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NGFI-B Nuclear Orphan Receptor Nurr1 Interacts with p53 and Suppresses Its Transcriptional Activity

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Abstract

Nurr1 is a member of the NGFI-B nuclear orphan receptor family which includes two other members, Nur77 and Nor-1. Nurr1 is essential for the development and survival of dopaminergic neurons. It was reported that Nurr1 has antiapoptotic functions, however, the mechanisms by which Nurr1 mediates these effects remain unknown. Here, we show that overexpression of Nurr1 decreases Bax expression whereas knockdown of Nurr1 increases Bax expression. Nurr1 also interacts with p53 and represses its assembly. Furthermore, Nurr1 represses p53 transcriptional activity in interaction-dependent and dose-dependent manners. Moreover, Nurr1 protects cells from doxorubicin-induced apoptosis. These findings provide evidence that Nurr1 promotes cell survival through its interacting with and repressing p53, thus implicating that Nurr1 may play an important role in carcinogenesis and other diseases. (Mol Cancer Res 2009;7(8):1408–15)

Introduction

Nurr1 (NR4A2) is a nuclear receptor belonging to a superfamily in which its members have no known ligands, so-called orphan nuclear receptors. The members of the NGFI-B subfamily, including Nurr1, Nur77 (NR4A1, NGFI-B), and Nor-1 (NR4A3), have a high degree of conservation in either DNA-binding domains (DBD) or ligand-binding domains (1, 2). Nurr1 is not only predominantly expressed in the brain but is also detected in osteoblasts (3), liver (4), and the pituitary gland (5). Nurr1 has been identified as a transcription factor activating target gene expressions such as tyrosine hydroxylase (6-8), aromatic L-amino acid decarboxylase (9), dopamine transporter (10), vesicular monoamine transporter-2 (9), and vasoactive intestinal peptide (11). The transcriptional activity of Nurr1 is important for the development, maintenance, and maturation of dopaminergic neurons. Nurr1 and Nur77 have similar structural characteristics in which these two transcription factors could both form heterodimers with retinoic X receptors (RXR) to regulate transcription by RXR interaction with its ligand (12, 13). Nur77 and Nor-1 were reported to be necessary for T-cell antigen receptor–induced apoptosis in thymocytes and T-cell hybridomas (14-16). Nur77 also has the ability to induce apoptosis in tumor cells (17). In the human breast cancer cell line, MCF-7, Nor-1 acts as a mediator to perform proapoptotic functions under the stimulation of A23187, an inducer of endoplasmic reticulum (ER) stress (4). Nur77-mediated apoptosis involves both its transcriptional activities to up-regulate the gene expressions responsible for promoting apoptosis (18, 19) and its translocation from the nucleus to the cytosol to convert Bcl-2 function from antiapoptosis to proapoptosis (20). Interestingly, Nur77 functions in both positive and negative regulation of apoptosis depending on the cellular context and different external signals. For example, Nur77 survives cells in tumor necrosis factor–induced cell death (21) and prevents A20B cells from ceramide-induced cell death (22). It is most likely that Nur77 exerts its antiapoptotic effects by functioning in the nucleus (21, 23).

To date, Nurr1 has been reported to have effects on antiapoptosis (24, 25). Increased expression of Nurr1 by prostaglandin E_2 inhibits apoptosis induced by serum starvation (25). In colorectal cancer tissues, Nurr1 expression is significantly increased in the proliferative crypt compartment (25). Down-regulation of Nurr1 induces intrinsic apoptosis, and low expression of Nurr1 has been implicated to be responsible for the "nontransformed" phenotype of HeLaHF cells (24), a revertant variant isolated from HeLa cells, in which tumor suppressor genes, such as p53, IGFBP-3, and DKK-1, are activated (26, 27).

In our present studies, we show that Nurr1 has an ability to down-regulate the expression of proapoptotic protein Bax, which is directly transactivated by tumor suppressor p53 (28). Nurr1 interacts with p53 and represses its transcriptional activities. Furthermore, Nurr1 represses doxorubicin-induced cell death. These data suggest that Nurr1-mediated negative regulation of p53 might be a fundamental mechanism for its protective function to survive cells.

Results

Nurr1 Down-Regulates Bax and Protects Cells from Doxorubicin-Induced Apoptosis

As it was reported that Nurr1 has antiapoptotic properties and its low expression is responsible for p53 activation in HeLaHF cells (24), we examined whether overexpression of

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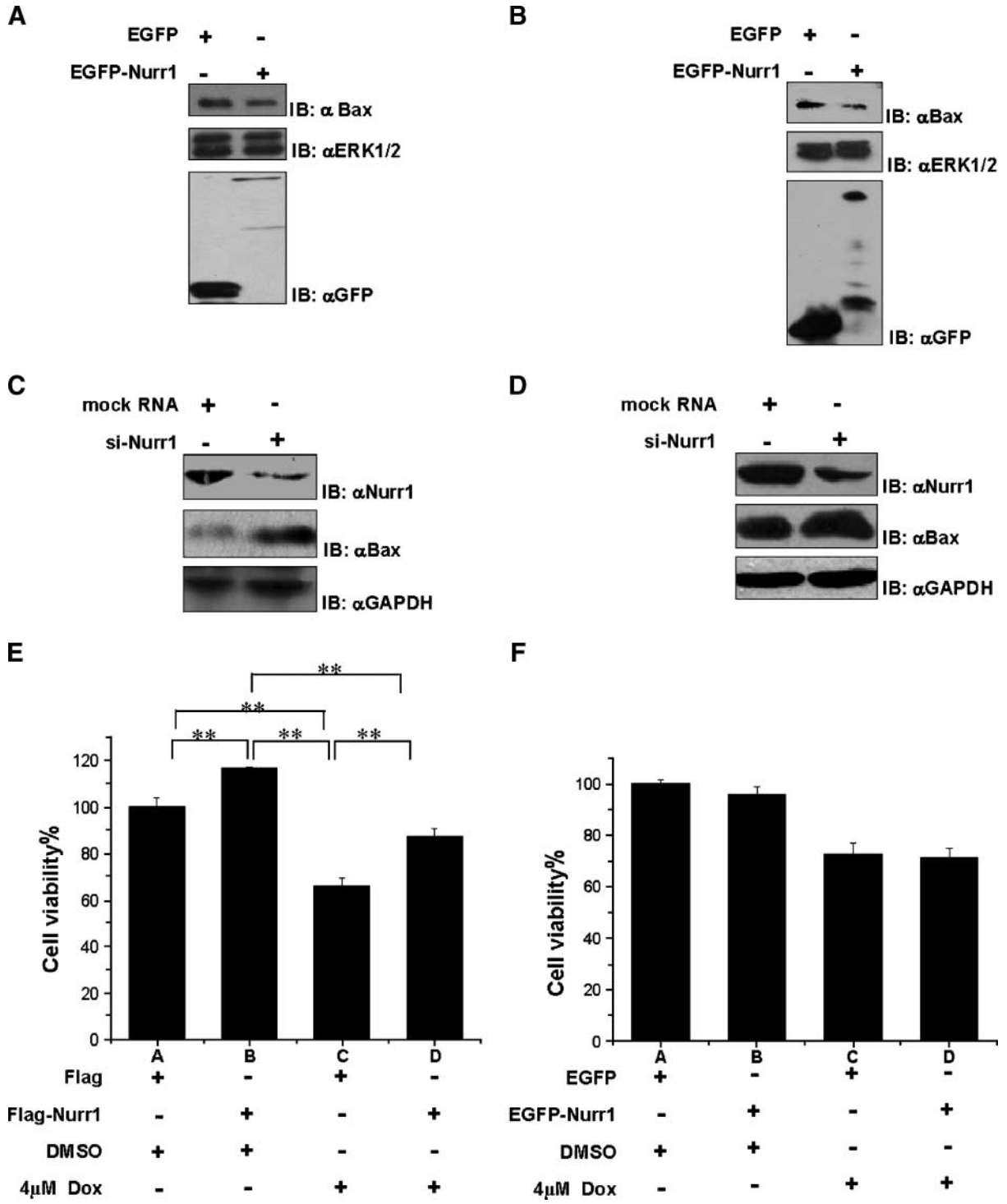

FIGURE 1. Down-regulation of Bax by Nurr1. **A.** Nurr1 down-regulates the expression of Bax in N2a cells. N2a cells were transfected with EGFP-Nurr1 or EGFP. Forty-eight hours after transfection, cells were collected and subjected to immunoblot analysis using anti-GFP antibody, anti-GAPDH antibody, or anti-Bax antibodies. **B.** Nurr1 down-regulates the expression of Bax in HEK293 cells. Experiments similar to **A** were done using HEK293 cells. **C** and **D.** Knock-down of Nurr1 increases Bax levels. HEK293 cells (**C**) and A549 cells (**D**) were transfected with oligonucleotides against Nurr1 mRNA or an irrelevant oligonucleotide (as a control). The cell lysates were collected 72 h after transfection, followed by immunoblot analysis using anti-GAPDH antibody, anti-Bax antibodies, or anti-Nurr1 antibodies. **E.** Nurr1 protects cells from doxorubicin-induced cell death in HCT116 $p53^{+/+}$ cells. HCT116 $p53^{+/+}$ cells were transfected with Flag or Flag-Nurr1. After 24 h, doxorubicin was added to the culture medium to a final concentration of 4 μmol/L, and an equivalent DMSO was added as a control. MTT assay was done 24 h after doxorubicin treatment. Quantification of absorbance and calculations of relative ratios were done. Columns, mean; bars, SE. Data from six independent experiments (*, $P < 0.05$; **, $P < 0.01$ by one-way ANOVA). **F.** Nurr1 failed to protect cells from doxorubicin-induced cell death in HCT116 $p53^{-/-}$ cells. Experiments similar to **E** were done using HCT116 $p53^{-/-}$ cells that were transfected with EGFP or EGFP-Nurr1. MTT assay was done 24 h after doxorubicin treatment.

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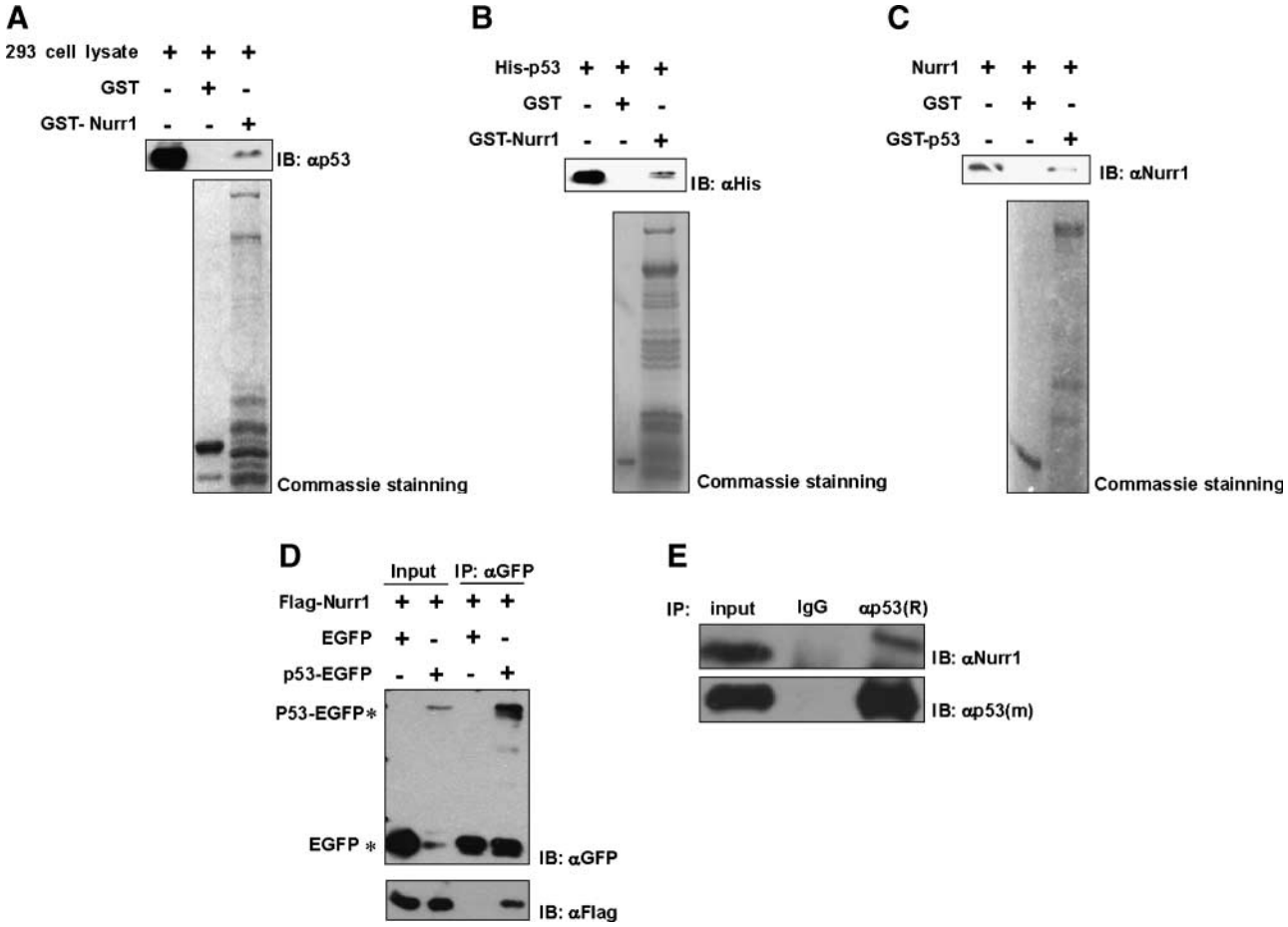

FIGURE 2. Interactions between Nurr1 and p53. **A.** Nurr1 interacts with endogenous p53 expression in HEK293 cells. The supernatants of cell extracts from HEK293 cells were incubated with GST or GST-Nurr1 coupled to glutathione-agarose beads. Immunoblot analysis was done with anti-p53 antibody. **B** and **C.** Nurr1 interacts with p53 *in vitro*. GST or GST-Nurr1 coupled to glutathione-agarose beads were incubated with His-p53 expressed from *E. coli*. The bound proteins were detected using anti-His antibody (**B**). Experiments similar to **B** were done using anti-Nurr1 antibodies (**C**). **D.** Coimmunoprecipitation of Flag-Nurr1 and p53-EGFP. Extracts from HEK293 cells cotransfected with EGFP or p53-EGFP and Flag-Nurr1 were immunoprecipitated with anti-GFP antibody. The immunoprecipitants were subjected to immunoblot analyses with anti-GFP antibody or anti-Flag antibody. **E.** Coimmunoprecipitation of endogenous p53 and Nurr1. Extracts from HEK293 cells were immunoprecipitated with anti-p53 antibodies and rabbit IgG (as a control). The immunoprecipitants were subjected to immunoblot analyses with anti-Nurr1 antibodies or anti-p53 antibody.

Nurr1 affects Bax levels. We transfected neuro2a (N2a) cells with enhanced green fluorescent protein (EGFP)-Nurr1. Forty-eight hours after transfection, cells were collected and subjected to immunoblot analysis. Endogenous Bax levels were lower in the cells transfected with EGFP-Nurr1 than in the cells transfected with EGFP (Fig. 1A). The same results were obtained using HEK293 cells that were transfected with EGFP-Nurr1 or EGFP (Fig. 1B). To further investigate the effects of Nurr1 on Bax expression, HEK293 cells and A549 cells were transfected with small interfering RNAs (siRNA) against Nurr1 or control siRNAs. In both HEK293 cells (Fig. 1C) and A549 cells (Fig. 1D), knockdown of Nurr1 increased endogenous Bax levels. These results suggest that Nurr1 negatively regulates Bax expression. We next examined the viability of cells that were transfected with EGFP or EGFP-Nurr1 after treatment with or without doxorubicin for 24 hours. In HCT116 $p53^{+/+}$ cells (Fig. 1E), but not in HCT116 $p53^{-/-}$ cells (Fig. 1F), overexpression of Nurr1 increased cell viability with or without doxorubicin treatment; meanwhile, the decrease of cell viability caused by doxorubicin treatment was blocked by Nurr1. These data suggest that Nurr1 promotes cell survival and protects cells from doxorubicin-induced cell death in a p53-dependent manner.

Nurr1 Interacts with p53 In vitro *and* In vivo

As both Nurr1 and p53 are nuclear, and we found that Nurr1 decreases the Bax level, we tested whether there was a direct interaction between Nurr1 and p53. We therefore performed glutathione *S*-transferase (GST) pull-down assays. GST-Nurr1 coupled to glutathione-agarose beads interacted with endogenous p53 from HEK293 cell extracts, but GST alone did not (Fig. 2A). Meanwhile, GST-Nurr1 pulled down His-p53 expressed from *Escherichia coli* (Fig. 2B), and GST-p53 also pulled down His-Nurr1 expressed from *E. coli* (Fig. 2C), suggesting that Nurr1 interacts directly with p53 *in vitro*. To further examine the possible interactions between Nurr1 and p53 in

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cells, we performed coimmunoprecipitation experiments. HEK293 cells were cotransfected with Flag-Nurr1 and p53-EGFP. When p53-EGFP was immunoprecipitated from the supernatants of the cell lysates using anti-GFP antibody, co-transfected Flag-Nurr1 was coimmunoprecipitated (Fig. 2D). However, no Flag-Nurr1 was coimmunoprecipitated by anti-GFP antibody in HEK293 cells cotransfected with EGFP and Flag-Nurr1 (Fig. 2D). Furthermore, when endogenous p53 was immunoprecipitated from HEK293 cells using anti-p53 antibodies, endogenous Nurr1 was coimmunoprecipitated (Fig. 2E). These data suggest that Nurr1 interacts with p53 both *in vitro* and *in vivo*.

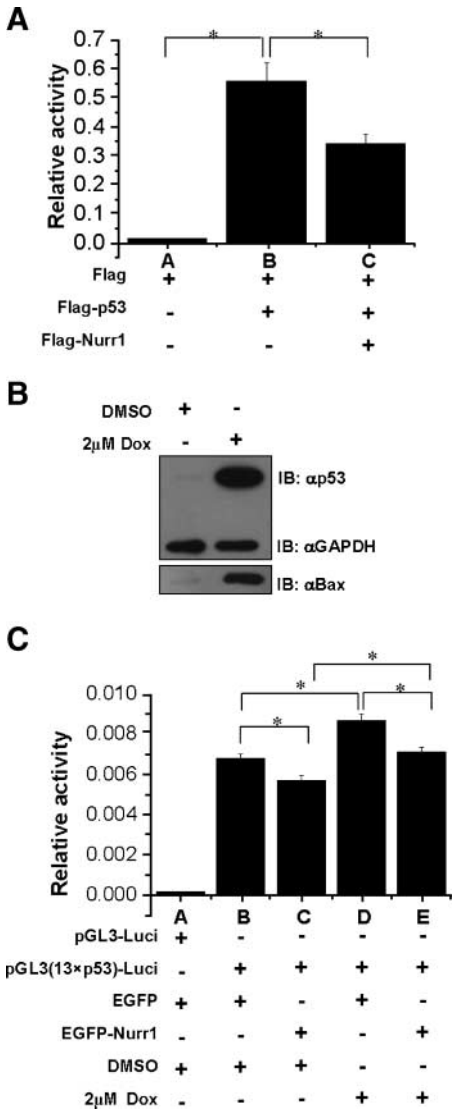

FIGURE 3. Nurr1 suppresses p53 transcriptional activity. **A.** Repression of p53 transcriptional activity by Nurr1. H1299 cells were cotransfected with pGL(13×p53)-Luciferase construct with or without a fixed amount of FLAG-p53, along with Flag or Flag-Nurr1. The total amount of plasmid DNA was kept constant by the addition of empty plasmid. *Renilla* expressing vector pRL-CMV was used as an internal transfection control. Quantification of luciferase activities and calculations of relative ratios were done. Columns, mean; bars, SE. Data from six independent experiments (*, $P < 0.05$ versus control analyzed by one-way ANOVA). **B.** Increase of p53 expression by doxorubicin. Doxorubicin was added to HCT116 $p53^{+/+}$ at a final concentration of 2 μmol/L. Twenty-four hours after incubation, cell lysates were subjected to immunoblot assay using anti-p53 antibody, anti-GAPDH antibody, or anti-Bax antibodies. **C.** HCT116 $p53^{+/+}$ cells were cotransfected with pGL(13×p53)-Luci and EGFP or EGFP-Nurr1. Plasmids expressing pGL3-Luci were used as a control. Twenty-four hours after transfection, doxorubicin was added into the culture medium to a final concentration of 2 μmol/L. Twenty-four hours later, quantification of luciferase activities and calculations of relative ratios were done. Columns, mean; bars, SE. Data from six independent experiments (*, $P < 0.01$ by one-way ANOVA analysis).

Nurr1 Suppresses the Transcriptional Activity of p53

Because Nurr1 interacts with p53, we further examined whether Nurr1 affects p53 transcriptional activities using reporter gene assays. A luciferase reporter construct containing 13 repeats of the binding sites for p53 (13×p53-Luci) was used in these experiments. We cotransfected pGL(13×p53)-Luci and p53 with or without Nurr1 into H1299 ($p53^{-/-}$) cells. As shown in Fig. 3A, induced Flag-p53 significantly activated the luciferase reporter gene expression; however, cotransfection of Flag-Nurr1 greatly decreased Flag-p53 transcriptional activity (Fig. 3A). These data suggest that Nurr1 represses p53-mediated transcriptional activity on p53-response elements in the promoter. In HCT116 $p53^{+/+}$ cells, doxorubicin treatment induced an increase of p53 and Bax (Fig. 3B); therefore, we examined whether Nurr1 blocks doxorubicin-induced p53 transcriptional activity. We cotransfected pGL(13×p53)-Luci with or without Nurr1 into HCT116 $p53^{+/+}$ cells and treated these cells with or without doxorubicin. Endogenous p53 significantly activated the luciferase reporter gene expression, but EGFP-Nurr1 repressed its transcriptional activity (Fig. 3C, *columns B* and *C*). Doxorubicin treatment increased the reporter gene expression driven by p53 response elements in the promoter; however, EGFP-Nurr1 inhibited doxorubicin-induced reporter gene expression (Fig. 3C, *columns D* and *E*). These data suggest that Nurr1 has an intrinsic effect on suppressing the transcriptional activity of p53, independent of doxorubicin stimulation.

Nurr1 Suppresses p53 Self-Assembly and Inhibits p53-Mediated Transcriptional Activity in Interaction-Dependent and Dose-Dependent Manners

Because Nurr1 interacts with p53 and suppresses p53-mediated transcriptional activity, we next investigated how Nurr1 suppresses the transcriptional activity of p53. We performed GST pull-down assays to identify which domain of Nurr1 interacts with p53. The deletion mutants of Nurr1 fused to GST were created (Fig. 4A). GST-Nurr1 DBD (amino acids 263-352) interacted with His-p53 expressed in pET-15b (Fig. 4B). However, GST-Nurr1 (amino acids 1-262, the NH_2 terminus before DBD), as well as GST-Nurr1 ligand-binding domain (amino acids 353-598), did not interact with p53 (Fig. 4B). These results suggest that p53 interacts with the DBD of Nurr1. Based on these results, we further investigated whether Nurr1 represses the transcriptional activity of p53 depending on the DBD of Nurr1. We cotransfected H1299 $p53^{-/-}$ cells with p53 and 13×p53-Luci, along with Flag-tagged Nurr1 or Nurr1ΔDBD. As shown in Fig. 4C, Flag-Nurr1ΔDBD failed to repress the activation of luciferase reporter gene expression, suggesting that the DBD of Nurr1, which interacts with p53, is necessary for Nurr1 to repress p53 transcriptional activity.

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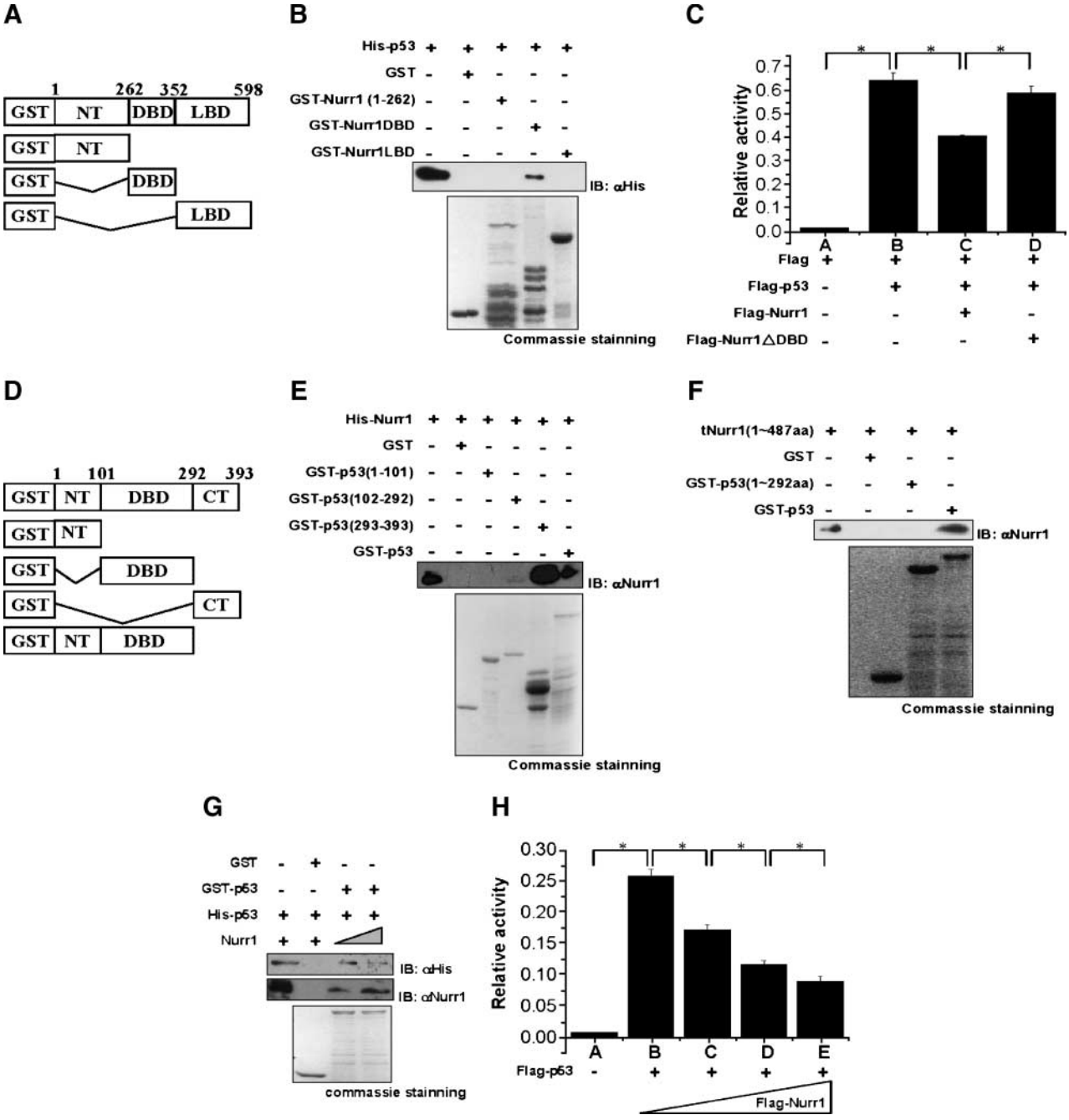

FIGURE 4. Nurr1 suppresses p53-mediated transcriptional activity in interaction-dependent and dose-dependent manners. **A.** Schematic representation of deletion mutants of Nurr1 and full-length Nurr1 fused to GST. **B.** Identification of the domains in Nurr1 interaction with p53 using GST pull-down assays. GST or deletion mutants of GST-Nurr1 coupled to glutathione-agarose beads were incubated with His-p53 expressed in *E. coli*. After incubation, the bound proteins were subjected to immunoblot analysis with anti-His antibody. **C.** H1299 cells were cotransfected with pGL(13×p53)-Luciferase construct with or without a fixed amount of FLAG-p53, along with Flag-Nurr1 or Flag-Nurr1ΔDBD. Quantification of luciferase activities and calculations of relative ratios were done. Columns, mean; bars, SE. Data from six independent experiments (*, $P < 0.05$ by one-way ANOVA analysis). **D.** Schematic illustration of the full-length and deletion mutants of p53 fused to GST. **E** and **F.** Identification of the domains in p53 interaction with Nurr1 using GST pull-down assays. GST, GST-p53 (amino acids 1-101), GST-p53 (amino acids 102-292), GST-p53 (amino acids 293-393), GST-p53 (amino acids 1-292), or GST-p53 coupled to glutathione-agarose beads were incubated with His-Nurr1 expressed from *E. coli*. After incubation, the bound proteins were subjected to immunoblot analysis with anti-Nurr1 antibodies. **G.** Nurr1 interferes with p53 self-assembly by competitive interaction with p53. GST or GST-p53 coupled to glutathione-agarose beads were incubated with both His-p53 and increased amounts of His-Nurr1. After incubation, the bound proteins were subjected to immunoblot analyses with anti-His antibody or anti-Nurr1 antibodies. **H.** Nurr1 inhibits p53 transcriptional activity in a dose-dependent manner using reporter gene assays. H1299 cells were cotransfected with pGL(13×p53)-Luciferase construct with or without a fixed amount of FLAG-p53, along with Flag-Nurr1. The amounts of plasmids expressing pGL(13×p53)-Luciferase and renilla are equal (*lanes A-E*), and the amounts of Flag-p53 are equal (*lanes B-E*). The amounts of Flag-Nurr1 are 0 μg (*lanes A* and *B*), 0.1 μg (*lane C*), 0.5 μg (*lane D*), and 2 μg (*lane E*). The total amount of plasmid DNA was kept constant by the addition of plasmid expressing Flag. Quantification of luciferase activities and calculations of relative ratios were done. Quantification of luciferase activities and calculations of relative ratios were done. Columns, mean; bars, SE. Data from six independent experiments (*, $P < 0.01$ by one-way ANOVA analysis).

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To investigate which domain of p53 interacts with Nurr1, we created different deletion mutants of p53 fused to GST (Fig. 4D) and performed GST pull-down assays. As shown in Fig. 4E and F, the full-length and COOH terminus of p53 (amino acids 293-393) interacted with Nurr1, but the NH_2 terminus and the DBD of p53 did not, suggesting that the COOH terminus of p53 interacts with Nurr1. As the COOH terminus of p53 contains the tetramerization domain (amino acids 325-356) for oligomerization, and the tetrameric p53 is the active form for DNA binding or protein-protein interaction (29, 30), we performed competitive binding assays to identify whether Nurr1 influences p53 assembly. Fixed amounts of GST-p53 coupled to glutathione agarose beads were incubated with fixed amounts of His-p53 and increased amounts of Nurr1. His-p53 or Nurr1 that was bound to GST-p53 was detected using immunoblot analysis. As shown in Fig. 4G, increased input of Nurr1 increased the amount of Nurr1 that was bound to GST-p53, but decreased the amount of His-p53 that was bound to GST-p53, suggesting that Nurr1 interferes with p53 self-interactions. To investigate whether a high amount of Nurr1 represses p53 transcriptional activity more effectively, we performed reporter gene assays with different amounts of Nurr1. We cotransfected H1299 cells with pGL(13×p53)-Luci and p53, along with different amounts of Nurr1. As shown in Fig. 4H, the addition of more amounts of Nurr1 resulted in lower luciferase reporter gene expressions being presented, suggesting that Nurr1 represses p53 transcriptional activity in a dose-dependent manner.

Discussion

Nurr1, Nur77, and Nor-1 are orphan nuclear receptors of the NGFI-B subfamily. Nurr1 is highly expressed in the substantia nigra pars compacta and the ventral tegmental area (31). It has been reported that Nurr1 is a transcription factor essential for the development and maintenance of the dopaminergic neurons (32). As members of the NGFI-B subfamily, Nurr1, Nur77, and Nor-1 share some structural and functional similarities. In lung cells, cadmium-induced apoptosis is mediated by Nur77, and apoptosis is reversed by the dominant negative Nur77 (33). It was also reported that Nur77 has an antiapoptotic effect (21, 22). It seems that nuclear Nur77 inhibits apoptosis by its effects on transcriptional regulation (21). However, it mediates apoptosis if it translocates from the nucleus to the cytosol, by inverting Bcl-2 from cell protector to killer, leading to mitochondrial cytochrome *c* release (20).

Recently, it was reported that Nurr1 has antiapoptotic effects. The homozygous Nurr1 mutant ($Nurr1^{-/-}$) mice died soon after birth due to abnormal differentiation of precursory dopamine neurons (34). These results indicate that Nurr1 sustains the survival of dopamine neurons whereas loss of Nurr1 induces consequent death. In cultured HeLa and M14 cells, knockdown of Nurr1 increases the number of apoptotic cells (24). By microarray analyses, overexpression of Nurr1 down-regulates caspase-3 and other apoptotic factors in neural stem cells (35). It was also reported that Nur77 inhibits ceramide-induced up-regulation of Bax (22).

In our present study, we show that Nurr1 interacts with p53 and inhibits p53 transcriptional activity. This interaction is mediated by the DBD of Nurr1 and COOH-terminal of p53 containing the tetramerization domain. Importantly, it has been proven that Nurr1 represses the up-regulation of doxorubicin-induced Bax (Fig. 3C) and protects cells from doxorubicin-induced apoptosis (Fig. 1E). Taken together, we propose that Nurr1, at least in part, survives cells by regulating p53 transcriptional activity in the nucleus.

p53 is a well-known tumor suppressor in cell cycle arrest and cell death, and p53 functions as a transcription factor in complicated cell circumstances. Like other transcription factors, such as a forkhead transcription factor and member of the FH box class O (FOXO3a; ref. 36) and cyclic AMP response element binding protein (CREB; ref. 37), p53 recruits many regulating factors on its COOH terminus or DBD to modulate its transcriptional activity (38, 39). For instance, the physical and functional interaction of PIAS-like protein hZimp10 and p53 has been documented, and it is identified as a coactivator of p53 (40). Brn-3a and Brn-3b have also been shown to interact with p53 and modulate p53 transcriptional activity (41, 42). Our present study shows that Nurr1 interacts with the tetramerization domain of p53 and represses p53 activity by interfering with p53 self-assembly, which is important for the transcriptional activation of p53. Consequently, the decrease of p53 transcriptional activity results in the down-regulation of Bax and the increase of cell viability. It is well known that tumor growth depends on the imbalance between prosurvival and proapoptotic pathways, as we have shown that Nurr1 has protective properties to survive cells by inhibiting p53 transcriptional activity; therefore, it may contribute to tumorigenesis. p53 is not only a tumor suppressor, but has also been identified to be involved in neuronal cell death in the drug-induced mouse model with Parkinsonism (43, 44). Nurr1 is ubiquitously expressed in brain and other tissues. It is therefore possible that Nurr1 plays an important role in the survival of dopamine neurons and other tissue cells by suppression of the p53-Bax pathway.

Materials and Methods

Recombinant DNAs

Full-length p53 constructed in p3×flag-myc-cmv-24, pET-15b, pEGFP-N1, and pGEX-5×-1 were described previously (45). Nurr1 was created by cloning a PCR product, using pCMX-Nurr1 (kindly donated by Dr. Thomas Perlmann) as a template with primers 5′-CAGGATCCCCATGCCTTGTGTTC and 5′-CGCCTCGAGAAAGGTAAAGTGTC, into pGEX-5×-1, pEGFP-C2 or p3×flag-myc-cmv-24. Deletion mutants of p53 encoding amino acids 1 to 101, 102 to 291, and 292 to 393, and deletion mutants of Nurr1 encoding amino acids 1 to 262, 263 to 352, and 353 to 598 were created by subcloning PCR products into pGEX-5×-1. The PCR products were amplified with following primers, respectively: 5′-CGCGGATCCAAATGGAGGAGCCGCAG and 5′-CCGCTCGAGCTTTTCTGGGAAGGGAC for pGEX-5×-1-p53(1-101); 5′-CGGGATCCAAATGTACCAGGGCAGC and 5′-CCGCTCGAGCTTTTCTTGCGGAGATT for pGEX-5×-1-p53(102-291); 5′-CGGGATCCAAATGGAGCCTCACCAC and 5′-CCGCTCGAGTGTTTGTCTGAGTCAGGC for pGEX-5×-1-p53(292-393); 5′-CAGGATCCCCATGCCTTGTGTTC and 5′-GCGCTCGAGCAGCCCCTCGTTGGAG

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for pGEX-5×-1-Nurr1(1-262); 5′-GCGGATCCATCTGTGCG-CTGTGTGTGG and 5′-CGCCTCTGGGCTCTTCGGTTTCG for pGEX-5×-1-Nurr1(263-352); 5′-GCGGATCCATCAG-GAGCCCTCTCCC, and 5′-CGCCTCGAGAAAGG-TAAAGTGTC for pGEX-5×-1Nurr1(353-598).

Cell Culture, Transfection, and Treatment

H1299 cells, A549 cells, HCT116 $p53^{+/+}$, and HCT116 $p53^{-/-}$ cells were cultured in DMEM with 10% fetal bovine serum. N2a cells and HEK293 cells were grown in DMEM with 10% calf serum. The expression plasmids were transfected into cells using LipofectAMINE2000 (Invitrogen). Forty-eight hours after transfection, the transfected cells were collected for immunoblot analyses or immunoprecipitation assays. For doxorubicin (Sigma) treatment, cells were treated with doxorubicin at a concentration of 2 or 4 μmol/L 24 h after transfection. After incubation for 24 or 48 h, the reporter gene assay, immunoblot assays or 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assays were manipulated.

GST Pull-Down Assays

GST or GST-fused proteins expressed from *E. coli* were incubated with 20 μL of glutathione agarose beads (Pharmacia) for 20 min at 4°C. The beads coupled with GST or GST-fused proteins were incubated with HEK293 cell lysates or proteins expressed from *E. coli* for 1 h at 4°C. Bound proteins were subjected to immunoblot analysis with antibodies.

Immunoprecipitation Assays and Immunoblotting Assays

HEK293 cells cotransfected with plasmids expressing EGFP or p53-EGFP and Flag-Nurr1 were collected 48 h after transfection. The supernatants were incubated with monoclonal anti-Flag antibody (Sigma) for 1 h at 4°C. After incubation, protein G Sepharose was used for precipitation. The immunoprecipitants were eluted with SDS sample buffer for immunoblot analysis. Proteins were separated by 12% or 15% SDS-PAGE and then transferred onto polyvinylidene difluoride membranes (Millipore). The following primary antibodies were used: mouse monoclonal anti-Flag antibody and anti–glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody (Chemicon); anti-GFP antibody, anti-p53 antibody, rabbit polyclonal anti-Bax antibodies, and anti-ERK1/2 antibodies (all from Santa Cruz Biotechnology). Sheep anti-mouse or anti-rabbit IgG-HRP (Amersham Pharmacia) was used as the secondary antibody. The proteins were visualized using an ECL detection kit (Amersham Pharmacia).

Luciferase Reporter Gene Assay

H1299 $p53^{-/-}$ cells or HCT116 $p53^{+/+}$ cells were cotransfected with pGL(13×p53)-Luci (reporter construct which contains an array of 13 repeats for p53 binding sites upstream of the luciferase gene) and a fixed amount of Flag-p53 (or EGFP-Nurr1), along with Flag (or EGFP), Flag-Nurr1, or Flag-Nurr1ΔDBD, respectively. Renilla expressing plasmid pRL-CMV was cotransfected to normalize the variations. Total amount of plasmid DNA was kept constant by the addition of empty plasmid. Cell lysates were prepared using Passive Lysis Buffer (Promega) 48 h after transfection. Both Firefly and Renilla activities were measured with Dual Luciferase Reporter Systems using Veritas Microplate Luminometer according to the instructions of the manufacturer. The absolute values of firefly luminescence were normalized to those of Renilla and the ratios were presented as mean ± SE with six transfections.

Nurr1 siRNA Knockdown

Double-stranded oligonucleotides designed against the region starting from nucleotide 1588 of human Nurr1 cDNA were synthesized by Shanghai GenePharma (Shanghai, China). The sequences of these duplexes were sense 5′-GAUUGUAAAUU-GUCUCAAATT-3′, and antisense 5′-UUUGAGACAAUUUA-CAAUCTT-3′. Meanwhile, irrelevant oligonucleotides were used as controls. When cells reached 40% to 50% confluence, the transfection was done with OligofectAMINE (Invitrogen) according to the instructions of the manufacturer. Briefly, a mixture of Opti-MEM medium (Invitrogen) with OligofectAMINE was incubated for 5 min and then incubated with siRNA for a further 30 min at room temperature to allow the complex formation. At 12 h posttransfection, the culture medium was replaced with fresh complete medium. Cells were harvested 72 h after transfection, followed by further analyses.

MTT Assay

HCT116 $p53^{+/+}$ cells or HCT116 $p53^{-/-}$ cells were grown in DMEM with 10% fetal bovine serum in 24-well plates. Twenty-four hours after doxorubicin treatment, the culture medium was refreshed by MEM without phenol red and fresh 3-(4,5)-dimethylthiahiazo(z-y1)-3,5-di-phenytetrazoliumromide (MTT) to a working concentration of 0.5 mg/mL. Three hours after incubation, the reaction was stopped and the formazan crystals dissolved by 0.04 N of HCl-isopropyl alcohol. The absorbance of wells was measured by a photometer at 570 nm with a reference filter of 630 nm. The data were normalized to control and the ratios were presented as mean ± SE with three transfection experiments.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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