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Defining an N-terminal activation domain of the orphan nuclear receptor Nurr1☆

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Abstract

Nurr1 is an orphan nuclear receptor essential for the development of midbrain dopaminergic neurons. Activation of Nurr1 depends on two so-called activation functions (AFs) situated in the N- and C-terminal regions, respectively. The region important for activation within the C-terminal domain has been shown to promote activation in a highly cell-type specific fashion in the absence of added exogenous ligands. In contrast, the region in the N-terminal domain (AF1) has been much less characterized. Here we mutagenized the N-terminal domain of Nurr1 to define essential activation regions. The results identified a short core activation region localized close to the N-terminus of Nurr1. In addition, cell-type specific influences by other signaling pathways were analyzed by mutagenesis of specific conserved phosphorylation sites. The results indicate that mitogen-activated protein kinase activity (MAPK) positively influences Nurr1 AF1-dependent transcriptional activation via a conserved phosphorylation site outside the core activation region.

Keywords: Nuclear receptor; Orphan receptor; Transcription; Activation function; Phosphorylation

Nuclear receptors (NRs) 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 [1,2]. Nurr1 (NR4A2), an orphan NR lacking an identified ligand, is mainly expressed in the central nervous system (CNS), where it plays a critical role for the development of dopamine (DA) cells. Nurr1 continues to be expressed in mature DA cells, which are those cells that degenerate in patients with Parkinson's disease. Recent data have indicated essential functions in mature DA neurons. Notably, mutations in the human Nurr1 gene have been identified in familial cases of Parkinson's disease providing additional compelling evidence for a clinically relevant role of Nurr1 in the adult human brain [3]. In addition to Nurr1, two closely related orphan receptors, NGFI-B (NR4A1) and Nor1 (NR4A3), are also expressed in the central nervous system and in several peripheral tissues in both overlapping and distinct regions. The NGFI-B/Nurr1/Nor-1 subgroup is unique within the large family of NRs by being encoded by immediate early genes that are rapidly induced by various stimuli, for example growth factors [4–6]. Thus, these three orphan receptors are likely to exert both unique and redundant functions in vivo.

Nurr1, as well as its close relatives NGFI-B and Nor1, can bind to DNA as monomers recognizing a consensus binding site referred to as NGFI-B response element (NBRE; AAAGGTCA) and can function as a constitutively active transcription factor. In addition, Nurr1 can

☆ *Abbreviations:* Nurr1, nur77 related; RXR, retinoid X receptor; IPG, Immobilized pH gradient.

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also form heterodimers with the retinoid X receptor (RXR) and bind to direct repeats of the consensus half-site sequence AGGTCA spaced by five nucleotides [7,8]. RXR is permissive to ligand induced activation in these heterodimers. In contrast, RXR is unable to respond to cognate ligands in many other heterodimeric complexes [7–10]. Finally, Nurr1 and its close relatives NGFI-B and Nor1 have also been shown to bind and activate transcription as homodimers [11].

Two major regions important for transcriptional activation (AF1 and -2) have been identified in NRs, situated in the N-terminal (AF1) and C-terminal (AF2) regions, respectively. Nurr1 contains an unusually large N-terminal domain, which has been shown to mediate transcriptional activation [12]. In addition, AF2 has also been shown to mediate cell-type specific activation. In other NRs, ligand-binding results in repositioning of the AF2 core α-helix enabling interactions with so-called co-activators [13]. Recent structural analyses have shown that Nurr1 also contains a corresponding α-helix. However, structural features of the Nurr1 core α-helix are clearly distinct and it seems unlikely that it mediates interactions with "classical" NR co-activators [14]. Interestingly, these structural studies have also demonstrated that the Nurr1 ligand-binding domain lacks a cavity for ligand binding thus defining Nurr1 as a ligand-independent orphan NR [14].

Several NRs are phosphoproteins, and phosphorylation at specific positions in NRs has been demonstrated to influence, e.g., DNA binding, protein stability, and regulation of activation/repression [15]. Both positive and negative regulation of NR activity has been associated with phosphorylation of the AF-1 domain, e.g., via activation of the mitogen-activated protein kinase (MAPK) pathway [16–23]. Several distinct MAP kinase signal transduction pathways have been described in mammalian cells, each leading to activation of either the extracellular-regulated kinases (ERKs), c-Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK), p38 proteins or ERK5, and ERK3 isoforms. In addition to MAPK, both cyclin-dependent protein kinase and $pp90^{rsk1}$ have also been shown to phosphorylate the N-terminal domains of certain NRs [24–26]. In NGFI-B, a phosphorylation at a site in the hinge region in between the DNA- and ligand-binding domains negatively regulates DNA-binding activity [24,27–31].

Only very limited information is available on the function of the N-terminal domains in orphan receptors. It is of particular interest to understand the function of this region in Nurr1, since it is unusually large and may constitute the main activating region. The aim of the present study was to delineate important regions within the N-terminal domain for transcriptional activity and to assess the potential influence by kinase pathways.

Materials and methods

Plasmids. The luciferase reporters used in transient transfection assays contain three copies of the NBRE or four copies of the Gal4-binding site (upstream activating sequence, UAS), cloned upstream of the herpes simplex virus thymidine kinase gene minimal promoter [7]. pCMX-Nurr1, containing the coding cDNAsequence of Nurr1, was cloned into expression vector pCMX. pCMX-Gal4-Nurr1–262 (amino acid 1–262) was cloned by PCR in-frame after the sequence encoding the DNA-binding domain of yeast Gal4 (amino acid 1–147) [12]. Truncated derivatives of the Nurr1 N-terminal domain were also generated by PCR and fused to Gal4. Mutant pCMX-Gal4-Nurr1–262 (S126A, T132A) was generated by site-directed mutagenesis (Gene-Editor In Vitro Site-Directed Mutagenesis System, Promega) using primer (5′-TAC AAG CCC TCT GCG CCC CCG ACA CCC AGC GCC CCG AGC TTC CAG GTG-3′). N-terminal truncations of pCMX-Nurr1 were cloned by PCR. pCMX-Nurr1(84–598)D589A has a specific point mutation in AF2, an alanine substitution in position 589. All mutations were confirmed by sequencing.

Transient transfection assay. Human chorion carcinoma JEG-3 cells (ATCC) were maintained in minimal essential medium supplemented with 10% fetal calf serum. Transfections were performed by the calcium phosphate method as described previously [7]. Each well was transfected with 100 ng reporter plasmid, 100 ng receptor expression vector, and 200 ng pCMX-β gal reference plasmid containing a bacterial β-galactosidase gene. After 6–7 h, the medium was replaced by fresh medium. The cells were harvested after 36-h incubation and lysed and extracts were assayed for luciferase and β-galactosidase activity in a luminometer/photometer reader (Lucy-1, Anthos). Human embryonic kidney 293 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. Transfections were performed as for JEG-3 cells except that the cells were incubated with the calcium phosphate precipitate until harvest. All experiment was carried out a minimum of three times. Error bars represent SEM.

Immunohistochemistry. Cells were plated and transfected as described for transient transfection assay and fixed three days later with 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS), for 30–45 min at room temperature (RT). Blocking was performed for 1 h at RT with 3% BSA in PBS, 0.1–3% Triton X-100. Cells were incubated overnight at 4 °C with primary Nurr1 antibody [32], diluted at 1:2000 in PBS, 1–3% Triton X-100. After washing with PBS, cells were incubated for 1 h at RT with secondary antibody, CY3-conjugated goat-anti-rabbit at 1:200 dilution (Jackson ImmunoResearch Laboratories, USA). After rinses in PBS immunoreactivity was visualized by fluorescence microscopy and photographed. The pictures were assembled using PhotoShop (Adobe Systems, USA).

DNA-binding assay. The DNA-binding experiments were carried out as described in [12]. Briefly, proteins were made by coupled in vitro transcription and translation in rabbit reticulocyte lysates (TNT, Promega). The indicated species of proteins were incubated with binding buffer. The probe was ^{32}P-labeled by a fill-in reaction with the Klenow fragment, After addition of the probe, the reactions were incubated on ice for 30 min. Protein–DNA complexes were resolved by electrophoresis on 4% non-denaturing polyacrylamide gel in 0.25× TBE running buffer. After electrophoresis, the gel was dried for autoradiography. The NBRE sequence, which was used as probe, is as follows: agcttgagttttaAAAGGTCAtgctcaattt.

Protein extraction, two-dimensional gel electrophoresis, and Western blot. Proteins were extracted from 293 cells according to [33]. Protein concentration of nuclear extracts was determined by the Bradford assay (Bio-Rad). As much as 1–2 μg of nuclear extract was mixed with 2-D buffer (125 μl total volume, 2-D buffer containing 7 M urea, 2 M thiourea, 0.2% (w/v) dithiothreitol, 4% (w/v) Chaps, and 0.5% (v/v) IPG Buffer, pH 6–11 (Amersham Bioscience)). One hundred and twenty-five microliters of the reaction mixture was loaded onto a pH 6–11 7 cm 2-D IPGstrip (Amersham Bioscience) and focused according

to the manufacturer's instructions. Following focusing, the strip was reduced, alkylated, and subjected to SDS–PAGE as instructed using a Bio-Rad mini Protean gel apparatus (Bio-Rad). Proteins from the resulting gel were transferred to Immobilon-P, and Nurr1 protein was detected by Western blot using a polyclonal serum raised against the C-terminal part of the protein (Santa Cruz Biotechnology) and revealed using ECL Plus reagent (Amersham Bioscience) according to the manufacturer's recommendations.

Results and discussion

Activation by the N-terminal AF1 was studied by fusing the entire N-terminal domain of Nurr1 (amino acids (aa) 1–262) to the DNA-binding domain of the yeast transcription factor Gal4 [Gal4-Nurr1(1–262)]. Transfection in human embryo kidney 293 cells and in human chorion carcinoma JEG-3 cells showed that this Nurr1 derivative was efficiently activating a luciferase reporter gene containing three Gal4 DNA-binding sites in both tested cell lines (Fig. 1). In contrast, as described previously, the ligand-binding domain of Nurr1, fused to Gal4 [Gal4-Nurr1(353–598)], activated the reporter only in 293 cells [12].

A number of truncated derivatives of the Nurr1 N-terminal domain were generated and fused to the Gal4 DNA-binding domain (Fig. 2A). Western blots verified that all Gal4 derivatives were expressed at approximately equal levels in transfected 293 cells (data not shown). Derivatives with progressive deletions from the N-terminus indicated that the first 52 amino acids are dispensable for activation (Fig. 2A). Further deletions to

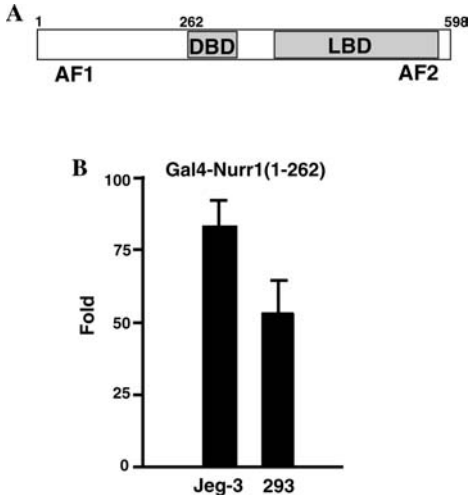

Fig. 1. The Nurr1 N-terminal (AF1) activation. (A) A schematic illustration of the Nurr1 sequence including the different domains. (B) JEG-3 cells and 293 cells were transfected with a luciferase reporter plasmid containing four upstream activating sequence (UAS) binding sites (UASx4-tk-luc) and with Gal4-Nurr1(1–262) expression vector. Cells were harvested and lysed, and cell extracts were assayed for luciferase and β-galactosidase activity. Values were computed as fold induction after normalization to β-galactosidase activities.

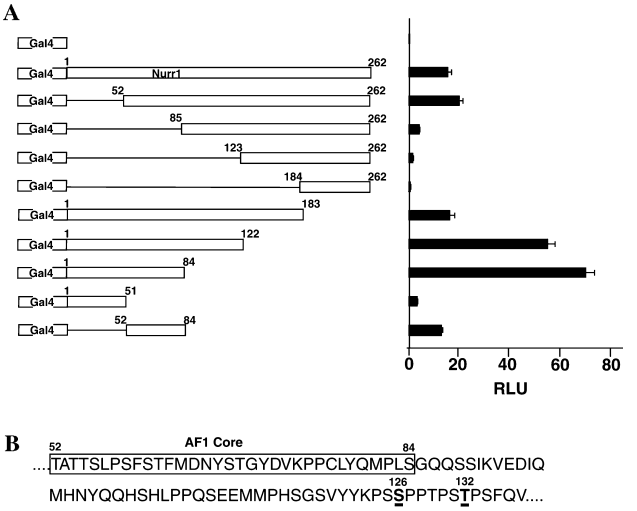

Fig. 2. Functional characterization of the Nurr1 N-terminal by mutagenesis is showing a core activating region, AF1. (A) Alignment showing the series of mutated derivatives of the amino-terminal region of Nurr1 fused to Gal4, and their activating potential in JEG-3 cells. JEG-3 cells were transfected with the UASx4-tk-luc reporter plasmid and, as indicated, with expression vector for Gal4-Nurr1(1–262) and mutated derivatives. Cells were harvested, lysed, and assayed for luciferase and β-galactosidase activity. Relative light units (RLU) were computed after normalization to β-galactosidase activities. (B) Part of the Nurr1 N-terminal sequence. The AF1 core is marked by a rectangle. Amino acids, serine 126 and threonine 132, are underlined and marked in bold.

amino acid 85, 123, and 184, respectively, almost completely abolished activation. Interestingly, deletions from the C-terminal end identified an inhibitory influence on AF1 activity. Thus, while truncation of amino acids 184–262 did not influence activity, a derivative truncated at amino acid 122 activated transcription more efficiently than the complete domain (1–262) fused to Gal4 (Fig. 2A). However, deletion to amino acid 51 severely reduced activation, even below the level of the full-length N-terminal domain. Together these results identify a core activation domain situated between amino acids 52 and 84 (Figs. 2A and B). Indeed, this region resembles a corresponding region in NGFI-B which in previous studies have been shown to contribute to transcriptional activation [24,34].

N-terminal truncations were analyzed in the context of the full-length receptor. As seen in Fig. 3A, deletion of the N-terminal core activation region diminished activation in 293 cells. Remaining activity is due to activation by the C-terminal domain, as shown previously [12]. This is further shown in Fig. 3B using a specific point mutation of AF2. Transcriptional activity is almost abolished when the AF2 mutation is introduced in a derivative of Nurr1 also containing the N-terminal deletion of the AF1 core. Further N-terminal deletions to 164 and 183 almost completely abolished activation. This complete loss of activation was unexpected since approximately 50% of activation by the full-length

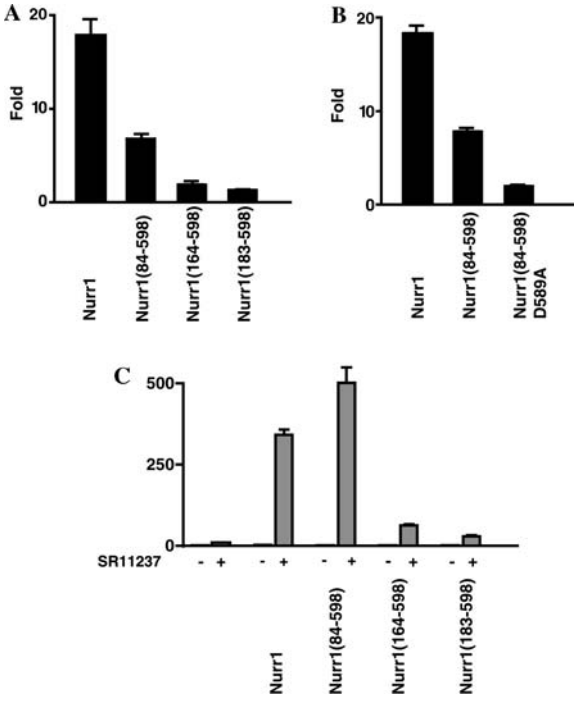

Fig. 3. N-terminal Nurr1 (AF1) dependent activity in the full-length receptor. (A) Activating potential by mutated Nurr1 derivatives in 293 cells. 293 cells were transfected with the NBREx3-tk-luc reporter plasmid and, as indicated, with expression vectors for Nurr1(WT) or mutated Nurr1 derivatives. Cells were harvested and lysed and cell extracts were assayed for luciferase and β-galactosidase activity. Values were computed as fold induction after normalization to β-galactosidase activities. (B) AF-2 mutation decreases activation further. 293 cells were transfected as in A with expression vector as indicated. Here introducing a specific point mutation of AF2, D589A, into the Nurr1 N-terminal mutation. (C) Activating potential by mutated Nurr1 derivatives as heterodimerization partners of RXR in JEG-3 cells. JEG-3 cells were transfected with a luciferase reporter plasmid containing three βRE-binding sites (βREx3-tk-luc) and, as indicated, with expression vectors for Nurr1(WT) or mutated Nurr1 derivatives. Cell culture was treated with (+) or without (−) the synthetic ligand SR11237. Cells were harvested, lysed, and assayed as in (A). Relative light units (RLU) were computed after normalization to β-galactosidase activities.

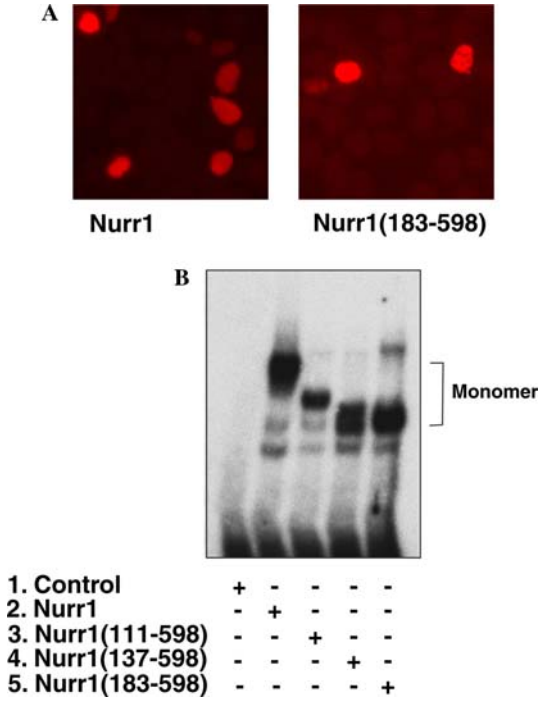

Fig. 4. Nuclear localization and DNA-binding of the N-terminal mutants of Nurr1. (A) Nurr1 and mutated derivatives, protein expression. JEG-3 cells were transfected with Nurr1 and Nurr1(183–598). Immunoreactivity was visualized by fluorescence microscopy using an anti-Nurr1 antibody. (B) DNA-binding analyses of Nurr1 and mutated derivatives. A gel mobility shift assay was performed in which in vitro transcribed and translated Nurr1 and Nurr1 mutant proteins were incubated with ^{32}P-labeled NBRE probes and subsequently run on a 4% non-denaturing polyacrylamide gel. The position of the monomeric complexes is indicated.

Nurr1 depends on the C-terminal AF2 domain [12]. Moreover, these previous studies indicated that the Nurr1 AF1 activity was dispensable for the ability of the C-terminal domain to promote activation. This prompted us to further analyze the N-terminal truncations also in the context of Nurr1-RXR heterodimers. Both Nurr1 N-terminal deletion mutants were almost entirely inactive also as heterodimers with RXR in the presence of a synthetic RXR ligand (SR11237) (Fig. 3C). Thus, truncation of the 164 N-terminal amino acids abolishes activity both as monomers and as heterodimers with RXR. All mutants truncated from the N-terminus, including Nurr1(183–598), were expressed and localized to the nucleus (Fig. 4A and data not shown). Moreover, DNA-binding analyzed by gel mobility shift assays demonstrated that all mutants bind DNA as efficiently as wild-type Nurr1 (Fig. 4B and data not shown). In conclusion, therefore, although all of the truncated mutants are expressed, localize to the cell nucleus, and bind to DNA in vitro, N-terminal truncations beyond amino acid 137 yield proteins that are inactive in reporter gene assays both as monomers and as heterodimers with RXR. Thus, in the context of the full-length Nurr1, a region of the N-terminal domain is required for activation although the AF2 region is intact.

Two-dimensional gel electrophoresis experiment reveals that Nurr1 is likely a phosphoprotein (Fig. 5). A search for potential phosphorylation sites in the N-terminal domain of Nurr1 reveals a consensus site for serine/threonine mitogen-activated protein kinases (MAPK) in a region conserved between Nurr1, NGFI-B, and Nor1. We tested the potential importance of phosphorylation of this site by analyzing the activity of Gal4-Nurr1(1–262) in the presence of PD98059, a selective MAPK (MEK) inhibitor. As seen from Fig. 6A, MAPK inhibition resulted in significant reduction of transcriptional activation. Notably, this inhibition was only observed in JEG-3 cells and not in 293 cells indicating a cell-specific dependence on MAPK activity

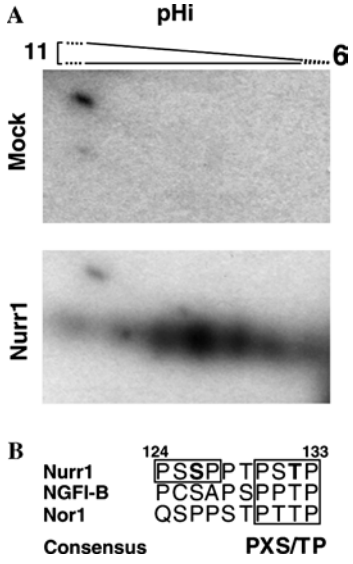

Fig. 5. Two-dimensional gel electrophoresis analysis of Nurr1 protein. (A) Nurr1 protein is revealed as several spots consistent with the existence of multiple phosphorylated isoforms. One microgram of nuclear extracts from 293 cells transfected with either Nurr1 expression vector or empty vector (mock) was analyzed by two-dimensional gel electrophoresis using IPG strip, pH 6-11, for the first dimension and SDS–PAGE for the second dimension. Nurr1 protein was detected by Western blot with Nurr1-specific antibody. (B) Sequence alignment of the Nurr1/NGFI-B/Nor1 subgroup showing the potential MAPK consensus region.

(data not shown). A specific point mutation, replacing serine 126 and threonine 132 for alanines (Gal4-Nurr1(1–262)S126A,T132A), was introduced in the MAPK consensus phosphorylation site (Fig. 5B). This mutation inhibited activation to a similar degree as seen in the presence of the MAPK inhibitor (Fig. 6B). Interestingly, this mutation did not inhibit activation in 293 cells (Fig. 6C). Notably, PD98059 was entirely unable to further reduce the activity of Gal4-Nurr1(1–262)S126A,T132A indicating that phosphorylation at this site is required for full AF1-dependent activity (Fig. 6D). Finally, we wished to analyze the consequence of introducing these mutations in a derivative containing its own DNA-binding domain but lacking AF2. As seen in Fig. 6E, when the MAPK sites were mutated, in such a derivative, the reporter was less efficiently activated in JEG-3 cells.

The results presented here delineate a core region within the N-terminal region of Nurr1 as an important component of the AF-1 activation domain. This region is highly conserved in NGFI-B/Nurr1/Nor1 family members and has also been implicated in transcriptional activation of NGFI-B. Moreover, truncations defined a region, which in the context of the full-length Nurr1 abolishes its activity both as a monomer and as a heterodimer partner with RXR. The reason why the Nurr1(164–598) and Nurr1(183–164) fail to function in reporter gene assays both as monomers and heterodimers remains unclear. However, we can exclude the possibility that these mutants are not expressed, localize to the cell nuclei, or bind to DNA as demonstrated in Figs. 4A and B.

Our data indicate that Nurr1 AF-1 activity is in part dependent on a MAPK phosphorylation site. This conclusion is supported by several observations. First, the MAPK-specific inhibitor PD98059 diminished the activity of the N-terminal domain. Second, mutations in a conserved MAPK phosphorylation site localized in the N-terminal activation domain also diminished activity. Third, this mutated form of Nurr1 could not be further inhibited by PD98059. Members of the NGFI-B/Nurr1/Nor1 subfamily of NRs have been shown to be phosphoproteins. Interestingly, in NGFI-B an MAPK-dependent phosphorylation in the vicinity to the DNA-binding domain has been shown to negatively influence DNA binding and transcriptional activity. This region is highly conserved in Nurr1 and it seems likely that a similar inhibitory influence may be significant also in this NR. Thus, MAPK signaling pathways may impart both positive (AF-1) and negative (via inhibition of DNA-binding) influences on the transcriptional activity of Nurr1. Nurr1 is encoded by an immediate early gene and is rapidly upregulated as a consequence of several stimuli. Many of these stimuli have influences on MAPK and other signaling pathways. These findings together with previous studies indicate that the NGFI-B/Nurr1/Nor1 subfamily of NRs is subjected to intricate regulation by phosphorylation.

NGFI-B has been the most extensively analyzed member of the NGFI-B/Nurr1/Nor1 subfamily. Several phosphorylation events in this NR have been suggested to influence its activity by distinct mechanisms. Thus, DNA binding is negatively modulated via phosphorylation of a site in the hinge region linking the DBD to the putative LBD [24,29]. In addition, phosphorylation of NGFI-B Ser-105 (corresponds to Thr-129 in Nurr1) induces nuclear export [35]. Finally, experiments have indicated that phosphorylation of the NGFI-B N-terminal Ser-142 and Thr-145 (corresponding to Thr-129 and Thr-132 in Nurr1) positively regulates its transcriptional activity in a cell-type specific manner [36]. Thus, the opposite regulatory influence was noted as compared to what was described here for Nurr1. These results might suggest differences in the regulation of NGFI-B and Nurr1, respectively. Alternatively, cell-type specific effects may explain these apparent discrepancies. In any event, several studies help us to manifest the importance of phosphorylation in the regulation of these highly inducible NRs. How these signals act in concert to influence Nurr1 expression and activity is clearly an important challenge in understanding the function of this orphan NR.

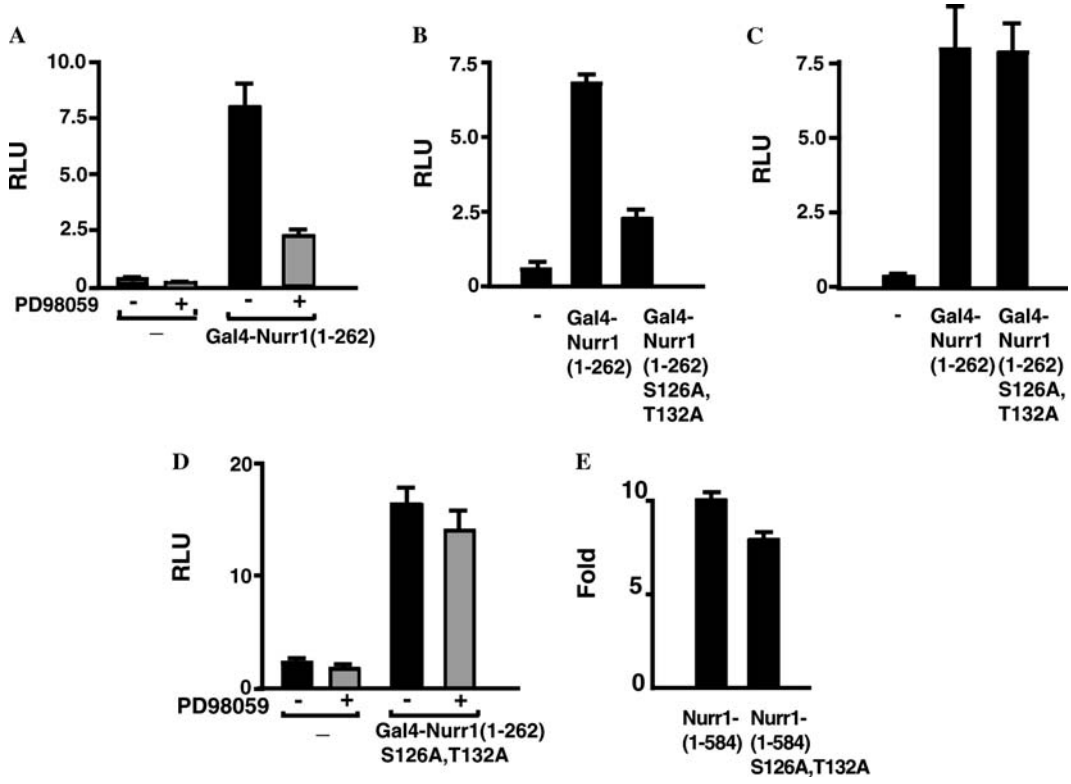

Fig. 6. MAPK Cell-type specific influence on transcriptional activation in JEG-3 cells. (A) Transcriptional activity of Gal4-Nurr1(1–262) in the presence of the selective MAPK (MEK) inhibitor, PD98059. JEG-3 cells were transfected with the UASx4-tk-luc reporter plasmid and, as indicated, with or without expression vector for Gal4-Nurr1(1–262). Six to seven hours after transfection, the medium was replaced by fresh media with (+) or without (−) the selective MAPK (MEK) inhibitor, PD98059. Cells were harvested 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) Transcriptional activity of a double mutant of a putative MAPK site in the N-terminal region of Nurr1 in the Gal4 fusion. JEG-3 cells were transfected with the UASx4-tk-luc reporter plasmid and, as indicated, with expression vectors for Gal4-Nurr1(1–262) or alanine mutated Gal4-Nurr(1–262)S126A,T132A. Cells were harvested, lysed, and assayed as in (A). (C) Transcriptional activity of a double mutant of a putative MAPK site of Nurr1 in 293 cells. The cells were transfected, harvest, lysed, and assayed as in (B). (D) Transcriptional activity of a double mutant of a putative MAPK site in the amino-terminal region of Nurr1 in the presence of the selective MAPK (MEK) inhibitor, PD98059. JEG-3 cells were transfected with the UASx4-tk-luc reporter plasmid and, as indicated, with expression vectors for Gal4-Nurr1(1–262) or alanine mutated Gal4-Nurr1(1–262)S126A,T132A. Cells were treated, harvested, lysed, and assayed as in (A). (E) Transcriptional activity of a double mutant of the putative MAPK sites in the amino-terminal region of Nurr1. JEG-3 cells were transfected with the NBREx3-tk-luc reporter plasmid and, as indicated, with expression vectors for Nurr1-584 or alanine mutated Nurr1-584(1–262)S126A,T132A. Cells were harvested, lysed, and assayed as in (A).

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