Multiple signaling pathways regulate the transcriptional activity of the orphan nuclear receptor NURR1

Paola Sacchetti*, Rodolphe Carpentier, Pascaline Ségard, Cécile Olivé-Cren1 and Philippe Lefebvre

INSERM U459, Faculté de Médecine de Lille, 59045 Lille, France and 1UMR CNRS 8009, LCOM—IFR 118, 59655 Villeneuve d’Ascq cedex, France

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ABSTRACT

The orphan nuclear receptor nurr1 (NR4A2) is an essential transcription factor for the acquisition and maintenance of the phenotype of dopamine (DA)-synthesizing neurons in the mesencephalon. Although structurally related to ligand-regulated nuclear receptors, nurr1 is functionally atypical due to its inability to bind a cognate ligand and to activate transcription following canonical nuclear receptor (NR) rules. Importantly, the physiological stimuli that activate this NR and the signaling proteins that regulate its transcriptional activity in mesencephalic neurons are unknown. We used an affinity chromatography approach and CSM14.1 cells of mesencephalic origin to isolate and identify several proteins that interact directly with nurr1 and regulate its transcriptional activity. Notably, we demonstrate that the mitogen-activated protein kinases, ERK2 and ERK5, elevate, whereas LIM Kinase 1 inhibits nurr1 transcriptional activity. Furthermore, nurr1 recruits ERK5 to a NBRE-containing promoter and is a potential substrate for this kinase. We have identified amino acids in the A/B domain of nurr1 important for mediating the ERK5 activating effects on nurr1 transcriptional activity. Our results suggest that nurr1 acts as a point of convergence for multiple signaling pathways that likely play a critical role in differentiation and phenotypic expression of dopaminergic (DAergic) neurons.

INTRODUCTION

Neurons of the ventral midbrain area synthesizing the neurotransmitter dopamine (DA) are implicated in disease states where dopaminergic (DAergic) neurotransmission is deregulated. Parkinson’s disease is a result of a severely reduced DAergic tone due to the degeneration of nigrostriatal neurons. In contrast, elevated DAergic signaling is observed in attention-deficit hyperactivity disorders and drug addiction (1). Although, these disorders have tremendous impact on human health, their aetiologies are still unclear and therapeutic tools remain to be developed.

The transcription factor nurr1 [NR4A2; (2)] is highly expressed in DAergic neurons of the midbrain (3) and knock-out experiments in mice revealed that nurr1-mediated transcriptional activities are limited primarily to the midbrain DA neurons (4–6). Nurr1 is thought to play a key role in maintenance of a DAergic phenotype via regulation of DA neuron-specific genes [tyrosine hydroxylase (7–9); DA transporter (DAT) (10,11)]. It also promotes survival of DA neurons by protecting them from toxic insults (12). Thus, nurr1 serves as a DA neuron-specific transcription factor whose activities could be regulated to modulate DAergic neurotransmission in Parkinson’s disease and other disorders.

Nurr1 is an atypical member of the nuclear receptor (NR) superfamily comprising mostly ligand-activated receptors, like glucocorticoid, estrogen and retinoic acid receptors, which regulate gene expression via recognition of specific DNA-binding sequences (13). Nurr1 shares with other NRs a common structural organization: the N-terminus contains an activation function 1 (AF1) involved in ligand-independent transcriptional activity, and is the target for regulatory post-translational modifications (14). The central region of the protein consists of the highly conserved DNA-binding domain

*To whom correspondence should be addressed at Molecular Neurobiology, MBB, Karolinska Institutet, Stockholm, Sweden. Tel: +46 8 5248 7656; Fax: +46 8 341960; Email: paola.sacchetti@ki.se

Present address:
Rodolphe Carpentier and Philippe Lefebvre, INSERM U545, Lille; Institut Pasteur de Lille, Département d’Athérosclérose, 59019 Lille, France

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(DBD) that allows recognition of specific DNA sequences [hormone response elements (HREs)]. In the C-terminus, generally identified as the ligand binding domain (LBD), are located the dimerization interfaces for homo- and/or heterodimerization between NRs, the ligand binding pocket for the cognate ligand and the AF2, a highly conserved module that confers the ability to recruit co-activators (CoAs) and to induce transcriptional activity in response to agonists [reviewed by (13)].

Upon binding of a cognate ligand, NRs undergo a conformational change inducing the loss of interaction with represive proteins [known as co-repressors—CoRs; reviewed by (13)] in favor of CoAs, promoting transcriptional activation. Unlike other NRs, the amino acid sequence of the AF2 domain of nurr1 is not conserved (15) and its ability to trans-activate reporter genes appears to be constitutive (15,16). This receptor does not possess a typical ligand binding pocket. In fact, crystallographic studies show that nurr1 assumes the conformation of an active receptor even in the absence of a ligand and is thought to lack a classic CoA binding interface (17). In addition, the nurr1 DBD recognizes an extended HRE (NBRE—AAAGGTCA) and consequently can trans-activate genes acting also as a monomer, unlike other NRs that require formation of homo- or heterodimers (15,16,18).

Experiments reported to date show that nurr1 is an atypical NR whose transcriptional activity is independent of its interaction with classical NR CoAs, such as p160-related factors, CBP/p300 or the mediator complex. Only the rexinoid receptor RXR and the repressor PIAS tors, CBP/p300 or the mediator complex. Only the rexinoid receptor RXR and the repressor PIAS appear to be constitutive (15,16). This receptor does not possess a typical ligand binding pocket. In fact, crystallographic studies show that nurr1 assumes the conformation of an active receptor even in the absence of a ligand and is thought to lack a classic CoA binding interface (17). In addition, the nurr1 DBD recognizes an extended HRE (NBRE—AAAGGTCA) and consequently can trans-activate genes acting also as a monomer, unlike other NRs that require formation of homo- or heterodimers (15,16,18).

Here, we report the screening of immortalized CSM14.1 mesencephalic cells and the consequent identification of novel regulators of nurr1 activity. Notably, we establish that several protein kinases, which belong to signaling pathways known to regulate neuronal differentiation and cell survival, are capable of modulating nurr1 transcriptional activity. Our results suggest that these novel modulators are potentially responsible for regulating nurr1-mediated processes in DAergic neurons, such as gene expression and neuroprotection.

MATERIALS AND METHODS

Plasmids

Glutathione S-transferase (GST)–nurr1 fusion proteins were constructed using PCR-generated fragments of mouse pCMX-nurr1 subcloned into pDEST15 plasmid (Gateway) following manufacturer’s instructions. The GST–LBD construct coded for aa 327–598, while a Smal restriction of GST-A/B domain (1–262 amino acid) was used to subclone plasmid GST-A/BA183 (1–183 amino acid). Constructs were verified by automated sequencing and western blot. Plasmids NBre3xKLuc, pCMX-nurr1 and pCMX- nurr1AF2 have been described elsewhere (16), while the HA-tagged full-length human nurr1 was kindly donated by M.N. Castel (Aventis Pharma). Nurr1 mutants ΔA[Q7-P58], ΔB[151-P127], ΔC[Y121-P169], ΔD[R166-Q180], ΔE[L368-W420], ΔF[426-G467], ΔH[Q473-R515], ΔI[K522-Q571], S89A, T168A and S177A were obtained by site-directed mutagenesis of plasmid pCMX-nurr1 using the Quick Change Kit (Stratagene) following manufacturer’s instructions. Following the same procedures, pCMV-HA-ERK5 (obtained from J. Dixon) was mutated by site-directed mutagenesis to obtain the kinase mutant forms, pHA-ERK5 AEF and pHA-ERK5 K83M. Wild-type (wt) ERK2 (pHA-ERK2), its constitutively active form (pCMV5-ERK2 L73P-His) and the catalytic inactive form (pCMV-HA-ERK2 K52R) were kindly provided by R. Davis, M. A. Emrick and P. Crespo, respectively. The following plasmids were kind gifts: GST-LIM (I. Briche) coding for a LIM domain; pcDNA3-LIM-Kinasel (LIMK1) (G. N. Gill) and pcDNA3-HA-LIMK1 kinase domain (H. Betz) coding, respectively for full-length wt and the kinase domain only of LIMK1; pEBG-BMK1, pFlag-BMK1/ERK5 wt and AEF (J. D. Lee); pMyC-HA-MEK5 wt, dominant negative (dn) (K106M) and constitutively active (ca) (DD; M. Cobb) and ca RasL61 (R. Davis).

RT–PCR

Total RNA extraction and nurr1 amplification using specific primers were carried out as described previously (16).

Cell lines and transfections

PC12 cells were grown and transfected as described previously (16). For statistical analysis, ANOVA and post tests were performed using GraphPad prism software. CSM14.1 cells (21) were grown at 33°C in 5% CO2 humidified atmosphere in DMEM high glucose (Invitrogen) containing 10% FBS (BioWhittacker) and supplemented with 2 mM l-glutamine, 1000 U/ml penicillin and 100 μg/ml streptomycin (Invitrogen). For IPs, cells were plated (3.5 × 10^6 cells) in 10 cm plates and transfected with LipofectAMINE reagent (Invitrogen).

Preparation of CSM14.1 nuclear extracts—5 × 10^8 CSM14.1 cells were washed with phosphate-buffered saline (PBS) and harvested by trypsin. Pellet was resuspended in 4X Packet Cell Volume (PCV) buffer A [20 mM Tris–HCl (pH 7.9), 10 mM KCl, 0.2 mM EDTA, 0.2 mM EGTA, 1.5 mM MgCl2, 350 mM sucrose, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM DTT, antiprotease cocktail (Sigma)] and cells were allowed to swell on ice for 15 min. After centrifugation, pellet was resuspended in 2 PCV buffer A and dounced 15 times with pestle B. After centrifugation, the nuclear pellet was resuspended in 0.5 PCV buffer A, 20% glycerol and then 0.4 PCV buffer B [20 mM Tris–HCl (pH 7.9), 1 M KCl, 0.5 mM PMSF, 1 mM DTT and antiprotease cocktail] was added dropwise. Nuclear fraction was incubated for 45 min with gentle rocking at 4°C, centrifuged and dialyzed overnight at 4°C against buffer C [20 mM Tris–HCl (pH 7.9), 100 mM KCl, 0.2 mM EDTA and 20% glycerol]. The extract was centrifuged and protein concentration was estimated by the Bradford Assay. Enrichment of the nuclear fraction was verified by using specific antibodies against nuclear proteins (TFIIB, RXR, SRC-1 and PARP-1) and no contamination by these proteins was observed in the cytosolic fraction.
GST–pulldown assays

GST–LIM fusion protein was expressed in *Escherichia coli* BL21(DE3)pLysS following induction with 0.1 mM isopropyl-β-D-galactopyranoside (IPTG) for 3 h at 20°C. Soluble bacterial lysates were added to glutathione Sepharose beads (Amersham Biosciences), while 10 µg of the purified GST–ERK2 fusion protein (Cell Signaling Technology, Inc.) were added to glutathione Sepharose beads. GST–ERK5 fusion protein was produced by transfecting CSM14.1 cells with pEBG-BMK1/ERK5 and protein extraction was performed as in co-immunoprecipitation and added to glutathione Sepharose beads. For binding assays, GST fusion proteins bound to the Sepharose matrix were incubated with 35S-labeled proteins (TNT Quick coupled system, Promega) for 3 h with gentle rocking at 4°C. GST–pulldown assays were performed as in co-immunoprecipitation and added to glutathione Sepharose beads. For binding assays, GST fusion proteins bound to the Sepharose matrix were incubated with 35S-labeled proteins (TNT Quick coupled system, Promega) for 3 h with gentle rocking at 4°C in GST-binding buffer [20 mM Tris–HCl (pH 7.9), 180 mM KCl, 0.2 mM EDTA, 0.05% NP-40, 0.5 mM PMSF and 1 mM DTT] containing 1 mg/ml BSA. After extensive washing, resine-bound proteins were resolved by SDS–PAGE. Gels were dried and exposed for quantification with a Storm 860 phosphorimager (Molecular Dynamics). Binding values obtained were normalized against the input in each gel and values (n = 3–5) were averaged and standardized compared to wt binding (100%). For pulldown assays with nuclear extracts, GST and GST-nurr1 proteins underwent double purification after which purity and quantification of GST fusion protein were estimated by silver nitrate staining. Binding with CSM14.1 nuclear extracts was performed as described previously (22) overnight at 4°C and samples were resolved by 6–15% gradient SDS–PAGE. Following Colloidal Blue staining, proteins were analyzed by mass spectroscopy.

Immunoblotting

GST-pulldown samples obtained using nuclear extracts were resolved on 9% SDS–PAGE and transferred onto nitrocellulose membranes (Hybond-C, Amersham Biosciences). Immunodetections were carried out as described previously (16) using antibodies directed against RXRα (sc-553; Santa Cruz Biotechnology), ERK (610 123; BD Biosciences Pharmingen) and horseradish peroxidase (HRP)-conjugated secondary antibodies (Sigma).

Co-immunoprecipitations and DNA-IP assays

CSM14.1 cells were transfected with a HA-tagged nurr1 encoding plasmid and a second plasmid encoding for the appropriate partner when necessary (see figure legend). Cells were harvested in IP lysis buffer [20 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β-Glycerolphosphate, 1 mM Na₂VO₄ and 1 µg/ml Leupeptin], Whole-cell extracts (1 mg) were incubated overnight at 4°C with 25 µl anti-HA agarose conjugated beads (A-2095; Sigma). After extensive washing, samples were resuspended in sample buffer and resolved by SDS–PAGE. Detection of HA-nurr1 was determined by immunoblotting using an anti-HA antibody (H-9658; Sigma), while the co-immunoprecipitated proteins were revealed using antibodies raised against ERK, BMK1/ERK5 (07–039; Upstate) and LIMK1 (sc-5576).

For DNA-IP assays, CSM14.1 and PC12 cells were transfected with HA-nurr1 and pGL3-NBRE3xtkLuc or pGL3-tk-Luc plasmids. After 36 h of post-transfection, the cells were washed in PBS containing 1 mM MgCl₂ and fixed in formaldehyde (1% final concentration) for 15 min at 37°C, and the reaction was stopped by addition of 200 mM glycine at room temperature. Cells were washed twice in PBS/MgCl₂ and scrapped in 1 ml ice-cold PBS. Cells were lysed in 0.6 ml lysis buffer [1% SDS, 10 mM EDTA, 50 mM Tris–HCl (pH 8.1), 0.5 mM PMSF and 1× protease inhibitors (Sigma)]. DNA was sheared by sonication and cell debris were eliminated by centrifugation. Supernatant was diluted 10-fold in ChIP buffer [0.01% SDS, 1.1% Triton X-100, 2 mM EDTA, 20 mM Tris–HCl (pH 8.1), 150 mM NaCl and protease inhibitors] followed by immunoclearing with 100 µl of 50% protein A-agarose slurry (16–125, Upstate) for 4 h at 4°C. Immunoprecipitations were performed overnight at 4°C using 2–4 µg of specific antibodies [anti-nurr1 (sc-990, Santa Cruz) and anti-BMK1/ERK5 (Upstate)]. Complexes were collected by incubation at 4°C for 2 h with 40 µl of 50% protein A-agarose slurry in ChIP buffer plus 100 µl salmon sperm DNA and 2 mg ovalbumin per ml. Beads were washed twice with low salt buffer [0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris–HCl (pH 8.1) and 50 mM NaCl], once with LiCl Buffer [0.25 M LiCl, 1% IGEPAL-CA630, 1% sodium deoxycholate, 1 mM EDTA and 10 mM Tris–HCl (pH 8.1)] and twice with TE Buffer [10 mM Tris–HCl (pH 8.1) and 1 mM EDTA]. Immune complexes were eluted overnight at 65°C in elution buffer containing 0.1 M NaHCO₃ and 1% SDS. DNA was purified with the QIAquick Spin kit (Qiagen) and amplified by PCR using RVprimer3 and GLprimer2 (Promega) as upstream and downstream primers, respectively. Conditions were optimized to ensure linearity of the PCR.

In vitro phosphorylation assays

GST fusion proteins (A/BΔ183 and GST only) were expressed in *E.coli* and purified as described above. CSM14.1 cells were lysed in IP lysis buffer and 400 µg of total proteins were incubated overnight at 4°C with an anti-ERK5 antibody followed by addition of 60 µl of a 50% slurry of Protein A-agarose for 2 h. Immunoprecipitates were incubated with 1 µg of purified GST fusion proteins and 5 µCi of [γ-32P]ATP in 50 µl of kinase buffer [25 mM Tris–HCl (pH 7.5), 5 mM β-Glycerolphosphate, 2 mM DTT, 0.1 mM Na₂VO₄ and 10 mM MgCl₂] for 30 min at 30°C. Kinase reactions were terminated by addition of SDS sample buffer and samples were resolved by SDS–PAGE. Equal loading of fusion proteins was verified by Coomassie blue staining of the SDS gel.

RESULTS

Isolation of nurr1-interacting proteins from CSM14.1 mesencephalic cells

Preliminary studies using *in vitro* pulldown assays showed that the NR nurr1 does not interact with most known NR CoAs [P. Sacchetti and P. Lefebvre, unpublished data; (17)]. These data, in addition to the observation that nurr1
transcriptional activity is cell type-specific, suggested that nurr1 might interact with tissue-specific regulators present only in nurr1-expressing cells. To identify novel modulators of nurr1 activity, we examined a cell type closely related to DAergic cells. The immortalized cell line, CSM14.1, derived from primary foetal rat mesencephalic cells, has been shown previously to express tyrosine hydroxylase and nurr1 (23). Nurr1 mRNA was expressed at high levels in CSM14.1 cells and modulated by forskolin as reported previously [Supplementary Figure 7; (24)], while it was not endogenously expressed in PC12 cells. We tested the ability of nurr1 to increase expression levels of a reporter construct encoding for a luciferase gene under the control of three canonical nurr1 binding sites [NBRE3xkLuc; (16)]. In CSM14.1 cells, nurr1 was able to activate transcription of a reporter construct, while nurr1-expressing cells of mesencephalic origin express a functional nurr1 protein as well as its transcriptional partners.

We set up an affinity chromatography technique to identify nurr1-interacting proteins in nuclear extracts of CSM14.1 cells. We engineered two nurr1 bait proteins (Figure 1A), one coding for the N-terminus including the AF1 domain (GST-A/BΔ183; 1–183 amino acid), and the other for the C-terminus including the AF2 domain (GST–LBD; 327–598 amino acid). We then performed GST-pulldown assays by co-incubating the GST fusion proteins and CSM14.1 nuclear extracts, and the bound proteins were resolved by SDS–PAGE. Evaluation of the differential protein profiles retained by the three GST fusion proteins was first performed by silver stained gradient gels (Figure 1B). Only few bacterial contaminants were retained non-specifically by the GST bait alone or GST-nurr1 proteins in the absence of nuclear extracts. Subsequently, identical experiments were performed and the corresponding gels were Colloidal blue stained for MALDI-TOF/MS mass spectroscopy analysis (Supplementary Figure 8). Bands of interest representing proteins that were specifically retained by the nurr1 fusion proteins were excised from Colloidal blue stained gels (corresponding arrow heads, shown on silver nitrate stained gel; Figure 1B) and analyzed by MALDI-TOF/MS mass spectroscopy for identification of protein fragments as detailed in Supplementary Data. Proteins identified by mass spectroscopy analysis were classified according to their functional role, as shown in Supplementary Table 1. Strikingly, a large number of the factors identified were members of signaling pathways (ERK, Akt and LIMK1) and several were transcription factors, most of which were NRs [RXRα, peroxisome proliferator-activated receptor γ (PPARγ) and farnesoid X receptor (FXR)]. We also identified potential repressors NR co-repressor 1 (NCoR-1 and Mxi1) and a protein implicated in DNA repair mechanisms (PARP-1). Several of the potential interactions were subsequently confirmed through independent in vitro experiments (Supplementary Figure 9) and thus, proteins like Poly (ADP-ribose) Polymerase-1 (PARP-1), NCoR-1, PPARγ, glucocorticoid receptor (GR; R. Carpentier and P. Lefebvre, manuscript in preparation), FXR and the transcription factor signal transducer and activator of transcription (STAT3) were identified as new potential transcriptional partners of nurr1.

**Figure 1.** Identification of nurr1-interacting proteins in cells of mesencephalic origin. (A) Schematic representation of the N- and C-termini of nurr1 fused to GST and used as baits in GST-pulldown experiments. (B) Silver nitrate staining of pulldown samples obtained after incubation of immobilized GST–nurr1 fusion proteins with (+) or without (−) CSM14.1 nuclear extracts (1.5 mg). GST alone was used as control for non-specific interactions. Samples were separated on 6–15% gradient one-dimension (1D) gels before staining. Multiple identical experiments (n = 7) were performed and analyzed by 1D gel in strictly comparable conditions. Gels with similar electrophoretic profiles (n = 4) were used to excise Colloidal blue stained protein bands which were further analyzed by mass spectroscopy after tryptic digestion. A representative silver stained gel is shown and arrows correspond to bands of interest excised from Colloidal blue stained gels for further analysis. (C) Detection of identified partners on 1D gels. After transfer to nitrocellulose membranes of GST-pulldown samples obtained using 300 μg of CSM14.1 nuclear extracts, western blots were performed using the specified antibodies (RXR and ERK) to detect newly identified interacting factors. Values on the left inside denote molecular weights.

**In vitro validation of nurr1 interactions with LIMK, ERK1/2, ERK5**

Mass spectroscopy analysis provided a list of new potential partners of nurr1 that needed to be further validated. To confirm the identity of proteins of interest, we performed western blots on the affinity chromatography eluates, when specific antibodies were available. Using an RXRα-specific antibody,
an interaction between RXRα and the nurr1 GST–LBD fusion protein was detected (Figure 1C), thus providing validation for our screening system. Using an antibody capable of recognizing different forms of mitogen-activated protein kinases (MAPK), a differential profile was observed with GST–LBD versus GST-A/B protein (Figure 1C). Both A/B and LBD domains interacted with ERKs (44/42 kDa), whereas the LBD only interacted with big-mitogen-activated kinase 1 BMK1/ERK5 (ERK5, 85 kDa). None of the proteins tested were found to interact non-specifically with the GST bait.

The question whether these proteins interact directly with nurr1 was addressed by GST-pulldown experiments using in vitro radiolabeled proteins and nurr1 fusion proteins (see Figure 1A). Radiolabeled full-length LIM Kinase 1 (LIMK1) interacted with both nurr1 domains LBD and A/B in similar manner, but not with GST alone (Figure 2A). Similarly, the C-terminal part of LIM that encodes for its kinase domain displayed a strong interaction with both LBD and A/B domains of nurr1, as shown in Figure 2A. Nurr1 also interacted with a LIM domain-containing module (similar to the LIMK1 module), when we incubated a GST-LIM construct overexpressed in bacteria and radiolabeled nurr1 (Figure 2B). Thus, LIMK1 participates in a direct and concomitant protein–protein interaction with nurr1 via its LIM and kinase domains. We thought to verify that full-length nurr1, and not only its separately expressed domains, were capable of interacting with the proteins identified. We radiolabeled nurr1 and incubated it with GST fusion constructs encoding for potential partners (Figure 2B). A strong specific interaction was observed between nurr1 and ERK2. Since the presence of ERK5 in the chromatography eluates was confirmed by western blotting (Figure 1C), we tested its interaction with nurr1 and obtained a positive signal (Figure 2B).

**Figure 2.** Nurr1 interacts directly with multiple proteins identified by MS analysis. (A) 35S-labeled LIMK1 or kinase domain of LIMK1 were incubated in the presence of bacterially expressed GST alone (−) and GST–nurr1 fusion proteins (LBD or A/BΔ domain) for 3 h. The first lane corresponds to 10% input. Protein interactions were analyzed by SDS–PAGE and assayed by autoradiography. (B) 35S-labeled full-length nurr1 was incubated with ERK2, LIM domain, ERK5 fused to GST or GST alone (−) expressed, separated and assayed as in (A). (C–E) In vivo interactions between nurr1 and several signaling proteins. Co-immunoprecipitations with the anti-HA antibody of cellular extracts of CSM14.1 cells co-transfected with HA-tagged human nurr1 and empty vector (−) or expression plasmids of indicated proteins (+): (C) pcDNA3-LIMK1 and detected with anti-LIMK antibody, (D) pCMV-ERK2ca and visualized with pan-ERK antibody and (E) pFlag-ERK5 wt and detected with anti-BMK1/ERK5 antibody. Input represents levels of GST-LIMK, ERK2 and ERK5 in CMS14.1 cells.

**Nur1 interacts with LIM kinase 1, ERK2 and ERK5 in intact CSM14.1 cells**

We sought to establish that the observed physical interactions detected in our in vitro experiments also occurred in intact
mesencephalic cells. We performed co-immunoprecipitation studies using CSM14.1 cells co-transfected with an expression plasmid coding for HA-tagged full-length human nur1, and plasmids encoding for the proteins identified through our screening. An empty vector was used as negative control. After overexpression of wt LIMK1, we observed an interaction between LIMK1 and nur1 (Figure 2C). A strong interaction was detected between nur1 and the constitutively active form of ERK2 (ERK2 ca; Figure 2D), suggesting that the catalytic activity of ERK2 is important for the interaction with nur1. We also performed experiments in vivo with ERK5 and observed an interaction with nur1 in CSM14.1 cells (Figure 2E). Identical results were obtained when similar experiments were performed in the human embryonic kidney 293-T cell line (data not shown).

**Nurr1 has distinct binding sites for different kinases**

We further dissected the A/B and LBD regions of nur1 to identify the specific binding domains implicated in the interaction with these protein kinases. Several minimal deletion mutants in the A/B (ΔA-D) and LBD (ΔF-I) domains of nur1 were engineered (Figure 3A), in vitro radiolabeled and tested by GST-pulldown experiments with LIM domains of LIMK1, wt ERK2 and wt ERK5 proteins. As summarized in Figure 3A and shown in the representative experiments in Figure 3B, deletion of the region spanning from amino acid 1 to 52 of nur1 did not affect its interaction with any of the kinases. Interestingly, deletion of the AF1 core domain (amino acid 52–84; ΔB) partially altered the physical binding of nur1 with both MAPKs (ERK2 and ERK5), but not with LIMK1. Deletion of amino acid 166–180 (ΔD) severely impaired the binding of nur1 to LIMK1 and partially to ERK2, but did not interfere with ERK5 interaction (Figure 3C). On the other hand, the mutant ΔF (deleted from amino acid 368 to 420) was the only one to lose its ability to bind to ERK5, validating the previously observed binding of ERK5 to the LBD and not to the A/B domain of nur1 (Figure 1C). The mutant ΔD also showed a lower binding to LIMK1, suggesting that this hinge region may play an important role in protein–protein interactions. Mutations in the C-terminal domain of nur1 (AG-ΔAF2) mostly affected the interaction of nur1 with LIMK1, in particular, as shown in Figure 3, more dramatic effects were obtained with mutants ΔH and ΔI. Loss of the AF2 domain (nur1-ΔAF2) affected only the interaction with ERK5. Taken together, these data confirms that nur1 interacts via distinct and separate binding sites to the different protein kinases identified by affinity chromatography, arguing for a specificity of the binding of Nurr1 to these novel partners.

**ERK2 and LIMK1 are co-regulators of nur1**

Given that nur1 transcriptional activity is not regulated by a cognate ligand and known CoAs, we postulated that post-translational modifications could play a major role in modulating its activity. Thus, we were interested in characterizing the effects of the protein kinases identified on nur1 activity. We tested the ability of the interacting factors to alter nur1 function in PC12 cells which do not express nur1 and have been used extensively to study nur1 functional activity (16). Cells were transiently transfected with the NBR3xtkLuc reporter construct and we studied the effects of over-expressing different proteins on nur1 transcriptional activity. As previously shown (16), the reporter construct is devoid of activity in this cell line and overexpression of the different kinases tested did not alter its basal activity (see insets Figure 4A and B).

As shown in Figure 4A, overexpression increasing amounts of wt ERK2 did not strongly alter the effect of nur1 on the NBR3xtkLuc construct. In contrast, the constitutively active form of ERK2 (ERK2 ca) was able to further enhance the nur1-induced luciferase activity in a dose-dependent manner. These results together with the Co-IP shown in Figure 2D suggest that the catalytic activity of ERK2 is essential for the interaction of the kinase with nur1. To validate the role of ERK2 activation in the regulation of nur1 activity, we used a kinase-dead form of ERK2 (K52R) containing a mutation that disrupts ATP-binding and thus prevents phosphorylation of substrates (25). Contrary to the effects observed with the ca and wt forms of ERK2, increasing doses of the K52R mutant did not further enhance the nur1-induced activity of the NBR3x construct (Figure 4A). Thus, these results suggest that a functional ERK2 kinase activity is required to affect nur1 transcriptional activity. Conversely, overexpression of wt LIMK1 protein strongly repressed the transcriptional effects producing up to 45% inhibition of the nur1-induced luciferase activity (Figure 4B), without affecting the basal activity of the NBR3x construct alone (inset). Thus, our functional experiments strengthen the co-immunoprecipitation results (Figure 2C and D) and underscore the existence of a physical and functional link between nur1 and the catalytic activities of ERK2 and LIMK1.

**Big-mitogen kinase 1/ERK5 enhances nur1 transcriptional activity in vivo**

Our screening also identified the MAPK/ERK kinase 5 (MEK5) as a potential modulator of nur1 activity (Supplementary Table 1). Co-immunoprecipitation and GST-pulldown experiments established an interaction between nur1 and ERK5 (Figures 2 and 3), the direct substrate of MEK5. We therefore tested whether overexpressing components of the MEK5/ERK5 signaling pathway altered nur1 activity. Increasing doses of wt ERK5 did not affect the basal activity of the NBR3xtkLuc construct in the absence of nur1 (Figure 5A). Whereas nur1-induced activation of the luciferase construct was significantly enhanced by wt ERK5 in a dose-dependent manner. We then tested the effects of MEK5 on the transcriptional activity of nur1 using MEK5 wt, ca and dn forms (Figure 5B). As observed previously, none of the constructs activated non-specifically the NBR3xtkLuc reporter, while nur1 alone or in the presence of ERK5 increased luciferase activity. Cotransfection of nur1, ERK5 and wt MEK5 as well as its ca form significantly enhanced luciferase levels, whereas the dn MEK5 had no significant effect (Figure 5B). To verify that ERK5 is indeed the conduit for transduction of MEK5 signal, we used a Flag-tagged wt ERK5 construct (Flag-ERK5 wt) as well as a ERK5 mutant lacking both activation sites for MEK5 (Flag-ERK5 AEF). In the presence of wt ERK5, constitutively active MEK5 was able to enhance nur1-induced luciferase expression (Figure 5C). However, the level of
Luciferase expression due to the coexpression of nurr1 and the ERK5 AEF mutant did not change in the presence of MEK5 ca, suggesting that MEK5 could modulate nurr1 activity essentially through ERK5 activation. Surprisingly, the unphosphorylated AEF mutant was still capable of enhancing nurr1 activity, even in the absence of MEK5 contribution (Figure 5C). It has been previously suggested that, in non-activating conditions, ERK5 AEF could act as the

Figure 3. Mapping of the domains of interaction between nurr1 and LIMK1, ERK2, ERK5. (A) Schematic representation of the nurr1 deletion mutants used for GST-pulldown assays and summary of the quantification of obtained interactions with the kinases. Symbols represent quantification of binding interactions ($n = 3$–$5$). Intensity of binding was standardized to that of wt (100%); $+$, binding values $<$80%; $\pm$, 79% $\times$ 60%; $\times$, $<$59%. (B) Representative GST-pulldown assays obtained by incubating $^{35}$S-labeled pCMX-nurr1 wt or deletion mutants (wt, AA-I, AF2) with bacterially expressed GST alone (–) and GST-kinase fusion proteins (wt GST-LIM domain, ERK2 or ERK5).
When 

ERK5 wt, AEF and the kinase-dead ERK5 mutant K83M

were co-expressed nurr1 and different forms of ERK5 (HA-ERK5 wt, AEF and the kinase-dead ERK5 mutant K83M) and measured NBRE luciferase levels (Figure 5D). Wt ERK5 and the AEF mutant further enhanced nurr1-induced luciferase activity, with the mutant showing stronger activating effects on nurr1 activity (the HA-tagged AEF construct showed higher effects on nurr1 activity than the Flag-tagged construct; compare Figure 5C and D). Importantly, the kinase-dead mutant had no effects at any of the doses used.

Hence, the basal activity of ERK5 seems sufficient to affect nurr1 function without excluding that, in particular conditions, activation of MEK5 could further contribute to the modulation of nurr1 transcriptional activity.

To clarify the mechanism by which ERK5 transduces its activating properties on nurr1, we asked if the transcriptional effects observed were correlated with an interaction between nurr1 and ERK5 on DNA. Because of the lack of direct nurr1 binding response elements in the promoters of DAergic target genes, we used the NBRE reporter construct for our DNA immunoprecipitation assays, a system previously used to explore NRs transactivation mechanisms (27). Thus, we tested the recruitment of the two proteins on the NBRE-containing reporter plasmid by DNA immunoprecipitation assay (Figure 6A) using antibodies specific to nurr1 and ERK5. Immunoprecipitation followed by PCR amplification of the DNA fragment encompassing the three NBRE response elements showed that nurr1 and ERK5 were both strongly bound to this response element in vivo, suggesting that ERK5 interacts with DNA-bound nurr1 and acts as transcriptional activator. This recruitment was not observed on a promoter deprived of the nurr1 binding sites (pGL3-tk-Luc; Figure 6A). Identical DNA immunoprecipitation results were obtained in CSM14.1 and PC12 cells, confirming our functional studies obtained in the later cell type (Figure 5). These studies suggested that the kinase activity of ERK5 was involved in enhancing nurr1 activity. Thus, we wondered if nurr1 could be a new substrate for this kinase and tested the ability of ERK5 to phosphorylate GST–nurr1 fusion proteins in vitro. Therefore, we immunoprecipitated endogenous ERK5 from CSM14.1 cells and co-incubated GST fusion proteins coding for the A/B domain of nurr1 with the precipitated kinase in the presence of radioactive ATP. As shown in Figure 6B, nurr1 A/B domain was significantly phosphorylated by ERK5, while the GST protein alone was not a substrate for this kinase. We attempted to activate the endogenous MEK-ERK5 pathway by stimulating CSM14.1 cells with EGF for 15 min and verify the phosphorylation state of nurr1. These data would suggest once again that the basal activity of ERK5 is responsible for the phosphorylation of nurr1. To establish which amino acids were implicated in ERK5-mediated phosphorylation, several residues in the A/B domain of nurr1 were mutated to alanines. First, we observed that all point mutations had a high basal activity which was comparable to wt nurr1 plus ERK5 (Figure 6C) and that, similarly to wt nurr1, the S89A mutant response was significantly enhanced in the presence of ERK5. On the other hand, the activity of the T168A mutant was only slightly increased in the presence of the kinase, while the S177A mutant lost completely its capacity to be activated by ERK5. Thus, residues T168 and S177 appeared to be functional determinants for ERK5-mediated phosphorylation, as suggested by the recruitment of ERK5 to the NBRE, the phosphorylation of nurr1 N-terminal by this same kinase and the capacity of MEK5 and ERK5 to increase nurr1 transcriptional activity.
DISCUSSION

DAergic neurons are essential for the appropriate functioning of brain areas controlling motor behavior, motivation and the reward system, and diseases inducing imbalances in DAergic neurotransmission are very debilitating (1). The orphan NR nurr1 is a transcription factor highly expressed in mesencephalic DAergic neurons and it plays a major role in the development and maintenance of the phenotype of these cells. However, how nurr1 is tightly regulated and controls DAergic neuronal function is presently unknown. Target genes implicated in DAergic phenotype specification and directly bound by nurr1 still remain to be identified. For example, the DAT has been described as a nurr1-target gene, but its activation occurs via an indirect mechanism (10).

Nurr1 has a high tissue-specific activity and does not recruit classical NR CoAs, at least in vitro [P. Sacchetti and P. Lefebvre, unpublished data; (15,17)], suggesting that nurr1 transcriptional activity might be regulated by interactions with, and/or modifications induced by, tissue-specific factors. The well characterized nurr1-RXR heterodimers (16,19) do not control nurr1-regulated expression of phenotypic marker genes, e.g. DAT (10), thus rendering necessary the identification of other partners to understand the transcriptional mechanisms used by nurr1 to regulate gene expression in DA cells. To identify proteins involved in the regulation of nurr1 transcriptional activity in neuronal cells, we used an affinity chromatography system and embryonic cells of mesencephalic origin (CSM14.1) that endogenously express

Figure 5. Nurr1 transcriptional activity is regulated by the MEK5/ERK5 signaling pathway. (A) Dose-dependent induction by wt ERK5 of NBRE3xtkLuc activity in the presence of 25 ng of nurr1 in PC12 cells transfected with increasing doses of wt HA-ERK5. (B) Analysis of luciferase activity induced by MEK5 wt, ca and dn in the absence or presence of pCMX-nurr1 and wt HA-ERK5. (C) Comparison of transcriptional activity between wt and dn ERK5. PC12 cells were transfected with NBRE3xtkLuc, pCMX-nurr1, wt Flag-ERK5 or Flag-ERK5 AEF in the presence or absence of MEK5 ca. (D) Comparison of transcriptional activity induced by wt, AEF and kinase-dead K83M ERK5. Cells were transfected as in (A). Luciferase activity was normalized and results are expressed as described in Figure 4. Data are the means ± S.E. (bars) of 3–6 experiments.
nurr1 and RXR and we also identified novel interactions with NRs and other transcription factors in CSM14.1 cells. Using this affinity chromatography screen coupled to mass spectroscopy, we have confirmed the interaction between nurr1 and endogenous ERK5, which was further confirmed by GST-pulldown experiments (Supplementary Figure 9). However, the mass spectroscopy analysis identified new potential dimer partners of nurr1 pertaining to the NR superfamily, like the PPARγ, glucocorticoid receptor (GR; R. Carpentier and P. Lefebvre, manuscript in preparation) and FXR, and using several techniques (western blot, GST-pulldown and Co-IPs) we confirmed these physical interactions (Supplementary Figure 9) and are currently evaluating the functional roles of these dimers.

However, this screening method enabled us to identify not only direct binding partners (Figure 2), but also proteins that at first glance were unlikely to interact directly with a transcription factor, like MEK and p21-activated kinase, also known as PAK (Supplementary Table 1). It is possible that these proteins interact with direct binding partners of nurr1 and still participate in the modulation of nurr1 activity as components of a larger multiprotein complex, as previously exemplified by the identification of the DRIP–TRAP complex (22,28).

Together with the closely related nur77 and nor1, nurr1 constitutes the NGFI-B subfamily of NRs (29). Given that nur77 is highly phosphorylated [(14,30,31) and references therein], nurr1 could be a phosphoprotein as well. Several of the identified nurr1-interacting factors are signaling proteins pertaining to phosphorylation cascades that could modify nurr1 via post-translational modifications. Therefore, we focused on analyzing in depth the effects of a selected group of kinases and their capacity to alter transcriptional activity on a canonical nurr1 response element.

The kinase LIMK1 is known to interact with p57kip2, a cyclin-dependent kinase inhibitor essential during embryogenesis. This interaction induces translocation of the complex LIMK1/p57kip2 to the nucleus, thus limiting the cytoplasmic activity of the kinase, and targeting its action to the nucleus (32). Interestingly, both nurr1 and p57kip2 are involved in cell cycle arrest and differentiation and a direct interaction between these two proteins has been recently demonstrated, which results in inhibition of nurr1 transcriptional activity (33). We report here a direct interaction between LIMK1 and nurr1, suggesting the possible existence of a complex consisting of these three proteins. Since LIMK1 (Figure 5B) and p57kip2 (33) have an inhibitory effect on nurr1 transcriptional function, it is conceivable that the nuclear translocation of the LIMK1–p57kip2 complex plays a regulatory role in nurr1 activity. Thus, the existence of a LIMK1/p57kip2–nurr1 protein complex and the characterization of its effects in nurr1-mediated gene expression deserve deeper analysis. In addition, LIM-containing proteins can recruit the Sin3A–histone deacetylase co-repressor complex (34) and inhibit gene transcription, a mechanism well described for NRs (13) that could participate in the inhibition of nurr1 activity by LIMK1.

MAP kinases are essential for neuronal differentiation and neurotrophin signal transduction (35), and activation of the MEK/ERK cascade is involved in regulation of several transcription factors, including those belonging to the NGFI-B family. In particular, ERK2 phosphorylates the nur77 receptor in vitro (36) and in vivo (37), and MEK mutants can alter nur77 transcriptional responses on specific response elements (30). Further, the pharmacological MEK
inhibitors (PD98059 and U0126) have been shown to repress nur1 transcriptional activity (38) and in our studies they were the only molecules able to inhibit Ras-induced nur1 activation (P. Sacchetti and P. Lefebvre, unpublished data). We show here a direct in vivo physical and functional interaction between nur1 and ERK2 that seems to rely on the activation status of ERK2. The two proteins bind each other and our data support the existence of a MAPK docking site in nur1, located between amino acids 160 and 188, which is involved in ERK2–nur1 interaction. The existence of a docking site on the substrate recognized by MAPKs seems to be an essential prerequisite for guiding the specificity of interaction between kinase and substrate. However, Co-IPs evidenced a much stronger interaction of nur1 with the constitutively active form of ERK2, suggesting that in vivo the activation status of ERK2 is important to stabilize the interaction with nur1. This conclusion is further supported by our functional experiments that show an enhanced nur1 activity in the presence of a catalytically active ERK2 (wt and ca ERK2), but not in the presence of a kinase-dead mutant (K52R). The existence of a bona fide docking site on nur1 would suggest a direct protein–protein interaction between this transcription factor and the kinase; however, none of the data exclude the existence of a possible intermediate protein conveying the signal from ERK2 to nur1. Interestingly, ERK2 is capable of interacting directly with PAK2 and p85 βPIX (Supplementary Table 1) and this multimeric complex is activated via the Ras/MEK/ERK cascade in response to bFGF (39), a stimulus involved in mesen-
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Interestingly, ERK2 is capable of interacting directly with PAK2 and p85 βPIX (Supplementary Table 1) and this multimeric complex is activated via the Ras/MEK/ERK cascade in response to bFGF (39), a stimulus involved in mesencephalic cell development. Our studies showed that nur1 can interact in vitro and in vivo with p85 βPIX, even if this interaction does not seem to affect nur1 transcriptional response on a NBRE element (data not shown). Additionally, the post-translational modifications induced by these signaling molecules could affect nur1 interactions with cofactors, and changes in the phosphorylation state of nur1 could thus be directly implicated in recruitment of CoAs and CoRs.

It remains to be determined if ERK2 is involved in regulating the basal activity of nur1 or a stimulus-induced activity and to define which cellular signal activates ERK2 to affect nur1. In this regard, recent studies in DAergic primary cultures have linked the DA-induced activation of the ERK pathway to the activation of nur1 and highlighted the importance of this step in the development of DAergic neurons (40).

An interesting link between nur1 and neuroprotection is delineated by the interaction that we identified between nur1 and the MAP kinase, ERK5. In fact, ERK5 is known to play a critical role in neuronal survival (41) and recent studies have established its involvement specifically in neuroprotection of DAergic neurons resulting from oxidative stress (J. E. Cavanaugh et al., 34th Annual Meeting Soc. Neurosci., abstr. 221.3, 2004). Importantly, overexpression of nur1 results in cell resistance to MPTP, a selective DAergic neurotoxin used to mimic neuronal degeneration in Parkinson’s disease (42), and its reduced expression renders animals more susceptible to this neurotoxin (12), suggesting a neuroprotective role for nur1 in DAergic cells. Thus, the direct interaction that we observed between nur1 and ERK5 suggests a potential mechanism through which nur1 prevents neurotoxic responses in DA neurons. Our data establish the role of the MEK-ERK5 pathway on the regulation of nur1. Activation of MEK, which in turns activates ERK5, does enhance the ERK5-induced nur1 activity, while no increase is observed in the presence of a dn MEK5 or of the unphosphorylated ERK5 AEF, in which amino acids phosphorylated by MEK5 are mutated. In addition, an inhibition of nur1 transcriptional activity was observed when using the pharmacological MEK inhibitors PD98059 and U0126 (data not shown), molecules that block not only ERK1/2-, but also ERK5 activation by upstream kinases. Thus, the inhibitory effect of these compounds on nur1 activity observed by us and others [data not shown; (38)] may be explained by the inhibition of the MEK5/ERK5 signaling cascade. Taken together, these data suggest that activation of the MEK-ERK5 pathway could result in enhanced nur1 transcriptional responses. However, the AEF form, which cannot be activated, is still capable of stimulating nur1 as potently as the wt form, suggesting that activation by MEK is not necessary for ERK5 to regulate nur1 activity. Additionally, the phosphorylation status of nur1 is not further enhanced after activation of this signalling pathway by EGF, and nur1 transcriptional activity is left unaffected only by the kinase-dead form of ERK5 (K83M). Thus, ERK5 basal activity could be sufficient to modulate nur1 responses. Similarly, the basal activity of ERK5 has been shown recently to be sufficient in mediating survival of leukaemia cells, by affecting the oncogenic variant v-Abl of the tyrosine kinase c-Abl (26). Thus, it would be plausible for ERK5 and nur1 to cooperate in homeostatic conditions to ensure maintenance and survival of DAergic neurons; while, under stressful conditions, activation of MEK5 would result in further enhancement of the cooperative effects of these proteins.

Could nur1 be a direct substrate for ERK5? The two proteins can interact in vivo and this interaction is mediated through a region in the nur1 LBD (amino acids 360–420) in vitro. The A/B domain of nur1 is highly phosphorylated in the presence of ERK5 and mutations of two amino acids in this same domain decrease significantly the ERK5-mediated nur1 transcriptional response. The specificity and efficiency of action of MAPKs is usually conferred through docking interactions between the kinases and their substrate. Thus, ERK5 would dock on its substrate and phosphorylate the acceptor residues, which are different from the docking residues (43). The lack of similarity of the nur1 360–420 amino acid region to the canonical MAPK docking site and the lack of a definitive proof for a direct phosphorylation of S177 and T168 by ERK5 leave open the possibility that nur1 is not a direct substrate for ERK5. Although we do not favor this hypothesis in light of our current data, these effects could be indirect and mediated through a previously characterized ERK5 substrate, like the transcription factors MEF2 (43). However, we were not able to detect a direct interaction between nur1 and MEF2C, as tested by GST-pulldown (data not shown), leaving open the search for a potential mediator of the effects of ERK5 on nur1 transcriptional activity.

Taken together, our data identify ERK5 as the first described transcriptional activator of nur1. In fact, the recruitment of ERK5 on a nur1 response element provides a direct proof of the implication of this kinase in the modulation of nur1 activity at the transcriptional level.
At the physiological level, activation of this oxidative stress-related pathway is of particular interest in the context of neuroprotection of DAergic neurons against toxic insults. Stimulation of ERK5 and the consequent enhancement of nurr1 activity could be aimed at altering expression of genes related to cell protection. Clearly, confirmation of this hypothesis would necessitate the identification of new nurr1-target genes implicated in cell survival.

In summary, using our screening methodology we have identified signaling cascades that regulate nurr1 activity (ERK2, LIMK1 and MEK5/ERK5) as well as potential functional partners of nurr1 in transcription and survival/apoptosis. Initially, we had hypothesized that tissue-specific factors would regulate nurr1 transcriptional activity. However, our results point to a complex network of signaling pathways participating in the modulation of nurr1 activity, and most likely, the integration of their combinatorial action results in the observed in vivo cell-specific functions of this transcription factor. Future studies should focus on testing the ability of the newly identified regulators to alter the expression of DAergic genes modulated by nurr1 directly or indirectly, like DAT and TH, in their appropriate cell context. Clearly, these data would validate the relevance of nurr1 activity could be aimed at altering expression of genes related to cell type. Clearly, confirmation of this hypothesis would necessitate the identification of new nurr1-target genes implicated in cell survival.

SUPPLEMENTARY DATA
Supplementary Data are available at NAR Online.

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