Author's response to reviews

Title: Expression of a novel short isoform of the kidney disease protein podocin in human kidney

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Author's response to reviews: see over
Dear Editor,

Thank you for giving us the opportunity to resubmit an improved version of our manuscript. We took the reviewers' concerns and advice seriously and believe that the quality of the manuscript improved significantly. In particular, we realized from the questions from reviewer 2, that the mass spectrometry part was not very conclusive and convincing. For this revision we changed the experimental settings and the method of analysis. Therefore, the part of the paper dealing with mass spectrometry is completely new. However, the results/conclusion did not change, namely the unambiguous identification of the podocin short isoform using mass spectroscopy. This result is a major advance over previous reports. Antibody-based methods of detection are prone to false positive results (unspecific binding), especially when a weakly expressed isoform has to be identified. This is the reason why, although the corresponding cDNA was known since some time, the expression of the short podocin isoform on protein level was questioned in the field. We therefore believe that the unambiguous identification on protein level is exciting and justifies publication. Markus Rinschen helped with this analysis which is the reason that we added him as an author.

Except for some minor grammatical and spelling changes, changes in the manuscript are highlighted in yellow.

Please find below a point-by-point response to the referees' concerns.

With kind regards,

Martin Höhne
Referee 1

Major points

1 This in-flame exon deletion was originally reported by Horinouchi I et al (Kidney International 2003) and has been more extensively studied by Relle M et al (Mod Pathol, 2011). The whole sequence of podocin short variant was shown in the previous paper (Relle M et al) as exactly same as in this paper. Therefore, despite of some additional biochemical and immunocytochemistry data provided in the current paper, the podocin short variant is not truly “novel” protein.

There have been earlier reports on this short isoform, and both references (Horinouchi et al, 2003; Relle et al, 2011) are cited in our manuscript. We still believe that our manuscript adds some novel aspects, in particular the unambiguous existence of the short isoform on protein level using mass spectrometry. But we agree that the term “novel isoform/protein” was not appropriate and removed this term from all occurrences in the manuscript.

2 The relevance of N-glycosylation of short variants remains unclear, if they are mostly localized within ER and are hardly trafficked to the Golgi, the key organelle which organizes the generation of mature sugar chains. Expression in different systems (host cells and/or vector promoters) may be helpful to evaluate the cellular functions of short variants.

We agree with the referee that the relevance of N-glycosylation is not completely clear. However, this part of the manuscript is rather descriptive. We put our observations in the context of published data. The first observation is the retention of the short isoform mainly in the ER. This observation is put into context with the fact that many known mutations of podocin are trafficked to the ER as well. It is interesting, that the physiological localization of the podocin isoform is the same as the pathogenic subcellular localization of podocin mutants. The second observation is the fact, that a significant proportion of the short isoform is N-glycosylated. This is interesting because of the report from Kadurin et al (2009), that stomatin, a protein whose membrane topology is considered similar to the podocin membrane topology, is also N-glycosylated in a stomatin point mutation. Again it seems that the physiologic podocin short isoform has some features that are known from pathogenic variants of other proteins. However, elucidating the physiologic role of the podocin short isoform is beyond the scope of the current manuscript. To better convey out point of view to the reader we rephrased the corresponding part of the manuscript (lines 252-261).

It is interesting to note that deleting parts of the PHB domain as it occurs in Pod
short has a similar effect on the protein membrane topology as a single point mutation in a region preceding the PHB domain as in stomatin
P47S. It has been shown that also in wildtype stomatin, the formation of the hairpin loop protein was not 100% efficient and that a certain pool of the wildtype protein was also N-glycosylated [17]. Because of the low abundance compared with the hairpin loop form, a physiological function for the transmembranous form was considered rather unlikely [17]. For Pod
short, it seems that a considerable fraction is glycosylated and hence present in the non-hairpin loop conformation. However, the functional implication of these observations and the physiological role of Pod
short remains to be elucidated.
Minor points

1 In Abstract/conclusion page 2, it might be better to emphasize what are the new aspects of short variant of podocin more specifically from the current observations e.g., glycosylation, floating property, ER localization. etc, instead of giving an inclusive remark of “the functional implication of this isoform is still elusive”

We rephrased the conclusion part according to the reviewer’s suggestion (lines 52-56).

Conclusions: A second isoform of human podocin is expressed in the kidney. This isoform lacks part of the PHB domain. It can be detected on protein level. Distinct subcellular localization suggests a physiological role for this isoform which may be different from the well-studied canonical variant. Possibly, the short isoform influences lipid and protein composition of the slit diaphragm complex by sequestration of lipid and protein interactors into other cell compartments.

Referee 2

- MAJOR COMPULSORY REVISIONS

1. A figure showing the immunoprecipitation results (podocin-binding proteins) should be added.

We added the co-IP results to the paper. The experiments are described in lines 230-236 and the results are shown in the new figure 4.

Due to its interaction with several other slit diaphragm proteins and its localization to detergent resistant membranes, podocin is a central organizer of the slit diaphragm protein complex [5, 16]. We examined the interaction of the Podshort with several known slit diaphragm interactors in co-immunoprecipitation experiments. Despite the fact that the short isoform has a different subcellular distribution, we could not observe any difference between the interaction of the canonical and the short podocin isoform with CD2AP, TRPC6, NEPH1 and nephrin (Fig. 4).

2. The larger canonical isoform of podocin should be also subjected to mass spectrometry and the basepeak chromatogram should be reported. The differences of tryptic peptide analysis between canonical and short isoforms should be showed and marked.

Thanks to the comments of referee 2 (this comment here and the ones below), we became aware of the fact that our initial mass spectrometry results were not presented in an easily understandable way and were not as conclusive as they should have been. We therefore removed the old mass spectrometry data and replaced it with newer data. However, the general conclusion remains unchanged: the unambiguous identification of the podocin short isoform on protein level from human glomerular lysate. We hope to convince the referees and editor with the new data and its representation.

The new methods (lines 116-160)
Human glomerular samples were prepared from healthy kidney tissue of tumor nephrectomies by a sieving technique described elsewhere [9]. The study protocol was approved by the independent ethics committee of Cologne University and all patients provided written informed consent.

Human glomerular samples were denatured by boiling in Lämmli buffer for 5 minutes, and unsoluble components pelleted by centrifugation. Soluble protein content was separated by standard SDS-page gel electrophoresis (4-20% Gradient gel) and stained with colloidal Coomassie brilliant blue dye. Gel segments at the molecular weight expected for the podocin protein (canonical isoform: 42.2 kDa; short isoform: 34.4 kDa) were excised from the gel and subjected to mass spectrometry.

Peptide isolation and Mass Spectrometry

Tryptic in-gel digest: Following electrophoresis the gel was washed thoroughly in water. The area of interest was cut out and minced using a scalpel. After destaining with 50% 10 mM NH₄HCO₃ / 50% ACN at 55°C and dehydration in 100% ACN gel pieces were equilibrated with 10 mM NH₄HCO₃ containing porcine trypsin (12.5 ng/µl; Promega) on ice for 4 hours. Excess trypsin solution was removed and tryptic hydrolysis was performed for 4 hours at 37°C in 10 mM NH₄HCO₃. The supernatant was collected and further extraction steps were performed. After acidification with 5% TFA, gel pieces were excised twice with 1% TFA and then with 60% ACN/40%H₂O/0.1% TFA followed by a subsequent two-step treatment using 100% ACN. The supernatant and the extractions were combined and concentrated using a SpeedVac concentrator (Christ). Prior to nano-LC-MS/MS analysis the peptides were desalted using STAGE Tip C18 spin columns (Proxeon/Thermo Scientific) as described elsewhere [10]. Eluted peptides were concentrated in vacuo and then re-suspended in 0.5% acetic acid in water to a final volume of 10 µl.

Nano-LC ESI-MS/MS Mass Spectrometry: Experiments were performed on a LTQ Orbitrap Discovery mass spectrometer (Thermo Scientific) that was coupled to an EASY-nLC nano-LC system (Proxeon/Thermo Scientific). Intact peptides were detected in the Orbitrap at 30,000 resolution in the mass-to-charge (m/z) range 350-2000. Internal calibration was performed using the ion signal of (Si(CH₃)₂O)₆H at m/z 445.12003 as a lock mass. For LC-MS/MS analysis, up to five CID spectra were acquired following each full scan. Aliquots of the samples were separated on a 15 cm, 75 µm reversed phase column (Proxeon/Thermo Scientific). The gradient used for liquid chromatography is described elsewhere in more detail [11].

Peptide and Protein Identification: The search algorithm Sequest as implemented in the Proteome Discoverer software (Thermo Scientific) was used for protein identification. To identify the proteins contained in the excised gel area, MS/MS data were searched using the canonical sequence database of the Homo sapiens reference proteome provided by the UniProt Consortium using the target-decoy strategy. The sequence of the predicted short isoform of podocin was added to the database. The maximum of two modification was allowed per peptide. Oxidation of methionine residues was used as a variable modification and carbamidomethylation of cysteine residues as a fixed modification. For Orbitrap data, 10 ppm mass tolerance was allowed for intact peptide masses and 0.8 Da for CID fragment ions detected in the linear ion trap. Peptides were subsequently filtered to match a FDR < 0.01. Protein identifications were based on at least 2 peptides. Ion chromatograms were extracted using the NHLBI in-house software QUOIL which extracts ion chromatograms for identified peptides [12]. Isotope patterns were visualized using the MaxQuant Viewer software [13].
One crucial limitation of mass spectrometry based identification in human glomerular samples is the low purity of a preparation using the classical sieving technique as well as the limited amount of starting material. We therefore used lysates of HEK293T cells transfected with the canonical and short podocin isoform, respectively, as reference samples. These samples were processed in parallel to the glomerular sample and analyzed by mass spectrometry subsequent to the glomerular lysates as described in the method section.

Within the sample containing the short isoform of podocin, we unambiguously identified the isoform-specific peptide with the sequence LQTLIEPFHEVALDSVTcIWGIK. This triple-charged peptide carried a carbamidomethylation at the cysteine residue (m/z = 890.47). The MS2 spectrum for this peptide is depicted in figure 2B.

Next, we analyzed whether the specific mass corresponding to this isoform-specific peptide (+/-10ppm) was also found within a very limited time window (+/- 1 min) in the MS1 precursor chromatogram of the glomerular sample or the sample obtained from HEK cells expressing the canonical podocin isoform. As expected, the peptide mass was absent in the sample obtained from HEK293T cells transfected with the canonical isoform whereas many other peptides matching to podocin were by far more abundant in this sample. However, the mass corresponding to the short isoform specific peptide was also found in the human glomerular sample (Fig 2C). In addition, the MS1 precursor isotope pattern confirmed the presence of a triple charged peptide mass within both samples (Fig. 2 D). Combining these results, we report evidence on protein level for the existence of a shorter isoform of human podocin.

- MINOR ESSENTIAL REVISIONS

• TITLE

1. The word “novel” should be avoid: the data showed by Dr. Relle and colleagues (Modern Pathology, 2011; 24: 1101–1110) using RT–polymerase chain reaction and immunoblotting followed by sequence analysis, are the real first evidence to prove the expression of a “novel” podocin isoform.

and

2. Podocin is a “kidney protein” not a “kidney disease protein”.

We agree that both “novel” and “kidney disease protein” were not appropriate. We changed the title accordingly:

Characterization of a short isoform of the kidney protein podocin in human kidney

• METHODS

3. Reagents and Plasmids section: The authors should report at least the name of kit used for site direct mutagenesis.
We did not use a kit for site directed mutagenesis, but a modified strategy published by Wang et al. (Wang W, Malcolm BA: **Two-stage PCR protocol allowing introduction of multiple mutations, deletions and insertions using QuikChange Site-Directed Mutagenesis**. *BioTechniques* 1999, **26**:680–682.)

We changed the text accordingly and added the reference (line 92):

> a modified QuikChange site directed mutagenesis approach [8] was

4. **Immunoprecipitation section: The origin of human glomeruli samples should be reported;**

We added the required information (lines 116-119):

**MS sample preparation**

Human glomerular samples were prepared from healthy kidney tissue of tumor nephrectomies by a sieving technique described elsewhere [9]. The study protocol was approved by the independent ethics committee of Cologne University and all patients provided written informed consent.

5. **Immunoprecipitation section: The expected molecular weight of the short isoform should be specified;**

This comment refers to the immunoprecipitation section from the old mass spec protocol. In the new version this information is now added to the section “MS sample preparation” (lines 123-124):

> segments at the molecular weight expected for the podocin protein (canonical isoform: 42.2 kDa; short isoform: 34.4 kDa) were excised from the gel and subjected to mass spectrometry

6. **Immunoprecipitation section: “CID” definition should be reported in the List of Abbreviations**

CID - Collision-induced dissociation has been added to the List of Abbreviations

**• RESULTS AND DISCUSSION, THIRD PARAGRAPHS**

7. The authors state: “The low amount of starting material for MS analyses resulted in elution of the mass 1078.2191 for about 15 seconds (Fig. 2C)”. Anyway, the elution peak of m/z 1078.2191 reported in figure 2 was at 40.02 minutes. Could you please clarify this point?

8. The authors state: “Taking the high mass accuracy of m/z 1078.2191 with the predicted peptide into account and the fact that the corresponding MS/MS spectrum could only be found in Podshort, we report initial evidence on the protein level for the existence of a shorter isoform of human podocin (Fig. 2B)”. Figure 2B shows the basepeak chromatogram of the eluting peptides from the specific band of short podocin isoform, thus I think that the previous authors’ statement referring to figure 2 should be avoid. This sentence should be restated if mass spectrometry analysis of canonical isoform is not reported.
These comments refer to the mass spectrometry data/results that have been replaced with a new experiment.

- **FIGURES**

9. *Figure 3C: the authors should indicate in figure legend what are lane 1, 2, 3, ...etc.*

We added the required information to the legend of figure 3 (lines 384-386):

> Fractions 1-7 were collected from the top and analyzed by Western blot. Both isoforms fractionate into the DRMs (fractions 1 and 2, as identified by flotillin staining).