nurr1 activated a reporter gene containing three NBREs upstream of a minimal thymidine kinase promoter several times more efficiently than both NGFI-B and Nor1 (Fig. 1A). In contrast, Nurr1 was much less active in human chorion carcinoma JEG-3 cells, whereas NGFI-B and Nor1 showed similar levels of activity as in 293 cells. The efficient transactivation by Nurr1 monomers in 293 cells was also apparent when expressing a derivative of Nurr1 (Gal4-Nurr1) containing the DNA binding domain of the yeast transcription factor GAL4 fused to the LBD of Nurr1 (Fig. 1B). This is interesting to note, since previous studies have demonstrated that in NGFI-B monomers, activation depends on AF1 within the amino-terminal domain of the receptor, while the carboxyl-terminal AF2 domain is dispensable for transcriptional activation (43, 44). Thus, in contrast, GAL4-NGFI-B and GAL4-Nor1 were significantly less active in JEG-3 cells, and in JEG-3 cells monomers of the GAL4-fused LBDs were not translated (Fig. 1B). These results demonstrate that the carboxyl-terminal domain of Nurr1 contains a region that contributes to activation in a cell type-dependent fashion. Consistent with this conclusion, a deleted version of Nurr1 (Nurr1(1–583)) lacking the carboxyl-terminal AF2 core, activated the NBRE reporter gene with diminished efficiency (Fig. 1C). Additional experiments demonstrated that the residual activity (approxim-
The hydrophobic amino acids (asterisks in Fig. 2A) at a position that in ligand-dependent receptors is a highly conserved glutamic acid. This glutamic acid is important for association with coactivators and transcriptional activation by several NRs (15–19, 21, 46–48). To more carefully analyze the structural requirements of the AF2 core region of Nurr1 for transactivation in 293 cells, we substituted each AF2 core amino acid with alanine in a series of mutated Nurr1 derivatives (Fig. 2A). These mutations were introduced into full-length Nurr1 and into hybrid receptor derivatives containing the mutated carboxyl-terminal domains fused to the Gal4 DNA binding domain. As shown in Fig. 2B, several of the residues are of importance for full activation by Nurr1. Notably, two mutations (D589A and F592A) reduced activation to the same extent as when the entire AF2 core is deleted (compare with Nurr1(1–583)). Consistent with this result, analysis of Gal4 hybrid mutants demonstrated that alanine substitutions of Asp<sup>589</sup> and Phe<sup>592</sup> resulted in almost complete abolishment of transactivation by the carboxyl-terminal domain (Fig. 2C). In contrast, three of the Gal4 derivatives (I588A, L591A, and L593A) showed decreased but not completely abolished activity (Fig. 2C). Interestingly, two of the mutants (I587A and K590A) consistently showed a slightly increased activity both in the context of the full-length Nurr1 and in Gal4 fused receptor derivatives. Together these results define specific structural requirements of the Nurr1 AF2 core region and further emphasize its critical role for transcriptional activation in 293 cells.

**Nurr1 AF2 Core Is Not Required for Ligand Activation of Nurr1-RXR Heterodimers**—We next wished to analyze if the functional integrity of the AF2 core region is essential for ligand-induced activation by Nurr1-RXR heterodimers. When Nurr1 is expressed in, for example, JEG-3 or 293 cells, heterodimerization with endogenous RXR leads to efficient RXR ligand-dependent activation of reporter plasmids containing DR-5 response elements such as the retinoic acid response element, ßRE (31). Ligand-dependent RXR activation is promoted equally well by wild-type Nurr1 as by all of the generated AF2 core mutants including D589A and F592A, which abolished AF2-dependent transactivation by Nurr1 monomers in 293 cells (Fig. 3A). In contrast, a mutation introduced into the AF2 core of RXR diminished ligand-induced reporter gene activation of Nurr1-RXR heterodimers (data not shown). Thus, unlike several other heterodimers that require the intact AF2 core regions of both dimerization partners, a functionally intact Nurr1 AF2 core is not required for efficient activation by Nurr1-RXR heterodimers.

Surprisingly, we found that the truncated Nurr1 derivative (Nurr1(1–583)) lacking the AF2 core region showed diminished activation in parallel experiments (Fig. 3B). As demonstrated by a gel shift analysis, GST-RXR pull-down of <sup>35</sup>S-labeled Nurr1 derivatives and a mammalian two-hybrid experiment using the truncated Gal4-Nurr1 derivative as a bait, dimerization between Nurr1 and RXR is abolished in the absence of the Nurr1 AF2 core region (Fig. 3, C and D, and data not shown). This indicates an unusual amino acid requirement for heterodimerization between Nurr1 and RXR compared with other RXR heterodimers. In contrast, the amino acid substitutions in the AF2 core mutants D589A and F592A, which perturbed constitutive Nurr1 transactivation, did not affect dimerization (Fig. 3C).

**Nurr1 Activation Is Not Promoted by SRC-1**—Since Nurr1 AF2 has a clear activation potential, we wished to analyze how co-expression of NR coactivators influence activation by Nurr1. SRC-1 has previously been shown to interact with several NRs (41). However, SRC-1 expression did not influence NBRE-specific reporter gene induction by Nurr1 in 293 cells, whereas activation by RXR of a ßRE reporter gene in response to the
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RXR ligand SR11237 was significantly enhanced in the presence of SRC-1 (Fig. 4, A and B). In addition, a VP16 fused derivative of SRC-1 did not interact with Gal4-Nurr1 as revealed by a two-hybrid experiment, while efficient interaction with Gal4-RXR was detected in control experiments (Fig. 4C). In parallel experiments, the coactivators ACTR and cAMP-response element-binding protein-binding protein were also unable to promote Nurr1 activation (data not shown). Collectively, these data indicate the existence of distinct coactivators for AF2-dependent Nurr1 activity (see "Discussion").

Adenovirus E1A Promotes AF2-dependent Activation in JEG-3 Cells—The prominent AF2-dependent activity of Nurr1 in 293 cells suggests that factors or ligands are present that contribute to cell-specific activation. Conditioned medium from 293 cell cultures did not promote activation by Nurr1 in JEG-3 cells, indicating that other factors influence the activity of Nurr1 (data not shown). Since 293 cells are stably transformed with the adenoviral oncoprotein E1A, we were interested to analyze its ability to modulate the activity of Nurr1. Interestingly, E1A and Gal4-Nurr1 co-expression in JEG-3 cells resulted in efficient activation of a reporter gene containing promoter binding sites for Gal4 (Fig. 5). However, E1A did not appear to promote the interaction with SRC-1 (data not shown). Mutation of the Nurr1 AF2 core (F592A; Fig. 2) diminished activation and demonstrated that E1A could promote AF2-dependent transcriptional activation. E1A also potentiuated activation of Gal4-NGFI-B and Gal4-Nor1, albeit to lesser extents. Thus, stably expressed E1A apparently contributes to AF2-dependent activation in 293 cells and can influence the capacity of the AF2 region to mediate transcriptional activation in cells in which the LBD of Nurr1 is normally inactive.

E1A Is Not an Obligatory Requirement for AF2-dependent Nurr1 Activity—Nurr1 is primarily expressed in neuronal cells of the embryonic and adult central nervous system. To determine if the carboxyl-terminal domain of Nurr1 can promote activation also in cells that do not express E1A, we analyzed Nurr1 activation in c17.2 cells, a neuroblast-like cell line derived from the embryonic mouse cerebellum (49). Nurr1 activated a NBRE reporter gene in c17.2 cells with a similar efficiency as in 293 cells (data not shown), and, importantly, mutations within the AF2 core diminished activation (data not shown). In addition, Gal4-Nurr1 is highly active in these cells and exhibited the similar profile of activation as was demonstrated in 293 cells with mutated Gal4-Nurr1 derivatives (Fig. 6). Thus, the truncated derivative Gal4-Nurr1(1–583) as well as the missense mutations D589A and F592A abolished activation. Furthermore, reduced activity was observed when amino acids Ile588, Leu591, and Leu593 were mutated. Interestingly, the two mutations resulting in slightly increased activation in 293 cells (I587A and K590A) resulted in strongly increased activation compared with the wild-type Gal4-Nurr1 derivative. The ability of the Nurr1 AF2 to mediate activation was also observed in two additional neural cell lines (mouse mesencephalic cell line MN9D and human neuroblastoma SHSY5Y; data not shown). Taken together, the results demonstrate that endogenous factors expressed in several cell lines of neural origin can promote AF2 dependent Nurr1 activation and provide a basis for potent cell type-selective Nurr1 transcriptional activity.

DISCUSSION

In this study, we provide evidence demonstrating that the LBD of Nurr1 can be transcriptionally active depending both on cell type and mode of DNA binding. Furthermore, cell type-specific AF2-dependent activation can be attributed to the expression of the oncoprotein E1A in 293 cells. In neural cell
FIG. 3. The Nurr1 AF2 core is not required for ligand-induced activation of Nurr1-RXR heterodimers. A, Nurr1 AF2 core mutants are functional as heterodimerization partners of RXR. 293 cells were transfected with a luciferase reporter plasmid containing three βRE binding sites (βRE×3-tk-luc) and, with expression vectors for Nurr1 (WT) or mutated Nurr1 derivatives as indicated. Cell cultures were treated with (+) or without (−) the synthetic RXR ligand SR11237. The cells were harvested after a 36-h incubation and lysed, and cell extracts were assayed for luciferase and β-galactosidase activity. Relative light units (RLU) were computed after normalization to β-galactosidase activities. B, deletion of the Nurr1 AF2 region abolishes activation by heterodimers. JEG-3 cells were transfected with the βRE×3-tk-luc reporter and with expression vectors for Nurr1 (WT) or Nurr1-(1–583), as indicated. Cell cultures were treated with (+) or without (−) 1 μM of the synthetic RXR ligand SR11237. The cells were harvested, lysed, and assayed as in A. RLU were computed after normalization to β-galactosidase activities. C, deletion of the Nurr1 AF2 core region disrupts heterodimerization with RXR. JEG-3 cells were transfected with the UAS×4-tk-luc reporter plasmid and, as indicated, with Gal4-Nurr1, Gal4-Nurr1-(1–583), Gal4-Nurr1D589A, Gal4-Nurr1F592A, and VP16-RXR expression vectors. The cells were harvested after a 36-h incubation and lysed, and cell extracts were assayed for luciferase and β-galactosidase activity. Relative light units were computed after normalization to β-galactosidase activity. D, truncation of the Nurr1 AF2 core abolishes heterodimerization with RXR in vitro. A gel mobility shift assay was performed in which in vitro transcribed and translated Nurr1 and Nurr1,(1–583) proteins were incubated with βP-labeled βRE probes as indicated and subsequently run on a 4% nondenaturing polyacrylamide gel. The positions of monomeric and dimeric complexes are indicated.

lines, other factors were demonstrated to mimic the activity of E1A. Taken together, the results indicate a regulatory potential of the Nurr1 carboxyl-terminal domain and suggest that natural and synthetic Nurr1 ligands, yet to be identified, may modulate the activity of Nurr1 in vivo.

Activation by NRs is mediated by two distinct regions localized within the amino- and carboxyl-terminal regions (AF1 and AF2), respectively. While the AF1 region is structurally divergent, the AF2 region is evolutionary conserved, indicating a general mechanism for transcriptional activation. Accordingly, in ligand-dependent receptors, the ability to undergo a shift between inactive and active states is critically dependent on the AF2 region. Our demonstration that the Nurr1 AF2 can mediate transcriptional activation raises questions of whether Nurr1 can be switched between different conformations by analogy to ligand-dependent receptors. We found that activation by Nurr1 monomers depends strictly on cell type. Thus, it seems likely that the Nurr1 carboxyl-terminal domain is in an “active” conformation selectively in cells in which the AF2 region mediates activation. Such a conformation could be induced by several mechanisms. For example, a ligand may be synthesized specifically in cell types in which Nurr1 AF2 is active. Although experiments using conditioned cell culture media in transfection assays have not provided evidence supporting the existence of Nurr1 ligands (data not shown), the possibility of their existence cannot be excluded, since ligands could be trapped inside cells and signal by an “intracrine” mechanism (50, 51). Moreover, an alternative mechanism may involve post-translational modifications, such as phosphorylation, that could mimic the effect of a ligand and induce an active conformation. Furthermore, interacting proteins, specifically expressed in certain cell types, could potentially influence the conformation of Nurr1.

An alternative possibility is that the conformation of the Nurr1 carboxyl-terminal domain is constitutively folded in an active conformation both in cells in which the AF2 is active and inactive. This would imply that cells in which Nurr1 is active express a co-factor(s) that enables the Nurr1 AF2 region to mediate activation. Alternatively, interaction with the Nurr1 AF2 may require a unique post-translational modification of a coactivator, occurring specifically in cells in which Nurr1 AF2 is active. In conclusion, the possible existence of specific coactivators and/or endogenous ligands provide important incentives to elucidate the mechanism underlying AF2-dependent activation.
enabling them to associate with Nurr1 or, alternatively, may induce the expression of a receptor-selective coactivator. Perhaps more likely (and supported by results from GST pull-down experiments; data not shown) is that E1A stimulates Nurr1 AF2-dependent activation by a similar mechanism as has been suggested for RAR and TR, where a direct interaction with E1A promotes transcription via contacts with components of the basal transcription machinery (53, 54).

Although a role for Nurr1 in E1A-mediated cellular transformation or adenoviral infection is less likely, the results demonstrate the potential of the Nurr1 AF2 in cell type-specific transcriptional activation. The similar activation profile that was demonstrated in both 293 cells and in neural cell lines, as indicated, with Gal4-Nurr1, Gal4-NGFI-B, Gal4-Nor1, Gal4-Nurr1(D589A), and E1A (2 ng). The cells were harvested after a 36-h incubation and lysed, and cell extracts were assayed for luciferase and $\beta$-galactosidase activity. Relative light units were computed after normalization to $\beta$-galactosidase. Values are calculated as $\pm$ fold induction over reporter alone.

Additional clues regarding coactivator dependence and mechanisms for activation are derived from analysis of the primary amino acid composition of the Nurr1/NGFI-B/Nor1 AF2 regions. A positively charged side chain (Lys$^{590}$) is a conserved and unique feature of the Nurr1/NGFI-B/Nor1 AF2 core regions. In most other NRs, a glutamic acid is situated at this position, and in ligand-dependent receptors, this residue is critically involved in activation (15-19, 21, 46-48). Specifically, crystallographic analyses of liganded peroxisome proliferator-activated receptor $\gamma$, TR, and estrogen receptor LBDs in complexes with coactivator fragments from SRC-1 (peroxisome proliferator-activated receptor $\gamma$) and GRIP-1 (TR and endoplasmic reticulum), respectively, have suggested a crucial role suggesting a strategy to generate dopamine cells for cell transplantation therapy in Parkinson's disease (55).
for this residue for coactivator interactions (5, 6, 56). Together with a highly conserved lysine in helix 3, the glutamic acid helps to position the LXXLL coactivator motif into the coactivator binding site within the LBD (5, 6, 20, 56). Interestingly, Nurr1, NGFI-B, and Nor1 have a related arrangement of charged amino acid side chains; however, the positions of the residues are reversed (positively charged lysine in helix 12 and a negatively charged glutamic acid in helix 3). Although results using mutated receptors (K590A and K590E; Fig. 2 and data not shown) did not indicate a critical role of Lys590, it remains possible that this arrangement of charged amino acid side chains serves a crucial role under other conditions, e.g. dependent on promoter context, cell type, or the presence of a specific Nurr1 ligand.

In marked contrast, our results demonstrate that Lys590 has an inhibitory influence on the Nurr1 AF2, since amino acid substitutions result in receptors with increased activity. This effect was particularly evident when analyzing the mutated derivatives in the neural cell line c17.2. These somewhat unstructured substitutions result in receptors with increased activity. This effect was particularly evident when analyzing the mutated derivatives in the neural cell line c17.2. These somewhat unstructured substitutions result in receptors with increased activity. This effect was particularly evident when analyzing the mutated derivatives in the neural cell line c17.2. These somewhat unstructured substitutions result in receptors with increased activity. This effect was particularly evident when analyzing the mutated derivatives in the neural cell line c17.2. These somewhat unstructured substitutions result in receptors with increased activity. This effect was particularly evident when analyzing the mutated derivatives in the neural cell line c17.2. These somewhat unstructured substitutions result in receptors with increased activity. This effect was particularly evident when analyzing the mutated derivatives in the neural cell line c17.2. These somewhat unstructured substitutions result in receptors with increased activity. This effect was particularly evident when analyzing the mutated derivatives in the neural cell line c17.2. These somewhat unstructured substitutions result in receptors with increased activity. This effect was particularly evident when analyzing the mutated derivatives in the neural cell line c17.2. These somewhat unstructured substitutions result in receptors with increased activity. This effect was particularly evident when analyzing the mutated derivatives in the neural cell line c17.2. These somewhat unstructured substitutions result in receptors with increased activity. This effect was particularly evident when analyzing the mutated derivatives in the neural cell line c17.2. These somewhat unstructured substitutions result in receptors with increased activity. This effect was particularly evident when analyzing the mutated derivatives in the neural cell line c17.2. These somewhat unstructured substitutions result in receptors with increased activity. This effect was particularly evident when analyzing the mutated derivatives in the neural cell line c17.2. These somewhat unstructured substitutions result in receptors with increased activity.

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Transcriptional Activity of the Nurr1 COOH-terminal Domain