A Novel Domain Regulating Degradation of the Glomerular Slit Diaphragm Protein Podocin in Cell Culture Systems

Markus Gödel¹,², Benjamin N. Ostendorf¹,², Jessica Baumer¹, Katrin Weber¹, Tobias B. Huber¹,²

1 Renal Division, University Hospital Freiburg, Freiburg, Germany, 2 BIORR Centre for Biological Signalling Studies, Albert-Ludwigs-University Freiburg, Freiburg, Germany

Abstract

Mutations in the gene NPHS2 are the most common cause of hereditary steroid-resistant nephrotic syndrome. Its gene product, the stomatin family member protein podocin represents a core component of the slit diaphragm, a unique structure that bridges the space between adjacent podocyte foot processes in the kidney glomerulus. Dislocation and misexpression of slit diaphragm components have been described in the pathogenesis of acquired and hereditary nephrotic syndrome. However, little is known about mechanisms regulating cellular trafficking and turnover of podocin. Here, we discover a three amino acids-comprising motif regulating intracellular localization of podocin in cell culture systems. Mutations in this motif led to markedly reduced degradation of podocin. These findings give novel insight into the molecular biology of the slit diaphragm protein podocin, enabling future research to establish the biological relevance of podocin turnover and localization.

Introduction

Podocytes are specialized epithelial cells constituting an essential part of the glomerular filtration barrier. They form a delicate network of cell extensions, so called primary and secondary processes that enwrap the glomerular capillaries. Interdigitating secondary processes are connected by a specialized cell junction, the slit diaphragm. Orderly composition of the slit diaphragm is essential for various cellular functions of the podocyte such as cell survival, polarity and cytoskeletal organization [1,2]. Over the last decade, much progress has been made in identifying the molecular makeup of the slit diaphragm [3–5].

NPHS2 is the most frequently affected gene in steroid-resistant nephrotic syndrome. Mutations in NPHS2 are responsible for about 50% of familial (autosomal recessive) and up to 20% of sporadic cases [4,6,7]. So far, expression of its gene product, the PHB-domain containing protein podocin, has only been shown in the glomerular podocyte and testis Sertoli cells [8]. In the podocyte, podocin localizes to the slit diaphragm, where it is assumed to act as an intracellular scaffold protein, assembling slit diaphragm components in lipid raft associated microdomains [9,10]. Podocin is a membrane-attached protein. It is predicted to form a hairpin like structure, with both N- and C-terminus residing in the cytoplasm. Several disease causing NPHS2 mutations were shown to interfere with podocin intracellular trafficking [11].

Various forms of injury to the glomerular filter trigger a common pathophysiological pathway inducing podocyte foot process effacement. Subcellularly, effacement is accompanied by the dislocation and degradation of slit diaphragm associated proteins such as nephrin and podocin [12–14]. It is therefore assumed that the spatiotemporal regulation of slit diaphragm components plays an essential role in the homeostasis of glomerular function [15]. The comprehensive knowledge of molecular events affecting slit diaphragm stability and degradation will be helpful in identifying novel therapies to maintain function and size selectivity of the glomerular filter in nephrotic disease. Recently, mechanisms such as ubiquitination and phosphorylation have been shown to participate in regulating nephrin endocytosis and degradation [16–19]. However, despite its significance at the slit diaphragm the mechanisms regulating the turnover of podocin remain unknown. It was therefore the aim of this work to investigate into these mechanisms in order to provide the basis for future research projects defining the biological relevance of podocin turnover and localization for podocyte physiology. Using a cell culture-based approach we were able to map a three amino acids comprising domain influencing subcellular localization and subsequent degradation of podocin.
Materials and Methods

Reagents and Plasmids

Marine podocin, human transferrin-receptor and pLXSN plasmids have been described previously [10,20,21]. All truncated or mutated variants of podocin were generated using standard cloning techniques. Solely N-terminally tagged fusion constructs of podocin (Flag, V5) were used for this study. Fusion proteins of podocin with a CD7-CD16 header were generated using a vector kindly provided by G. Walz [22]. A cDNA construct encoding eGFP-CD63 was kindly provided by D. Cutler. All newly synthesized constructs were verified by automated sequencing.

For immunofluorescence, primary antibodies were obtained from Santa Cruz (anti-CD16 mouse mAb, sc-51525), Sigma (anti-Flag rabbit pAb, F7425), Serotec (anti-V5 mouse mAb, MCA-1360), Chemicon Millipore (anti-V5 rabbit pAb, AB3792), Cell Signaling (anti-EEA1 rabbit pAb, 2411; anti-calnexin rabbit pAb, 2433) and Molecular Probes (anti-golgin97 mouse mAb, A-21270). Nuclear staining reagents and fluorophore-conjugated secondary antibodies were obtained from Invitrogen (Hoechst 33342, H3570; Alexa Fluor 488 donkey anti-rabbit, A21296; Alexa Fluor 488 donkey anti-mouse, A21292; Alexa Fluor 555 donkey anti-mouse, A31570; Alexa Fluor 555 donkey anti-rabbit, A31572). Lysotracker Red DND-99 was obtained from Invitrogen (L-7528).

For western blot, antibodies were obtained from Sigma (anti-Flag A31570; Alexa Fluor 555 donkey anti-rabbit, A31572; Alexa Fluor 488 donkey anti-mouse, A1978) and HRP-conjugated secondary antibodies were obtained from Dako.

Cell culture

HEK 293T and HeLa cells were grown in Dulbecco’s modified Eagle's medium (DMEM) supplemented with 10% FBS. A human podocyte cell line was provided by M. Saleem (Children’s Renal Unit, Bristol Royal Hospital for Children, University of Bristol, UK) and was cultured as described previously [23].

Generation of Transgenic Podocyte Cell Lines

Transgenic podocyte cell lines were generated using the pLXSN-vector expression system as previously described [21]. Briefly, retrovirus was produced by transfection of HEK 293T cells with 2.5 µg of pMD-G, 7.5 µg of pMD-Gp and 10 µg of the retroviral transfer vector pLXSN. The supernatant was harvested, centrifuged, filtered and cultured podocytes were transduced three times.

Immunofluorescence and Protein Internalisation

HeLa cells were transfected using Lipofectamine 2000 by Invitrogen and stained as described previously [10]. Briefly, HeLa cells or podocytes were fixed in 4% PFA for 3 minutes or in methanol at −20°C for 10 minutes when using anti-Flag antibody. They were then washed with PBS, permeabilised with 0.01% Triton-X in PBS and blocked using 5% BSA in PBS. Incubation periods with primary and secondary antibodies were followed by multiple washing steps after which the cells were mounted in Prolong Gold Antifade (Invitrogen) and subjected to immunofluorescence microscopy using an Apotome microscope (Zeiss).

To label acidic organelles cells were incubated with 50 nM Lysotracker Red DND-99 for 35 minutes at 37°C, 5% CO₂, washed twice with room temperature PBS, followed by fixation in 4% PFA for 10 minutes. Afterwards immunofluorescence was performed as described above.

Transient overexpression in human podocyte cell culture was performed by using Lonza Nucleofector technology with the primary epithelial cell transfection kit. 2 µg of plasmid DNA were electroporated using Nucleofector I program T-20 according to the manufacturer’s standard protocol.

To demonstrate protein internalization cells were serum starved for 1 hour and incubated with primary antibody at 4°C for 20 minutes (anti-CD16 mouse mAb, Santa Cruz sc-51525 1:1000 in DMEM and 20 mM HEPES). Following three washings they were incubated at either 4°C for controls or at 37°C for 20 minutes. Antibody remaining extracellularly was stripped in a 2 minute incubation step (0.5% acetic acid, 0.5 M NaCl, pH 3) and cells were subsequently washed and fixed. Further treatment was performed as described above.

Protein Stability Assay

Transgenic podocyte cells were treated with 20 µg/ml cycloheximide in DMEM for the times as indicated, lysed in buffer A (8 M urea, 100 mM NaH2PO4, 10 mM Tris, 1% Triton X-100, pH 8.0) and analyzed by western blot. Densitometry of western blots was performed using ImageJ software and podocin levels were normalized to actin levels.

FACS

HEK 293T cells were seeded on poly-L-lysine coated slides (poly-L-lysine diluted 1:1 with H2O; Sigma P4707, 0.01% solution) and transfected with CD16-tagged constructs of podocin also expressing GFP driven by an internal ribosomal entry site (IRES). At 24 hours after transfection cells were serum starved for one hour, cooled on ice for 15 minutes and incubated with anti-CD16 antibody (anti-CD16 mouse mAb, Santa Cruz sc-51525 1:750 in DFH (10% FBS and 20 mM HEPES in DMEM)). Subsequent to extensive washing with DFH they were incubated with fluorophore-conjugated anti-mouse antibody for 20 minutes (anti-mouse Alexa 633, Invitrogen (A21050) 1:500 in DFH), washed again, resuspended in 5 mM EDTA/PBS and separated with cell strainers (BD Biosciences). FACS analysis was performed using a FACS Calibur machine (BD Biosciences). Gates were set to include GFP-positive cells only.

Lipid Raft Preparation

Lipid rafts were isolated by sucrose density centrifugation as described previously [10]. Briefly, HEK293T cells were transfected using the calcium phosphate method and homogenized by 20 strokes in a Dounce homogenizer in 850 µl of TNE buffer (130 mM NaCl, 5 mM EDTA, 10 mM Tris, pH 8.2, proteinase inhibitors) in the presence of 1% Triton X-100. The lysates were incubated on ice for 20 min and centrifuged for 10 min at 3000 g at 4°C. The supernatant was adjusted to 50% sucrose and pipetted at 4°C for

Computational Protein Structure Prediction

Protein Structure Prediction was carried out using the I-TASSER algorithm [24].

Statistics

Data are expressed as means ± standard errors of the mean (SEM) of n experiments. All experiments were performed at least 3 times. Data analysis was performed using GraphPad Prism 5 statistical software.
Podocin Localizes to the Endosomal Compartment

Previously, podocin has been shown to reside in intracellular, vesicle-like structures next to its canonic membranous localization [25]. Consistently, in addition to the localization to the plasma membrane, the immunofluorescence signal of V5-tagged podocin in transiently transfected HeLa-cells also revealed podocin localizing to intracellular vesicles. In order to characterize those structures we co-stained V5-tagged podocin with markers for different cellular compartments. Little colocalization could be observed with markers for the secretory pathway, calnexin and golgin-97 (Fig. 1a and b), which stain the endoplasmatic reticulum and Golgi apparatus respectively. Also, only limited colocalization could be appreciated with EEA1 as marker for the early endosomal compartment (Fig. 1c). In contrast, vesicular podocin could be shown to partly reside in acidic organelles using fixed Lysotracker probes (Fig. 1d). Podocin demonstrated a more pronounced colocalization with co-expressed, Gfp-tagged CD63 (also known as LAMP3), a member of the tetraspanin superfamily localizing predominantly to the plasma membrane and vesicles of the late endosomal and lysosomal compartment (Fig. 1e) [26]. We therefore concluded that a fraction of podocin resides in the late endosomal compartment positive for CD63/LAMP3. In agreement, the immunofluorescence signal of transiently expressed, V5-tagged podocin in podocytes revealed a similar pattern of intracellular and plasma membrane podocin distribution and colocalization with Gfp-tagged CD63/LAMP3 (Fig. 1f). Intracellular localization of podocin seems not to be influenced by N-terminal tagging as perfect colocalization of tagged and untagged podocin could be demonstrated and untagged podocin was also shown to localize to Gfp-CD63/LAMP3-positive vesicles (supplemental Fig. 1a, b).

The C-Terminus Regulates Plasma Membrane Fraction and Internalization of Podocin

Various domains of podocin have been shown to mediate specific functions, such as interaction with nephrin, plasma membrane association and lipid raft binding [10,27]. However,
no function of the C-terminus distal from the PHB-domain has been described, although mutations in this region have been found in patients suffering from steroid-resistant nephrotic syndrome [28]. We hypothesized that this region, podocin286–385, might participate in the regulation of podocin localization. For a comparison of the subcellular localizations of podocin wild type and a truncation lacking the C-terminus, podocin1–285, we overexpressed these proteins in HeLa-cells. Strikingly, podocin1–285 localized in a distinct way from podocin wild type, staining mainly the plasma membrane and only few intracellular vesicles, suggesting a role of podocin286–385 in regulating podocin localization (Fig. 2A).

The analysis of podocin cell surface expression is complicated by the lack of extracellular domains. To address, whether

Figure 2. A C-terminal domain regulates plasma membrane localization of podocin. A. Immunofluorescence for a transiently expressed, Flag-tagged truncation of podocin (podocin1–285) reveals increased plasma membrane localization in comparison with podocin wild type in HeLa-cells (b and a respectively). B. Schematic representation of constructs consisting of the extracellular domain of CD16 and the transmembrane domain of CD7 fused to different parts of podocin. C. Immunofluorescence using anti-CD16 antibody showed primarily membranous staining patterns for CD16-7- and CD16-7-podocin1–285 (a, b). In contrast, immunofluorescence for CD16-7-podocin286–385 revealed multiple vesicular structures (c), thereby proving analogous localization of CD16-fusion constructs and V5/Flag-tagged constructs.
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increased plasma membrane expression was a result of reduced internalization rather than increased shuttling to the plasma membrane, we created constructs consisting of either podocin1–285 or podocin286–385 fused to the extracellular domain of human CD16 and the transmembrane domain of CD7 (schematic representation in Fig. 2B). We first compared the subcellular localization of these constructs in steady state to the V5/Flag-tagged constructs in order to evaluate their applicability for studying podocin trafficking. CD16-CD7-podocin1–285 localized in a fashion analogous to the Flag-tagged construct. CD16-CD7-podocin286–385 mainly localized to intracellular vesicles in contrast to our control construct CD16-CD7, which localized mainly to the plasma membrane, thereby supporting the hypothesis that podocin286–385 harbors an internalization promoting motif (Fig. 2C). A fusion construct of CD16-CD7 with podocin full length was mainly retained in the endoplasmatic reticulum and was not used for further studies (supplemental Fig. S2 a). For a dynamic analysis of podocin internalization, live HeLa cells expressing the aforementioned constructs were exposed to CD16 antibody in the cell culture media, thereby labeling only podocin-fusion constructs with their CD16-tag on the extracellular surface. After washing, cells were incubated at 37°C to allow internalization of the antibody or at 4°C for controls. After stripping remaining antibody from the extracellular surface, cells were fixed. No internalized fraction of CD16-CD7 alone or CD16-CD7-podocin1–285 could be detected after 20 minutes at either 4°C or 37°C (supplemental Fig. S2 b–e). In contrast, in cells expressing CD16-CD7-podocin286–385 vesicular structures were observed. No staining was detectable in 4°C controls (supplemental Fig. S2, f and g). These data indicate that podocin286–385 efficiently mediates a signal for internalization.

Podocin340–350 Regulates Subcellular Localization of Podocin

In order to specify the region within podocin286–385 responsible for determining its subcellular localization, we stained various truncated versions of podocin in HeLa-cells. This approach revealed that the truncations podocin1–310, podocin1–335 and podocin1–340 all exhibited a pattern comparable to podocin1–285 (Fig. 3a, b; supplemental Fig. S3a, b), localizing primarily to the plasma membrane and bearing little colocalization with CD63. In contrast, the truncations podocin1–350, podocin1–365 and podocin1–377 exhibited a more vesicular pattern similar to podocin wild type, colocalizing significantly with CD63 (Fig. 3c, d; supplemental Fig. S3c, d). These results suggest a role of podocin340–350 in regulating podocin plasma membrane expression.

PodocinTVV339,340,341 Regulates Localization and Degradation of Podocin through a Lipid Raft-Independent Mechanism

For a more precise mapping of the domain regulating podocin internalization we generated four Flag-tagged podocin variants with clusters of three amino acids mutated to alanine within podocin335–350. Immunofluorescence of these constructs overexpressed in HeLa cells revealed podocinTVV339,340,341AAA to localize similarly to podocin1–285, whereas the other three mutants podocinEKP335,336,337AAA, podocinPLP343,344,345AAA and podocinDML347,348,349AAA exhibited a staining pattern comparable to podocin wild type (Fig. 4A). We confirmed the localizations of podocin wild type, podocin1–285 and podocinTVV339,340,341AAA in transgenic podocytes stably expressing the construct to be analogous to the patterns seen in HeLa cells (Fig. 4B). Thus, podocinTVV339,340,341 seems to be responsible for the regulation of plasma membrane expression of podocin. In order...
to quantify the difference in cell surface expression we used a FACS based approach. Mutated plasmids were transiently expressed in HeLa cells and localization of mutants was assessed by immunofluorescence using anti-Flag antibody. In contrast to the other mutants, podocin TVV339,340,341AAA was shown to localize in a predominantly membranous pattern similar to podocin1–285 (a–d). B. Immunofluorescence of Flag-tagged podocin wild type, podocin1–285 and podocin TVV339,340,341AAA confirmed localization in transgenic differentiated podocytes to be analogous to HeLa cells (a–c). C. 293T-cells were transfected with plasmids expressing either CD16-7-*, CD16-7-podocin286–385 or CD16-7-podocin286–385 TVV339,340,341AAA and cell surface expression was analyzed by FACS. As transfected cells also expressed Gfp driven from an internal ribosomal site from the same vector, gates were set to include Gfp-positive cells only. Cell surface expression of CD16-7-* and CD16-7-podocin286–385 was comparable (b), while less CD16-7-podocin286–385 could be detected at the plasma membrane, consistent with a role of podocin TVV339,340,341AAA in regulating internalization of podocin (a).

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Figure 4. Podocin TVV339,340,341 regulates the surface expression of podocin. A. Amino acid-triplets within podocin335–350 were mutated using a Quickchange approach. Mutated plasmids were transiently expressed in HeLa cells and localization of mutants was assessed by immunofluorescence using anti-Flag antibody. In contrast to the other mutants, podocin TVV339,340,341AAA was shown to localize in a predominantly membranous pattern similar to podocin1–285 (a–d). B. Immunofluorescence of Flag-tagged podocin wild type, podocin1–285 and podocin TVV339,340,341AAA confirmed localization in transgenic differentiated podocytes to be analogous to HeLa cells (a–c). C. 293T-cells were transfected with plasmids expressing either CD16-7-*, CD16-7-podocin286–385 or CD16-7-podocin286–385 TVV339,340,341AAA and cell surface expression was analyzed by FACS. As transfected cells also expressed Gfp driven from an internal ribosomal site from the same vector, gates were set to include Gfp-positive cells only. Cell surface expression of CD16-7-* and CD16-7-podocin286–385 was comparable (b), while less CD16-7-podocin286–385 could be detected at the plasma membrane, consistent with a role of podocin TVV339,340,341AAA in regulating internalization of podocin (a).

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resistant membranes, indicating intact recruitment of the mutant to lipid rafts (Fig. 5B, b).

In order to assess the localization of the TVV339,340,341 motif in the 3D configuration of podocin we performed a structure prediction analysis using the I-Tasser algorithm [24]. This showed podocinTVV339,340,341 to be exposed on the protein surface, thereby being accessible for presumptive interaction partners regulating podocin localization and degradation (Fig. 5B, c).

Discussion

Spatiotemporal regulation of the slit diaphragm proteins nephrin and podocin is an essential mechanism in glomerular homeostasis and injury. Recent studies have focused on the mechanisms regulating the internalization of nephrin: PKCα was shown to mediate nephrin endocytosis via the regulation of an interaction between nephrin and β-arrestin2 [17,30,31]. In certain human glomerulopathies and in a CD2AP-deficiency model, nephrin internalization was also found to be stimulated by ubiquitination [16,19,32]. Moreover, Qin and co-workers proposed a raft-mediated endocytic pathway for nephrin following its phosphorylation [18]. However, no signals have been characterized mediating turnover cues for podocin.

We demonstrated that next to the plasmamembrane a considerable amount of podocin localizes to the late endosomal compartment, where it colocalizes with CD63/LAMP3 partially overlapping with acidic organelles. We then proposed a role of the C-terminus in regulating plasma membrane expression of podocin, suggested by markedly increased plasma membrane...

Figure 5. PodocinTVV339,340,341 regulates the turnover of podocin through a lipid raft-independent mechanism. A. Differentiated podocytes stably expressing Flag-tagged podocin, podocin1–285 or podocinTVV339,340,341AAA were exposed to the translation inhibitor cycloheximide for the times as indicated and analyzed per western blot using anti-Flag antibody (a). Actin levels detected by anti-actin antibody served as loading control. Podocin1–285 and podocinTVV339,340,341AAA were shown to be more stable than podocin wild type, consistent with a regulatory role of podocinTVV339,340,341 in its degradation. (b) Summarizes the results of three experiments. Podocin levels were normalized to actin levels. B. (a) shows a schematic comparison between the PHB-domain proteins podocin and stomatin. UMAUF ET AL proved a motif partially overlapping with podocinTVV339,340,341 to play a crucial role in lipid raft binding. (b) HEK293T cells were transfected with the plasmids as indicated, lysed in 1% TX-100 on ice and subjected to flotation gradient centrifugation to prepare detergent-resistant membranes (DRM). In contrast to the control protein transferrin receptor, both podocin wild type and podocinTVV339,340,341AAA were detected in DRM. C. Graphical representation of the structure prediction analysis of podocin using the I-Tasser algorithm revealed exposed position of podocinTVV339,340,341.

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localization and reduced internalization of a truncation lacking the C-terminus. To further dissect the region mediating this effect we used several truncation variants as well as podocin Quickchange mutants in immunofluorescence based assays, a FACS-based approach and degradation assays to provide evidence that podocinTV339,340,341 represents the domain responsible for mediating degradation signals and plasma membrane localization. Since podocin is a raft-associated molecule [10], a raft-dependent endocytic pathway has been suggested for nephrin [18] and a region of the podocin related protein stomatin partially overlapping with podocinTV339,340,341 was revealed to mediate lipid raft binding [29], we tested for the association of podocinTV339,340,341AAA with lipid rafts. This revealed the presence of both podocin wild type and the mutant in lipid rafts, excluding the possibility of podocinTV339,340,341AAA exerting a change to turnover kinetics due to defect lipid raft binding. To support biological relevance consolidated findings obtained by use of easily

Disruption of a three amino acids-comprising motif led to increased plasma membrane expression and reduced degradation of podocin through a lipid raft-independent mechanism. Relating this mechanism to turnover events in glomerular maintenance and injury could lead to an extended understanding of proteinuric diseases and provide novel therapeutic options.

Supporting Information

Figure S1 N-terminal tagging of podocin does not seem to influence its subcellular localization in HeLa cells as there is a perfect overlap with overexpressed untagged podocin stained with a podocin specific antibody. Untagged podocin also displayed significant colocalization with eGFP tagged CD63/LAMP3 (a and b respectively).

Figure S2 A C-terminal domain regulates the internalization of podocin. HeLa cells transiently expressing the constructs as indicated were incubated on ice with anti-CD16 antibody. Following a 20 minute incubation period with medium at 4°C versus 37°C remaining extracellular antibody was stripped and the cells were fixed and permeabilized. Immunofluorescence revealed no internalized fraction for control cells at 4°C (b, d, f). In contrast to both CD16-7- and CD16-7-podocin1–285 (c and e) an internalized fraction could be detected with podocin1–285 and podocinTV339,340,341 after incubation at 37°C (g). A construct of CD16-CD7 fused to podocin wild type full length stained with anti-CD16 in permeabilized cells revealed a staining pattern indicating retention in the endoplasmatic reticulum (a).

Figure S3 Subcellular localization of different podocin truncations. A–D. Various truncations of Flag-tagged podocin were coexpressed with eGfp-tagged CD63 in HeLa-cells. Immunofluorescence using anti-Flag antibody revealed a primarily membranous staining pattern for podocin1–310 and podocin1–335 similar to podocin1–363 (a and b). In contrast, podocin1–363 and podocin1–377 were shown to localize similarly to podocin wild type (c and d).

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Author Contributions

Conceived and designed the experiments: MG BNO TBH. Performed the experiments: BNO JB KW MG. Analyzed the data: MG BNO TBH. Contributed reagents/materials/analysis tools: TBH. Wrote the paper: BNO MG TBH.
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