Phosphoproteomic Analysis Reveals Regulatory Mechanisms at the Kidney Filtration Barrier

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ABSTRACT

Diseases of the kidney filtration barrier are a leading cause of ESRD. Most disorders affect the podocytes, polarized cells with a limited capacity for self-renewal that require tightly controlled signaling to maintain their integrity, viability, and function. Here, we provide an atlas of in vivo phosphorylated, glomerulus-expressed proteins, including podocyte-specific gene products, identified in an unbiased tandem mass spectrometry–based approach. We discovered 2449 phosphorylated proteins corresponding to 4079 identified high-confidence phosphorylated residues and performed a systematic bioinformatics analysis of this dataset. We discovered 146 phosphorylation sites on proteins abundantly expressed in podocytes. The prohibitin homology domain of the slit diaphragm protein podocin contained one such site, threonine 234 (T234), located within a phosphorylation motif that is mutated in human genetic forms of proteinuria. The T234 site resides at the interface of podocin dimers. Free energy calculation through molecular dynamic simulations revealed a role for T234 in regulating podocin dimerization. We show that phosphorylation critically regulates formation of high molecular weight complexes and that this may represent a general principle for the assembly of proteins containing prohibitin homology domains.


The kidney filter consists of three layers: fenestrated endothelial cells, the glomerular basement membrane, and podocytes.¹ Damage to any of these compartments becomes clinically evident as proteinuria and the development of kidney disease.² Of particular importance for the regulation of podocyte biology through signaling is the slit diaphragm, a specialized intercellular junction that bridges the 40-nm gap in between foot processes of neighboring podocytes. It also serves as a signaling platform regulating podocyte function. Mutations in genes encoding for components of the slit diaphragm, such as nephrin,³ podocin,⁴ CD2AP,⁵ and TRPC6,⁶,⁷ are important causes of genetic forms of proteinuria. Alteration of these proteins results in defective signaling causing podocyte dysfunction, progressive glomerulosclerosis, and kidney failure. The

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slit diaphragm protein complex is a lipid-multiprotein supercomplex.\textsuperscript{8} Of central importance to the integrity and function of the protein complex is the prohibitin homology (PHB) domain protein podocin,\textsuperscript{9} which forms multimeric complexes and is required to control signal transduction through associated transmembrane proteins.\textsuperscript{10,11}

Signaling processes governing podocyte function, integrity, and survival largely depend on signaling processes involving phosphorylation.\textsuperscript{12,13} Comprehensive analyses of the signaling events in podocytes \textit{in vivo} have been hampered by the fact that interference with these signaling cascades by genetic deletion often results in massively disrupted and dysfunctional podocytes. One of the primary aims of this study was to use phosphoproteomics to analyze thousands of phosphorylation sites in native murine glomeruli within single samples. Within this study, we show that this approach allows the introduction of new concepts into signaling processes at the kidney filtration barrier.

RESULTS

Phosphoproteomic and Proteomic Analyses of Murine Glomeruli

We freshly isolated murine glomeruli to obtain a comprehensive dataset of \textit{in vivo} phosphorylated glomerular proteins. The isolated glomeruli showed preservation of podocyte microstructure as demonstrated by light and electron microscopy, even after maintaining them for several minutes \textit{ex vivo}.\textsuperscript{14} We performed immunoblotting of protein lysates to validate the purity of the preparation. In the glomerular fraction, there was an enrichment of podocin and nephrin and a de-enrichment for two tubular markers, Na-K-ATPase and Tamm–Horsfall protein (Supplemental Figure 1A). We also verified the solubilization of detergent-resistant glomerular proteins such as nephrin and podocin by a lysis buffer containing 8 M urea (Supplemental Figure 1B).\textsuperscript{15}

We performed both proteomic and phosphoproteomic profiling of the murine glomerular lysates and identified 4671 expressed proteins based on previously published peptide and protein identification criteria.\textsuperscript{16} We confirmed the majority of previously mass spectrometry (MS)–based identified proteins in murine glomeruli (Figure 1A). In addition, this study comprises >90% of all proteins, which are significantly more highly expressed in podocytes than in nonpodocyte glomerular cells, based on a previous study\textsuperscript{16} (termed podocyte-specific proteins) (Figure 1B).

The phosphoproteomic analysis identified 2449 phosphoproteins. These phosphoproteins covered a substantial percentage of podocyte-specific proteins as well as proteins shown to be expressed in glomeruli previously (Figure 1C).\textsuperscript{16} We found 6868 phosphorylated residues. Of these phosphorylation sites, 1717 were not found in the version of the PhosphositePlus database released on March 2013.\textsuperscript{17} We found that 4079 of these sites were unambiguously localized with high confidence (localization score>$0.75$). These phosphorylation sites were used for further analysis and are accessible online in the GloPhos database (https://helixweb.nih.gov/ESBL/Database/GloPhos/GloPhos.htm). The phosphorylation sites showed a typical distribution of serine, threonine, and tyrosine phosphorylated proteins, with most phosphorylation sites residing on serine residues (Supplemental Figure 2A). The majority of proteins were found to contain one or two phosphorylated residues (Supplemental Figure 2B). Phosphoserines and phosphothreonines localized to substantially different protein classes than phosphotyrosines (Figure 1D, protein classes based on Panther classification). Phosphoserines and phosphothreonines were predominantly localized on structural proteins, transcription factors, and cytoskeleton-associated proteins. Tyrosines, however, mainly localized to receptors, kinases, and cytoskeletal proteins. The list of tyrosine phosphorylated proteins is depicted in Supplemental Table 1. Gene ontology (GO) term analysis using a previously published mouse phosphorylation atlas as background revealed overrepresentation of phosphorylation sites on proteins involved in key functions in the glomerulus such as organization of polarity, cell–cell contacts, and cytoskeleton (Supplemental Tables 2 and 3).

We performed a categorization of phosphorylation motifs using a binary decision tree algorithm.\textsuperscript{18} Most serine and threonine phosphorylation sites were either part of basophilic ([RK]-x-x-[ST]) or of proline-directed motifs ([ST]-P) (Figure 2A). Position-weighted matrices for the three major kinase motifs (proline-directed, acidic, and basophilic) are depicted in Figure 2B. This finding is representative of a variety of phosphorylation datasets obtained with similar approaches.\textsuperscript{18–21} Using the PhosphoSitePlus repository,\textsuperscript{17} we extracted respective kinases of basophilic and proline-directed phosphorylation motifs, indicating that substrates of protein kinase A, protein kinase C (PKC), extracellular signal-regulated kinase, and cyclin–dependent kinase 1 (CDK1), CDK2, and CDK5 are most frequent (Figure 2, C and D). We also analyzed the phosphorylation sites for conservation across multiple species using the CPhos algorithm (Supplemental Figure 3).\textsuperscript{22}

Phosphorylation Regulates Podocyte-Specific and Slit Diaphragm–Associated Proteins

We next generated a list of 48 phosphoproteins known to be specifically expressed in podocytes.\textsuperscript{16} The 146 confident phosphorylation sites corresponding to these proteins are outlined in Supplemental Table 4. In the overall dataset, we also found phosphorylation sites on additional several \textit{bona fide} podocyte-specific proteins such as phospholipase A2 receptor,\textsuperscript{23} FAT1,\textsuperscript{24} nephrin, Pdlim2,\textsuperscript{25} and Lats26 (Slc43a1) (Supplemental Table 5). Representation of different classes of phosphorylation motifs within these candidates was similar compared with the total dataset (Figure 3, A and B; data not shown).

The podocyte phosphoprotein synaptopodin (\textit{Syp}) was one of the proteins with the highest number of phosphorylation sites ($n=18$) in the entire glomerular dataset and in the
podocyte-specific protein population (Figure 3, A and B). Phosphorylation affected all parts of the protein (Figure 3C). Interestingly, most of the phosphorylated residues were localized on the C-terminal tail of synaptopodin comprising amino acids 684–909 (UniProt ID: Q9355-2). Of note, all phosphorylation sites identified on synaptopodin were proline directed (Figure 3D). Figure 3E depicts the most frequent residues surrounding all 121 serines on synaptopodin and shows a particular frequent localization of proline after these residues.16

Figure 1. Analysis of the glomerular proteome and phosphoproteome obtained by LC-MS/MS. (A) Venn diagram indicating the overlap of protein identifications between this study and the previous murine glomerular proteome by Boerries et al.16 (B) Venn diagram indicating the overlap of protein identifications between proteins obtained in this study and proteins determined as podocyte specific in this study. (C) Bar graph indicating the number of glomerular and podocyte-specific proteins found as nonphosphoprotein or phosphoprotein. (D) Classification of glomerular phosphoproteins identified by LC-MS/MS. Protein classification is performed using the Panther database. The horizontal axis shows the relative abundance of serines, threonines, and tyrosines.
We were able to identify 16 phosphorylation sites on major slit diaphragm proteins (nephrin, Neph1, podocin, TrpC6, and Cd2ap) (Table 1). Kinase preferences or binding motifs attributed to these phosphorylation sites were analyzed using the Scansite software package (Table 1).27

Biologic Relevance of PHB Domain Phosphorylation of Podocin

To show the relevance of these novel phosphorylation sites, we focused on the phosphorylation sites found on the slit diaphragm protein podocin. We identified phosphorylation sites localized quite centrally in the PHB domain of podocin (T234) and at the distal C terminus (S362, S382) (Figure 4A).

Mutations of the podocin gene NPHS2 are a major cause of steroid-resistant nephrotic syndrome and ESRD requiring dialysis in children and are therefore of major importance in medicine.28 To prioritize the phosphorylation sites for further studies, we analyzed the frequency of known patient point mutations associated with these diseases surrounding the distinct phosphorylation sites within a 11 amino acid sequence window as obtained from the Human Gene Mutation Database (HGMD) (March 2013)29 (Figure 4B). Regions with >5 point mutations within an 11 amino acid window are significantly enriched for the occurrence of mutations (P<0.05). It appeared that human T232 (the site corresponding to murine T234) resides in a region, which is frequently affected by mutations. In fact, the residue itself is affected in a patient with sporadic kidney disease (heterozygous T232I mutation).30 Residue T232 of human podocin is part of a highly conserved basophilic phosphorylation motif (H-R-X-X-T). This phosphorylation motif is affected by two further nonconservative amino acid mutations in this patient group (R229L and H228D)31 (Figure 4C). In addition, the polymorphism R229Q (risk allele) also affects this motif.28,30 By contrast, only one patient mutation resided close to the human residue number S360 and none close to S380 (Figure 4C).

It has been postulated that phosphorylation sites with high biologic significance often represent a common phosphorylation site in different members of a domain protein family.32 To further substantiate this hypothesis, we analyzed the occurrence of PHB domain phosphorylation at the domain region corresponding to murine podocin T234. The algorithm prioritizes post-translational modifications within protein domains by searching for statistical enrichment of phosphorylation sites with actual proteomic evidence compared with random sampling of phosphorylation sites across the entire domain.32 The analysis revealed that phosphorylation sites on PHB domains were significantly overrepresented within the domain area aligning with the murine podocin residue T234 (Figure 4D). Thus, it represents a “phosphorylation hotspot,”32 which supports its likeliness to play a pivotal role for PHB domain function.32

In Silico Characterization of Podocin T234 Phosphorylation

The quaternary structure of the homologous PHB domain of stomatin has recently been solved: two PHB domains of stomatin form a banana-shaped dimer via main chain interaction of residues 196–199.33 The residues responsible for interaction of dimers are well conserved in human and mouse podocin as well as in MEC-2, the podocin ortholog in Caenorhabditis elegans (Figure 5A). Moreover, the basophilic phosphorylation motif within the PHB domain is also conserved.

Figure 2. Analysis of phosphorylation motifs based on confidently localized phosphorylation sites. (A) Classification of phosphorylation motifs in four classes based on a binary decision tree. (B) Position-weighted matrices of the three main phosphorylation motifs (proline-directed, acidic, and basophilic). (C and D) Abundance of known substrates for basophilic (C) and proline-directed (D) phosphorylation sites as indexed within the PhosphoSitePlus database. CamK2, calmodulin-dependent kinase 2; SGK, serum glucocorticoid-regulated kinase 1; mTOR, mammalian target of rapamycin; JNK, c-Jun N-terminal kinase.
with some differences (Figure 5A). We used this information to visualize the structural context corresponding to murine podocin T234 in murine stomatin S161. Interestingly, the residue S161 localized on the PHB domain of the first dimer resided very close to the residue S161 on the PHB domain of the second protein (Figure 5B). The distance between both serine hydroxyl groups is 9.8 Å (Figure 5C). Thus, we hypothesized that this phosphorylation site is involved in regulating dimerization of the protein.

To further substantiate the effect of phosphorylation on PHB domain interaction in silico, we performed molecular dynamics simulations to calculate free energies using the CHARMM algorithms. We could rely on the previously published crystal structure of stomatin PHB domain dimers (Figure 5, B and C; Protein Data Bank ID 4FVF) to generate a homology model of podocin PHB domains (Supplemental Figure 4, A and B). The dimerization and phosphorylation of monomers and dimers are represented by a thermodynamic cycle (Supplemental Figure 4C). According to Hess’s law, the effect of phosphorylation on dimerization was calculated by the difference in free energy changes during phosphorylation of monomers and dimers, which can be directly simulated (equation in Supplemental Figure 4C). Both banana-shaped dimers are predicted to bend more upon phosphorylation (Figure 6A) (10° bending for stomatin, 4° bending for podocin in the phosphorylated state). The calculated free energy changes due to phosphorylation for monomer and dimers, as well as for the dimerization, are listed in Table 2. These results predict that phosphorylation promotes stomatin dimerization ($\Delta \Delta G = -6.1 \pm 1.4$ kcal/mol) but prevents podocin from forming dimers ($\Delta \Delta G = 8.1 \pm 2.2$ kcal/mol). Consistent with this prediction, the phosphorylation-dependent change in dipole moment was negative for podocin (−16 debyes) and positive for stomatin dimer formation (+92 debyes) (Table 3). The reason for the differential effect of phosphorylation on interaction is the result of a negatively charged (E235) residue representing podocin, but not in stomatin (Figure 6B). This residue is predicted to repulse phosphate groups localized at the other protein domain. The force at the interaction surface is thereby strong enough to overcome all opposite forces and prevents dimer formation.

Effect of Podocin T234 Phosphorylation on Multimerization

To further corroborate our in silico findings, we performed biochemical analysis of native stomatin and podocin protein complexes using the blue native gel electrophoresis (BN-PAGE) technique. We introduced phosphoablating (stomatin S161A, podocin T234A) or phosphomimicking (stomatin S161D, podocin T234D) mutations into the full-length protein. Both phosphoablating and phosphomimicking mutants of stomatin were equally expressed (Figure 7A). As previously published, stomatin forms multimeric megadalton complexes (Figure 7A). Consistent with the results obtained in silico, stomatin showed an increased multimerization when the

![Figure 3](image-url)
A phosphomimicking mutation S161D was present (lack of low molecular mass complexes, with a molecular mass \(< 1000 \text{kDa}\)) (Figure 7A). In contrast and consistent with the simulations, podocin showed a lower multimeric complex when the T234D mutation was introduced (Figure 7B). We confirmed these findings in coimmunoprecipitation assays. Compared with

### Table 1. Phosphorylation sites on slit diaphragm proteins

<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>Name (UniProt ID)</th>
<th>Phosphopeptide Sequence</th>
<th>Site</th>
<th>Scansite Prediction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nphs1</td>
<td>Nephrin (Q9QZS7)</td>
<td>LAEEIS<em>EAG5</em>EEDR</td>
<td>S1112, T1115, S1119</td>
<td>Casein kinase sites</td>
</tr>
<tr>
<td></td>
<td></td>
<td>LAEEISEKT*EAGSEEDDR</td>
<td>T1115&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Casein kinase site</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TEAG5*EEDDR</td>
<td>S1119</td>
<td>Casein kinase site</td>
</tr>
<tr>
<td>Nphs2</td>
<td>Podocin (Q9QZS)</td>
<td>SLT*EILLER</td>
<td>T234</td>
<td>AGC family kinase site</td>
</tr>
<tr>
<td></td>
<td></td>
<td>AQGS*INYPSSSKPVEPLNPK</td>
<td>S362</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>KKDS*PML</td>
<td>S382</td>
<td></td>
</tr>
<tr>
<td>Trpc6</td>
<td>Short transient receptor potential channel 6 (Q61143)</td>
<td>FGI5GS*HE6L5K</td>
<td>S814</td>
<td>Casein kinase site</td>
</tr>
<tr>
<td>Cd2ap</td>
<td>CD2-associated protein (Q9JLQO)</td>
<td>S*VDLDAFVAR</td>
<td>S458</td>
<td>Casein kinase site</td>
</tr>
<tr>
<td></td>
<td></td>
<td>FNGGHS<em>PTQS</em>PEK</td>
<td>S510, S514</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>AEADDGKRNS*VDELRI</td>
<td>S580</td>
<td>ERK D-domain binding</td>
</tr>
<tr>
<td></td>
<td></td>
<td>VETVNREPLTMH*SDREEDTASISTATR</td>
<td>T581</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>VETVNREPLTMH*SDREEDTASISTATR</td>
<td>S584, T590</td>
<td>Casein kinase site</td>
</tr>
<tr>
<td>Kirrel</td>
<td>Neph1 (Q80W68)</td>
<td>VETVNREPLTMH*SDREEDTASISTATR</td>
<td>S584, S592</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>VETVNREPLTMH*SDREEDTASISTATR</td>
<td>S592, T595</td>
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<tr>
<td></td>
<td></td>
<td>LSH*SIGNYQLNTYSR</td>
<td>S676&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
<td></td>
<td></td>
<td>FSYTSQHS*DYGQR</td>
<td>S775</td>
<td>PDK1 binding motif</td>
</tr>
</tbody>
</table>

Asterisks (*) indicate the localization of phosphate. ERK, extracellular signal-regulated kinase; PDK1, phosphoinositide-dependent protein kinase 1.

<sup>a</sup>Ambiguous phosphorylation site due to localization score < 0.75, appropriate spectrum on manual examination.

<sup>b</sup>Ambiguous phosphorylation site (S676 versus S675) upon manual examination of spectrum.

Figure 4. Phosphorylation sites discovered on murine podocin and its corresponding human sites. (A) Murine and corresponding human residue numbers are mapped to the structure of podocin (residue numbers based on Pfam entry [http://pfam.sanger.ac.uk/]). (B) Frequency of described point mutations within the podocin protein. All currently known mutations associated with either FSGS or nephrotic are extracted from the HGMD. The plot shows the frequency of found mutations within an 11-amino-acid sequence window surrounding the respective residue number. (C) An 11-amino-acid sequence window of murine podocin phosphosites. Mutations or risk alleles previously described in patients with either FSGS or nephrotic syndrome are mapped to the sequence. (D) Phosphorylation enrichment analysis of PHB/Band7-like protein domains. The position of aligned murine Podocin T234 residue corresponds to a phosphorylation hotspot within the PHB domain. Asterisk (*) indicates significant enrichment.
the phosphoablating mutant (F.T234A), phosphomimicking mutant (F.T234D) interacted to a lesser degree with V5.T234A podocin (Figure 7, C and D). When both T234 residues (on V5 and FLAG-tagged mutants) were mutated to D, there was also a marked decrease in homophilic interactions (Figure 7, C and D). Fyn was used as a similar-sized protein not interacting with podocin.39 Converse results were obtained for the respective phosphomimicking stomatin mutants (Figure 7, E and F).

Atypical PKC (aPKC) is a master regulator of podocyte biology and is critically involved in regulating cellular polarization and differentiation.11,40 We tested whether aPKC may regulate podocin phosphorylation at T234. To this end, we cotransfected wild-type murine podocin with active (T410E) or inactive (T410A) aPKC and subjected whole cell lysates to phosphoproteomic analysis. Label-free quantification revealed an increased phosphorylation of T234 with the active version of the kinase (Supplemental Figure 5). The phosphorylation at S382, the second discovered site in this setting, was not increased by cotransfection of the active kinase.

**DISCUSSION**

Phosphoproteomic approaches have been applied to the kidney with a focus on the tubular system. These studies enhanced the understanding of epithelial function in the tubular system41–44 and are cornerstones for hypothesis-driven studies in many laboratories.45–47 Here, we present the first available atlas of phosphorylated residues of proteins present in native murine glomeruli revealed by an advanced MS-based method.

The phosphopeptides analyzed in this study may derive from any glomerular cell type, including mesangial cells. However, we believe that this approach is not only more feasible but is also superior to single-cell separations and long flow-sorting procedures because these may dramatically alter phosphorylation patterns in any cell type. We performed bioinformatics analysis to retrieve phosphorylation sites in podocyte-specific proteins and added several bona fide podocyte-specific proteins to this list. Phosphorylation motifs at least for the 146 phosphorylation sites on known podocyte-specific proteins were not strikingly different compared with nonpodocyte proteins (data not shown). This suggests that global baseline kinase activity in the different cell types is comparable. Hence, differences in morphology and cell cycle most likely result from signaling within microdomains such as the foot processes. Our study is also consistent with the concept of different responsibilities for tyrosine and serine/threonine phosphorylation sites in maintaining cell morphology in general (Figure 1D, Supplemental Table 2).
Table 2. Free energy changes (kcal/mol) of phosphorylation for monomer and dimer proteins

<table>
<thead>
<tr>
<th>System</th>
<th>ΔG1 (kcal/mol)</th>
<th>ΔG2 (kcal/mol)</th>
<th>ΔG3 (kcal/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Podocin</td>
<td>−272.6±0.8</td>
<td>−537.1±2.1</td>
<td>8.1±2.2</td>
</tr>
<tr>
<td>Stomatatin</td>
<td>−275.3±0.4</td>
<td>−556.7±1.6</td>
<td>−6.1±1.4</td>
</tr>
</tbody>
</table>

Figure 6. In silico analysis of podocin phosphorylation on T234 through molecular dynamics. (A) Result of molecular dynamics simulation predicting bending of the banana-shaped dimer by 10° (stomatatin, conformations before and after phosphorylation are colored green and purple, respectively) as well as 4° (podocin, conformations before and after phosphorylation are colored yellow and red, respectively). (B) Details at the interface explaining different effect of phosphorylation on podocin and stomatin dimerization. Protein backbones are shown as helical ribbons for helices and as arrowed ribbons for β-strands with arrows toward the C terminus. Positively and negatively charged residues are colored blue and red, respectively. The side chains of S161 in stomatin and T234 in podocin, as well as the phosphate groups attached to them, are shown as sticks. The differential effect of phosphorylation on free energy can be explained by the existence of a negatively charged residue, E235 in podocin, but not in stomatin. The negative charge of E235 repulses the phosphate group so that the phosphorylated podocin has less tendency to be dimerized (indicated by gray arrows).

Our analysis revealed that synaptopodin is among the phosphoproteins with highest number of phosphorylation sites within the podocyte and the glomerulus (Figure 3, A and B). The majority of sites localized within the C terminus of the kidney-specific isoform and were mostly proline directed, a finding that may be attributed to the large number of proline residues within this protein (Figure 3, C–E). The C terminus is one of the main interaction sites for α-actinin-4, a regulator of podocyte cytoskeletal dynamics. Mutations in the ACTN4 gene encoding for α-actinin-4 are a known cause of genetic forms of kidney disease and hypertension. It is highly conceivable that dynamic phosphorylation at this region (by proline-directed kinases, such as extracellular signal-regulated kinase and CDK1, CDK2, or CDK5) may be involved in regulating this interaction and their modulation of the podocyte cytoskeleton.

The podocyte slit diaphragm is a structure with a direct pathophysiologic effect. This dataset yielded 16 previously undescribed phosphorylation sites on five major slit diaphragm proteins: Neph1, nephrin, podocin, CD2AP, and Trpc6. Interestingly, Neph1, nephrin, Trpc6, and CD2AP all had phosphorylation sites within acidic regions and may thus be targets of acidic kinases such as casein kinases, a kinase class not implicated in glomerular biology. S814 at TrpC6 appears not to be phosphorylated by casein kinase 2. The previously described tyrosine phosphorylation site on nephrin was not detected, which may either be the result of the not optimal access of trypsin to this site or due to a low baseline phosphorylation because several studies have shown that nephrin tyrosine phosphorylation is inducible upon ligation of the extracellular domain.

We focused on studying phosphorylation of the PHB domain protein podocin. Mutations in the podocin gene (NPHS2) are a major cause of steroid-resistant nephrotic syndrome in children. Three coherent lines of evidence suggest a major importance of the phosphorylation site T234 on murine podocin function. First, this site is highly conserved and localizes within a conserved functional protein domain. Within the PHB domain, this phosphorylation site resides within a phosphorylation hotspot, a protein domain region with significantly enriched evidence for phosphorylation (Figure 4D). Such regions have been shown to be important for protein functions in general. Second, mutation of the phosphorylation site T232 in humans was recently found in a patient with FSGS. This mutation (T232I) is nonphosphorylatable. In addition, the functional basophilic phosphorylation motif (H-R-x-x-T) is affected by two other patient mutations (R229L, H228D) and the frequent risk polymorphism R229Q. Genetic data obtained from the model organism C. elegans point to a similar direction. There is a strong similarity of the mechanosensory machinery in C. elegans and the complex organization of the slit diaphragm.

The PHB domain protein MEC-2 is a crucial member of this complex responsible for touch sensitivity. In fact, a dominant mutation (T246I) in the PHB domain of MEC-2, affecting the same area of the domain as T232I in podocin (Figure 3A),
enhances touch insensitivity. The phosphorylation site itself, however, is not conserved in C. elegans (Figure 5A). Third, based on the crystal structure of the homologous PHB domain of stomatin, this site shows an extremely prominent localization close to the interface responsible for the interaction with other PHB domains (Figure 5, B and C). Using in silico and in vitro methods, we show that this phosphorylation site is involved in organizing podocin multimeric complexes. Interestingly, the homologous phosphorylation sites on stomatin (within protein domains) have an opposite effect on protein multimerization. Phosphorylation induced dimerization for stomatin, whereas phosphorylation reduced dimerization for podocin. This was predicted by molecular dynamics simulations (Figure 6, Tables 2 and 3) and was confirmed for the respective full-length protein using native gels (Figure 7, A and B).

The PHB domain phosphorylation site discovered in this study may prove to be a modulator of the oligomerization status of podocin at the slit diaphragm in vivo, thereby altering resistance and mechanics in the podocyte in response to various intracellular and extracellular stimuli affecting AGC kinases. In fact, aPKC, a kinase with relevance for podocyte biology, can induce phosphorylation of this site when coexpressed with podocin in human embryonic kidney (HEK293T) cells (Supplemental Figure 5).

Taken together, we use a combination of complex tissue isolation, liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS)–based phosphoproteomics, and computational analysis with subsequent in vitro and in silico experiments to understand molecular signaling mechanisms in kidney health and disease.

CONCISE METHODS

Isolation of Murine Glomeruli

Murine glomeruli were obtained as previously described. Briefly, 6- to 8-week-old female Bl6N wild-type mice were euthanized by cervical dislocation and the kidneys were excised and perfused with HBSS containing magnetic beads. Subsequently, kidneys were digested with collagenase for 1 minute at 37°C and purified by sieving and a strong magnet. Isolated glomeruli were washed two times and were pelleted down using spin of 1000 rpm for 3 minutes at 4°C. Kidneys, glomeruli, and lysates were kept on ice during the whole procedure. Glomeruli were suspended in 200 μl lysis buffer (8 M urea and 50 mM ammonium bicarbonate) containing protease and phosphatase inhibitor cocktails (Thermo Fisher Scientific, Bremen, Germany).

<table>
<thead>
<tr>
<th>Table 3. Dipole moments (debyes) of stomatin and podocin monomers and dimers before and after phosphorylation</th>
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<tbody>
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<tr>
<td></td>
</tr>
<tr>
<td>Monomer</td>
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<tr>
<td></td>
</tr>
<tr>
<td>Dimer</td>
</tr>
<tr>
<td>Dimerization change</td>
</tr>
</tbody>
</table>

Ph, phosphorylation of the respective residue.

Phosphopeptide and Peptide Purification

Phosphopeptides were purified using a protocol previously used for phosphoproteomic studies with some modifications. One and one half milligrams of protein was reduced using 5 mM dithiothreitol and alkylated using 50 mM of iodoacetamide in the dark. Urea was diluted to a concentration of 1.5 M and trypsin was added at a 1:50 w/w ratio. Digestion was performed at 37°C for 16 hours. Aqueous peptide solution was desalted using Oasis HLB columns (Waters Corporation, Milford, MA). Briefly, peptides were fractionated using a polysulfoethyl A column (4.6 mm internal diameter ×20 cm length, 5-μm particle size, 300-Å pore size) on a Finnigan HPLC machine. Strong cation exchange chromatography was conducted at a 1 ml/min flow rate using the following flow gradient: 100% solvent A and 0% solvent B for 2 minutes; 0%–20% solvent B for 40 minutes; 20%–100% solvent B for 5 minutes; and 100% solvent B held for 5 minutes (solvent A: 5 mM KH2PO4, 25% acetonitrile (ACN) pH 2.7; solvent B: 5 mM KH2PO4, 25% ACN, 350 mM KCl, pH 2.7). Seven to nine fractions were collected. Phosphopeptides were enriched using FeNTA Immobilized metal ion affinity chromatography (IMAC) columns (Thermo Fisher Scientific). The IMAC elutes containing phosphopeptides were subjected to further MS analysis. After cleanup using ZipTips (Millipore, Bonn, Germany), samples were submitted to LC-MS/MS analysis. One half of the sample was analyzed using an LTQ Orbitrap XL, and the other half was analyzed using a Q Exactive mass spectrometer. IMAC flowthroughs containing mainly nonphosphorylated peptides were analyzed on an LTQ Orbitrap XL mass spectrometer only to enhance proteomic coverage.

MS

One half of the respective phosphopeptide samples were analyzed using an LTQ Orbitrap XL mass spectrometer and collision induced dissociation (CID) fragmentation. Analyses using reversed-phase liquid chromatography coupled to nanoflow electrospray tandem MS were carried out using an EASY nLC-II system (Proxeon/Thermo Fisher Scientific) with a 150 mm C18 column (75 μm internal diameter; Dr. Maisch GmbH, Ammerbuch, Germany) coupled to a LTQ Orbitrap Discovery mass spectrometer (Thermo Fisher Scientific). Sonication on ice (1.5 minutes, 0.5-second pulses) was performed and the supernatant was saved for further analysis. This cycle was repeated twice in order to ensure the maximal amount of solubilization of membrane and nuclear proteins. All three lysates were pooled and protein amount was determined using a commercial bicinchoninic acid assay. We saved 40 μl for immunoblot analysis. The experiment was repeated twice. All animal procedures were performed according to protocols approved by federal agencies.

Figure 7. Characterization of PHB domain phosphorylation in vitro. (A) BN-PAGE analysis of Stomatin S161A and stomatin S161D multimeric complexes. Lower molecular weight (LMW) complexes are marked by LMW. The same lysates are subjected to SDS-PAGE followed by immunoblotting. (B) BN-PAGE analysis of podocin T234A and T234D multimeric complexes. The same lysates are subjected to SDS-PAGE followed by immunoblotting. All BN-PAGE analyses are performed in triplicate. (C and D) Coimmunoprecipitation
Exactive mass spectrometer using higher-energy collisional dissociation. The other half of the samples was analyzed using a Q were isolated and fragmented in the linear ion trap using CID fragmentation. The dynamic exclusion option was enabled (1 minute). The spectra in data-dependent mode and automatically switched between using m/z 250 – 445.12003 as a lock mass. The mass spectrometer acquired data in a dependent mode and automatically switched between MS and MS/MS acquisition. Signals with unknown charge state were excluded from fragmentation. The dynamic exclusion option was enabled (1 minute). The five most intense ions (charge state z=2) were isolated and fragmented in the linear ion trap using CID fragmentation. The other half of the samples was analyzed using a Q Exactive mass spectrometer using higher-energy collisional dissociation (HCD) fragmentation as described.38,39 Briefly, enriched phosphopeptides were loaded on a reversed-phase column packed in-house with C18-AQ 13 μm resin material (Dr. Maisch GmbH) via an Easy nLC nanoflow ultra-HPLC system (Thermo Fisher Scientific). Separation was performed by a linear gradient from 5% to 60% buffer B (80% ACN and 0.1% formic acid) at a flow rate of 250 μl/min over 150 minutes. Survey scan MS spectra were measured in the Orbitrap (m/z 300–1750) with a resolution of 70,000 (at m/z 400) after accumulation of 10^6 ions. The 10 most intense peptides (charge state z=2) were isolated and fragmented in the octopole collision cell by HCD.

**Biinformatic Analyses**

Raw files were searched using MaxQuant (version 1.3.0.5). Raw file spectra were searched against the mouse UniProt reference database using the target-decoy strategy. Mass accuracy was 20 ppm in the first search and 4.5 ppm in the second search with the deisotoping option enabled. For fragment ions, mass tolerance was 0.5 Da for CID data and 20 ppm for HCD data. Fixed modifications were carboxyamidomethylation of cysteines (+57 Da). Variable modifications were phosphorylation at S,T,Y (+80 Da) and oxidation of methionine (+16 Da) with a number of four allowed modifications. Protein, peptide, and site false discovery rate (FDR) were adjusted to <0.01. All peptides that passed these criteria were taken into consideration. A protein was defined as expressed with one or more unique peptides matched to the sequence.16 Class I phosphorylation sites with a localization score >0.75 were termed as confident and were used for further analysis.

All raw files and corresponding search results were uploaded to the ProteomeXchange/PRIDE repository (ProteomeXchange accession: PXD000266; http://www.ebi.ac.uk/pride). All phosphorylation data are made accessible at the online GloPhos database (https://helixweb.nih.gov/ESBL/Database/GloPhos/GloPhos). Protein categories were obtained from the Panther database based on gene symbols of the respective proteins (http://www.panther.org).

Further analyses of bioinformatics data were performed using Perseus and Microsoft Excel, as well as previously published National Heart, Lung, and Blood Institute (NHLBI) in-house software.60,61 GO terms were annotated via the Perseus package using UniProt accessions as the reference. For enrichment analysis, a Fisher’s exact test with correction for multiple testing (Benjamini–Hochberg FDR) was performed. A cutoff of Benjamini–Hochberg FDR of 0.02 (for serines/threonines) or 0.05 (for tyrosins) was chosen. The five GO terms with highest enrichment were selected (Supplemental Tables 2 and 3).

As a background dataset, we selected the following: a dataset containing all phosphoproteins containing serine and threonine phosphorylation in a comprehensive mouse tissue phosphoproteomic study (Supplemental Table 2);12 and a dataset containing all phosphoproteins containing tyrosine phosphorylation in the same study (Supplemental Table 3). The latter two datasets were extracted from PhosphoSite (http://www.phosphosite.org).17 All protein entries with multiple phosphorylation sites were counted as one phosphoprotein in the respective analysis. The CPhos program was used for analysis of site conservation with default settings for mouse datasets.22

Phosphorylation enrichment analysis of PHB/Band7-like protein domains was performed as previously described.32 For this analysis, we used 198,773 phosphorylation sites derived from previously published MS experiments across 15 species obtained from the PTMFunc database (http://www.ptmfunc.com). All proteins containing a Band_7 PFAM domain were aligned to a representative Band_7 containing protein (the mouse Flot2, UniProt: FLOT2_MOUSE) and 30 phosphosites from these proteins were mapped to the Flot2 sequence. We then used a sliding window of 10 amino acids and random sampling to identify regions within the Band_7 containing Flot2 protein that are enriched for phosphosites. Any 10 amino acid peptide with a significant (P<0.01) enrichment of phosphosites compared with random distribution of sites was defined a potential regulatory region or regulatory hot spot. Enrichment for mutations within the podocin protein was performed using the same method. Mutations were retrieved from the HGMD database (March 2013, professional version). Eleven amino acid windows surrounding residues were tested. Randomizations were performed 10,000 times. Enrichment of mutations was considered significant when P<0.05.
Structure Visualization and Free Energy Calculation

Sequences were aligned using the ClustalWX algorithm. Structures (Protein Data Bank ID 4FVF) were visualized using the Cn3D program obtained from the National Center for Biotechnology Information website (http://www.ncbi.nlm.nih.gov). Distance between phosphorylatable serine hydroxyl groups was measured using J Mol (http://www.jmol.org). Molecular dynamics were simulated using the CHARMM program.35,36 We used the perturbation method to calculate free energy differences of a series of 154 windows along the path linking the state before and after phosphorylation in both directions. In each free energy simulation, 1540-ps simulations were performed. Both molecular dynamics simulation and force momentum–based self-guided Langevin dynamics (SGLDfp) simulation methods were utilized.62 For SGLDfp simulations, we used a local average time of 0.2 ps, a guiding factor of 1.0, and a friction constant of 1/ps.

Cell Culture and Coimmunoprecipitation, and Immunoblotting

HEK293T cells were maintained in DMEM supplemented with 10% FBS. HEK293T cells were transiently transfected separately with FT234A, FT234D podocin constructs, and F.Fyn constructs cloned into a pcDNA6.2 vector using the calcium phosphate method. For each of these dishes, one additional dish was transfected either with V5.T234A podocin or V5.T234D podocin (cloned into a pcDNA6.2 vector). Two days after transfection, cells were harvested with ice-cold PBS. Cells were lysed in a 1% Triton X-100 buffer (1% Triton X-100, 20 mm Tris–HCl, pH 7.5, 50 mm NaCl, 50 mm NaF, 15 mm Na4P2O7, 2 mm NaN3, 50 mM imidazole/HCl pH 7.0, 50 mM KPi buffer pH 7.4, 10% urea buffer for later Western blot analysis (i.e., lysate). Lysates containing V5-tagged protein (either V5.T234A or V5.T234D, respectively) were subsequently pooled and some volumes were added to the F9-tagged protein lysate containing FLAG-tagged protein to reduce expression variability in V5-tagged bait protein. Pooled lysates were incubated for 1 hour at 4°C with anti-FLAG (M2) antibody covalently coupled to agarose beads (Sigma-Aldrich). The beads were washed five times with lysis buffer with 0.25% sodium deoxycholate added, and bound proteins were resolved by SDS–PAGE, blotted onto polyvinylidene fluoride membranes, and probed with polyclonal rabbit anti-V5 antibody or anti-FLAG antibody (1:2000 dilution). Protein bands were visualized with enhanced chemiluminescence. Densitometry of raw band intensities was performed using the ImageJ/FIJI suite. Statistics were performed as two-tailed t tests.

Blue Native Gels

A 3%–13% BN-PAGE was performed as described by Wittig et al.37 with the following modifications. We solubilized 100 µg whole cells in solubilization buffer (50 mM NaCl, 5 mM 6-aminohexanoic acid, 50 mM imidazole/HCl pH 7.0, 50 mM KPi buffer pH 7.4, 10% glycerol) with 4 g/g protein digitonin (1.0% w/v) or 2.5 g/g protein. The beads were washed at 4°C with anti-FLAG (M2) antibody covalently coupled to agarose beads. The supernatant was preserved and diluted with 2× SDS–PAGE sample buffer for later Western blot analysis (i.e., lysate). Lysates containing V5-tagged protein (either V5.T234A or V5.T234D, respectively) were subsequently pooled and some volumes were added to the F9-tagged protein lysate containing FLAG-tagged protein to reduce expression variability in V5-tagged bait protein. Pooled lysates were incubated for 1 hour at 4°C with anti-FLAG (M2) antibody covalently coupled to agarose beads (Sigma-Aldrich). The beads were washed five times with lysis buffer with 0.25% sodium deoxycholate added, and bound proteins were resolved by SDS–PAGE, blotted onto polyvinylidene fluoride membranes, and probed with polyclonal rabbit anti-V5 antibody or anti-FLAG antibody (1:2000 dilution). Protein bands were visualized with enhanced chemiluminescence. Densitometry of raw band intensities was performed using the ImageJ/FIJI suite. Statistics were performed as two-tailed t tests.

Quantitative Analysis of Podocin Phosphorylation in HEK293T Cells

For quantitative phosphoproteomics analysis of podocin phosphorylation in HEK293T cells, 5 µg F. murine podocin cloned into a pcDNA6.2 vector was cotransfected with either active PKCz (T410E) or inactive PKCz (T410A). These plasmids were obtained from Addgene (Addgene 10801 and 10804).65 Cells were lysed in 8 M urea buffer and subjected to phosphopeptide enrichment with IMAC columns as described above in the phosphopeptide purification section and analyzed with an LTQ Orbitrap XL machine as described above in the MS section. Raw data were searched using the Sequest search algorithm implemented in the Proteome Discoverer platform. Data were further analyzed using the NHLBI in-house QUOIL program for visualization, normalization, and label-free quantification of MS1-precursor ion intensities as previously described (n = 3).66

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DISCLOSURES

None.

REFERENCES


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