Nuclear Import and Export Signals Control the Subcellular Localization of Nurr1 Protein in Response to Oxidative Stress*

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Background: Little is known about the regulation of transcription factor Nurr1.

Results: We identified a bipartite NLS and two NES signals implicated in its subcellular trafficking, and we report that oxidative stress induces nuclear export of Nurr1.

Conclusion: Nurr1 shuttles between the cytoplasm and nucleus, and its subcellular localization is modified by stress.

Significance: Nurr1 subcellular localization will help understand its function under physiopathological conditions.

Orphan receptor Nurr1 participates in the acquisition and maintenance of the dopaminergic cell phenotype, modulation of inflammation, and cytoprotection, but little is known about its regulation. In this study, we report that Nurr1 contains a bipartite nuclear localization signal (NLS) within its DNA binding domain and two leucine-rich nuclear export signals (NES) in its ligand binding domain. Together, these signals regulate Nurr1 shuttling in and out of the nucleus. Immunofluorescence and immunoblot analysis revealed that Nurr1 is mostly nuclear. A Nurr1 mutant lacking the NLS failed to enter the nucleus. The Nurr1 NLS sequence, when fused to green fluorescent protein, led to nuclear accumulation of this chimeric protein, indicating that this sequence was sufficient to direct nuclear localization of Nurr1. Furthermore, two NES were characterized in the ligand binding domain, whose deletion caused Nurr1 to accumulate predominantly in the nucleus. The Nurr1 NES was sensitive to CRM1 and could function as an independent export signal when fused to green fluorescent protein. Sodium arsenite, an agent that induces oxidative stress, promoted nuclear export of ectopically expressed Nurr1 in HEK293T cells, and the antioxidant N-acetylcysteine rescued from this effect. Similarly, in dopaminergic MN9D cells, arsenite induced the export of endogenous Nurr1, resulting in the loss of expression of Nurr1-dependent genes. This study illustrates that Nurr1 shuttling between the cytosol and nucleus is controlled by specific nuclear import and export signals and that oxidative stress can unbalance the distribution of Nurr1 to favor its cytosolic accumulation.

Transcription factor Nurr1 (NOT/NR4A2) together with Nur77 (NGIF-B/NR4A1) and Nor-1 (MINOR/NR4A3) constitute the orphan receptor subfamily of the steroid nuclear hormone receptors (1). These three receptors trans-activate target genes through monomer binding to a consensus nerve growth factor-responsive element (NBRE) sequence (AAAGGTCA) or homodimer binding to the palindromic NurRE sequence (AAAT(G/A)(C/T)CA) (2, 3). Unlike most other nuclear receptors, crystal structure and NMR data indicate that orphan receptors can function as ligand-independent transcription factors because the putative ligand binding domain is occupied by several bulky hydrophobic side chains (4, 5). Nurr1 can also heterodimerize with the 9-cis-retinoic acid receptor to mediate retinoid signaling (6, 7).

Nurr1 participates in a variety of biological processes such as development and survival of dopaminergic neurons (8), regulation of macrophage inflammatory genes (9), and progression of cancer cells by suppressing apoptosis (10–12). Nurr1 is expressed predominantly in the substantia nigra, ventral tegmental area, and limbic areas of the brain and is responsible for acquisition and maintenance of neuronal dopaminergic phenotypes. It controls dopamine metabolism by inducing the expression of tyrosine hydroxylase, monoamine vesicular transporter 2 (VMAT2), l-aromatic amino acid decarboxylase (AADC), etc. (13, 14). In humans, mutations in the Nurr1 gene have been associated with neurological diseases, including Parkinson disease, schizophrenia, and manic depression (14–17). In animal models, homozgyous Nurr1 knock-out mice are lethal due to lack of mesencephalic dopaminergic neurons, which are known to degenerate in Parkinson disease, and heterozygous mice exhibit high vulnerability to the Parkinson-toxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (8, 14, 18). Nurr1 also

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4 The abbreviations used are: NBRE, nerve growth factor-responsive element; AADC, aromatic l-amino acid decarboxylase; CRM1, chromosomal region maintenance 11 EGFP, enhanced green fluorescent protein; NAC, N-acetylcysteine; NES, nuclear export signal; NLS, nuclear localization signal; Nurr1, nuclear receptor related 1; Nur77, nuclear receptor 77; VMAT2, vesicular monoamine oxidase; ANOVA, analysis of variance.
modulates the inflammatory response in midbrain astrocytes and microglia, suggesting a role in protection against these processes that are intimately linked to neurodegeneration (19). Other functions associated with Nurr1 include regulation of osteocalcin in osteoblasts (20, 21), aldosterone synthase in adrenal cortex (22), and aromatase in ovarian granulosa cells (23). Moreover, Nurr1 participates in development of some colorectal, bladder, and lung cancers (24–26). Therefore, a characterization of physiological and pathological mechanisms that regulate Nurr1 transcriptional activity may provide better evidence on the relevance of this transcription factor as a new therapeutic target for these diseases.

Nurr1 is regulated primarily by phosphorylation by ERK or AKT, sumoylation by protein inhibitor of activated STAT-γ, or dimerization with glucocorticoid or retinoid receptors (6, 16, 27–31). However, little is known about a possible mechanism involved in regulation of its subcellular localization under both basal and stimulus-induced conditions. Cytoplasm-nucleus and nucleus-cytoplasm trafficking through nuclear pores is mediated by a variety of nuclear importin and exportin proteins (32) that recognize the NLS (33) or NES (34) on cargo proteins and facilitate their transport in a Ran GTPase-dependent manner. In the case of nuclear export, CRM1 (chromosome region maintenance-1) has been characterized as a nuclear exportin for many transcription factors (35), including Nur77 (36), but so far there is no evidence indicating that Nurr1 might be a cargo for this exportin. Post-translational modifications such as phosphorylation, acetylation, and oxidation may alter the cytoplasmic to nuclear ratios of proteins (3, 19–24). Among these modifications, oxidative stress has been associated with significant alterations in nuclear transport (37) and may be of great relevance in neurodegenerative diseases and other pathologies.

In this study, we have characterized two NLS and two NES, which are responsive to CRM1 in Nurr1. We also provide evidence that oxidative stress provokes nuclear export of Nurr1. These results uncover a new mechanism of regulation of nuclear/cytoplasm shuttling of Nurr1 that explain how oxidative stress participates in loss of Nurr1 transcriptional activity.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Reagents**—Human embryonic kidney (HEK) 293T cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) (Sigma) supplemented with 10% fetal bovine serum (FBS) (Invitrogen) and 80 μg/ml gentamycin. MN9D is an immortalized dopaminergic neuronal cell line derived from mouse mesencephalon. MN9D cells were cultured in high-glucose DMEM, supplemented with 10% FBS and 80 μg/ml gentamycin. Human neuroblastoma SH-SY5Y cells were grown in DMEM containing 10% FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin. Sodium arsenite, leptomycin B, and N-acetylcysteine (NAC) were from Sigma.

**Plasmids**—Vector pCI-HA-CRM1 was provided by Dr. Alan Diehl (University of Pennsylvania, Philadelphia). Vectors pGL3-NBRE3×Luc and pGL3-TkLuc were provided by Dr. Philippe Lefebvre (INSERM Institut Pasteur de Lille, Lille, France). pcDNA3.1-Nurr1-V5His was generated using Nurr1 wild-type fragment from vector pCMX-Nurr1 provided by Dr. Kazuhiro Umesono (Kyoto University, Kyoto, Japan). pCMX-Nurr1 was used as a template to generate Nurr1 PCR insert with oligonucleotides that introduce EcoRV and NotI restriction sites at both ends (Table 1, pcDNA3.1-Nurr1-V5His). Nurr1 NES and NLS mutants were generated with the Gene Tailor site-directed mutagenesis system (Invitrogen) using as template pcDNA3.1-Nurr1-V5His. Oligonucleotides to generate Nurr1 NES1*, Nurr1 NES2*, and Nurr1 NES1/2*, Nurr1 NLS1*, Nurr1 NLS2*, and Nurr1 NLS1/2* mutants are described in Table 1. pEGFP-NES1, pEGFP-NES2, pEGFP-NLS1, and pEGFP-NLS2 were generated introducing sequences of NES1, NES2, NLS1, and NLS2 on pEGFP-C1 with the oligonucleotides shown in Table 1. Forward and complementary oligonucleotides were annealed at a final oligonucleotide concentration of 100 μM at 80 °C for 2 min. The mixture was then slowly cooled to 4 °C with a thermal profile of 1 °C/min. 50 μM of double-stranded oligonucleotide was 5′-end-phosphorylated using T4 polynucleotide kinase (Promega, Fitchburg, WI) with 1 mM ATP. Cloning was performed using restriction enzymes BamHI/BglII.

**Luciferase Assays**—HEK293T cells were seeded in 24-well plates (75,000 cells per well), cultured for 16 h, and transfected with luciferase reporter plasmid pGL3-NBRE3×Luc and pGL3-TkLuc using the calcium phosphate method. After 24 h of transfection, cells were lysed and assayed for luciferase activity with the luciferase assay system (Promega), according to the manufacturer’s instructions. Relative light units were measured in a GLOMAX 96-microplate luminometer (Promega).

**Preparation of Nuclear and Cytosolic Extracts**—MN9D, SH-SY5Y, and HEK293T cells were seeded in p100 plates (2 × 10⁶ cells/plate). HEK293T cells were transfected with calcium phosphate, and after 24 h, cells were treated with 100 μM sodium arsenite for 30, 60, or 90 min. Cytosolic and nuclear fractions were prepared as described previously (38). Briefly, cells were washed with cold PBS and harvested by centrifugation at 1100 rpm for 10 min. The cell pellet was resuspended in 3 pellet volumes of cold buffer A (20 mM HEPES, pH 7.0, 0.15 mM EDTA, 0.015 mM EGTA, 10 mM KCl, 1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 20 mM NaF, 1 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 1 μg/ml leupeptin) and incubated in ice for 30 min. Then the homogenate was centrifuged at 500 × g for 5 min. The supernatants were taken as the cytosolic fraction. The nuclear pellet was resuspended in 5 volumes of cold buffer B (10 mM HEPES, pH 8.0, 0.1 mM EDTA, 0.1 mM NaCl, 25% glycerol, 1 mM phenylmethylsulfonyl fluoride, 20 mM NaF, 1 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 1 μg/ml leupeptin). After centrifugation in the same conditions indicated above, the nuclei were resuspended in loading buffer containing 0.5% SDS. The cytosolic and nuclear fractions were resolved in SDS-PAGE and immunoblotted with the indicated antibodies.

**Immunocytochemistry**—HEK293T cells were seeded in 24-well plates (75,000 cells per well) on poly-d-lys-covered slides, cultured for 16 h, and transfected with calcium phosphate. After 24 h, cells were treated with 100 μM sodium arsenite for 30 or 60 min. Then cells were washed with cold PBS and fixed in 4% paraformaldehyde for 15 min at room temperature. After three 5-min washes with PBS, cells were permeablized with
0.25% Nonidet P-40 for 10 min. The slides were incubated with primary antibodies for 1.5 h at 37 °C in a humidified chamber. Then cells were washed three times with PBS and incubated with secondary antibodies for 45 min under the same conditions. To visualize the nuclei, cells were stained with DAPI. The fluorescence images were captured using appropriate filters in a Leica DMIRE2TCS SP5 confocal microscope (Nussloch, Germany). The lasers used were Ar 488 nm for green fluorescence, Ar/He 543 nm for red fluorescence, and Ar 351 nm/364 nm for UV fluorescence. Densitometric quantification of fluorescence intensity was performed with the ImageJ version 1.46r software. Several confocal pictures taken with a ×40 objective, accounting for a total of 100 cells per experimental condition, were analyzed with two threshold density levels as follows: one for the most fluorescent compartment (nucleus or cytoplasm) and another for the whole cell. These measurements allowed calculation of an average density of nuclear or cytoplasmic fluorescence. The integrated densities of nuclei or cytoplasm were then divided by the integrated densities of the whole cells to obtain the mean percentage of nuclear or cytoplasmic Nurr1 ± S.E. Primary antibodies used in immunocytochemistry were mouse anti-V5 (Invitrogen) and rabbit anti-HA (Abcam, Cambridge, UK). Secondary antibodies were Alexa Fluor 488-conjugated goat anti-mouse IgG and Alexa Fluor 546-conjugated goat anti-rabbit IgG (Molecular Probes).

**Immunoblotting**—The primary antibodies used were anti-V5 (Invitrogen), anti-HA (Abcam), anti-GAPDH (Merck), and anti-Nurr1 (sc-990), anti-actin (sc-1616), and anti-lamin B (sc-6217) (Santa Cruz Biotechnology). Cells were washed once with cold PBS and lysed on ice with RIPA lysis buffer (150 mM NaCl, 25 mM Tris-HCl, pH 7.5, 1% Nonidet P-40, 1% sodium deoxycholate, 0.1% SDS, 1 mM phenylmethylsulfonyl fluoride, 1 mM NaF, 1 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 1 μg/ml leupeptin, 1 mM EGTA). Precleared cell lysates were resolved in SDS-PAGE and transferred to Immobilon-P membranes (Millipore, Billerica, MA). These membranes were analyzed using the primary antibodies indicated above, and the appropriate peroxidase-conjugated sec-

### Table 1

| Plasmid               | Template                | Primers (forward and reverse)                      |
|-----------------------|-------------------------|---------------------------------------------------|
| pCDNA3.1-Nurr1-       | pCDNA3.1-Nurr1-V5His    | 5'-ATAAAAACTGCCAGTGAGCAGCGCGCGAGGCGGAAATGCTTGACTGCTACT-3'  |
| NLS1-V5His            |                         | 5'-GTCCAAGTGGCAGTTTTATTAGCTTAAAC-3'                |
| pCDNA3.1-Nurr1-       | pCDNA3.1-Nurr1-V5His    | 5'-GGGTGCGACGAGCAGGTAAAGGCGCAGGCGGAGGTGTCTTTACCTCGCCGCAGCGAGGACCCAGG-3'  |
| NLS2-V5His            |                         | 5'-TAACGTGCTGGCGAACACCTTCTTTAAAC-3'                |
| pCDNA3.1-Nurr1-       | pCDNA3.1-Nurr1-LS1-V5His| 5'-GGTGGCGACGAGCAGGTAAAGGCGCAGGCGGAGGTGTCTTTACCTCGCCGCAGCGAGGACCCAGG-3'  |
| NLS1/2-V5His          |                         | 5'-TAACGTGCTGGCGAACACCTTCTTTAAAC-3'                |
| pCDNA3.1-Nurr1-NES1-V5His| pCDNA3.1-Nurr1-V5His | 5'-TTGAAACTCGCTTCTCTTAAGAACTTTGTCGCGCGAGCATAGGTA-3'  |
|                         |                         | 5'-TTCCTGGAAGCTTTAGCTTAAACAGGACCCAGGACCCAGGACCCAGGACCCAGGACCCAGG-3'  |
| pCDNA3.1-Nurr1-NES2-V5His| pCDNA3.1-Nurr1-V5His | 5'-GGACCTCCCTTGACACAGAGCGCAGCTTGTACACGGAAAGGGAAGACTTG-3'  |
|                         |                         | 5'-GCCCTTGCGCTGAAAGGGGCTGCAGGATCTTG-3'                |
| pCDNA3.1-Nurr1-NES1-NES2-V5His| pCDNA3.1-Nurr1-V5His | 5'-GGACCTCCCTTGACACAGAGCGCAGCTTGTACACGGAAAGGGAAGACTTG-3'  |
|                         |                         | 5'-GCCCTTGCGCTGAAAGGGGCTGCAGGATCTTG-3'                |
| pEFGP-NES1             | pEFGP-C1                | 5'-GAATTCTGGACACAAGCTTCTCTCGGCCTGCTGCTGCTCAGACTTA-3'  |
|                         |                         | 5'-GAATTCTGGACACAAGCTTCTCTCGGCCTGCTGCTGCTGCTCAGACTTA-3'  |
| pEFGP-NES2             | pEFGP-C1                | 5'-GAATTCTGGACACAAGCTTCTCTCGGCCTGCTGCTGCTGCTCAGACTTA-3'  |
|                         |                         | 5'-GAATTCTGGACACAAGCTTCTCTCGGCCTGCTGCTGCTGCTCAGACTTA-3'  |
RESULTS

Nurr1 Is a Predominantly Nuclear Protein—In preliminary experiments, we analyzed the subcellular distribution of Nurr1 in two dopaminergic cell lines, SH-SY5Y and MN9D, of human and murine origin, respectively. As shown in Fig. 1, A and B, subcellular fractionation assays demonstrated that a majority of Nurr1 is located in the nucleus, and a minor fraction is present in the cytosol in both cell types. We generated a V5-tagged version of mouse Nurr1 to identify and characterize import and export signals. In HEK293T cells transfected with an expression vector for Nurr1-V5, subcellular fractionation assays (Fig. 1C) and immunofluorescence staining (Fig. 1D) indicated that ectopically expressed Nurr1 exhibits a similar distribution as endogenous protein in dopaminergic cells, with a tendency to be located at the nucleus. Densitometric quantification of confocal images of Nurr1-transfected cells further indicated that more than 80% of Nurr1 is localized in the nucleus under basal homeostatic conditions (Fig. 1E).

Identification of Nuclear Localization Signals in Nurr1—Analysis of the Nurr1 sequence tentatively suggested one bipartite nuclear localization signal (NLS) (termed here NLS1) conforms to the consensus structure of typical NLS (ZZX_10–20ZZZ, where Z is Lys or Arg and X is any amino acid), as those found in other nuclear proteins such as nucleoplasmin, p73, and Nrf2 (Fig. 2B) and also in other orphan receptors (Fig. 2C). Moreover, this sequence is conserved among Nurr1 of different vertebrates (Fig. 2D). In addition, we found a putative atypical NLS (termed here NLS2) consisting of scattered Arg and Lys residues in a small stretch of 12 amino acids at positions 338–350, also in the DNA binding domain (LBD), ligand binding domain, and a minor fraction is present in the cytosol in both cell types. We generated a V5-tagged version of mouse Nurr1 to identify and characterize import and export signals. In HEK293T cells transfected with an expression vector for Nurr1-V5, subcellular fractionation assays (Fig. 1C) and immunofluorescence staining (Fig. 1D) indicated that ectopically expressed Nurr1 exhibits a similar distribution as endogenous protein in dopaminergic cells, with a tendency to be located at the nucleus. Densitometric quantification of confocal images of Nurr1-transfected cells further indicated that more than 80% of Nurr1 is localized in the nucleus under basal homeostatic conditions (Fig. 1E).

Identification of Nuclear Localization Signals in Nurr1—Analysis of the Nurr1 sequence tentatively suggested one bipartite nuclear localization signal at positions 287–314, located within the DNA binding domain (Fig. 2A). This putative NLS (termed here NLS1) conforms to the consensus structure of typical NLS (ZZX_10–20ZZZ, where Z is Lys or Arg and X is any amino acid), as those found in other nuclear proteins such as nucleoplasmin, p73, and Nrf2 (Fig. 2B) and also in other orphan receptors (Fig. 2C). Moreover, this sequence is conserved among Nurr1 of different vertebrates (Fig. 2D). In addition, we found a putative atypical NLS (termed here NLS2) consisting of scattered Arg and Lys residues in a small stretch of 12 amino acids at positions 338–350, also in the DNA binding domain (Fig. 2, A and D). This sequence was also conserved in other orphan receptors (Fig. 2C). To determine whether these NLSs are functional, we made point mutants of Nurr1-V5,
where some Lys or Arg residues had been mutated to Ala to generate Nurr1-NLS1*, Nurr1-NLS2*, and Nurr1-NLS1/2* carrying mutations in the putative NLS1, NLS2, or both, respectively (Fig. 3A). HEK293T cells were transfected with these constructs and analyzed by immunofluorescence (Fig. 3B and C) and cell fractionation (Fig. 3D and E). Both approaches indicated that wild-type Nurr1 was mostly nuclear, although all NLS mutants were mostly cytosolic. In additional experiments, HEK293T cells were co-transfected with expression vectors for Nurr1 and Nurr1-NLS mutants and a luciferase reporter construct carrying three tandem sequences of the Nurr1-responsive element (NBRE-3×Luc). As shown in Fig. 3F and G, wild-type Nurr1 activated this reporter in a dose-dependent manner. By contrast, the Nurr1-NLS2* mutant did not activate this reporter even though it was expressed to a similar level as wild-type Nurr1. These results indicate that both regions NLS1 and NLS2 are part of a bipartite NLS and that both sequences are required for nuclear localization of Nurr1.

We made chimeric proteins with EGFP fused at its carboxyl-terminal end with either NLS1 or NLS2 to generate EGFP-NLS1 and EGFP-NLS2 (Fig. 4A). As shown in Fig. 4B and C, in HEK293T-transfected cells, EGFP was localized at both the nucleus and cytoplasm with higher levels in the nucleus. EGFP-NLS1 exhibited a similar distribution as EGFP alone. However, EGFP-NLS2 was strictly nuclear and accumulated in nuclear bodies. These results indicate that although both NLS1 and NLS2 are required to drive Nurr1 to the nucleus (Fig. 3), the NLS2 sequence is strong enough to translocate at least EGFP.

Identification of Nuclear Export Signals in Nurr1—An in silico search for nuclear export signals with the NetNes program (Center for Biological Sequence Analysis, Technical University of Denmark) suggested two putative NES in the Nurr1
sequence at positions 443–445 (NES1) and 568–577 (NES2) (Fig. 5A). Both sequences conform to the consensus motif L(1–3)X(2–3)L where L is Leu and X is any amino acid. These sequences are similar to functional NES found in other proteins subjected to nuclear/cytoplasm shuttling such as IκB-α, TFIIIA, hDM2, p53, p73, PKI-α, and Nrf2 (Fig. 5B). Moreover, both putative NES are conserved in orphan receptors Nur77 and Nor-1 (Fig. 5C) and in Nurr1 from several vertebrates, including human, mouse, rat, Xenopus, and fish (Fig. 5D).

Nuclear export of proteins depends in many cases on exportin CRM1. Therefore, to determine whether the two putative NES sequences are functional, we generated V5-tagged point mutants of Nurr1, where critical Leu and Ile residues had been changed to Ala to generate Nurr1-NES1*, Nurr1-NES2*, and Nurr1-NES1/2* (Fig. 6A). HEK293T cells were co-transfected with expression vectors for these proteins and for HA-tagged CRM1. Cell fractionation (Fig. 6, B–F) and immunofluorescence (Fig. 6, G–K) assays indicated that wild type and point mutants were mostly nuclear. However, in the presence of CRM1, wild-type Nurr1 was redistributed to the cytosol, whereas mutants in NES1, NES2, or both remained mostly nuclear (Fig. 6, F and K). These results indicate that both NES sequences participate in nuclear export of Nurr1.

To further determine the relevance of each NES, we made chimeras of EGFP fused at the carboxyl terminus with either NES1 (EGFP-NES1) or NES2 (EGFP-NES2) (Fig. 7A). HEK293T cells were co-transfected with expression vectors for these proteins and analyzed by immunofluorescence (Fig. 7, B and C). EGFP was mostly nuclear as expected and insensitive to CRM1-mediated export. By contrast, EGFP-NES1 was already cytoplasmic even in the absence of ectopically expressed HA-CRM1, suggesting that this NES is so strong that it can be exported with endogenous exportins. EGFP-NES2 yielded a mixed cytoplasmic and nuclear pattern and required overexpression of HA-CRM1 to be exported efficiently to the cytoplasm. These results indicate that both NESs are functional but that NES1 is the strongest.

Oxidative Stress Provokes Nuclear Exclusion of Nurr1—Oxidative stress affects the subcellular localization of various proteins. Therefore, we determined whether the localization of Nurr1 is susceptible to regulation by sodium arsenite, which interacts with sulfhydryl groups, depletes glutathione, and is a well established inducer of oxidative stress (40). HEK293T cells were transfected with V5-tagged wild-type Nurr1. After transfections, cells were maintained in serum-free medium for 16 h and then treated with sodium arsenite (100 μM). Under these conditions, Nurr1 was redistributed to the cytoplasm, whereas untreated cells were mostly nuclear (Fig. 8A). These results indicate that oxidative stress provokes nuclear exclusion of Nurr1.

With the aim of determining whether the localization of Nurr1 is susceptible to regulation by sodium arsenite, HEK293T cells were transfected with V5-tagged wild-type Nurr1. After transections, cells were maintained in serum-free medium for 16 h and then treated with sodium arsenite (100 μM). Under these conditions, Nurr1 was redistributed to the cytoplasm, whereas untreated cells were mostly nuclear (Fig. 8A). These results indicate that oxidative stress provokes nuclear exclusion of Nurr1.

**FIGURE 4.** NLS2 but not NLS1 is sufficient to confer nuclear import capacity. A, fusion proteins used for analysis of the subcellular localization of EGFP. Bar indicates 10 μm. C, densitometric quantification of the distribution of Nurr1 from representative cell fields according to B. Values correspond to the mean percentage of nuclear fluorescence ± S.E. (n = 100 cells). ***, p < 0.001 versus EGFP as determined with a Student’s t test.**

**FIGURE 5.** Putative NES1 and NES2 sequences in Nurr1. A, primary structure of mouse Nurr1 depicting amino acid sequences between residues 443–452 and 568–577 corresponding to putative NES1 and NES2, respectively. B, comparison of putative NES1 and NES2 from mouse Nurr1 with NESs from other nuclear proteins. C and E, comparison of putative NES1 and NES2 from Nurr1 with other mouse orphan receptors. D and F, phylogenetic conservation of putative NES1 and NES2. The highlighted residues correspond to the putative sites that conform to the consensus sequence for an NES.
FIGURE 6. CRM1 requires NES1 and NES2 to export Nurr1. A, outline of the amino acid substitutions in Nurr1-NES1* and Nurr1-NES2*. B–E, HEK293T cells co-transfected with expression vectors for HA-tagged CRM1 and either V5-tagged wild-type Nurr1 (B), Nurr1-NES1* (C), Nurr1-NES2* (D), and Nurr1-NES1/2* (E). F, densitometric quantification of protein levels from representative blots like those in B–E. Cytosolic and nuclear fractions were normalized with β-actin and lamin B, respectively, and then represented as fold of change versus the Nurr1 group without CRM1. Data are means ± S.E. (n = 3). *** p < 0.001 versus the group of nuclear Nurr1 without transfection of CRM1 according to a Student’s t test. G–J, immunofluorescence staining of HEK293T cells transfected with the indicated expression vectors. Bar indicates 10 μm. K, densitometric quantification of the subcellular distribution of Nurr1-NES mutants from representative cell fields according to G–J. Values correspond to the mean percentage of nuclear fluorescence ± S.E. (n = 100 cells). *** p < 0.001 versus the group of nuclear Nurr1 without transfection of CRM1 as determined with a Student’s t test.
conditions, sodium arsenite induced a time-dependent translocation of Nurr1 to the cytosol that was abrogated when cells were preincubated with the oxidant blocker NAC (10 mM, 3 h) (Fig. 8, A–C). Consistently, arsenite reduced the transcriptional activity of Nurr1 toward the NBRE-driven luciferase reporter, and NAC reversed this constraint (Fig. 8D). These results suggest that oxidative stress induces the nuclear exclusion of Nurr1, either by preventing import or accelerating export, resulting in loss of its transcriptional activity.

NES2 Participates in the Redox-dependent Export of Nurr1—To analyze in more detail these two possibilities, we compared the subcellular distribution of V5-tagged wild-type Nurr1 and the NES mutants Nurr1-NES1*, Nurr1-NES2*, and Nurr1-NES1/2* following 30, 60, and 90 min of incubation with arsenite (Fig. 9). Sodium arsenite induced a time-dependent translocation to the cytosol of wild-type Nurr1-V5 (Fig. 9, A and E) and Nurr1-NES1* (Fig. 9, B and F). By contrast, Nurr1-NES2* (Fig. 9, C and G) and Nurr1-NES1/2* (Fig. 9, D and H) were unresponsive to sodium arsenite. These results indicate that oxidative stress favors export of Nurr1 through the use of the NES2 sequence.

To gain further insight into this effect, we analyzed the subcellular distribution of the same mutants by immunofluorescence (Fig. 10, A–D and E). Wild-type Nurr1-V5 redistributed to the cytosol after treatment with sodium arsenite (100 μM, 60 min). Similarly, Nurr1-NES1*, harboring NES2 but not NES1, also responded to sodium arsenite, rendering Nurr1 cytosolic. By contrast, Nurr1-NES2* and NES1/2*, both lacking NES2, were resistant to sodium arsenite–induced translocation. Hence, these results indicate that NES2, but not NES1, is sensitive to redox regulation.

Nuclear Export of Nurr1 Results in Loss of Dopaminergic Phenotype—The functional relevance of arsenite-induced changes in the subcellular localization of Nurr1 was addressed in the dopaminergic cell line MN9D. As shown in Fig. 11, A and C, in this cell line endogenous Nurr1 redistributed to the cytosol in response to arsenite with similar kinetics as found for ectopically expressed Nurr1-V5 in HEK293T cells. Moreover, this change was prevented by preincubating the cells with the CRM1 inhibitor leptomycin B, further demonstrating that the

FIGURE 7. NES1 and NES2 are sufficient to confer export capacity to EGFP. A, fusion proteins used for analysis of the subcellular localization of EGFP. B, subcellular localization of EGFP fusion proteins in HEK293T cells co-transfected with HA-tagged CRM1 and EGFP and EGFP-NES1 and EGFP-NES2 as indicated. Bar indicates 10 μm. C, densitometric quantification of the subcellular distribution of EGFP fusion proteins from representative cell fields according to B. Values correspond to the mean percentage of nuclear fluorescence ± S.E. (n = 100 cells). ***p < 0.001 versus EGFP without transfection of CRM1. ###p < 0.001 versus EGFP with transfection of CRM1, as determined with a Student’s t test.

FIGURE 8. Sodium arsenite induces Nurr1 nuclear export that is reversed by NAC. HEK293T cells were transfected with expression vector for Nurr1-V5, serum-starved for 16 h, and treated with vehicle (0.1% ethanol) or NAC (3 h, 10 mM). Then cells were treated with sodium arsenite (As, 100 μM) for the indicated times. A and B, protein blots of cytosolic and nuclear fractions of cells treated with vehicle or NAC, respectively. C, densitometric quantification of cytosolic and nuclear protein levels from representative blots of A and B. Cytosolic and nuclear fractions were normalized with GAPDH and lamin B, respectively, and then represented as fold of change versus the group at 0 min with vehicle. Values correspond to the mean ± S.E. (n = 3). Statistical analysis was performed with one-way ANOVA followed by Newman-Keuls multiple comparison test. ***p < 0.001 versus nuclear Nurr1 at time 0 min. ###p < 0.001 versus cytosolic Nurr1 at time 0 min. D, effect of sodium arsenite and NAC on the Nurr1-dependent regulation of NBRE-3Luc. Cells were co-transfected with expression vector for Nurr1 and either pGL3-NBRE3Luc or pGL3-TkLuc as a control. Then cells were placed in serum-free medium and pretreated with vehicle or NAC (1 h) prior to addition of sodium arsenite. After 16 h, cells were analyzed for luciferase activity. Each point is the mean ± S.E. (n = 3). Statistical analysis was performed with a two-way ANOVA followed by Bonferroni post hoc test. *p < 0.05 and **p < 0.001 comparing vehicle versus 5 mM NAC-treated groups. #p < 0.01 and ##p < 0.001 comparing vehicle versus 10 mM NAC-treated groups.
redistribution is a consequence of accelerated export (Fig. 11, B and C). Finally, we analyzed in this dopaminergic cell line the expression of three Nurr1-regulated genes, namely tyrosine hydroxylase, vesicular monoamine transporter 2 (VMAT2), and AADC. As shown in Fig. 11, D–F, the messenger RNA levels of these three genes dropped to about 50% after 6 h in the presence of arsenite. Taken together, these results indicate that oxidative stress promotes the nuclear export of Nurr1 resulting in reduction of Nurr1-regulated gene expression and loss of the dopaminergic phenotype.

DISCUSSION

The regulation of transcription factor Nurr1 is ill-defined. Like the other orphan nuclear receptors, it appears to function independently of ligands. So far, Nurr1 has been reported to be regulated by dimerization and post-translational modifications (41, 42). In this study, we report for the first time that Nurr1 is also regulated at the level of subcellular localization. Nurr1 has a bipartite NLS, which we termed NLS1/NLS2, that is implicated in its nuclear localization. We also report the identification of two NES signals involved in its nuclear export to the cytosol. It is not clear why Nurr1 contains more than one NLS and one NES. However, several other proteins enclose multiple import and export signals (43). In connection with the orphan receptors, it has been reported that Nur77, a related member of the orphan receptor family, possesses two NLSs and three NES signals that are implicated in its redistribution from the nucleus to the cytoplasm (44). Of note, it has been reported recently that Nur77 is exported from the nucleus by the exportin CRM1 (36). The presence of several regulatory signals may confer superior versatility to integrate alternative mechanisms of subcellular regulation.

Nurr1 presented a predominantly nuclear localization, although there was a minor fraction in the cytosol. This led us to identify two NES signals that respond to the exportin CRM1, which we termed NES1 and NES2. These two NES appear to be conserved in other orphan receptors (44). It has been reported that stimulation with NGF induces export of the Nur77/retinoic acid receptor dimer to the cytosol by using this NES (44). Nurr1 mediates the expression of a battery of genes involved in dopaminergic cell differentiation, modulation of inflammation, and cytoprotection (14, 19, 45). Such events are down-regulated by oxidative stress. Therefore, we speculated that oxidant conditions might inhibit Nurr1 transcriptional activity. In this study, we found that arsenite, a typical inducer of oxidative stress that depletes glutathione levels (40), led to nuclear export of Nurr1. Indeed, NES2 acted as a redox sensor. In fact, this effect was abrogated by the oxidant blocker NAC.

Our results demonstrating arsenite-induced nuclear export of Nurr1 are consistent with a recent report showing cytoplas-
mic localization of Nurr1 in glutamate-treated neurons (46). Moreover, it has been reported that Nurr1 exhibits a cytoplasmic localization in some bladder tumors (26). Although those studies did not explore the molecular mechanisms involved in nucleus-cytoplasm shuttling of Nurr1, they provide further evidence on pathological mechanisms that lead to the nuclear exclusion of Nurr1.

In agreement with the response of Nurr1 to sodium arsenite as reported here, Nur77 is translocated from the nucleus to the cytosol by H2O2 treatment in HEK293 cells, as well as by 6-hydroxydopamine in dopaminergic SH-SY5Y cells (47). Similar results have been found for Nor1, the other orphan receptor member (46). Most likely, the mechanism of export in response to oxidative stress is conserved in these proteins and involves the use of the conserved NES (47). The molecular mechanisms that convert this NES in a redox sensor for nuclear export are not clear. One possibility is the participation of nearby cysteines that, through disulfide bonding, might induce conformational...
changes that expose NES2. This has been suggested for the transcription factor Nrfr2 (48). However, we have not found cysteine residues that could likely participate in such a regulatory mechanism. Alternatively, phosphorylation of critical residues might increase the affinity of NES2 for CRM1. Indeed, it has been reported that CRM1 together with Ran-GTP participates in the export of several phosphoproteins (49), and evidence indicates that Nurr1 is regulated by phosphorylation (16). Further work will be required to determine how phosphorylation and other post-translational modifications of Nurr1 govern its subcellular localization.

Nuclear export of Nur77 leads to its mitochondrial translocation (50). However, we have not detected Nur1 in mitochondria (data not shown), and most likely this function of Nur77 is related to sensitization of cells to oxidative stress-induced cell death (47, 50). In the case of Nur1, we propose that nuclear export is just a mechanism to shut down the expression of its target genes.

We speculate that this switch-off system could play an important role in the pathogenesis of several neurodegenerative disorders like Parkinson disease, where oxidative stress is very relevant. In fact, two genetic variants of Nurr1 (deletion of Thr-291 and point mutation T245G) have been described in patients with Parkinson disease (51). These mutations result in a marked decrease in Nurr1 mRNA levels and subsequent loss of the dopaminergic phenotype. In line with this hypothesis, MN9D cells lost their Nur1-dependent gene expression in response to arsenite-induced oxidative stress. Future studies will be required to determine whether oxidant inhibition of the nuclear localization of Nur1 may participate in loss of dopaminergic phenotype in pathologies like Parkinson disease or contribute to tumor development.

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