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Recommended Citation
Miner, Jeffrey H.; Morello, Roy; Andrews, Kaya L.; Li, Cong; Antignac, Corinne; Shaw, Andrey S.; and Lee, Brendan, "Transcriptional induction of slit diaphragm genes by Lmx1b is required in podocyte differentiation." The Journal of Clinical Investigation. 109, 8. 1065-1072. (2002).
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Transcriptional induction of slit diaphragm genes by Lmx1b is required in podocyte differentiation

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Received for publication August 13, 2001, and accepted in revised form February 18, 2002.


Introduction

Podocytes are specialized cells of the renal glomerulus with both epithelial and mesenchymal characteristics. They lie atop the glomerular basement membrane (GBM) in Bowman’s space and enwrap the glomerular capillaries with long, interdigitated foot processes. Podocytes are receiving increased attention and recognition as critical components of the kidney’s ultrafiltration barrier (1, 2). They not only synthesize and secrete GBM components but also assemble the glomerular slit diaphragms, which are thought to be the kidney’s ultimate size-selective filtration barrier (3, 4).

Several genes have recently been shown, through either positional cloning or gene knockout approaches, to be involved in podocyte function. Most of these genes encode structural proteins that are important in formation, function, and/or maintenance of foot process architecture and slit diaphragm integrity (2, 5, 6). However, two encode transcription factors presumed to regulate expression of genes in podocytes. The first, Pod1, encodes a basic-helix-loop-helix protein expressed in podocytes and in other cell types in the kidney and elsewhere (7). The phenotype of the Pod1−/− kidney is complex, but it is clear that podocytes are developmentally arrested (8). This suggests that Pod1 normally activates expression of genes important for podocyte maturation, but specific downstream genes have not been identified. The second transcription factor, Lmx1b, encodes a LIM-homeodomain protein (9). Mutations in LMX1B cause nail-patella syndrome (NPS), an autosomal dominant disease with skeletal abnormalities, nail hypoplasia, and nephropathy. Expression of glomerular basement membrane (GBM) collagens is reduced in Lmx1b−/− mice, suggesting one basis for NPS nephropathy. Here, we show that Lmx1b−/− podocytes have reduced numbers of foot processes, are dysplastic, and lack typical slit diaphragms, indicating an arrest in development. Using antibodies to podocyte proteins important for podocyte function, we found that Lmx1b−/− podocytes express near-normal levels of nephrin, synaptopodin, ZO-1, α3 integrin, and GBM laminins. However, mRNA and protein levels for CD2AP and podocin were greatly reduced, suggesting a cooperative role for these molecules in foot process and slit diaphragm formation. We identified several LMX1B binding sites in the putative regulatory regions of both CD2AP and NPHS2 (podocin) and demonstrated that LMX1B binds to these sequences in vitro and can activate transcription through them in cotransfection assays. Thus, LMX1B regulates the expression of multiple podocyte genes critical for podocyte differentiation and function. Our results indicate that reduced levels of proteins associated with foot processes and the glomerular slit diaphragm likely contribute, along with reduced levels of GBM collagens, to the nephropathy associated with NPS.

LMX1B encodes a LIM-homeodomain transcription factor. Mutations in LMX1B cause nail-patella syndrome (NPS), an autosomal dominant disease with skeletal abnormalities, nail hypoplasia, and nephropathy. Expression of glomerular basement membrane (GBM) collagens is reduced in Lmx1b−/− mice, suggesting one basis for NPS nephropathy. Here, we show that Lmx1b−/− podocytes have reduced numbers of foot processes, are dysplastic, and lack typical slit diaphragms, indicating an arrest in development. Using antibodies to podocyte proteins important for podocyte function, we found that Lmx1b−/− podocytes express near-normal levels of nephrin, synaptopodin, ZO-1, α3 integrin, and GBM laminins. However, mRNA and protein levels for CD2AP and podocin were greatly reduced, suggesting a cooperative role for these molecules in foot process and slit diaphragm formation. We identified several LMX1B binding sites in the putative regulatory regions of both CD2AP and NPHS2 (podocin) and demonstrated that LMX1B binds to these sequences in vitro and can activate transcription through them in cotransfection assays. Thus, LMX1B regulates the expression of multiple podocyte genes critical for podocyte differentiation and function. Our results indicate that reduced levels of proteins associated with foot processes and the glomerular slit diaphragm likely contribute, along with reduced levels of GBM collagens, to the nephropathy associated with NPS.

The Journal of Clinical Investigation | April 2002 | Volume 109 | Number 8

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end-stage renal failure (12), so their importance for renal function is well recognized. Levels of \( \text{Col4a3} \) and \( \text{Col4a4} \) RNA and protein are reduced in \( \text{Lmx1b}^{-/-} \) glomeruli, and \( \text{LMX1B} \) binds to a site in their common regulatory region (13). Thus, reduced expression of \( \text{COL4A3} \) and \( \text{COL4A4} \) in NPS patients is a likely consequence of \( \text{LMX1B} \) haploinsufficiency. This reduction would contribute to the nephropathy which, like Alport syndrome, is characterized by the presence of distinct GBM abnormalities.

Here, we have characterized the \( \text{Lmx1b}^{-/-} \) podocytes in detail. We found significant morphological defects as well as defects in podocyte gene expression, suggesting that \( \text{Lmx1b}^{-/-} \) podocytes do not differentiate properly. Our results indicate that reduced levels of proteins associated with foot processes and the glomerular slit diaphragm likely contribute, along with reduced levels of GBM collagens, to the nephropathy associated with \( \text{LMX1B} \) haploinsufficiency in NPS.

**Methods**

**Electron microscopy.** Kidneys from newborn control and \( \text{Lmx1b}^{-/-} \) mice were fixed in 2% paraformaldehyde/2% glutaraldehyde in 0.15 M sodium cacodylate. They were then rinsed in cacodylate buffer, stained with osmium tetroxide and uranyl acetate, dehydrated, and embedded in Poly/Bed 812. Ultrathin sections were stained with uranyl acetate and lead citrate and viewed with an electron microscope. Reagents were obtained from Polysciences Inc. (Warrington, Pennsylvania, USA).

**Immunohistochemistry.** Kidneys were frozen in OCT compound and sectioned at 7 \( \mu \text{m} \). Sections were fixed in 2% paraformaldehyde in PBS for 10 minutes and rinsed in PBS. Primary antibodies were diluted in 1% BSA in PBS and applied for 1 hour. After a PBS rinse, fluorophore-conjugated secondary antibodies were applied for 1 hour. Sections were rinsed in PBS, mounted in 0.1 \( \times \) PBS/90% glycerol/1 mg/ml \( p \)-phenylenediamine, and viewed with a fluorescence microscope. Images were captured with a Spot 2 cooled color digital camera (Diagnostic Instruments Inc., Sterling Heights, Michigan, USA).

**Antibodies.** Antibodies obtained from generous colleagues were: rabbit anti-human nephrin (14, 15) from Karl Tryggvason (Karolinska Institute, Stockholm, Sweden); rabbit anti-chick integrin \( \alpha_3 \) (16) from C. Michael Dipersio (Albany Medical College, Albany, New York, USA); mouse anti-human synaptopodin (17) from Peter Mundel (Albert Einstein College of Medicine, Bronx, New York, USA); and rabbit anti-human \( \alpha \)-actinin-4 (18) from Alan Beggs (Children’s Hospital, Boston, Massachusetts, USA). Rat anti-mouse laminin \( \gamma 1 \) (MAB1914) was purchased from Chemicon International (Temecula, California, USA). Rat anti-mouse \( \alpha \)-actinin-4 (18) from Alan Beggs (Children’s Hospital, Boston, Massachusetts, USA); and rabbit anti-human podocin (22) were generated as described.
Secondary antibodies were conjugated to FITC (ICN Biomedicals Inc., Costa Mesa, California, USA) or Cy3 (Chemicon International).

In situ hybridizations. For Cd2ap in situ, kidneys were frozen fresh in OCT and sectioned at 12 µm. Digoxigenin-UTP-labeled sense and antisense Cd2ap riboprobes were hybridized to the sections and detected as described (23). The segment of mouse Cd2ap cDNA used to make the probes extended from nucleotides 430 to 926 (GenBank accession no. AF149092). For podocin in situ, kidneys were fixed in 4% paraformaldehyde in PBS and embedded in paraffin. Sectioning and in situ hybridization were carried out as described (24). The mouse podocin cDNA probe was homologous to nucleotides 250–632 of human podocin (GenBank accession no. AJ279254). The probe was generated by RT-PCR amplification of newborn mouse kidney cDNA using primers designed from a mouse expressed sequence tag (GenBank accession no. AW106985). Primers were: sense, 5′-CCGCCACCGTAGTGGACGTG-3′; antisense, 5′-CTCCATATTAAACATATTCTTG-3′. Antisense and sense probes were labeled with α-35S-UTP (ICN Biomedicals Inc.).

Electrophoretic mobility shift assay and transfections. Expression plasmids contained cDNAs encoding the full-length human LMX1B cDNA, its wild-type homeodomain, or the N246K mutant homeodomain in pcDNA3.1 (10). In vitro transcription/translation reactions were performed with unlabeled or 35S-labeled methionine using the TNT Quick Coupled Transcription/Translation System (Promega Corp., Madison, Wisconsin, USA). Labeled products were analyzed by PAGE to estimate efficiency of translation. Putative LMX1B binding sites (FLAT sequences) found in 5′ genomic sequences of CD2AP and NPHS2 (GenBank accession nos. AL355353 and AL160286, respectively) were generated by synthesis and annealing of two complementary oligonucleotides, as follows. CD2AP–1817: 5′-GATACGAACAAAGTTAATTAACAGTA-3′; antisense, 5′-CATCAGCTATATTAAATATAACAGCT-3′. Antisense and sense probes were labeled with α-35S-UTP (ICN Biomedicals Inc.).

Results

Aberrant foot process and slit diaphragm formation in Lmx1b–/– podocytes. Examination of glomeruli in Lmx1b–/– kidneys revealed an aberrant morphology at birth, including a dilated Bowman’s space and atypical cellular organization (data not shown). These abnormalities were similar to those visible in the initial report of the Lmx1b–/– phenotype (11). Lmx1b has been shown to positively regulate expression of Col4a3 and Col4a4 (13), genes that encode GBM collagens. However, reduced expression of only these genes cannot fully explain the glomerular abnormalities, because mice with inactivating mutations in Col4a3 and Col4a4 have unremarkable glomeruli at young ages (27–29). Here, we further investigated the nature of the glomerular defect in Lmx1b mutants, paying particular attention to podocytes, the specialized glomerular cell that expresses Lmx1b (13).

We performed an ultrastructural analysis of podocyte morphology in Lmx1b–/– and Lmx1b+/– glomeruli (Figure 1). Electron microscopy was used to evaluate the refined structure of podocytes that is so critical for proper efficiency. Luciferase and β-galactosidase activities were assayed as described previously (13) 16 hours after an initial 4-hour incubation with the transfection mixture.

Figure 2

Immunohistochemical analysis of podocyte gene expression in Lmx1b–/– and Lmx1b+/– glomeruli from newborn mice. No differences in levels of nephrin (a and b), laminin (Lam) α5 (c and d), or integrin α3 (e and f) were detected, suggesting that Lmx1b does not normally regulate their expression. G, glomerulus. Bar, 50 µm.
glomerular filtration (1). In comparison to control, Lmx1b−/− podocytes exhibited striking defects. Foremost was the lack of normal foot process formation. Though mutant podocytes were properly juxtaposed to the GBM, they only rarely elaborated processes (Figure 1, a, b, d, and e). Most mutant podocytes appeared quite similar to control podocytes present in immature glomeruli (Figure 1c), suggesting that there is an arrest in podocyte development at the stage when foot processes normally form. (The mutant podocytes examined were from the most mature glomeruli, so our interpretation was not confounded by comparing immature mutant podocytes to mature control podocytes.)

Another defect was observed in the morphology of those processes that did form: many did not have the flattened, side-by-side appearance typical of normal foot processes (Figure 1b, arrowheads). Instead, they had a somewhat stacked arrangement, with one process appearing to invaginate slightly beneath an adjacent one (Figure 1f, arrows). In addition, there was a lack of typical slit diaphragms between the processes. Slit diaphragms are specialized cell-cell junctions with characteristics of both adherens junctions (30) and tight junctions (31). They extend across the gap between foot processes and are thought to serve as the kidney’s ultimate filtration barrier. Slit diaphragms were visible in controls as electron-dense material lying between the foot processes parallel to the GBM (Figure 1b). In Lmx1b−/− mice, two classes of cell-cell junctions were observed: those between the rare processes, and those between adjacent podocyte cell bodies. For both classes, electron-dense material was not always observed in the spaces between the cells, and when it was, it was usually not parallel to the GBM (Figure 1f and data not shown). Thus, there were clearly defined morphological defects in Lmx1b−/− podocytes, suggesting perturbation of podocyte development. In addition, the nature of the defects suggests that renal function should be impaired. Consistent with this prediction, little if any urine was found in the bladders of Lmx1b−/− mice, and there were protein casts in a subset of tubules in the mutant kidney (data not shown). This indicates that what little urine was produced by Lmx1b−/− mice contained significant levels of protein, one hallmark of filtration barrier defects.

Aberrant podocyte gene expression in Lmx1b mutants. Based on these results, we hypothesized that Lmx1b, in addition to regulating collagen IV genes, also regulates podocyte genes encoding proteins involved in slit diaphragm and foot process formation. Over the last few years, several proteins from diverse families have been shown or hypothesized to be critically important for proper podocyte and/or slit diaphragm development, maturation, and function (2, 5, 6). It is unknown whether the genes encoding these proteins are regulated by Lmx1b. Those that are should exhibit reduced expression in Lmx1b−/− podocytes. We used immunohistochemistry to assay for expression of podocyte proteins thought to be important for filtration. The data shown were derived from the deepest glomeruli to ensure that only the most mature podocytes were assayed. Levels of several proteins were not significantly changed. These included nephrin, a component of the slit diaphragm (32–35); laminin α5 and γ1 chains, components of the GBM (20, 36); integrin α3, a podocyte receptor for laminin in the GBM (37); and α-actinin-4, a cytoskeletal protein mutated in one form of familial focal segmental glomerulosclerosis (18) (Figures 2 and 3). Thus, the genes encoding these proteins are not likely to be directly regulated by Lmx1b on a transcriptional level. Slight reductions in levels of ZO-1, a cytoplasmic protein found associated with tight junctions and slit diaphragms (31), and synaptopodin, an actin-asso-
associated protein found in foot processes (38), were consistently observed (Figure 3, g and h; and data not shown). However, because the reductions were slight, we suspect that they are more likely attributable to the paucity of cell-cell junctions and foot processes (respectively) than to decreased gene expression.

In contrast to these results, levels of CD2AP, an adapter protein that binds to the cytoplasmic tail of nephrin at the slit diaphragm (39), and podocin, a transmembrane protein which has been localized to the slit diaphragm (22, 40), were drastically reduced in mutant podocytes (Figure 3). Reduction was apparent not only in the deepest, most mature glomeruli, but also in the very primitive S-shape and capillary loop stage glomeruli (data not shown). Importantly, CD2AP and podocin are known to have crucial roles in podocyte function. Mice lacking CD2AP and humans with homozygous or compound heterozygous mutations in NPHS2, the gene encoding podocin, exhibit nephrotic syndrome accompanied by foot process effacement (41, 42). We therefore conclude that the podocyte abnormalities observed in Lmx1b–/– kidneys are, at least in part, caused by reduced levels of CD2AP and podocin.

We next hypothesized that Lmx1b directly regulates transcription of the Cd2ap and Nphs2 genes. To begin to test this hypothesis, we performed in situ hybridization on sections of control and Lmx1b–/– kidneys with probes for Cd2ap and podocin mRNA. This was done to distinguish potential posttranscriptional effects on gene expression; rather than regulating Cd2ap and Nphs2 directly, Lmx1b might regulate expression of other genes whose products stabilize CD2AP and podocin. However, the in situ analysis revealed significant reductions in the levels of both Cd2ap and podocin mRNAs (Figure 4). The reduction was most evident for Cd2ap in early capillary loop structures, the stage at which CD2AP protein is first detectable in normal kidney (14). Importantly, the reduction was specific to podocytes; Cd2ap RNA and protein levels were not significantly reduced in mutant collecting ducts (Figure 4a and data not shown), consistent with the fact that Lmx1b is not normally expressed in collecting ducts (11, 13). Podocin RNA was essentially undetectable in Lmx1b mutant kidney but was easily detected in the control (Figure 4b). Semiquantitative RT-PCR demonstrated that total podocin mRNA levels were reduced by 75% (data not shown). Together, these data provide strong evidence that Lmx1b is involved in regulating transcription of Cd2ap and Nphs2. Consistent with this notion, Lmx1b is expressed in primitive structures in the nephrogenic zone (11, 13), at stages before the onset of Cd2ap and Nphs2 expression (14, 42).

LMX1B binds to putative regulatory elements in Cd2ap and Nphs2 promoters. If Lmx1b directly regulates transcription of Cd2ap and Nphs2, then their regulatory regions should contain LMX1B binding sites. Unfortunately, the regulatory regions of these genes have not yet been fully characterized. Nevertheless, we were able to retrieve genomic DNA sequences of human CD2AP and NPHS2 from GenBank. Candidate LMX1B binding sequences (FLAT elements) (25) were identified in both genes in the sequences flanking the first exons (Figure 5a), where transcriptional regulatory elements are expected to be located. In Cd2ap, five FLAT sites flanked the predicted first exon, at –2855, –1817, –1170, +874, and +1053 nucleotides relative to the ATG translational start codon. In Nphs2, three FLAT sites flanked the predicted first exon, at –1251, –825, and +1275 relative to the ATG.

To determine whether LMX1B is capable of binding to these elements, we performed EMSA. Oligonucleotides representing both strands of the three 5′ CD2AP sites and the –825 NPHS2 site were synthesized and annealed to make double-stranded FLAT sites. Oligonucleotides were end-labeled with 32P and incubated with either full-length...
LMX1B or its DNA-binding homeodomain. The mixtures were then separated by native PAGE and subjected to autoradiography (Figure 5b). All four sites were bound by the full-length protein and were bound even more efficiently by the homeodomain polypeptide. No significant mobility shifts were observed if a mutant homeodomain (N246K, described in an NPS patient) or a negative control was used. This suggests that binding of LMX1B to the FLAT sites was specific.

To further demonstrate specificity of LMX1B binding, additional assays with the CD2AP –1817 site were performed (Figure 5c). An excess of the unlabeled (cold) –1817 probe was incubated along with the 32P-labeled probe. The cold probe was able to compete for binding to both full-length LMX1B and the homeodomain fragment, as shown by the reduction in intensity of the shifted bands, but 300-fold excess of an unrelated cold site did not compete effectively. Finally, an excess of the CD2AP –1817 site was able to compete for LMX1B binding to a FLAT-E site from the COL4A3-COL4A4 regulatory region (13). Taken together, these data show that LMX1B can efficiently bind to potential regulatory elements from both CD2AP and NPHS2 in a dose-related and specific fashion, consistent with the hypothesized regulation of these genes by Lmx1b.

LMX1B upregulates a reporter construct containing NPHS2 –825 FLAT sites. LMX1B is considered a relatively weak transactivator that requires coactivators for optimal activity (43). The coactivators required for Lmx1b-mediated expression of Cd2ap and Nphs2 in podocytes are unknown. Nevertheless, we attempted to demonstrate LMX1B-mediated transactivation of the NPHS2 –825 FLAT site in heterologous cells.
Four copies of this site and its flanking sequences were cloned upstream of a minimal promoter and a luciferase reporter. A similar reporter with a mutated version of the site was also constructed. These were cotransfected into NIH 3T3 cells with either LMX1B expression plasmid or empty vector, together with a β-galactosidase expression plasmid to control for transfection efficiency. LMX1B upregulated expression from the wild-type FLAT site, but not from the mutant site (Figure 6). Together with our immunohistochemical, in situ, and gel shift data, these results strongly suggest that LMX1B is involved in the transcriptional regulation of NPHS2 and CD2AP in podocytes, providing an additional basis for the nephropathy observed in NPS.

Discussion

The fact that heterozygous mutations in LMX1B cause a glomerulopathy suggests that LMX1B regulates genes critical for podocyte function. Homozygous Lmx1b+/− mice are an excellent model system for defining which genes expressed in podocytes are regulated by Lmx1b, and therefore which genes might be underexpressed — perhaps only subtly as a consequence of haploinsufficiency — in NPS. We previously reported reduced levels of Col4a3 and Col4a4 protein and RNA in Lmx1b+/− glomeruli (13); here, we found greatly reduced levels of both CD2AP and podocin protein and RNA (Figures 3 and 4). As these four proteins are known to be required for normal glomerular function, the glomerulopathy in NPS could stem from a reduction in any or all of them. In addition, there may be other genes whose expression is regulated by Lmx1b and whose products are important for podocyte differentiation and function.

Our electron microscopic analysis of Lmx1b+/− podocytes (Figure 1) showed that Lmx1b is required for normal podocyte differentiation. Mutant podocytes appeared to be developmentally arrested: they did not elaborate normal foot processes, and cell-cell junctions did not have typical slit diaphragms. Both CD2AP and podocin have been localized to the slit diaphragm (22, 39, 40) and are necessary for normal glomerular function (41, 42). We therefore suggest that reduced levels of CD2AP and podocin contribute to the developmental arrest and conclude that they normally act cooperatively to promote foot process and slit diaphragm formation during glomerulogenesis. Indeed, podocin has recently been shown to interact directly with CD2AP (40).

The ultrastructure of the cell-cell junctions that form between the mutant podocytes bears some resemblance to that reported for homozygous Nphs1 (nephrin) mutant podocytes (44). Nephrin is a major component of the slit diaphragm (32–35), and mutations in NPHS1 cause congenital nephrotic syndrome of the Finnish type (45, 46). The fact that we found significant levels of nephrin protein in Lmx1b mutant podocytes (Figure 2) suggests that nephrin is not sufficient for normal slit diaphragm formation. As CD2AP and podocin interact with each other (40), and CD2AP interacts with nephrin (39, 41), CD2AP and podocin may cooperate to organize or stabilize nephrin. Interestingly, podocytes in mice lacking nephrin also have reduced numbers of foot processes and exhibit some effacement (44), but the severity of these defects is less than we observed in the Lmx1b+/− podocytes. Thus, proteins other than nephrin must coordinate foot process formation, and CD2AP and podocin are likely to be involved.

The demonstration of LMX1B binding sites in putative regulatory regions of the CD2AP and NPHS2 genes (Figure 5) and the ability of LMX1B to transactivate the NPHS2 –825 site (Figure 6) strengthen our conclusion that Lmx1b positively regulates their expression. We previously showed that CD2AP and nephrin begin to accumulate in differentiating podocytes at approximately the same stage and predicted that they would be coregulated (14). However, we did not find a significant reduction in nephrin mRNA (13) or protein (Figure 2) in the absence of Lmx1b. This is consistent with the fact that a 1.25-kb segment of the human nephrin promoter drives expression of a reporter in podocytes (47), but it lacks consensus LMX1B binding sites. It will be interesting to determine whether Pod1, a basic-helix-loop-helix protein required for podocyte development (7, 8), and WT1, a zinc finger transcription factor expressed in podocytes (48) and mutated in Denys-Drash syndrome and isolated diffuse mesangial sclerosis (49, 50), are involved in regulating expression of any of the genes, such as Nphs1, that do not appear to be regulated by Lmx1b (Figure 2 and data not shown). These transcription factors could each control expression of nonoverlapping sets of podocyte genes necessary for proper glomerulogenesis and podocyte function. Haploinsufficiency of LMX1B (as occurs in NPS) or POD1 could lead
to either subtly or significantly reduced expression of a number of podocyte genes. Over time or with the appropriate genetic or environmental cofactors, this could in turn lead to nephropathy.

Acknowledgments

We thank Alan Beggs, Michael D persio, Peter Mundel, and Karl Tryggvason for gifts of antibodies; and Lori LaRose and Marilyn Levy of the Washington University School of Medicine Electron Microscope Facility for assistance. This work was supported by grants from the NIH to J. H. Miner (R01DK053196 and P50DK045181), A. S. Shaw (R01DK058366), and B. Lee (P01HD022657 and R01AR044738) and from the Arthritis Foundation to B. Lee. R. Morello was supported by a fellowship from Telethon-Italy.