Podocyte-Specific Deletion of Murine CXADR Does Not Impair Podocyte Development, Function or Stress Response

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Abstract

The coxsackie- and adenovirus receptor (CXADR) is a member of the immunoglobulin protein superfamily, present in various epithelial cells including glomerular epithelial cells. Beside its known function as a virus receptor, it also constitutes an integral part of cell-junctions. Previous studies in the zebrafish pronephros postulated a potential role of CXADR for the terminal differentiation of glomerular podocytes and correct patterning of the elaborated foot process architecture. However, due to early embryonic lethality of constitutive \( \text{Cxadr} \) knockout mice, mammalian data on kidney epithelial cells have been lacking. Interestingly, \( \text{Cxadr} \) is robustly expressed during podocyte development and in adulthood in response to glomerular injury. We therefore used a conditional transgenic approach to elucidate the function of \( \text{Cxadr} \) for podocyte development and stress response. Surprisingly, we could not discern a developmental phenotype in podocyte specific \( \text{Cxadr} \) knock-out mice. In addition, despite a significant up regulation of CXADR during toxic, genetic and immunologic podocyte injury, we could not detect any impact of \( \text{Cxadr} \) on these injury models. Thus these data indicate that in contrast to lower vertebrate models, mammalian podocytes have acquired molecular programs to compensate for the loss of \( \text{Cxadr} \).

Introduction

The renal filtration barrier is composed of at least four layers, the endothelial glycocalyx, the endothelial fenestrae, the glomerular basement membrane and the slit diaphragm (SD) in between neighbouring podocyte foot processes [1]. This last part of the filter is composed of a variety of different types of intercellular junctions, forming a uniquely broad, permeable and still highly selective barrier. Beside slit-diaphragm specific components such as NEPHRIN,
NEPH1 and PODOCIN, adherens junctional proteins i.e. CADHERIN or CATENINS as well as tight junctional components i.e. JAM-A, OCCLUDIN, CINGULIN and ZO-1 have been identified and localized to this junction [2–5]. Interestingly several members across different junctional classes belong to the Immunoglobulin Superfamily (IgSF) of molecules: NEPHRIN, NEPH1 and JAM-A [6].

Work on normal and nephrotic rat glomeruli identified the coxsackie- and adenovirus receptor (CXADR) as another IgSF member of the podocyte SD, which was upregulated in puromycin aminonucleoside (PAN) treated rats [7, 8]. In addition, it was demonstrated that CXADR physically interacts with SD proteins such as PODOCIN [7]. Furthermore a morpholino based CXADR knock-down approach in zebrafish suggested a role for CXADR in the terminal differentiation of glomerular podocytes [9].

CXADR was identified in 1997 as the receptor mediating binding and uptake of Coxsackie type B viruses and adenovirus type 2 and 5 [10, 11]. Subsequently it was shown that CXADR is an integral component of tight-junctions and interacts with ZO-1, MAGI-1 and MUPP1 [12–14]. Constitutive Cxadr knock-out mice exhibited an early embryonic lethality between E11.5 and E13.5 due to heart abnormalities [15]. Further evaluation using an inducible conditional mouse genetic approach, revealed that loss of CXADR results in impaired electrical conduction between the cardiac atrium and ventricle, which was underlined by a functional interaction of CXADR with connexins [16].

To elucidate the precise role of CXADR for mammalian podocyte development, maintenance and stress response in vivo we analyzed podocyte specific conditional Cxadr knockout mice.

Materials and Methods

Animals

All animal experiments were conducted according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals, as well as the German law for the welfare of animals and were approved by local authorities [G-09/23 Regierungspräsidium Freiburg]. Mice were generated as previously described [16] and crossed using the Tg(hNPHS2-cre)295Lbh line [17]. Mice were housed in a SPF facility with free access to chow and water and a 12h day/night cycle. Breeding and genotyping was done according to standard procedures. To assess genetic influence on CXADR expression the well described Cd2ap−/− on a C57Bl6 background was used at the age of 4 weeks [18].

Developmental assessment

Individual age and sex matched animals from parallel litters on a C57Bl6/NCrl background were used. Control animals (Cxadr fl/fl) were compared with podocyte specific (Cxadr fl/fl hNphs2Cre) mice. Collection of spot urine samples in the respective home cages was performed between 7 to 9 am at defined time points as indicated. Urinary albumin and creatinine were measured using a fluorimetric albumin test kit (Progen, PR2005, Heidelberg, Germany) and enzymatic colorimetric creatinine kit (LT-SYS, Lehmann, Berlin, Germany) following the manufacturer’s instructions. Evaluation of proteinuria (expressed as the albumin to creatinine ratio) was performed as previously described [19–21]. Primary outcome was development of proteinuria.

Glomerular stress models

For some of the following stress experiments C57Bl6/NCrl mice were backcrossed to ICR mice (Taconic, New York, USA) for at least 6 generations as indicated in the result section. All other
experiments were performed on a C57Bl6/NCrl background. We used the following well established stress-models: A.) adriamycin, and B.) nephrotoxic serum (NTS). In each of these, control animals (Cxadr fl/fl) were compared with podocyte specific (Cxadr fl/fl’hnphs2Cre) mice. Animals were allocated to the two groups based on age and sex. A.) Adriamycin (Pharmacy, University Hospital Freiburg, Germany) was administered at a dose of 15 μg/g BW in 0.9% saline i.v. under isoflurane anesthesia after collection of control urine. Urine was again collected at 1, 2, 3, 4 and 5 weeks after injection. Mice were killed by cervical dislocation and kidneys harvested at 5 weeks after injection. B.) NTS of sheep origin was generously supplied by Pierre-Louis Tharaux (INSERM PARCC, Paris, France) and generated as previously described [22]. Briefly, mice were injected with 2μl/g BW NTS on day 0, 6μl/g BW on day 1 and 5μl/g BW on day 2. each time under isoflurane anesthesia. Urine was collected before the respective injection on day 0 and 2 and additionally on day 3 and 4. Due to the severity of the phenotype and in accordance with our animal proposal mice were killed by cervical dislocation on day 4 and kidneys were harvested.

Isolation of glomeruli and podocytes

We used the magnetic bead method described by Takemoto et al. 2006 with appropriate modifications [23]. Briefly, kidneys were dissected together with the abdominal aorta and transferred into dishes filled with 37°C pre-warmed Hank’s buffered salt solution (HBSS). Each kidney was perfused slowly through the renal artery with 4 ml 37°C warm bead solution and 1 ml bead solution plus enzymatic digestion buffer [containing: collagenase 300 U/ml (Collagenase Type II, Worthington, Lakewood, New Jersey, USA), 1 mg/ml pronase E (P6911, Sigma, Schnelldorf, Germany) DNase I 50 U/ml (A3778, Applichem, Darmstadt, Germany)]. Kidneys were minced into 1 mm³ pieces using a scalpel. After addition of 3 ml digestion buffer they were incubated at 37°C for 15 min on a rotator (100rpm). The solution was pipetted up and down with a cut 1000μl pipette tip every 5 min. After incubation all steps were performed at 4°C or on ice. The digested kidneys were gently pressed twice through a 100 μm cell-strainer and the flow through was washed extensively with HBSS. After spinning down, the supernatant was discarded and the pellet resuspended in 2 ml HBSS. These tubes were inserted into a magnetic particle concentrator and the separated glomeruli were washed twice. Podocytes were isolated as previously described [24].

Morphological analysis

Kidneys were perfusion fixed in 4% phosphate buffered paraformaldehyde (Sigma, Schnelldorf, Germany), embedded in paraffin and further processed for PAS staining. For ultrastructural transmission electron microscopy (TEM) analysis kidneys were fixed in 4% phosphate buffered paraformaldehyde plus 1% glutaraldehyde (Serva, Heidelberg Germany). Samples were post-fixed in 1% osmium tetroxide in the same buffer for 1 hour and stained en bloc in 1% uranyl acetate in 70% ethanol for 1 hour, dehydrated in ethanol, and embedded in Durcupan (Plano, Wetzlar, Germany). Thin sections were stained with lead citrate and examined in a Zeiss Leo-906 transmission electron microscope. For scanning electron microscopy samples were fixed with 4% glutaraldehyde for 4 days and were then subsequently dehydrated (EtOH 50, 70, 80, 90 and 100%; 1:1 EtOH and Hexamethyldisilazan (HMDS) (Sigma, Schnellendorf, Germany) for 1 hour and 30 minutes 100% HMDS, afterwards solvent was allowed to evaporate) and coated with Gold (Zeiss Semco Nanolab7, Polaron Cool Sputter Coater E 5100, Balzer Cpd 020) [25]. Image acquisition was performed using a Leo 1450 VP scanning electron microscope.
Western Blot and Immunofluorescence

Kidneys or isolated glomeruli were glass-glass-homogenized in lysis buffer (containing 20 mM CHAPS and 1% Triton X-100). After centrifugation (15,000x g, 15 min, 4°C) protein concentration was determined by DC Protein-Assay (Bio-Rad, Hercules, California, USA). Equal amounts of protein were separated on SDS page. For immunofluorescence kidneys were frozen in OCT compound and sectioned at 4–5 μm (Leica Kryostat, Leica, Wetzlar, Germany). The sections were fixed with 4% paraformaldehyde, blocked in PBS containing 5% BSA + 5% Normal Donkey Serum (Jackson Immunoresearch, Suffolk, Great Britain) and incubated for 45 min with primary antibodies as indicated. For co-stainings with CXADR initial fixation was performed using methanol at -20°C for 10 minutes. After several PBS rinses, fluorophore-conjugated secondary antibodies (Life Technologies, Darmstadt, Germany) were applied for 30 minutes. Image acquisition was done either using a confocal imaging set up (Zeiss LSM 510 upright microscope, Zeiss, Germany), equipped with a Plan-Apochromat 63x/1.4 Oil M27 objective or a epifluorescence widefield imaging setup (Zeiss Axioplan 2 upright + Axiocam MRc5 digital camera)). Image recording was performed with the appropriate software (Zen black Software or Axiosvision LE46—Zeiss). The following antibodies were used: anti-CXADR (HPA030411, Atlas Antibodies, Stockholm, Sweden), anti-CXADR (H300, sc15405, Santa Cruz Biotechnology, Heidelberg, Germany) anti-NIDODEN (Clone ELM1, Millipore, Darmstadt, Germany), anti-NEPHRIN (GP-N2, Progen, Heidelberg, Germany), anti-ZO-1 (Clone ZO1-1A12, Life Technologies, Karlsruhe, Germany), anti-β-Actin (Clone AC-15, Sigma, Schnelldorf, Germany), anit-CD2AP (generous gift by Andrey Shaw, Washington University, St. Louis, USA), FITC-LTG and Texas-Red-DBA (Vectorlabs, California, USA), Hoe33342 and respective Alexa-Fluor 2nd antibodies (all Life Technologies, Karlsruhe, Germany).

In-Situ Hybridization

A mouse kidney cDNA library served to clone a fragment of the coding sequence of mouse Cxadr. The following primers were used: ISHcxadrMluF CGCGGGACGCGTACCAGGG ACCACTGGACATT and ISHcxadrNotB1: CGCGGGGCCCCGCGCAGTCAAAG TCTTCA, yielding a 843 bp PCR product. PCR fragments were inserted into pBluescript SK+ vector (Invitrogen, Carlsbad, CA, USA) using Not I and Mlu I restriction sites. pBluescript SK+ vector was linearized and digoxigenin-(DIG)-labeled antisense riboprobes were generated using T7-RNA-polymerase (Ambion, Karlsruhe, Germany). For paraffin ISH sections, slides were progressively rehydrated and permeabilized with proteinase K for 3 min. After prehybridization (60 min), hybridization with DIG-UTP probes took place overnight in standard saline citrate (SSC; pH 4.5; containing 50% formamide) at 65–70°C. Specimens were then incubated with alkaline phosphatase-conjugated anti-DIG Fab fragments (Roche, Mannheim, Germany) at a dilution of 1:4,000 overnight at 4°C. Alkaline phosphatase was detected using chromogenic conversion of NBT/BCIP (Roche, Mannheim, Germany). To avoid drying up of the slides during hybridization, we placed them in a humidity chamber containing 5×SSC and 40% formamide. Slides were then progressively dehydrated, washed in xylol, and mounted.

Statistics

Data are expressed as mean ± SEM. Statistical comparisons were performed using the GraphPad Prism Software Package (Ver.6, GraphPad Software, La Jolla, California, USA) with two-tailed Student’s t-test or ANOVA including respective corrections where indicated. Differences with p values below 0.05 were considered significant.
Results

Cxadr is highly expressed during kidney development.

Cxadr expression was analyzed using in-situ hybridization. Beside a predominant CNS signal, Cxadr was strongly expressed in several epithelial tissues including gut, lung and kidney (Fig 1A, 1B, 1C and 1D). Within the kidney Cxadr was localized to glomerular podocytes and epithelial cells of several tubular segments including the proximal tubule and the thick ascending limb of Henle (Fig 1E and 1F +S1 Fig). Western blot analysis of 6 week old animals confirmed protein expression in heart, kidney, lung, brain and isolated murine primary podocytes (Fig 1G). P1 kidneys were used to analyze the Cxadr expression during glomerular maturation (Fig 1H, 1I, 1J and 1K): In the S-shaped body stage CXADR was first expressed in epithelial cells committed to become parietal epithelial cells and podocytes, but not in cells destined to become proximal tubular cells (Fig 1H). Podocytes and parietal epithelial cells continued to express CXADR while mesangial and endothelial cells never showed a detectable signal (Fig 1J and 1K + S1 Fig). From the early capillary loop stage to mature glomeruli CXADR co-localized with the SD protein NEPHRIN (Fig 1L and 1M). In addition, a strong linear signal of CXADR along Bowman’s capsule, representing CXADR expression in parietal epithelial cells, was observed (Fig 1L”, white asterisks). Interestingly, the expression of CXADR in podocytes vanished by 6 weeks of age, while parietal epithelial cell expression remained constantly high (S1C–S1E Fig).

Podocyte specific knock-out of Cxadr does not impair glomerular development.

We generated podocyte specific Cxadr deficient animals, using the established hNPHS2Cre system (Fig 2A). Western blot of glomerular lysates and immunofluorescence staining confirmed CXADR expression in wild-type animals, which was up-regulated upon NTS treatment and absent in podocyte specific Cxadr deficient mice (Fig 2B, 2C and 2D). The specificity of our conditional approach was demonstrated by the maintained robust parietal epithelial cell expression of CXADR in both genotypes. (Fig 2D”—white arrows). Using immunofluorescence we evaluated the abundance and distribution of the slit diaphragm proteins NEPHRIN and PODOCIN as well as of the tight junctional protein ZO-1. At all three developmental stages examined—capillary loop stage, early and mature glomerulus glomerulus—we neither found any decisive difference in staining intensity nor any difference in protein distribution within podocytes of control and podocyte–specific Cxadr knock-out animals (Fig 2E–2J, S2A–S2F Fig). We therefore conclude that absence of CXADR does not lead to any major detectable changes in NEPHRIN, PODOCIN and ZO-1, at least with our methods used.Albumin excretion between genotypes was only slightly significantly different at P2 but not on all other five tested time points (Fig 3A, n = 5–10 per time point and group). Similarly, light microscopy at week 3, as well as SEM and TEM did not show any obvious histological or ultrastructural abnormalities (Fig 2B–2E).
Adriamycin enhances podocyte specific CXADR expression, but lack of CXADR does not influence the course of Adriamycin induced FSGS.

Next we exposed control and podocyte deficient animals to adriamycin. This anthracycline antibiotic is known to cause proteinuria and a FSGS like phenotype when administered to mice on a sensitive genetic background (e.g. ICR, balb/c), while it does not cause an overt podocyte phenotype in resistant mouse strains (i.e. C57Bl6/NCrl) [26]. Hence, depending on the genetic background the adriamycin stress model allows to test for either sensitization or protection in the context of the respective gene knockout. Using immunofluorescence and western blot we...
CXADR and Podocyte

CXADR

NEPHRIN

Merge

CXADR

Detail

control

adriamycin

CXADR

NEPHRIN

Merge

CXADR

Detail

C3BL/6

adriamycin

ICR

backcross

C

D

E

F

G

H

I

J

K

CXadr WT

Cxadr flox/flox

*HmPHS2Cre

6 weeks

urine measurement

control

adriamycin

CXADR

β-ACTIN

43 kDa

40 kDa

OD - arbit. units

control

adriamycin

ACR (albumin to creatinine)

J

K

adriamycin injections

0 3 5 weeks

0 1 2 4 5 weeks

0.6 0.4 0.2 0.0

25 20 15 10 5

n.s. n.s. n.s. n.s. n.s.
were able to demonstrate that adriamycin upregulates CXADR in control podocytes (Fig 4A, 4B, 4D and 4E). Inversely to CXADR expression, the expression level of the slit diaphragm molecules NEPHRIN and PODOCIN was greatly reduced in both genotypes (Fig 4F and 4G–staining was performed on ICR backcrossed animals of both respective genotypes). This was in contrast to ZO-1 which showed virtually constant expression levels and equal distribution between health and disease in all mice (Fig 4H and 4I). However, despite profound up-regulation of CXADR on both genetic backgrounds we could not identify any clinical functional difference between genotypes over the course of five weeks (Fig 4C, 4J and 4K; C57Bl6/NCrl background: control group n = 3, Cxadr fl/fl/ChNphs2Cre n = 4; ICR background: control group n = 6, Cxadr fl/fl/ChNphs2Cre n = 7).

Nephrotoxic serum (NTS) enhances podocyte specific CXADR expression, but lack of CXADR does not influence the course of NTS induced disease.

Subsequently we examined whether CXADR up-regulation is limited to toxic nephrotic states or whether this also applies to genetic or immunological conditions. Indeed, using an established genetic model of nephrotic syndrome (Cd2ap knockout animals) and sheep derived nephrotoxic serum (NTS), we could demonstrate that in both conditions tested, CXADR was strikingly up-regulated (Fig 5A–5C, 5E and 5F + S3A and S3B Fig). We therefore treated wild-type and Cxadr podocyte deficient animals with NTS, but again despite marked proteinuria within 4 days after administration of NTS, we could not detect any difference between respective genotypes (Fig 4D, 4G, 4H and 4I; control group n = 6, Cxadr fl/fl/ChNphs2Cre n = 6).

Discussion
Podocyte function and maintenance is regulated by precisely orchestrated cell-cell contacts. The discovery of the molecular composition of the SD has profoundly changed our understanding of glomerular filtration and function. However, the dynamic changes, molecular make up and intermolecular interactions at the SD remain incompletely understood. Here we identify the IgSF member CXADR to be specifically regulated and expressed in developing and injured mammalian podocytes. CXADR shows an early and specific expression in precursors of podocytes and parietal epithelial cells, starting in the S-shape body phase during glomerular development. Yet, while podocyte specific expression vanishes over the first six weeks of life, expression in parietal epithelial cells stays prominent through-out life. Given the potential role of parietal epithelial cells for podocyte regeneration and repair, this might indicate that Cxadr is a marker of immature podocytes which is lost during podocyte differentiation [27]. This loss of CXADR is actually paralleled by the formation of the slit-diaphragm out of a tight-junction based precursor [1]. Developmental reduction of Cxadr expression levels seems to be a common theme and has been described for brain and heart before [28, 29].
Interestingly, and in contrast to previously published results in the zebrafish pronephros [9], conditional podocyte specific knock-out in mice does not lead to a developmental phenotype. Obviously, it is known from previous studies that the hNPHS2 promotor is only active from approximately embryonic day E 14.0 onwards when glomerular development proceeds from the S-shaped body towards the early capillary loop stage [30]. As mentioned, CXADR is already highly expressed in the S shaped stage and hence early essential roles might be missed by a relatively late recombination event. Yet, hNPHS2-Cre mediated knock-out seems efficient with regard to CXADR protein reduction, as we could not detect any protein anymore in late capillary loop stages of glomerular development in respective knock-out animals.

As shown with different types of toxic, genetic and immunological glomerular injury models, Cxadr expression is robustly upregulated during podocyte injury. It could therefore be used as a podocyte stress marker, similarly to down regulation of SESTRIN in parietal epithelial cells [31, 32]. Interestingly, upregulation of CXADR during disease has been previously described in the heart under various conditions i.e. autoimmune inflammation, infarction and dilated cardiomyopathy [29, 33, 34]. A potential functional role of CXADR up regulation in response to glomerular injury is unfortunately not being revealed by our data. In fact, our analysis indicates that presence or absence of podocyte CXADR does not change the course of injury or repair, at least within the models and time lines evaluated in this study. In addition expression and distribution of other decisive slit diaphragm and tight junctional molecules was not changed. This in our opinion at least allows two conclusions: 1. There seems to be a redundancy with regard to Super Ig cell adhesion molecules possessing only 2 extracellular IgG domains and other members expressed in podocytes could potentially take over CXADR’s function [24, 35]. 2. ZO-1 based tight junctions forming during podocyte injury do not rely on additional CXADR expression and form stable cell-cell contacts themselves.

As CXADR expression within the glomerulum is much more prominent in parietal epithelial cells, future studies might be warranted to address the role of CXADR in these specialized epithelial cells of Bowman’s Capsule. This not only applies to their unchallenged physiologic state but also to any changes which occur in glomerular diseases such as rapid progressive glomerulonephritis, where proliferation and crescent formation of parietal epithelial cells are distinguished hallmarks.

Supporting Information

S1 ARRIVE Checklist. Supplemental Information ARRIVE. A completed ARRIVE checklist concerning all animal experiments in this manuscript is given.

S1 Fig. Non-podocyte renal expression of CXADR. (A&B) CXADR was expressed in proximal as well as distal parts of the renal tubular system, as demonstrated by co-labeling with either LTG or DBA-lectins. Here CXADR localizes clearly to cell-cell contacts of renal tubular epithelial cells. (C-E) Immunofluorescence at different developmental time points demonstrated a reduction of podocyte CXADR expression, whereas a strong signal was still present in

Fig 5. Nephrotoxic serum (NTS) enhances podocyte specific CXADR expression, but lack of CXADR does not influence the course of NTS induced disease. (A-C) On both d4 and d5 after NTS injection CXADR was increased in glomerular podocytes as shown in co-labeling experiments with NEPHRIN (control: white arrows—parietal epithelial cells, NTS: arrow heads—podocytes). As can be easily depicted from the NEPHRIN specific panel (A”, B”, C”) NEPHRIN abundance is greatly reduced during the course of the disease, underlining the severity of the chosen stress model. (D) Schematic of the injection scheme and follow-up using urine collections. (E&F) Western blot using decapsulated glomerular lysates was used to quantify CXADR expression which was increased two fold 5 days after NTS injection. (G&H) As shown with immunoflourescence stainings CXADR induction was absent in podocyte specific Cxadr knock-out animals. (I) Proteinuria developed similarly in wild-type and knock-out animals showing no functional differences.

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parietal epithelial cells of adult animals.

(TIF)

**S2 Fig. Developmental expression of the bona fide slit diaphragm proteins NEPHRIN and PODOCIN in control and podocyte specific Cxadr-/- mice.** Assessment of NEPHRIN and PODOCIN expression at different developmental stages—capillary loop, early glomerulus, mature glomerulus—in control (A-A”, C-C”, E-E”) and podocyte specific Cxadr-/- animals (B-B”, D-D”, F-F”) was performed using immunofluorescence. Loss of CXADR does not lead to changes in abundance and distribution of neither NEPHRIN nor PODOCIN.

(TIF)

**S3 Fig. CXADR is also upregulated in podocytes in the Cd2ap-/- model of genetic glomerular disease.** (A&B) CD2AP knockout animals exhibited a clear upregulation of CXADR expression in podocytes as demonstrated by immunofluorescence staining of CXADR and respective co-labeling with NEPHRIN.

(TIF)

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**Author Contributions**

Conceived and designed the experiments: CS TBH FG. Performed the experiments: CS OK AB MR MH FG. Analyzed the data: CS FG TBH. Contributed reagents/materials/analysis tools: UL MG PLT. Wrote the paper: CS TBH FG.

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