FHL2 Protein Is a Novel Co-repressor of Nuclear Receptor Nur77

Kondababu Kurakula, Erik van der Wal, Dirk Geerts, Claudia M. van Tiel, and Carlie J. M. de Vries

From the Departments of Medical Biochemistry and Human Genetics, Academic Medical Center, University of Amsterdam, 1105 AZ Amsterdam, The Netherlands

Background: Nur77 is an orphan nuclear receptor involved in vascular disease, of which the regulation of activity is poorly understood.

Results: FHL2 binds Nur77 and represses its transcriptional activity by inhibition of Nur77 association with DNA.

Conclusion: FHL2 acts as a co-repressor of Nur77.

Significance: Our data suggest that interaction of FHL2 with Nur77 plays a pivotal role in vascular disease.

The three members of the NR4A orphan nuclear receptor subfamily Nur77, Nur1, and NOR-1, regulate a variety of biological functions including vascular disease and metabolism. In this study, we identified Four and a half LIM domains protein-2 (FHL2) as a novel interacting protein of NR4A nuclear receptors by yeast two-hybrid screen and co-immunoprecipitation studies. Each of the four LIM domains of FHL2 can bind Nur77, and both the amino-terminal domain and the DNA binding domain of Nur77 are involved in the interaction between FHL2 and Nur77. FHL2 represses Nur77 transcriptional activity in a dose-dependent manner, and short hairpin RNA-mediated knockdown of FHL2 results in increased Nur77 transcriptional activity. ChIP experiments on the enolase3 promoter revealed that FHL2 inhibits the association of Nur77 with DNA. FHL2 is highly expressed in human endothelial and smooth muscle cells, but not in monocytes or macrophages. To substantiate functional involvement of FHL2 in smooth muscle cell physiology, we demonstrated that FHL2 overexpression increases the growth of these cells, whereas FHL2 knockdown results in reduced DNA synthesis. Collectively, these studies suggest that association of FHL2 with Nur77 plays a pivotal role in vascular disease.

Nuclear receptors of the NR4A subfamily have been described as early response genes that are induced by diverse extracellular signals in a wide range of tissues and cultured cells (1, 2). The NR4A subfamily includes three members: Nur77 (NR4A1, NGFI-B), Nur1 (NR4A2, NOT), and NOR-1 (NR4A3, MINOR) (2, 3). The NR4A members contain highly conserved DNA binding domains (DBD; 97% homology) and carboxyl-terminal ligand binding domains (LBD; 60–65% homology). The amino-terminal domain, however, is less conserved and shows a higher degree of variability among the NR4As with only 20–30% amino acid sequence homology (4–7). NR4A nuclear receptors are implicated in the regulation of a wide range of biological processes including cell differentiation, proliferation, and apoptosis (8–10). Recent data demonstrated that NR4A nuclear receptors are also involved in the regulation of genes involved in adipogenesis, metabolic disease, inflammation, and vascular disease (1, 11–13). NR4A receptors have been shown to bind the consensus NBRE sequence (AAAGGTCA) as monomers (14) or the palindromic NurRE sequence (TGATATTX6AAAGTCCA) as homodimers and heterodimers in promoters of target genes (2, 15). We and others have demonstrated that NR4A nuclear receptors are significantly induced in human vascular smooth muscle cells (SMCs) upon atherogenic stimulation and in vascular endothelial cells in response to proliferative and inflammatory signals (16–18).

Structural studies have revealed that the putative ligand binding pocket of Nur1 is tightly packed with bulky aromatic and hydrophobic residues, leaving no cavity for binding of any ligand (19, 20). To date, no physiological ligand for the members of this subfamily has been identified, and therefore they are classified as orphan nuclear receptors (17). However, several agonists have been reported that may modulate the expression and/or transcriptional activity of NR4A nuclear receptors in unconventional ways (11, 15). It has also been shown that the transcriptional activity of NR4A nuclear receptors is strongly regulated at the level of protein expression and/or post-translational modification, including protein-protein interactions with co-activators and co-repressors (15, 21). Further studies showed that the amino-terminal domain contains the activation function 1 region of the NR4A subfamily, which is necessary for optimal transcriptional activity and recruitment of co-factors (1, 20).

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1 Both authors contributed equally to this study.
2 To whom correspondence should be addressed: Dept. of Medical Biochemistry K1–113, Academic Medical Center, Meibergdreef 15, 1105 AZ Amsterdam, The Netherlands. Tel.: 31-205665152; Fax: 31-206915519; E-mail: c.j.devries@amc.uva.nl.

3The abbreviations used are: DBD, DNA binding domain; DCOH, dimerization co-factor for hepatocyte nuclear factor 1; FHL2, four and a half LIM domains protein-2; LBD, ligand binding domain; SMC, smooth muscle cell; SRF, serum response factor.
Four and a half LIM domains protein 2 (FHL2), also known as down-regulated in rhabdomyosarcoma LIM protein (DRAL), is the second member of the LIM-only subclass of the LIM protein superfamily (22–24). LIM domains contain cysteine-rich zinc-finger motifs, which mediate protein-protein interactions (25–28). FHL2 is a multifunctional protein that interacts with a large number of transcription factors and regulates a wide range of cellular functions, including gene expression, apoptosis, cell cycle, cytoskeleton modulation, and signal transduction (26, 28–31). FHL2 has been identified as a co-regulator for multiple transcription factors among which several nuclear receptors, including androgen receptor, estrogen receptor α and β, vitamin D receptor, thyroid receptor, retinoic acid receptor, retinoid-related orphan receptor α and γ, and retinoid X receptors (25, 26, 29, 30, 32). FHL2 is expressed predominantly in the heart and, to a lesser extent in other tissues, including the vessel wall (31, 33). FHL2 is not required for normal cardiac development and heart function, as was demonstrated in mice lacking FHL2, that are viable; however, these mice have been shown to develop enhanced cardiac hypertrophy following β-adrenergic stimulation (28, 29, 34).

In the current study, we identified FHL2 as a novel co-repressor of NR4A nuclear receptors. In particular, FHL2 interacts with NR4A nuclear receptors and inhibits the transcriptional activity of Nur77 involving the amino terminus and DBD. Expression analysis of FHL2 revealed strong expression in vascular SMCs and endothelial cells. Furthermore, functional studies show that FHL2 increases SMC proliferation. Altogether, these data suggest that the association of FHL2 with Nur77 in vascular cells plays a role in the regulation of vascular disease.

**EXPERIMENTAL PROCEDURES**

**Plasmids**—The cDNA encoding the amino-terminal domain of human (h)NOR-1 (GenBank D78579) amino acids 2–264 was cloned in yeast expression vector pGBKT7 (Clontech) for the yeast two-hybrid screen. Full-length hNur77 cDNA (GenBank D49728, bp 8–1947), hNurr1 cDNA (GenBank X75918, bp 73–2310), and hNOR-1 (bp 513–2872) were cloned into the lentiviral vector pRRL-cPPt-X2-CMV-PreSIN (35). hNOR-1, hNur77, and hNurr1 were amino-terminally epitope-tagged in pCMV-Myc (Clontech). Plasmids of 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 interacting proteins.

**Cell Culture and Transfection**—HEK293T cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) with 20 mM glucose, supplemented with 10% fetal bovine serum (FCS) and penicillin/streptomycin (Invitrogen). Human SMCs were explanted from umbilical cord arteries (37) and cultured in M199 medium (Invitrogen) supplemented with 10% FCS and penicillin/streptomycin. SMCs were used at passages 5–7 and were characterized by SM α-actin expression (1A4; DAKO) showing uniform fibrillar staining. Human umbilical vein endothelial cells were isolated from umbilical cords, at passages 1–3 and cultured in M199 medium supplemented with 20% FCS, endothelial cell growth supplement (Sigma), penicillin/streptomycin, L-glutamine, and heparin. THP-1 monocytes, PMA-induced macrophages and mouse bone marrow-derived macrophages were cultured in RPMI 1640 medium supplemented with 10% FCS, penicillin/streptomycin and L929 cell conditioned medium. HEK293T cells were transfected by the calcium phosphate method using CalPhos Mammalian Transfection Kit (Clontech).

**Co-immunoprecipitation Assays and Western Blot Analysis**—HEK293T cells were co-transfected with appropriate plasmids and incubated for 48 h. For optimal expression of the amino-terminal domain of Nur77 (amino acids 1–264), cells were treated with 25 μM MG-132, a proteasome inhibitor, for 8 h before cell lysis. The cells were lysed in lysis buffer (50 mM Tris–HCl, pH 7.4, 100 mM NaCl, 10% glycerol, 1% Nonidet P-40, 10 mM NaF, 1 mM Na3VO4) containing complete protease inhibitor mixture (Roche Applied Science). Cell lysates were precleared for 1 h at 4 °C with protein A-Sepharose (GE Healthcare) and then incubated overnight with the pulldown antibody and protein A-Sepharose. Immunoprecipitates were washed three times in lysis buffer, and bound protein was 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 (Licor Biosciences). Antibodies applied in this study were anti-HA (12CA5; Roche Applied Science), anti-Myc (Santa Cruz Biotechnology), and anti-Nur77 (M210; Santa Cruz Biotechnology).

**GST Pulldown Assay**—Escherichia coli-expressed glutathione S-transferase (GST)-FHL2 was bound by glutathione-Sepharose 4B (GE Healthcare), followed by the addition of in vitro-transcribed/translated Nur77. After 1 h of incubation at 4 °C, the beads were washed three times with PBS. The pulldown

**FHL2 Is a Co-repressor of Nur77**
FHL2 Is a Co-repressor of Nur77

complex was subjected to Western blot analysis using the appropriate antibodies.

Generation of Lentiviral Particles and Infection—Recombinant lentiviral particles encoding Nur77, FHL2, and shFHL2 were produced, concentrated, and titrated as described previously (3, 38, 39). SMCs were infected with recombinant lentiviruses for 24 h after which the medium was refreshed and the cells were cultured for another 72 h. Transduction efficiency was determined by immunofluorescence and real-time PCR. Immunofluorescence staining and the protein stability assay are described under supplemental Methods.

Luciferase Assays—HEK293T cells were transiently transfected with NurRE or NBRE luciferase reporter plasmids together with pCMV-Myc-Nur77 (deletion mutants) and pCMV-HA-FHL2 (deletion mutants) or pCMV-mock. pRL-TK Renilla reporter plasmid (Promega) was co-transfected as an internal control. SMCs were first transduced with recombinant lentiviral particles encoding Nur77 and FHL2 or shFHL2 followed by FuGENE6 (Roche Applied Science) transfection of NurRE reporter plasmid. Luciferase activity measurements were performed using the dual-luciferase reporter assay system (Promega) and Glomax Multi detection system (Promega) according to the manufacturer’s protocol. Each experiment (in duplicate) was repeated at least three times.

FHL2 Knockdown—Short hairpin RNA plasmids targeting FHL2 gene from the RNAi Consortium and Sigma Mission library (40) were obtained from Sigma-Aldrich. The human shRNA oligonucleotide sequences of FHL2 used were shFHL2#1, 5’-CCGGCCAGAAATGCAAGGACCAT-TCTGAGATGGTCTTCTTGGTTTTT-3’ and shFHL2#2, 5’-CCGGCGACTGTGTTTAACCTGAAGACCT-CGAGTTCTTACAGTTAAAGCAGTCGTTTTT-3’ (underlined sequences are target sequences). In all of our experiments both shRNAs gave similar results, therefore only the results using shFHL2#1 are shown.

Semiquantitative RT-PCR—Total RNA was prepared with Total RNA mini kit (Bio-Rad) from cells. cDNA synthesis was performed with iScript (Bio-Rad), followed by real-time PCR using the MyIQ system (Bio-Rad). Human FHL2 primers 5’-TGCTACTGCTGCTGACTGCTCTG-3’ (sense) and 5’-TCGTTATGCCACTGCCGTTCCTC-3’ (antisense) were used for real-time PCR. Acidic ribosomal phosphoprotein P0 was determined as an internal control for cDNA content of the samples.

Chromatin Immunoprecipitation Assay (ChiP)—ChiP assays were performed using the Magnify ChiP system (Invitrogen) according to the manufacturer’s instructions. The following primers were used to amplify the enolase3 promoter in PCR and semiquantitative PCR: 5’-GGGACTGCTTTCTTGGCACTCACT-3’ (sense) and 5’-TCGTTATGCCACTGCCGTTCCTC-3’ (antisense).

BrdU Incorporation Assay—SMCs were transduced with recombinant lentivirus particles encoding Nur77 and FHL2 or shFHL2. Four days after transduction, cells were seeded in a 96-well plate at a density of 3 × 10³ cells/well and incubated overnight. The cells were made quiescent by incubation in medium without FCS for 48 h, then FCS (10% v/v) was added and incubated for another 24 h. DNA synthesis was measured by the BrdU incorporation assay (Roche Applied Science) according to the manufacturer’s instructions. Briefly, the cells were incubated with BrdU for 4 h, fixed, and incubated with conjugated anti-BrdU antibody, and finally colorimetric analysis was performed with an ELISA plate reader. Each experiment (in quadruplicate) was repeated at least four times.

Statistical Analysis—Data are reported as mean ± S.D. and were analyzed with the unpaired Student’s t test. p values <0.05 were considered as significant.

RESULTS

FHL2 Interacts with NR4A Nuclear Receptors—To identify novel interacting proteins of NR4A nuclear receptors we employed a yeast two-hybrid screen. Because NR4A receptors are induced in activated SMCs, a cDNA library was generated from RNA derived from (activated) SMCs to identify novel interacting proteins in this cell type (8, 16, 37). The amino-terminal domain of Nur77 gave background in the yeast two-hybrid system; therefore, the amino-terminal region of NOR-1 was used as bait to screen the activated human SMC cDNA library. One of the positive clones in this screen encoded a protein of 279 amino acids with a predicted molecular mass of 32.2 kDa, which was identified as FHL2.

To corroborate the potential interaction between all three NR4As and FHL2, we performed co-immunoprecipitation experiments. HEK293T cells were co-transfected with expression vectors encoding full-length Nur77, Nur1, or NOR-1 and FHL2. Each NR4A nuclear receptor was efficiently co-immunoprecipitated with FHL2 from whole cell extracts using an anti-HA antibody (Fig. 1A and supplemental Fig. 1), and NR4A precipitation with an anti-Myc antibody resulted in pulldown of FHL2 (data not shown). Total cell lysates served as a control for input of NR4As and FHL2. These experiments revealed that FHL2 associates with all three NR4A receptors.

To analyze whether FHL2 binds directly to Nur77, GST pull-down experiments were performed with bacterially expressed GST-FHL2 and in vitro transcribed/translated Nur77. As shown in Fig. 1B, Nur77 was pulled down with GST-FHL2. However, it did not interact with the control fusion protein GST-DCOH. These data demonstrate direct interaction between FHL2 and Nur77.

Mapping of FHL2 Domains Interacting with Nur77—To identify which domain of FHL2 is responsible for the interaction with Nur77, we generated constructs encoding the individual LIM domains and performed co-immunoprecipitation assays. HEK293T cells were transiently transfected with full-length Myc-Nur77 and full-length HA-FHL2 or the separate HA-tagged FHL2 LIM domains. Protein expression in total lysates of the pulldown assay is shown in the upper two panels (Fig. 1D). Each of the FHL2 LIM domains was immunoprecipitated with anti-HA antibody and showed Nur77 binding as was detected by Western blotting (Fig. 1D, bottom panel). A reciprocal co-immunoprecipitation assay applying the anti-Myc tag antibody confirmed the interaction of HA-FHL2 and FHL2-
**FHL2 Is a Co-repressor of Nur77**

**A**  
Amino acids | FHL2 binding  
--- | ---  
Nur77 | 264 | 364 | 598  
N-Term | + | + | +  
DBD | DBD | DBD | C  
LBD | - | - | -  

**B**  
Input | Nur77 domains  
--- | ---  
Nurt | + | + | +  
LBD | - | - | -  
FHL2 | + | + | +  

**C**  
Input | N-Term domains  
--- | ---  
Nurt | + | + | +  
FHL2 | + | + | +  

**FIGURE 2. The amino-terminal domain and DBD of Nur77 interact with FHL2.** A, schematic diagrams Nur77 deletion mutants and their ability to interact with FHL2. B, HEK 293T cells were co-transfected with expression vectors encoding FHL2 and Nur77 domains, as indicated. Whole cell extracts were immunoprecipitated using the anti-HA antibody (IP: FHL2). Immunoprecipitated samples were resolved on 12% SDS-PAGE and analyzed by Western blotting with anti-HA antibody (WB: Nur77). C, HEK293T cells were transfected with the amino-terminal domain of Nur77 (N-Term) without or with FHL2. Prior to lysis the cells were treated with proteasome inhibitor MG-132 for 8 h. Whole cell extracts were incubated with anti-Myc antibody (IP: N-Term), subjected to SDS-PAGE, and analyzed by Western blotting with anti-HA antibody (WB: FHL2). The input represents 10% of the total cell lysate in all co-immunoprecipitation experiments. Data are representative of at least three independent experiments. Protein molecular mass marker (M) is shown in kilodaltons (kDa).

LIM domains with Nur77 (data not shown). These results demonstrate that a single LIM domain can already bind Nur77 and that the addition of other LIM domain does not affect the interaction between FHL2 with Nur77.

**Mapping of NR4A Domains Interacting with FHL2—**To assess which region of Nur77 interacts with FHL2, three Nur77 mutants (Fig. 2A) were assayed in co-immunoprecipitation experiments. HA-FHL2 co-immunoprecipitated full-length Nur77 and the DBD (amino acids 265–364), but not the LBD (amino acids 365–598) (Fig. 2B). The separate amino-terminal domain of Nur77 (amino acids 1–264) was not expressed efficiently in the cells unless the cells were treated with a proteasome inhibitor (MG132; see “Experimental Procedures”). The amino-terminal domain interacts with FHL2 as shown by specific pulldown of FHL2 with this domain (Fig. 2C). Similar results were obtained for Nur1 and NOR-1 (supplemental Fig. 2). These experiments demonstrate that FHL2 binds the amino-terminal region of all three NR4A members and their DBD and does not require intact NR4A for these interactions.

**Subcellular Localization of FHL2 and Nur77—**Having established that FHL2 interacts with Nur77, we performed immunofluorescence experiments to visualize subcellular localization of Nur77 and FHL2. FHL2 has been described to exhibit cell type-specific and stimulation-dependent subcellular distribution (33, 41). Nur77 is known to localize predominantly to the nucleus, but has also been shown to translocate to the mitochondria to provoke apoptosis in specific cancer cells (17). Here, we demonstrate that FHL2 protein is present throughout both cytoplasm and nucleus, whereas Nur77 protein is localized predominantly in the nucleus. FHL2 and Nur77 co-localize in the nuclear compartment in vascular SMCs (supplemental Fig. 3) and in HEK293T cells (data not shown). These data suggest that interaction between Nur77 and FHL2 occurs in the nucleus indicating that FHL2 may modulate Nur77 transcriptional activity.

**Inhibition of Nur77 Transcriptional Activity by FHL2—**To determine whether FHL2 modulates Nur77 activity, we co-expressed FHL2 and Nur77 along with luciferase reporter plasmids in HEK293T cells and SMCs. We observed that FHL2 significantly represses the transcriptional activity of Nur77 both for NBRE- and NurRE-mediated transcription in HEK293T cells (Fig. 3, A and B) and in SMCs (Fig. 3C). FHL2 inhibits the activity of Nur77 in a dose-dependent manner (supplemental Fig. 4). These results indicate that FHL2 represses both monomeric and dimeric Nur77-mediated transcriptional activity.

Based on the co-immunoprecipitation experiments, we proposed that each LIM domain interacts with Nur77, which prompted us to assess the effect of each LIM domain of FHL2 on Nur77 transcriptional activity. HEK293T cells were...
co-transfected with the individual LIM domains and FHL2 deletion mutants (for schematic representation of the different mutants, see supplemental Fig. 5). Subsequent measurements of Nur77 activity revealed that each LIM domain is sufficient to repress the activity of Nur77 except LIM0, which contains only half an LIM domain (Fig. 3D). Interestingly, the presence of multiple LIM domains in the same protein does not result in an additive or synergistic inhibition of Nur77 activity. Taken together, these data confirm that each domain of FHL2 is sufficient for interaction with Nur77 as well as for inhibition of the activity of this nuclear receptor.

Post-translational modifications such as phosphorylation, sumoylation, and most recently acetylation of NR4As have been demonstrated to be important in regulation of the activity and half-life of these nuclear receptors (42). To measure the potential impact of FHL2 on Nur77 protein stability, we cotransfected Nur77 and FHL2 and treated the cells with the protein synthesis inhibitor cycloheximide. Cells were harvested at different time points, and we performed Western blot analysis to measure Nur77 protein levels. The half-life of Nur77 protein was not influenced by FHL2 (supplemental Fig. 6). Based on these findings we concluded that FHL2 does not affect Nur77 protein stability.

**FHL2 Knockdown Increases Nur77 Activity**—To address further the effect of FHL2 on Nur77 activity, we suppressed FHL2 expression by FHL2-specific shRNA in human SMCs. SMCs were transduced with lentiviruses encoding Nur77 and FHL2 or shFHL2, and subsequently the cells were transfected with NurRE-Luc for luciferase activity measurements. The knockdown efficiency of FHL2 was determined by real-time RT-PCR (Fig. 4A) and Western blot analysis for FHL2 protein (Fig. 3B). After 72 h of transduction, a control shRNA (shCon) or a control shRNA (shCon) was used as a loading control. Consistent with gain-of-function experiments, knockdown of endogenous FHL2 expression resulted in increased Nur77 transcriptional activity (Fig. 4B). In Western blot analysis of FHL2 and Nur77, the data from semiquantitative PCR, with the appropriate controls. Data are representative of at least two independent experiments in two different batches of SMCs. Values represent mean ± S.D. *, p < 0.05.

**FHL2 Regulates Nur77 Target Genes**—Previous reports identified enolase3 as a target gene of Nur77 in liver (21). To address...
whether FHL2 regulates Nur77 target genes, we examined expression of enolase3 in human SMCs by real-time RT-PCR. As shown in Fig. 4D, we observed a strong induction of enolase3 expression by Nur77. When FHL2 was overexpressed together with Nur77, the induction of enolase3 was significantly inhibited. Furthermore, the induction of enolase3 by Nur77 was strongly induced upon knockdown of endogenous FHL2, confirming the role of FHL2 in regulation of Nur77 target genes. To understand the inhibitory effect of FHL2 on Nur77 transcriptional activity, we performed ChIP experiments on the enolase3 promoter (Fig. 4E). In the left panel of Fig. 4E, the PCR products after ChIP are visualized, showing that Nur77 binds to the enolase3 promoter and that this association is disturbed upon overexpression of FHL2. In the right panel of Fig. 4E, the semiquantitative PCR data of the ChIP analysis are shown, including all control IgG-ChIP data. Based on these results we conclude that FHL2 reduces binding of Nur77 to its NBRE in the enolase3 promoter, which reveals the mechanism of inhibition of Nur77 transcriptional activity by FHL2.

**FHL2 Is Expressed in Vascular Cells**—We and others reported that NR4A nuclear receptors are expressed in vascular cells such as SMCs, endothelial cells, and monocytes and macrophages (3, 11). FHL2 expression was demonstrated to be most abundant in heart; however, limited data are available on the expression pattern of FHL2 in the vessel wall (33). Therefore, we determined mRNA expression of FHL2 in different vascular cells by real-time RT-PCR. Interestingly, FHL2 expression is high in human umbilical endothelial cells and SMCs; however, no FHL2 expression was detected in THP-1 monocytes, THP-1 macrophages, or mouse bone marrow-derived macrophages (Fig. 5A).

**FHL2 Knockdown Inhibits DNA Synthesis in SMCs**—Nur77 has been shown to inhibit the proliferation of vascular SMCs (43, 44). Because both Nur77 and FHL2 are expressed in SMCs, we evaluated the impact of FHL2 on the antiproliferative effect of Nur77. Human SMCs were transduced with lentiviral particles encoding Nur77 and FHL2 or shFHL2. In line with published data, overexpression of Nur77 inhibits BrdU incorporation, whereas overexpression of Nur77 together with FHL2 increases DNA synthesis (Fig. 5B). In addition, knockdown of endogenous FHL2 expression significantly decreased DNA synthesis of FCS-stimulated SMCs (Fig. 5B). Furthermore, we examined the contribution of FHL2 on Nur77-inhibited SMC growth. Knockdown of Nur77 using shRNA resulted in enhanced proliferation of SMCs compared with expression of control shRNA. Overexpression of FHL2 did not influence these results, indicating that FHL2 is involved as expected, not only on Nur77 to modulate SMC proliferation (Fig. 5D). We confirmed these results in MTT assays (data not shown). Taken together, these data demonstrate that FHL2 is involved in the regulation of SMC proliferation involving its inhibitory interaction with Nur77.

**DISCUSSION**

We initiated the current study with the aim of identifying novel co-regulators that associate with NR4A nuclear receptors to delineate the underlying mechanism involved in regulation of NR4A nuclear receptor transcriptional activity. We provide compelling evidence that FHL2 interacts with Nur77 and inhibits its transcriptional activity. Previous studies revealed that Nur1 transcriptional activity may be linked to protein stability (45). We speculated that decreased transcriptional activity of Nur77 in the presence of FHL2 could be due to decreased protein stability. However, FHL2 has no influence on Nur77 protein stability. FHL2 represses Nur77 activity both in HEK293T cells and in vascular SMCs, suggesting that the inhibitory effect of FHL2 on Nur77 activity is independent of other tissue-specific co-regulators. Obviously, because FHL2 is not expressed in...
FHL2 Is a Co-repressor of Nur77

(activated) monocytes and macrophages the activity of Nur77 is not influenced by FHL2 in these cells.

Each LIM domain of FHL2 binds to Nur77 and represses Nur77-mediated activity independently and, interestingly, each LIM domain shows similar inhibitory capability as full-length FHL2. We observed no additive or synergistic effect when multiple LIM domains are present in the same FHL2 deletion mutant. Collectively, these results suggest that a single LIM domain of FHL2 is sufficient for interaction and optimal repression of Nur77 activity and that FHL2 does not serve as a protein platform for interaction of multiple proteins, but rather binds directly to Nur77, and this way prevents interaction with other co-regulators. Our ChIP experiments clearly demonstrate that FHL2 inhibits the association of Nur77 with its response element in the enolase3 promoter, which reveals the underlying mechanism of FHL2-mediated inhibition of Nur77 transcriptional activity.

NR4A nuclear receptors contain well conserved amino acid sequences in their carboxyl-terminal LBD and central DBD but a poorly conserved amino-terminal domain. Previous studies demonstrated that the amino-terminal domain of NR4A nuclear receptors is essential for the ligand-independent transcription activation and recruitment of co-regulators (1). In search for co-factors with high specificity for the individual NR4A receptors, we applied the amino-terminal domain as bait in our yeast two-hybrid screen. Unexpectedly, FHL2 binds and inhibits all three NR4As. We demonstrated that FHL2 binds to both the amino-terminal domain as well as the central DBD, which may explain the lack of NR4A specificity of FHL2. Previously, FHL2 has been shown to bind the nuclear androgen receptor in a LBD-dependent manner, whereas no interaction was observed with the LBD of NR4A nuclear receptors.

In the current study we demonstrate that FHL2 interacts with the nuclear receptor Nur77 in activated SMCs; because Nur77 has been shown to inhibit the proliferation of SMCs, we studied FHL2 function in SMC growth and observed enhanced SMC DNA synthesis upon overexpression of FHL2 (43, 44, 47). FHL2 has been demonstrated to inhibit the transcription factor serum-response factor (SRF) in vascular SMCs (31, 46). SRF recruits myocardin-like factors to the promoter of genes that serum-response factor (SRF) in vascular SMCs (31, 46). SRF has been demonstrated to inhibit the transcription factor SMC DNA synthesis upon overexpression of FHL2 (43, 44, 47). Previously, FHL2 has been shown to bind the nuclear androgen receptor in a LBD-dependent manner, whereas no interaction was observed with the LBD of NR4A nuclear receptors.

In the current study we demonstrate that FHL2 interacts with the nuclear receptor Nur77 in activated SMCs; because Nur77 has been shown to inhibit the proliferation of SMCs, we studied FHL2 function in SMC growth and observed enhanced SMC DNA synthesis upon overexpression of FHL2 (43, 44, 47). FHL2 has been demonstrated to inhibit the transcription factor serum-response factor (SRF) in vascular SMCs (31, 46). SRF recruits myocardin-like factors to the promoter of genes that contain a CaR response element and is crucial in maintaining the expression of SMC-specific genes like SM α-actin, calponin, and SM22α. FHL2 expression was shown to be induced by SRF to subsequently bind and inhibit this transcription factor in a negative feedback loop (46). Subsequently, it was demonstrated that FHL2 inhibits recruitment of the SWI/SNF chromatin-remodeling complex to DNA-bound SRF/myocardin (related factors), resulting in FHL2-mediated inhibition of expression of SMC-specific genes in response to bone morphogenetic protein-4 (31). In the latter study it was also shown that FHL2-deficient mice display abnormal vascular relaxation, which was explained by reduced expression of the contractile SMC proteins. We propose that the enhanced SMC growth in response to FHL2 can be explained, at least partly, by reduced transcriptional activity of both Nur77 and SRF in the presence of FHL2, which results in a phenotypic switch toward activated, proliferative SMCs with reduced expression of SMC-specific contractile proteins.

In summary, we identified FHL2 as a novel interacting factor of NR4A nuclear receptors in vascular SMCs. FHL2 represses the transcriptional activity of Nur77 through interaction with the amino-terminal and DBD of Nur77 and enhances SMC growth.

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