Glomerular Permeability Is Altered by Loss of P0, a Myelin Protein Expressed in Glomerular Epithelial Cells

Emmanuelle Plaisier,* Béatrice Mougenot,* Marie Christine Verpont,* Chantal Jouanneau,* Joan J. Archelos,† Rudolf Martini,† Donsctho Kerjaschki,§ and Pierre Ronco*

*Department of Nephrology, INSERM Unit 702, Tenon Hospital (AP-HP), University Pierre et Marie Curie, Paris, France; †Department of Neurology, University of Graz, Graz, Austria; ‡Department of Developmental Neurobiology, University of Würzburg, Würzburg, Germany; and §Department of Clinical Pathology, University of Vienna, Vienna, Austria

The myelin protein 0 (MPZ or P0) is a transmembrane glycoprotein that represents the most abundant myelin component. Mutations in the P0 gene are associated with one form of autosomal dominant demyelinating peripheral neuropathy, Charcot-Marie-Tooth disease type 1B (CMT1B). Because CMT1 may be associated with renal involvement, mostly focal segmental glomerulosclerosis, we hypothesized that P0 could be expressed in the kidney. P0 mRNA was detected by reverse transcriptase–PCR in the human and mouse renal cortex. P0 transcripts were identified by in situ hybridization at different stages of the mouse kidney development, especially in embryonic structures that give rise to the glomerulus. P0 protein was also detected by Western blot in human and rat glomerular extracts and in a human podocyte cell line using a monoclonal anti-P0 antibody. Immunogold electron microscopy disclosed a predominant staining of the membranes of intracellular vesicles in podocytes. P0 was also detected in the podocyte cell membrane, including at the foot processes. P0−/− mice exhibited mild growth retardation and demyelinating neuropathy similar to the one observed in patients with CMT1B. They also presented mild albuminuria, without significant ultrastructural change of the glomerular basement membrane or the podocytes. These results demonstrate that P0, the major myelin protein, is also expressed during nephrogenesis and in mature kidney, mostly in podocytes. They suggest that P0 gene mutations might be involved in renal diseases.


Glomerular epithelial cells, podocytes, are highly specialized cells that participate in the glomerular filtration barrier through the filtration slits bounded by the interdigitated foot processes and the synthesis of glomerular basement membrane (GBM) components. In the past few years, genetic studies of familial forms of nephrotic syndrome in human have expanded our knowledge on the cell biology of podocytes. They led to the identification of proteins that are functionally important in maintaining permselectivity of the glomerulus. These proteins include nephrin and podocin located at the slit diaphragm and the cytoskeleton-associated α-actinin 4 protein (1–3). In vitro experimental studies and knockout mouse models have further identified additional podocyte proteins, interacting mostly with nephrin and podocin for the maintenance of the slit diaphragm integrity. It is interesting that some of the podocyte proteins are electively coexpressed in the central nervous system. These include the cytoskeletal component synaptopodin, nephrin, densin, glomerular epithelial protein 1, the synaptic vesicle molecule Rab3A and its effector Rabphilin-3a, and the neuronal amino acid transporters CAT3 and EAAT2 (4–9). However, none of these proteins has been found in the myelinated peripheral nervous system, although focal segmental glomerulosclerosis (FSGS) has been reported in the most common familial form of demyelinating neuropathy, Charcot-Marie-Tooth (CMT) disease (10–12).

In this study, we investigated the renal expression and role of the myelin protein 0 (MPZ or P0). P0 is the most abundant protein of the peripheral nervous system (PNS), produced by Schwann cells. P0 is a small, integral membrane glycoprotein that has a single Ig-like extracellular domain and is responsible for membrane adhesion and compaction of the myelin membranes (13,14). Mutations in the P0 gene have been associated with hereditary peripheral demyelinating neuropathy, including CMT1B, Dejerine-Sottas disease, and congenital hypomyelination neuropathy (15). Expression of P0 outside the PNS has not been reported so far, except for thymic stromal cells (16). Here, we demonstrate that P0 is synthesized in the kidney and mostly localized in the podocytes from earlier stages of glomerulogenesis. Furthermore, knockout mice, whose neurologic phenotype is closely similar to the human disease (17), show increased glomerular permeability to albumin, suggesting a role for P0 in the control of glomerular permselectivity and its possible implication in CMT-associated renal disease.

Materials and Methods
Reverse Transcriptase–PCR
Total RNA was extracted from human adult kidney cortex (normal pole of cancer kidney) and mouse kidney using RNAwiz (Ambion Inc.,
Western Blot

Proteins from human and rat isolated glomeruli were extracted by lysis using a detergent-buffered lysis buffer (Complete Mini; Roche Diagnostic Corp., Indianapolis, IN). Protein extracts were also prepared according to the same procedure, from the human podocyte cell line established in our laboratory (18). Total protein extracts were run on a 12% SDS-PAGE gel and transferred to a nitrocellulose membrane (Immobilon-P; Millipore, Bedford, MA) by electrophoretic transfer. The proteins were immunoblotted with anti-P0 monoclonal antibody (mAb) (1:1000 diluted in 10% FCS/PBS) (20) or rabbit anti-nephrin antibody (1:1000 in 10% FCS/PBS; a gift from C. Antignac; INSERM Unit 574, Paris, France) (20) or rabbit anti-PO mAb (1:100 diluted in 10% FCS-PBS) and rabbit anti-podocin antibody (1:1000 in 10% FCS/PBS; a gift from F. Shimizu, Niigata, Japan) (21). After blocking unspecific binding in 10% skimmed milk PBS, membranes were incubated overnight with anti-P0 mAb (1:1000 in 10% FCS-PBS) and rabbit anti-PO mAb (1:100 diluted in 10% FCS-PBS) at 4°C. After washes in PBS, sections were incubated for 1 h with 10% FCS-PBS and incubated overnight with 2 h with alkaline phophatase–conjugated goat anti-mouse Ig (Chemicon International, Temecula, CA). The anti-P0 mAb was omitted in controls. Blots were developed using nitro blue tetrazolium and 5-bromo-4-chloro-indolyl phosphate reagents (Sigma-Aldrich, Steinhiem, Germany).

Double Immunofluorescence Labeling and Confocal Microscopy

For dual fluorochrome labeling, the slides were incubated simultaneously with anti-P0 mAb (1:100 diluted in 10% FCS-PBS) and rabbit anti-podocin antibody (1:1000 in 10% FCS/PBS; a gift from C. Antignac; INSERM Unit 574, Paris, France) (20) or rabbit anti-nephrin antibody (1:50 in 10% FCS/PBS; a gift from F. Shimizu, Niigata, Japan) (21). After washing in PBS, the slides were incubated with FITC-conjugated goat anti-mouse Ig antibody (1:200 diluted in 10% FCS-PBS; Sigma) and TRITC-conjugated goat anti-rabbit IgG (1:200; Sigma). Sections were examined with a Zeiss confocal microscope (Carl Zeiss Microscopy).

Immunogold Electron Microscopy

Immunogold electron microscopy was performed on ultrathin frozen sections of kidney biopsies from healthy subjects. Briefly, pieces of biopsies were fixed in 4% freshly prepared formaldehyde (by depolymerization of parafomaldehyde) and 0.1% distilled glutaraldehyde (Merk, Darmstadt, Germany) in 100 mM phosphate buffer (pH 7.2) for 6 to 12 h at 4°C, soaked in sucrose, and frozen and stored in liquid nitrogen. Ultrathin frozen sections were processed for indirect immunogold labeling, as described (22). A control experiment in which the first antibody was omitted was performed.

In Situ Hybridization

Mouse kidneys sampled at 17.5 dpc and in the neonate period were fixed in paraformaldehyde 4%, paraffin-embedded, and sectioned (5 μm). The mouse P0 riboprobes were synthesized from a 497-bp PCR product (spanning from 5 to 492 bp in mouse P0 cDNA), then subcloned into the pGEM-Teasy vector (Promega). The antisense probe was synthesized after digestion with PstI using the T7 polymerase, and the sense probe was synthesized after digestion with SphI using the Sp6 polymerase. The riboprobes were labeled with digoxigenin-11-UTP (Roche) according to the manufacturer’s instructions. In situ hybridization was carried out as described previously (23).

Knockout Mice Studies

The generation and neurologic phenotyping of P0<sup>−/−</sup> mice have been described previously (17). These mice are on a C57BL/6 background. All experiments were performed according to the European recommendations for the care and use of laboratory animals.

Mouse Genotyping. DNA was isolated from mouse tail, and mice were genotyped by PCR amplification with the following primers: mP0-Ex1(F) CCCCGCTCTCTCACCACCCAGACAGA and mP0-Ex1(R) GGGCAAGCATAGGCGGCTGGCG. A 1250-bp fragment was amplified in P0<sup>−/−</sup> mice.

Serum and Urine Analysis. Individual mice were housed overnight in a diuresis cage, and urine was collected after 24 h. Individual urine samples were analyzed by 10% SDS-PAGE, and gels were stained with Coomassie blue. Samples that were applied to the gel were normalized for creatinine content. Urine albumin and creatinine concentrations were assayed by ELISA and automated Jaffe method, respectively. Blood was collected from anesthetized mice by cardiac puncture.

### Table 1. Primer sequences and predicted product size

<table>
<thead>
<tr>
<th>Molecule</th>
<th>ID Number</th>
<th>Primer Sequences</th>
<th>Position</th>
<th>Product Size</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human protein myelin 0 (exons 2 to 3)</td>
<td>NM000530</td>
<td>CCGGCCCGACCTGTCGTT</td>
<td>125 to 143</td>
<td>356 bp</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CAGGCTGACCTAGAGGTCCTTGCACA</td>
<td>456 to 481</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>CGTGCATTCCAGCGCGCCTCCT</td>
<td>595 to 618</td>
<td>616 bp</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CCCCGGGGCGAGGTAGGGGGC</td>
<td>1187 to 1211</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>GGAATTGTAGCTCCGCGCGCTTCGAC</td>
<td>20 to 42</td>
<td>801 bp</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GCTTATGTCCCCCGCCGCCGCTAAT</td>
<td>800 to 821</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>CGGGGGTCTCTCTCACCAGCCCA</td>
<td>9 to 23</td>
<td>497 bp</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CCACCCGGAGGATGCCCGCCA</td>
<td>484 to 505</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>GGGTACGCTATCCGAGTGATGGCT</td>
<td>1 to 11</td>
<td>750 bp</td>
</tr>
<tr>
<td>Mouse protein myelin 0 (exons 1 to 6)</td>
<td>NM008623</td>
<td>GCTTACGCTGCGCGCTAAT</td>
<td>741 to 750</td>
<td></td>
</tr>
</tbody>
</table>

and serum samples were analyzed for creatinine by automated Jaffe
method.

**Histologic Analysis.** Kidneys were removed from killed mice, fixed in Dubosq-Brazil, dehydrated, and paraffin embedded. Sections were stained with Trichrome, silver stain, or periodic acid-Schiff. For electron microscopy, tissue samples from male P0+/+ and P0−/− mice were processed as described previously (8). For immunofluorescence, frozen kidney sections (3 μm) were incubated as above with the antipodocin antibody.

**Results**

Expression of P0 in mouse, rat, and human kidney was demonstrated by combined approaches including RT-PCR, Western blot, immunochemistry, immunoelectron microscopy, and in situ hybridization.

**RT-PCR**

RT-PCR analysis used different sets of primers (Table 1). The full-length P0 cDNA could be amplified from normal human and mouse kidney cortex, together with shorter cDNA, which encode different domains of P0 (Figure 1). Sequencing of the amplified PCR products revealed 100% identity with the peripheral nerve P0 cDNA from human and mouse, respectively.

**Immunoblotting**

The glomerular expression of P0 protein was detected by immunoblot using monoclonal mouse anti-P0 antibody raised against the extracellular part of the rat P0 protein (18). Blotting of extracts from isolated glomerular tissue and from a human podocyte cell line both revealed a single 30-kD band as described earlier in human peripheral nerve lysates (Figure 2A) (24). A doublet at approximately 28 kD was detected in rat glomerulus protein extracts, similar to the one observed with the nerve rat protein (Figure 2B) (19).

**Immunofluorescence**

Immunofluorescence of normal adult human kidney sections showed a glomerular positivity along the capillary wall with a pattern highly suggestive of podocyte staining (Figure 3). Extraglomerular staining was present also in vessel, specifically in endothelial cells. No tubular and interstitium labeling was present. Double staining was performed with anti-P0 antibody (green) and anti-nephrin and anti-podocin antibodies (red; Figure 4). P0 tightly co-localized with nephrin. Dual labeling with podocin also showed a co-localization along with an extension of P0 positivity at the periphery of podocin staining.

**Immunoelectron Microscopy**

The glomerular localization of P0 protein was determined precisely by electron microscopy. Gold particles were distributed in the podocyte cell body, especially in cytoplasmic vesicles, as well as in the nearby actin cytoskeleton (Figure 5A). Podocyte synthesis of P0 protein was attested by the dense labeling of endoplasmic reticulum (Figure 5B). Particles were also found in foot processes, being occasionally close to the slit diaphragm (Figure 5C). Sparse reactivity of the glomerular capillary endothelia was present, but no significant labeling was observed in GBM. Control experiment, in which the first antibody was omitted, was completely negative.
In Situ Hybridization

To analyze developmental expression of P0/MPZ during nephrogenesis, we conducted in situ hybridization in the 17.5-dpc and neonate mouse kidney. In the developing metanephric kidney, P0 transcript was detected in the peripheral cortex (Figure 6A) and in primitive nephronic structures that give rise to mature glomeruli including vesicles and S-shaped bodies (Figure 6B and insert). Antisense riboprobe also labeled the ureteric bud and the collecting ducts, although these structures were not stained by immunofluorescence in the adult kidney. No specific signal was observed with the sense probe (Figure 6C). Similar results were obtained in the neonate kidney (data not shown).

Renal Phenotyping of P0<sup>−/−</sup> Mice

To establish the potential role of P0 in glomerular permselectivity, we analyzed P0<sup>−/−</sup> mice that were previously established by one of us (17). These mice present with demyelinating neuropathy closely resembling the neurologic phenotype of patients with CMT1B and heterozygous P0 gene mutation. In addition to neurologic signs that appear during the first month of life, we observed a moderate growth retardation in P0<sup>−/−</sup> mice (22.4 ± 0.78 g; n = 7) compared with P0<sup>+/−</sup> (27.4 ± 1.42 g; n = 9; P < 0.05) and P0<sup>+/+</sup> mice (27.8 ± 1.48; n = 5; P < 0.05). As shown in Figure 7A, P0<sup>−/−</sup> mice exhibited significantly higher albuminuria/creatininuria ratio than P0<sup>+/−</sup> and P0<sup>+/+</sup>
mice, at both 4 to 6 mo and 12 mo of age. Urinary albumin excretion did not increase significantly at 12 mo, compared with earlier age. Electrophoresis of urinary proteins confirmed that albumin was predominant (data not shown).

For analyzing histologic lesions underlying increased urinary albumin excretion in P0−/− mice, mice were killed at 6 and 12 mo of age. Serum creatinine/body weight ratio was not significantly different between P0−/− (1.33 ± 0.18 μmol/L per g body wt; n = 7), P0+/− (1.05 ± 0.39 μmol/L per g body wt; n = 4), and P0+/+ mice (1.41 ± 0.17 μmol/L per g body wt; n = 7) at 6 mo. Kidney weight/body weight ratio was similar in P0−/−, P0+/−, and P0+/+ mice (0.0191, 0.0195, and 0.0183, respectively). No significant glomerular, vascular, or interstitial abnormality was noted by histologic examination of P0−/− kidney sections that were stained with hematoxylin eosin and Trichrome (data not shown). Immunofluorescence examination with anti-podocin antibodies of kidneys from P0−/− mice and age- and gender-matched P0+/+ littermates showed normal expression of the protein (data not shown). Electron microscopy that was performed at 4 and 12 mo of age showed no significant abnormality, including no foot process effacement, both in P0−/− mice and in their controls (Figure 7, B and C). These results are compatible with the entity referred to as “no change disease” previously described in experimental models and in cases of human nephrotic syndrome (25–27).

**Discussion**

In this work, we demonstrate the presence of P0 protein in human, rat, and mouse podocytes and increased urinary albumin excretion in mice that are deficient in this protein. These results were unexpected because P0 protein expression was thought to be limited to the peripheral myelinating Schwann cells (13). In the peripheral nerves, this transmembrane glycoprotein induces and maintains myelin compaction around nerve fibers through homophilic interactions of its Ig-like extracellular domains between opposed P0 proteins. Heterozygous P0 gene mutations in humans define CMT1B. This demy-
eliminating peripheral neuropathy is a consequence of uncompacted myelin sheaths, responsible for slowed nerve conduction velocity. In addition, P0 was described recently in thymic stroma cells, where its role remains elusive (16). Thymic expression may account for P0/MPZ central tolerance induction, as described for other tissue-specific antigens.

In addition to Schwann cells and thymic stroma cells, we show that full-length P0 mRNA transcripts are expressed in mouse and human kidney cortex and that expression of intact P0 protein can be detected by Western blot in human and rat glomeruli and in human cultured podocytes. Immunofluorescence study identified a glomerular staining with a pattern highly suggestive of podocyte labeling, as attested by partial and complete co-localization with podocin and nephrin, respectively. Extraglomerular labeling was also present in vessel endothelium but absent in tubule segments. Our immunogold results confirmed P0 synthesis in the endoplasmic reticulum and further highlighted P0 subcellular distribution within the podocyte cell body, mainly in vesicular structures and in foot processes, sometimes close to slit diaphragm. In situ hybridization in 17.5-dpc and neonate mouse kidneys disclosed P0 transcript in embryonic structures that give rise to mature glomerulus, including vesicles and S-shaped bodies, and also in the collecting duct. These results indicate that P0 is synthesized from the early stages of glomerular development and also transiently in ureteric bud–derived structures.

The identification of the myelin protein P0 in podocyte extends the range of proteins whose expression is shared by nervous cells and the glomerular epithelial cell. However, P0 to date is the only podocyte protein to be expressed in the PNS.

To investigate the role of P0 in glomerular epithelial cells and filtration barrier, we determined the renal functional and morphologic phenotype of P0−/− mice. Although their neurologic phenotype close to the human disease has been investigated extensively, there were no data on their renal function. Whereas heterozygous mice were normal, mice that lacked the P0 protein showed urinary albumin loss in the absence of ultrastructural alterations of podocyte foot processes or GBM. Focal areas of thickening of the GBM were detected in P0−/− mice but also in control. The renal phenotype of P0−/− mice seems to be closely similar to the one reported in DDR1−/− mice, in which the loss of discoidin domain receptor-1 in podocytes is associated with moderate high molecular weight proteinuria. Ultrastructural alterations of DDR1−/− glomeruli remain focal, with thickening affecting <4% of the GBM and adjacent loss of slit diaphragm (28). In contrast with P0−/− mice, synaptopodin-deficient mice do not show renal abnormalities as judged by electron microscopy and urinary protein excretion analysis, although podocytes normally express a large amount of synaptopodin (29). Albumin excretion rates were neither increased in Ptpo−/− mice that lack the glomerular epithelial protein 1, even after uninephrectomy, although scanning electron microscopy disclosed morphologic podocyte alterations (30). Overall, these data suggest that P0 plays a significant role in glomerular permselectivity. The expression of P0 mostly in vesicles in the podocyte cell body is compatible with a role in cell transport as suggested for Rab3 (8). Because the P0−/− mice are on C57Bl/6 background, which is known to confer resistance to glomerulosclerosis after nephron reduction or toxic injury (31), we are currently backcrossing the C57Bl/6−>P0−/− mice to other backgrounds that are more prone to the development of renal diseases.

P0−/− mice may represent a new model of albuminuria without detectable morphologic alteration of the glomerular filtration barrier, a condition that is more common than expected and now referred as “no change disease.” Even if most proteinuric renal diseases are associated with massive loss of podocyte foot processes, there are indeed human and experimental models of nephrotic-range proteinuria in which this podocyte abnormality is absent. In human, a familial form of nephrotic syndrome without podocyte alteration has been previously reported (25), and a sporadic case of massive proteinuria with only segmental effacement of foot processes affecting 20 to 30% of capillary loops has been observed (26). In the rat, injection of mAb 5-1-6 directed against nephrin induces massive proteinuria without any foot process damage (27). Altogether, these observations suggest that albuminuria of glomerular origin can occur in the absence of slit diaphragm alterations even at the electron microscope level.

Almost 20 cases of glomerular disease, mainly focal segmental glomerulosclerosis, have been reported in the course of familial demyelinating neuropathy. Genetic analysis, available in only two cases of CMT with FSGS, did not show mutations in PMP22, P0, and Connexin-32 genes, which are the three genes most commonly altered in CMT (12). Our results prompt the search for P0 mutation in cases of CMT with kidney diseases, as well as in autosomal familial forms of glomerulopathies that are not linked to most commonly mutated genes, including ACTN4, NPHS1, and NPHS2.

In conclusion, we have first identified in the murine and human glomerulus, P0, the major myelin protein of the PNS. Results that were obtained in P0−/− mice suggest that P0 plays some role in glomerular permselectivity, although this role remains to be defined.

Acknowledgments

This work was supported by grants from INSERM, University Paris 6, and AURA (Association pour l’Utilisation du Rein Artificiel and by EU contract QLG1-CT-2002-01215).

We thank Professor D. Vallat (University of Limoges, France) for helpful discussion and support and Philippe Fontanges for technical assistance.

This work was presented at the 38th Annual Meeting of the American Society of Nephrology, St. Louis, MO, November 2004.

References


31. Li-Jun Ma, Fogo AB: Model of robust induction of glomer Li-Jun Ma, Fogo AB: Model of robust induction of glomer Li-Jun Ma, Fogo AB: Model of robust induction of glomer
33. Archelos JJ, Roggenbuck K, Schneider-Schaulies J, Toyka Archelos JJ, Roggenbuck K, Schneider-Schaulies J, Toyka Archelos JJ, Roggenbuck K, Schneider-Schaulies J, Toyka
35. Archelos JJ, Roggenbuck K, Schneider-Schaulies J, Toyka Archelos JJ, Roggenbuck K, Schneider-Schaulies J, Toyka Archelos JJ, Roggenbuck K, Schneider-Schaulies J, Toyka