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Dual function of Pin1 in NR4A nuclear receptor activation: Enhanced activity of NR4As and increased Nur77 protein stability

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ABSTRACT

Nur77, Nurr1 and NOR-1 form the NR4A subfamily of the nuclear receptor superfamily and have been shown to regulate various biological processes among which are cell survival and differentiation, apoptosis, inflammation and metabolism. These nuclear receptors have been proposed to act in a ligand-independent manner and we aim to gain insight in the regulation of NR4A activity. A yeast two-hybrid screen identified the peptidyl-prolyl isomerase Pin1 as a novel binding partner of NR4As, which was confirmed by co-immunoprecipitation. Pin1 enhances the transcriptional activity of all three NR4A nuclear receptors and increases protein stability of Nur77 through inhibition of its ubiquitination. Enhanced transcriptional activity of NR4As requires the WW-domain of Pin1 that interacts with the N-terminal transactivation domain and the DNA-binding domain of Nur77. Most remarkably, this enhanced activity is independent of Pin1 isomerase activity. A systematic mutation analysis of all 17 Ser/Thr-Pro-motifs in Nur77 revealed that Pin1 enhances protein stability of Nur77 in an isomerase-dependent manner by acting on phosphorylated Nur77 involving protein kinase CK2-mediated phosphorylation of the Ser^{152}-Pro^{153} motif in Nur77. Given the role of Nur77 in vascular disease and metabolism, this novel regulation mechanism provides perspectives to manipulate Nur77 activity to attenuate these processes.

1. Introduction

The NR4A receptor family belongs to the nuclear hormone receptor superfamily and consists of three highly homologous mammalian members known as Nur77 (also indicated as NR4A1, NGFI-B, TR3), Nurr1 (NR4A2) and NOR-1 (NR4A3, MINOR, Nor1). The NR4A transcription factors were first described as early response genes expressed upon stimulation by multiple growth factors and have been shown to play a role in the regulation of metabolism in liver, skeletal muscle and adipose tissue and cardiovascular disease [1–3]. Basic cellular processes such as differentiation, apoptosis, proliferation and stress responses are modulated by NR4A nuclear receptors [4–6]. We have demonstrated that Nur77 and Nurr1 inhibit the formation of smooth muscle-rich vascular lesion formation and exhibit an anti-inflammatory function in macrophages [2,7–9].

Like all nuclear receptors, Nur77, Nurr1 and NOR-1 consist of a carboxy-terminal ligand-binding domain (LBD), an amino-terminal domain mediating ligand-independent coactivator recruitment and a central DNA-binding domain (DBD). This DBD is extremely similar among the NR4A receptors (94% amino acid sequence identity). The ligand-binding domains also show substantial amino acid similarity (approximately 65%), whereas the amino-terminal domains are more divergent (approximately 30% similarity) [10]. All three NR4A receptors bind as monomers to the response element NBRE (AAAGGTCA) or as NR4A heterodimers or homodimers to the palindromic NurRE (TGATATTTX6AAAGTCCA) in the promoters of specific target genes [11]. Nur77 and Nurr1, but not NOR-1, also form heterodimers with the Retinoid X Receptor in the presence of retinoids and thus can modulate the activities of a subclass of retinoid response elements [12]. NR4A receptors are referred to as orphan receptors, because as yet no ligands have been identified, which is in line with structural analyses of the ligand-binding domains of Nurr1 and Nur77 showing that the putative ligand-binding pocket is filled with bulky aromatic and hydrophobic residues [13,14]. These structural analyses also revealed that the canonical coactivator cleft, through which most nuclear receptors interact with the LxxLL motif of coregulators, is hydrophobic rather than hydrophilic in Nur77 and Nurr1. As a consequence, the interaction of NR4As with coregulators most likely does not involve such LxxLL motifs. For the NR4A subfamily the activation function (AF)-1 domain, which is localized in the amino-terminal domain, appears to be most important for transcriptional activation and cofactor recruitment. Direct interaction with the AF-1 domain of Nur77 has been shown for the

Abbreviations: CHX, cycloheximide; CK2, protein kinase CK2; DBD, DNA-binding domain; DCOH, dimerization cofactor for hepatocyte nuclear factor 1; GST, glutathione S-transferase; LBD, ligand-binding domain; Pin1, peptidyl-prolyl *cis/trans* isomerase 1; PPIase, Peptidyl-prolyl isomerase; pSer/pThr-Pro, phosphorylated proline-directed serine/threonine; TBB, 4,5,6,7-tetrabromobenzotriazole

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coactivators SRC-2, PCAF, p300, DRIP250 and TRAP220, as well as for the corepressors SHP and CRIF1. The corepressor SMRT has been shown to interact in a CaMKIV-dependent way with the ligand-binding domain of Nur77 [10,15–19].

Peptidyl-prolyl isomerases (PPIases) form an evolutionary conserved group of proteins that promote the *cis/trans* isomerization of the peptide bond preceding specific proline residues [20]. Peptidyl-prolyl *cis/trans* isomerase 1 (Pin1) belongs to the parvulin subfamily of PPIases and is unique in that it is the only PPIase that specifically recognizes phosphorylated proline-directed serine/threonine (pSer/pThr-Pro) motifs [21–23]. These Pin1-induced conformational changes have profound effects on the properties of many Pin1 substrates, like protein phosphorylation, enzyme activity, protein localization, protein interaction, transcriptional activity and protein stability [24–26]. This allows Pin1 to play an important role in a wide range of cellular processes such as regulation of the cell cycle [27,28], the cellular response to DNA damage [29], and in transcriptional regulation [30,31]. Pin1 is a protein of 163 amino acids and comprises an amino-terminal WW domain that is involved in protein–protein interaction and a carboxy-terminal PPIase domain [22,23]. A number of Pin1-interacting proteins are transcription factors, including c-Jun, NF-κB, p53, β-catenin, PPARγ and steroid receptor coactivator (SRC)3 [32,33].

In the current study, we sought to identify novel coregulatory proteins of the NR4A receptors and identified Pin1 as a novel coactivator of Nur77, Nurr1 and NOR-1, which has so far only been described for Nur77 [34]. Our yeast two-hybrid revealed four and a half LIM-domain 2 (FHL2) as a novel corepressor of NR4As [35]. We demonstrate that Pin1 interacts with and enhances the transcriptional activity of Nur77 in a PPIase-independent manner and enhances the stability of Nur77 protein in a PPIase-dependent manner by acting on the phosphorylated Ser^{152}-Pro^{153} motif in Nur77. Pin1 enhances the expression of the Nur77 target gene enolase 3. Altogether these data reveal a novel Pin1-mediated regulation mechanism of Nur77 activity and suggest that association of Pin1 with Nur77 plays a role in the regulation of vascular disease and metabolism.

2. Materials and methods

2.1. Plasmids

The cDNA encoding the N-terminal domain of hNOR-1 (Genbank D78579), coding for amino acids 2–290, was cloned into the pGBKT7 vector (Clontech). hNur77 cDNA (GenBank D49728, bp 8–1947), hNurr1 cDNA (Genbank X75918, bp 73–2310) and hNOR-1 (bp 513–2872) were cloned into the pRRL-cPPt-X2-CMV-PreSIN vector [36] and amino-terminally epitope tagged by cloning into the pCMV-Myc vector (Clontech). Pin1 was N-terminally tagged by cloning from the pGADT7 library vector into the pCMV-HA vector (Clontech), cloned into the pRRL-cPPt-X2-CMV-PreSIN vector and amino-terminally tagged with His6-GST by cloning into the pETM-30 vector. Plasmids encoding GST-CK2α and GST-CK2α′ have been described before [37] and were obtained via Addgene (plasmids 27083 and 27084). The luciferase reporter plasmids containing three copies of NurRE or NBRE have been described before [11,38]. Several mutants of Nur77 and Pin1 were generated by site-directed mutagenesis using the QuickChange site-directed mutagenesis method (Stratagene) according to the manufacturer's instruction. The primers used to generate the mutants are listed in Supplemental Tables S1 and S2. All constructs were verified by sequencing. Short hairpin RNA plasmids targeting CK2α and Ck2α′ genes from The RNAi Consortium (TRC) [39] were obtained from Sigma-Aldrich. The human shRNA oligo sequences are listed in Supplemental Table S3.

2.2. Yeast two-hybrid assay

A smooth muscle cell cDNA library was constructed from 0.5 μg of poly A+ RNA from activated smooth muscle cells [40] using the Matchmaker library construction and screening kit (Clontech) according to the manufacturer's instructions. The bait construct pGBKT7-NOR-1 was transformed into the *Saccharomyces cerevisiae* strain AH109 (Clontech) using the lithium-acetate method. Subsequently these yeast cells were transformed with the pGADT7-smooth muscle cell cDNA library and interacting clones were isolated on selective synthetic drop-out medium based on growth in the absence of tryptophan, leucine, and histidine in presence of 30 mM 3-amino-1,2,4-triazole (3-AT, Sigma). False positives were eliminated based on their interaction with the negative control (empty pGBKT7) vector. The plasmids of the positive clones were isolated and sequenced. The DNA sequences were then characterized by BLAST analysis against the NCBI database to determine the identity of the potential NOR-1 interacting proteins.

2.3. Cell culture

HEK293T cells were cultured in Dulbecco's Modified Eagle's medium supplemented with 10% (v/v) fetal bovine serum (FBS) and penicillin and streptomycin (Invitrogen). Human smooth muscle cells were explanted from umbilical cord arteries [40]. Cells were cultured in M199 containing 10% FBS and penicillin and streptomycin. Smooth muscle cells were used at passages 5 to 7 and were characterized by SM α-actin expression (1A4, DAKO) and showing uniform fibrillar staining.

2.4. Generation of lentiviral particles and transduction

Recombinant lentiviral particles encoding Nur77, shNur77 and Pin1 were produced, concentrated and titrated as described before [7]. Smooth muscle cells were incubated with recombinant lentivirus for 24 h after which the medium was refreshed and the cells were cultured for another 72 h. Transduction efficiency was determined by immunofluorescence.

2.5. Co-immunoprecipitation assay

HEK293T cells were seeded in a 6-well plate and co-transfected with 1 μg of the appropriate constructs by calcium phosphate precipitation using the CalPhos Mammalian Transfection Kit (Clontech). Medium was refreshed 24 h after transfection and cells were lysed 48 h after transfection in 400 μl ice-cold pull-down buffer (50 mM Tris–HCl, pH 7.4, 100 mM NaCl, 10% (v/v) glycerol, 1% (v/v) Nonidet P-40, 10 mM NaF, 1 mM Na_3VO_4 supplemented with Complete protease-inhibitor cocktail (Roche Applied Science)). Cell lysates were pre-cleared for 1 h at 4 °C with protein A-Sepharose (GE Healthcare) and then incubated overnight with the pull-down antibody and protein A-Sepharose. The precipitate was washed 3 times with pull-down buffer, and bound proteins were eluted by boiling in SDS-loading buffer before electrophoresis on 12% SDS-polyacrylamide gels. After protein transfer, PVDF membranes (Millipore) were incubated with appropriate primary antibodies and fluorescently conjugated secondary antibodies, followed by scanning using Odyssey Infrared Imaging System (LI-COR Biosciences GmbH). Antibodies applied in this study: anti-HA (12CA5; Roche Applied Science), anti-Pin1 (Calbiochem), anti-Nur77 (M210) and anti-c-Myc (both Santa Cruz Biotechnology).

2.6. GST pulldown

Escherichia coli-expressed glutathione *S*-transferase (GST)-His6-Pin1 was bound to glutathione Sepharose 4B (GE Healthcare), followed by the addition of Nur77, which was generated by *in vitro* transcription and translation using the TNT T7 Quick Coupled transcription/translation system according to the manufacturer's instructions (Promega). The bound proteins were resolved on 12% SDS-polyacrylamide gels and detected by Western blot analysis.