EXPERIMENTAL CELL RESEARCH 315 (2009) 76–96

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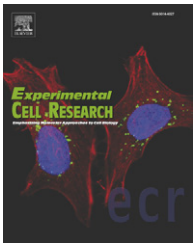

Research Article

The LIM-homeodomain transcription factor LMX1B regulates expression of NF-kappa B target genes

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ARTICLE INFORMATION

Article Chronology:
Received 4 September 2008
Revised version received
14 October 2008
Accepted 15 October 2008
Available online 28 October 2008

Keywords:
LMX1B
Gene expression profiling
NF-κB
ChIP assay
Transcription
siRNA-mediated knock-down
Chromatin
Knock-out mouse
Tet-off expression system
Luciferase reporter assay

ABSTRACT

LMX1B is a LIM-homeodomain transcription factor essential for development. Putative LMX1B target genes have been identified through mouse gene targeting studies, but their identity as direct LMX1B targets remains hypothetical. We describe here the first molecular characterization of LMX1B target gene regulation. Microarray analysis using a tetracycline-inducible LMX1B expression system in HeLa cells revealed that a subset of NF-κB target genes, including *IL-6* and *IL-8*, are upregulated in LMX1B-expressing cells. Inhibition of NF-κB activity by short interfering RNA-mediated knock-down of p65 impairs, while activation of NF-κB activity by TNF-α synergizes induction of NF-κB target genes by LMX1B. Chromatin immunoprecipitation demonstrated that LMX1B binds to the proximal promoter of *IL-6* and *IL-8 in vivo*, in the vicinity of the characterized κB site, and that LMX1B recruitment correlates with increased NF-κB DNA association. *IL-6* promoter–reporter assays showed that the κB site and an adjacent putative LMX1B binding motif are both involved in LMX1B-mediated transcription. Expression of NF-κB target genes is affected in the kidney of *Lmx1b*$^{-/-}$ knock-out mice, thus supporting the biological relevance of our findings. Together, these data demonstrate for the first time that LMX1B directly regulates transcription of a subset of NF-κB target genes in cooperation with nuclear p50/p65 NF-κB.

Introduction

LIM-homeodomain (LIM-HD) proteins are transcription factors that belong to the family of LIM proteins, originally named for the LIM domain-containing transcription factors Lin11, Isl1, and Mec3. To date, at least 12 LIM-HD transcription factors have been characterized in mammals (LHX1–9, ISL1–2, LMX1A and LMX1B). They play key regulatory functions in cell-type specification during development, and many of them are associated with human diseases (reviewed in [1,2]). LIM-HD proteins feature two LIM domains in their amino termini and a central homeodomain (HD). The HD is a highly conserved 60 amino acid domain that mediates the binding to specific DNA elements within target genes. The majority of the characterized HD recognizes AT-rich elements containing a 5′-TAAT-3′ core motif (or ATTA on the other strand) (reviewed in [3,4]). The LIM domain is a conserved cysteine- and histidine-rich zinc-coordinating domain of approximately 50–60 amino acids, consisting of two tandemly repeated zinc fingers. The LIM domain is a multifunctional protein–protein interaction domain. It mediates interactions with other transcription factors

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doi:10.1016/j.yexcr.2008.10.012

bound in the vicinity, as well as with transcriptional cofactors that do not bind to DNA (reviewed in [1]), and is thus responsible for positively or negatively regulating the transcriptional activity of LIM-HD proteins. Several cofactors of LIM-HD have been described, including the CLIM coactivators (CLIM1/LDB2 and CLIM2/LDB1/NLI) and the corepressor RLIM. LDB1 is a cofactor of multiple LIM-containing proteins, and plays essential functions in development (reviewed in [5]). It regulates transcription through protein interactions via its C-terminal LIM interaction domain (LID) [6–9]. The RING H2 zinc finger protein RLim is a negative transcriptional regulator of LIM domain-containing factors, through recruitment of histone deacetylase (HDAC) as well as through its ubiquitin ligase activity that targets both LDB1 and LIM-HD proteins for degradation [10,11].

The LIM-HD transcription factor LMX1B is expressed in multiple tissues, including kidney, brain, developing limb and eye, and cranial mesenchyme [12–21] (also reviewed in [2,22]). Gene targeting studies in mouse uncovered numerous functions of LMX1B. *Lmx1b* homozygous knock-out mice display limb defects, including dorsal-to-ventral conversion of the limbs and a lack of patella and nails [13]. These mice also display kidney defects, in particular abnormalities in the glomerular basement membrane, and impaired differentiation and maintenance of the podocytes, the cells specifically expressing LMX1B in the kidney [23], resulting in severe proteinuria and death [13,24–26]. In the brain, LMX1B is involved in the development of mesencephalic dopaminergic neurons [21] and of serotonergic neurons [17], and in the differentiation and migration of afferent sensory neurons [27]. It is also essential for the development of the tectum and the cerebellum [18]. Finally, LMX1B is required for the development of multiple tissues of the anterior segment of the murine eye [19].

Interestingly, the *Lmx1b* null mouse model mimics the human disease associated with *LMX1B* heterozygous mutations, nail–patella syndrome (NPS), also known as hereditary osteo-onychodysplasia (HOOD) and Fong's Disease. NPS is a rare autosomal dominant disorder characterized by various skeletal dysplasia (nail dysplasia, hypoplastic or missing patella, elbow dysplasia) in 70–90% of NPS patients, podocyte-associated nephropathy in 40% of NPS cases, open angle glaucoma in 10% of NPS patients, and often neuropathy (reviewed in [2,22,28]). Mutations in *LMX1B* concentrate within the exons encoding the LIM domains (LIM1 44%, LIM2 38%) and the HD (18%) [28], strongly supporting a role of LMX1B as a transcription factor and the importance of LIM domain-interacting cofactors.

LMX1B interacts through its LIM domains with the bHLH transcription factor E47 on the *insulin* gene minienhancer, resulting in synergistic gene activation [29,30]. LMX1B also interacts with the LDB1 cofactor *in vitro* [6–8,26,31]. Overexpression of LDB1 inhibits the synergistic activation by LMX1B and E47 in a reporter assay [7,32], very likely by competing for interaction to LMX1B LIM domains. We recently showed that LDB1 is essential for podocyte function *in vivo*, possibly as a cofactor of LMX1B transcriptional activity [26]. By contrast, E47 has no essential function in podocyte *in vivo*, and is thus an unlikely cofactor of LMX1B in the kidney [26]. Given LDB1 broad *in vivo* expression pattern and its role as a multifunctional adaptor protein, one would predict that specific cofactors of LMX1B exist *in vivo*, to regulate the tissue-specific expression of LMX1B target genes. However, tissue- and cell-specific LMX1B cofactors have not been identified thus far.

Immunohistochemical, *in situ* hybridization, and microarray analyses of *Lmx1b* null mice identified genes with deregulated expression (at RNA and/or protein level) in the various LMX1B-expressing tissues [13,18,19,24,25,33–35]. Thus, in the developing eye of *Lmx1b*$^{-/-}$ mice, *Foxc1* and *Foxc2* are upregulated and *keratocan* is absent [19]. In the developing tectum and cerebellum of *Lmx1b* null mice, expression of *Wnt1*, *En1, Pax2* and *Gbx2* is reduced while expression of *Fgf8* is abolished [18]. In the developing limb, loss of dorsoventral asymmetry is associated with an altered expression pattern of the *sFrp2*, *Six1* and *Six2* genes [33] and with up- and downregulation of multiple genes [34]. In the kidney of newborn *Lmx1b*$^{-/-}$ mice, expression of *Nphs2* (encoding podocin) is abolished, while expression of *Col4a3* and *Col4a4* (encoding the α(3) and α(4) chains of type IV collagen) and of *Cd2ap* is downregulated [13,24,25,35]. A/T-rich, so-called FLAT elements (FLAT-E: TAATTA, FLAT-F: TTAATA or TATTAA on the other strand) present in the promoter regions or introns of the genes encoding podocin, collagen IVα3 and IVα4, and CD2AP bind recombinant LMX1B *in vitro* [24,25,35], and drive LMX1B-mediated transcription of a reporter gene when present as multiple copies [24,35] but not from the native promoter [25]. In addition, expression of the genes encoding podocin, collagen IVα3 and IVα4, and CD2AP is not affected in the kidney of NPS patients [36]. Therefore, in the absence of *in vivo* molecular characterization of their regulation by LMX1B, it remains unclear whether deregulation of these genes in *Lmx1b* null mice is the consequence of an impaired or blocked differentiation program, or whether they represent *bona fide* LMX1B direct target genes.

We describe here the first molecular characterization of LMX1B target gene regulation *in vivo*. To identify putative LMX1B target genes, human LMX1B was expressed in HeLa cells using a tet-off inducible system. Genome-wide and quantitative RT-qPCR expression studies revealed that several NF-κB target genes (*IL-6*, *IL-8*, *IL-1β*, *IFN-β*, ...) and multiple interferon-stimulated genes (ISGs) were upregulated upon LMX1B expression. We found that while ISGs were induced in response to IFN-β, NF-κB target genes were directly activated by LMX1B. Chromatin immunoprecipitation assays demonstrated that LMX1B protein is recruited to the promoter of the NF-κB target genes *IL-6* and *IL-8*. Using NF-κB p65-specific siRNAs, we showed that induction of NF-κB target genes by LMX1B requires a functional p50/p65 NF-κB transcription factor. Interestingly, gene expression analysis in *Lmx1b* null mice revealed that the NF-κB targets induced by LMX1B in HeLa cells were upregulated in the kidney of *Lmx1b*$^{-/-}$ mice, suggesting LMX1B-mediated transcriptional repression of these genes in murine kidney, whereas they remained unaffected in a kidney tubuli-derived cell line (LLC-PK$_1$) upon LMX1B expression. Our data thus demonstrate for the first time that LMX1B regulates transcription of a subset of NF-κB target genes in cooperation with p50/p65 NF-κB, probably through recruitment of cell-specific LIM-interacting cofactors.

Materials and methods

Plasmids

The human LMX1B pcDNA3-derived (Invitrogen) expression vector (p3M/myc-LMX1B) used for co-transfection in luciferase reporter assays has been described [26]. The pUHD10-3/myc-