The Proteoglycan Syndecan 4 Regulates Transient Receptor Potential Canonical 6 Channels via RhoA/Rho-associated Protein Kinase Signaling

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Objective—Syndecan 4 (Sdc4) modulates signal transduction and regulates activity of protein channels. Sdc4 is essential for the regulation of cellular permeability. We hypothesized that Sdc4 may regulate transient receptor potential canonical 6 (TRPC6) channels, a determinant of glomerular permeability, in a RhoA/Rho-associated protein kinase-dependent manner.

Methods and Results—Sdc4 knockout (Sdc4−/−) mice showed increased glomerular filtration rate and ameliorated albuminuria under baseline conditions and after bovine serum albumin overload (each P<0.05). Using reverse transcription–polymerase chain reaction and immunoblotting, Sdc4−/− mice showed reduced TRPC6 mRNA by 79% and TRPC6 protein by 82% (each P<0.05). Sdc4−/− mice showed an increased RhoA activity by 87% and increased phosphorylation of ezrin in glomeruli by 48% (each P<0.05). Sdc4 knockout in cultured podocytes reduced TRPC6 gene expression and reduced the association of TRPC6 with plasma membrane and TRPC6-mediated calcium influx and currents. Sdc4 knockout inactivated negative regulatory protein Rho GTPase activating protein by 33%, accompanied by a 41% increase in RhoA activity and increased phosphorylation of ezrin (P<0.05). Conversely, overexpression of Sdc4 reduced RhoA activity and increased TRPC6 protein and TRPC6-mediated calcium influx and currents.

Conclusion—Our results establish a previously unknown function of Sdc4 for regulation of TRPC6 channels and support the role of Sdc4 for the regulation of glomerular permeability. (Arterioscler Thromb Vasc Biol. 2012;32:378-385.)

Key Words: receptors ■ signal transduction

Syndecan 4 (Sdc4), a member of the type I transmembrane heparan sulfate proteoglycan superfamily, is a major modulator of signal transduction and regulates localization and activity of proteins and channels.1–3 Sdc4 consists of an extracellular N-terminal domain with several heparan sulfate side chains, a single hydrophobic transmembrane domain, and a short C-terminal cytoplasmic domain. Several unique Sdc4 functions have been described, including binding of growth factors, modulation of the RhoA activity, modulation of the activity of ezrin (which cross-links the plasma membrane with actin cytoskeleton), and finally actin cytoskeleton organization.1–3 Recent reports implicate changes in Sdc4 with kidney diseases.4–6 Sdc4 is upregulated 26 times in mice with proteinuric kidney disease. Sdc4 transcript and protein levels are greatly elevated in glomerular disease.4–6 The question arises whether Sdc4 regulates major functions in podocytes, which form a crucial component of the glomerular filtration barrier. Podocytes are specialized cells in kidney glomerulus that cover the urinary surface of the filtering capillaries, normally preventing protein leakage into the urinary space.7 Recently, transient receptor potential canonical 6 (TRPC6) channels in podocytes have been recognized to regulate the glomerular filtration barrier and serve as an important determinant of glomerular permeability.8–12 Patients and mice with proteinuric kidney dis-
ease show an increased expression of native TRPC6 in podocytes.11,12 Thus, we reasoned that Sdc4 may regulate TRPC6 in podocytes and that this mechanism could be the underlying cause of prevalent proteinuric kidney diseases related to disturbed TRPC6 expression. In the present study, we show that Sdc4 regulates glomerular permeability in mice and major functions in podocytes by affecting RhoA/Rho-associated protein kinase activity and TRPC6 gene expression and function.

Materials and Methods

An extended Material and Methods section can be found in the supplemental material, available online at http://atvb.ahajournals.org.

Conditionally immortalized mouse podocytes (podocyte cell line E11), human endothelial cell line EA.hy926, human umbilical vein endothelial cells, and human embryonic kidney cells (HEK293) were cultured as described.13,14 Small interfering RNA (siRNA) knockdown of Sdc4 or TRPC6, isolation of RNA and cDNA synthesis, quantitative real-time reverse transcription–polymerase chain reaction, overexpression of Sdc4 in podocytes, immunoblotting of proteins and coimmunoprecipitation, quantitative in-cell Western assays of proteins, immunofluorescence, isolation of glomeruli, and electron microscopy were performed using standard techniques.15–17 The visualization of green fluorescent protein (GFP)– or yellow fluorescent protein (YFP)-tagged-TRPC6 in transfected cells, intracellular calcium measurements, and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry of isolated proteins have been described previously.18–22 Rho GTPase activating protein (RhoGAP), RhoA activity, podocyte membrane protein biotinylation and isolation, patch clamp measurements, and permeability assay were performed as described.23–25 Creation of Sdc4 <sup>−/−</sup> mice has been reported previously,26 and the Animal Use Committee for the Hannover Medical School (Niedersaechsisches Landesamt für Verbraucherschutz und Lebensmittelsicherheit, approval number 33.9-1E). Sdc4 <sup>−/−</sup> mice showed increased RhoA activity (F) (n=5; *P<0.05) and phosphorylation of ezrin (pEzrin), indicated by immunoblotting (G).

Figure 1. Characteristics of wild-type (syndecan 4 [Sdc4] <sup>+/+</sup>) and Sdc4 <sup>−/−</sup> mice. Both proteins (A and B) (Western blotting, n=3; *P<0.05) and transcripts (C) (quantitative reverse transcription–polymerase chain reaction, n=5; **P<0.01) of transient receptor potential canonical 6 (TRPC6) were reduced in glomeruli of Sdc4 <sup>−/−</sup> mice. Sdc4 <sup>−/−</sup> mice showed reduced urinary albumin excretion under baseline conditions (D) (n=9–10; *P<0.05) and after bovine serum albumin overload compared with Sdc4 <sup>+/+</sup> littermates (E) (n=4–5; *P<0.05). Sdc4 <sup>−/−</sup> mice showed increased RhoA activity (F) (n=5; *P<0.05) and phosphorylation of ezrin (pEzrin), indicated by immunoblotting (G).

Results

Sdc4<sup>−/−</sup> Mice Show Reduced TRPC6 Transcripts, Proteins, and Urinary Albumin Excretion

In the present study, we compared TRPC6 expression in glomeruli of Sdc4<sup>−/−</sup> mice<sup>26</sup> and wild-type (Sdc4<sup>+++</sup>) littermates. Our immunofluorescent results showed the expression of TRPC6 protein in glomerular podocytes, as evidenced by colocalization with podocyte-specific nephrin. TRPC6 protein expression (green) was reduced in Sdc4<sup>−/−</sup> compared with Sdc4<sup>+++</sup> mice (Supplemental Figure IA). Immunoblotting confirmed a significant reduction of TRPC6 protein by 82% in renal cortex of Sdc4<sup>−/−</sup> vs Sdc4<sup>+++</sup> mice. Notably, the protein expression of TRPC3, podocin, and nephrin did not differ between groups (Figure 1A and 1B). We also observed a significant 79% (P<0.05) reduction in TRPC6 mRNA, whereas the TRPC3 transcript was unchanged in Sdc4<sup>−/−</sup> mice (Figure 1C), providing further evidence for selective regulation of TRPC6 by Sdc4. Most importantly, under baseline conditions Sdc4<sup>−/−</sup> mice displayed reduced urinary albumin excretion (6.7±0.9 vs 3.9±0.4 g albumin/mol creatinine; P<0.05; Figure 1D). Furthermore, an increased albuminuria after bovine serum albumin overload was ameliorated in Sdc4<sup>−/−</sup> mice. Bovine serum albumin overload enhanced urinary albumin excretion 2.5±0.4-fold in Sdc4<sup>+++</sup> mice, whereas it enhanced urinary albumin excretion 13.4±5.2-fold in Sdc4<sup>++++</sup> littermates (P<0.05; Figure 1E). Sdc4<sup>−/−</sup> showed increased glomerular filtration rate, indicating healthy kidney function. Glomerular filtration rate was higher in Sdc4<sup>−/−</sup> compared with Sdc4<sup>++++</sup> littermates (164±12 µL/min vs 101±2 µL/min; n=5 each, P<0.05). As expected, Sdc4 mRNA was absent in the renal cortex of Sdc4<sup>−/−</sup> mice whereas Sdc3, Sdc2, and Sdc1 transcripts were

Statistical Analysis

Data are expressed as the mean±SEM. Comparisons between groups were analyzed using t test or ANOVA and Bonferroni multiple comparison test as appropriate. A 2-tailed probability value less than 0.05 was considered to indicate statistical significance.
Sdc4 Knockdown Selectively Reduces, Whereas Sdc4 Overexpression Increases TRPC6 Transcripts and Proteins Levels in Podocytes

In the podocyte cell line E11, we identified transcripts for TRPC6 and Sdc4 (Supplemental Figure IIA), the expression of TRPC6 and Sdc4 proteins, and the expression of podocyte-specific proteins, including nephrin, podocin, WT1, and synaptopodin. Podocyte-specific proteins were absent in 2 separate endothelial cell types, which were used as controls (Supplemental Figure IIB). Hence, we and others confirmed their unique cellular properties which characterize podocyte phenotype.13,20 Mass spectrometry verified that the detected proteins were TRPC6 (Mascot score 183) and Sdc4 (Mascot score 84; Supplemental Figure IIC). Administration of siRNA against Sdc4 reduced Sdc4 mRNA abundance by 51% (P<0.05) but did not affect Sdc3, Sdc2, or Sdc1 mRNA expression (Figure 2A). Knockdown of Sdc4 in vitro reduced TRPC6 mRNA by 25% (P<0.05), whereas TRPC3 was not affected (Figure 2B). In agreement with our results obtained in Sdc4−/− mice, knockdown of Sdc4 in cultured podocytes selectively reduced TRPC6 protein expression by 30% (P<0.05) but did not affect TRPC3 protein, as assessed by immunoblotting (Figure 2C), immunofluorescence assays of green fluorescent protein (GFP)-tagged TRPC6 (Figure 2D), and a quantitative in-cell Western assay (Supplemental Figure III). Control scrambled siRNA showed no effects on TRPC6 protein expression (101±2% of control). We observed a selectively increased TRPC6 protein expression in podocytes after transfection with Sdc4 full-length construct (Sdc4FL) but not with transfection with an Sdc4 construct lacking the cytoplasmic domain (Sdc4CY) (Supplemental Figure III). TRPC6 protein expression was enhanced by 40% (P<0.01) after Sdc4FL, whereas TRPC3 protein was not affected. Sdc4 protein was not different between the 2 groups (Supplemental Figure IB).

Sdc4−/− Mice Show Increased RhoA/ROCK Activity in Kidney Cortex

Sdc4 reportedly reduces RhoA activity via activation of p190RhoGAP-A, which is a negative regulator of active Rho GTPase activity.27,28 Consistent with these findings, Sdc4−/− mice showed an increase, 87% on the average (P<0.05), of RhoA activity in the renal cortex (Figure 1F). RhoA is known to activate the cytoskeleton-related protein ezrin by phosphorylation.29 In this regard, immunoblottings derived from freshly isolated glomeruli showed that phosphorylated ezrin was significantly increased by 48% in Sdc4−/− mice compared with Sdc4+/− littermates (Figure 1G). Taken together, Sdc4−/− mice were characterized by selectively reduced TRPC6 expression, reduced albuminuria, and increased RhoA/ROCK activity. Eckel et al recently reported that reduced TRPC6 ameliorates proteinuria in mice.30 Our present results give much experimental evidence that Sdc4 affects RhoA/ROCK signaling, which controls TRPC6 expression. This was further confirmed by our in vitro experiments using Sdc4 knockdown and overexpression in glomerular podocytes.
increased by 56% (P < 0.01) in podocytes after Sdc4FL expression. Thus, our results demonstrate that Sdc4 is a specific regulator of TRPC6, but not the other family member TRPC3, in podocytes.

**Sdc4 Knockdown Reduces, Whereas Sdc4 Overexpression Increases, TRPC6-Mediated Cation Influx and Currents in Podocytes**

TRPC6-mediated calcium influx into fluo-4-loaded podocytes was measured using confocal laser scanning microscopy. Calcium influx was induced by the known TRPC6 agonist flufenamic acid (FFA).31,32 siRNA against Sdc4 reduced the FFA-induced peak calcium influx by 58% (P < 0.01). In addition, increased TRPC6 channel density after transfection with Sdc4FL enhanced calcium influx by 111% (P < 0.01; Figure 3A). 1-Oleoyl-2-acetyl-sn-glycerol, a diacylglycerol analog, triggered calcium influx was reduced by 49% (P < 0.05) after Sdc4 knockdown. This reduction by siRNA was prevented when calcium influx was inhibited by the nonspecific TRPC blockers 2-aminoethoxydiphenylborane or SKF-96365, providing further evidence for the involvement of TRPC6 channels. As shown in Figure 3B to 3E, application of FFA caused increased cation currents showing a characteristic doubly rectifying the current-voltage relationship of TRPC6 channels in podocytes.33 Moreover, they could be blocked by 1 mmol/L gadolinium. Normalization of currents is shown in Figure 3E. siRNA against Sdc4 reduced the FFA-induced cation currents at 100 mV by 49% (P < 0.05). Figure 3F shows that 1-oleoyl-2-acetyl-sn-glycerol, a diacylglycerol analog, can trigger the TRPC-mediated currents in podocytes. These data indicate that Sdc4 knockdown reduces, whereas Sdc4 overexpression increases, TRPC6 channels and cation flux in podocytes.

**Sdc4 Regulates RhoA/ROCK Activity in Podocytes**

Next, we investigated the possibility of direct interactions between endogenous TRPC6 or GFP-tagged TRPC6 channel proteins and slit diaphragm proteins. In accordance with previous reports, in cultured podocytes, we observed coimmunoprecipitation of TRPC6, as well as GFP-tagged TRPC6, with the slit diaphragm protein nephrin.33 On the other hand, coimmunoprecipitation revealed that Sdc4 is not a glomerular slit diaphragm-associated protein (Figure 4A and 4B) but is associated with the cytoskeleton protein ezrin (Figure 4C). Previous studies showed that Sdc4 regulates RhoA activity via RhoGAP, which is a negative regulator of active RhoA.27 In accordance with these observations, knockdown of Sdc4 using siRNA against Sdc4 decreased the RhoGAP activity by 33% (P < 0.05), accompanied by a 41% (P < 0.01) increase in RhoA activity. In contrast, overexpression of Sdc4 using Sdc4FL, but not Sdc4CY, increased the RhoGAP activity by 30% (P < 0.05), accompanied by a 47% (P < 0.01) reduction in RhoA activity (Figure 4D and 4E). The effects of Sdc4 on RhoA activity after Sdc4 stimulation by fibronectin are summarized in Figure 4F. As shown in Figure 4G, administration of fibronectin reduced phosphorylation of ezrin by 50% (P < 0.01). siRNA against Sdc4 abolished the inhibitory effect of fibronectin. In contrast, overexpression of Sdc4 using Sdc4FL, but not Sdc4CY, augmented the inhibitory effects of fibronectin on ezrin phosphorylation (P < 0.01).
Sdc4 Facilitates the Insertion of TRPC6 Channels Into the Plasma Membrane of Podocytes via the RhoA Pathway

Using GFP-tagged TRPC6 and confocal laser scanning microscopy, we observed that TRPC6 is localized mainly to the plasma membrane of cultured podocytes (Supplemental Figure IVA and IVB). Knockdown of Sdc4 reduced TRPC6 protein found in the plasma membrane, whereas Sdc4FL, but not Sdc4CY, significantly increased the density of TRPC6 protein present in the plasma membrane (Figure 5A). As indicated above, in mice, Sdc4 modulated RhoA activity. Sdc4−/− mice showed increased RhoA activity and reduced TRPC6. Next, we established that Rho kinase agonist calpeptin reduced TRPC6 protein localized in the plasma membrane, whereas Rho-kinase inhibitors, Y27632, and fasudil increased it. We transiently transfected podocytes with YFP-tagged TRPC6 DNA and observed that calpeptin decreased podocyte monolayer permeability (Figure 5D) (n=3 each; P<0.05).

Figure 4. Syndecan 4 (Sdc4) is not attached to the slit membrane but interacts via RhoA pathway in podocytes. Coimmunoprecipitation showing that endogenous transient receptor potential canonical 6 (TRPC6) (A) and green fluorescent protein (GFP)-tagged TRPC6 (B) are associated with slit diaphragm protein nephrin but not with Sdc4. C, Coimmunoprecipitation showing that Sdc4 is associated with cytoskeleton protein ezrin. IP indicates primary antibody used for immunoprecipitation; WB, primary antibody used for Western blotting; eluate, immunoprecipitated proteins; lysate, total cell lysate control. n=3. RhoA activity is shown in podocytes cultured under control conditions, after Sdc4 knockdown, or Sdc4 overexpression. Rho GTPase activating protein (RhoGAP) activity (D) and RhoA activity at baseline (E) and after fibronectin (Fibro) administration (F) were compared between groups. *P<0.05, **P<0.01 compared with control. ##P<0.01 compared with Fibro alone. G, Phosphorylation of ezrin (pEzrin) in podocytes under control conditions, after Sdc4 knockdown or Sdc4 overexpression. Podocytes were stimulated with fibronectin. **P<0.01 compared with control.

Figure 5. Syndecan 4 (Sdc4) enhances the density of transient receptor potential canonical 6 (TRPC6) channels in the plasma membrane of podocytes via the RhoA-dependent pathway. A, Immunofluorescence showing that Sdc4 knockdown reduced, whereas Sdc4 overexpression increased, TRPC6 protein localized in the plasma membrane of podocytes. Sdc4FL indicates Sdc4 full-length construct; Sdc4CY, Sdc4 construct lacking cytoplasmic domain. Nephrin is shown for comparison. Scale bar = 5 μm. B, Rho kinase agonist calpeptin reduced TRPC6 protein localized in the plasma membrane, whereas Rho-kinase inhibitors, Y27632, and fasudil increased it. C, Biotinylation assay demonstrating that activation of Rho kinase by calpeptin reduced, whereas inhibition by Y27632 increased, plasma membrane-associated TRPC6 protein (n=5; **P<0.01). Transient transfection of podocytes with YFP-tagged TRPC6 plasmid DNA increased podocyte monolayer permeability (D) (n=3 each; P<0.05).
brane, thereby mimicking Sdc4 knockout. By contrast, 2 structurally independent Rho-kinase inhibitors, Y27632 and fasudil, significantly increased TRPC6 protein targeting to the plasma membrane of podocytes, mimicking Sdc4 over-expression (Figure 5B). These findings were supported by biotinylation assays (Figure 5C). Immunoblotting demonstrated that the Rho kinase agonist calpeptin reduced plasma membrane-associated TRPC6 protein by 55% \(P<0.05\), whereas the Rho-kinase inhibitor Y27632 increased plasma membrane-associated TRPC6 protein by 78% \(P<0.05\). Moreover, transient transfection of podocytes with YFP-tagged TRPC6 plasmid DNA significantly increased podocyte monolayer permeability by 47% compared with controls (transfected podocytes, 2.5\pm0.1; controls, 1.7\pm0.0; \(n=3\) each; \(P<0.05\); Figure 5D). Identical results showing that Sdc4 enhances density of TRPC6 channels in the plasma membrane by a RhoA-dependent pathway were observed in HEK cells transfected with YFP-tagged TRPC6 protein (Supplemental Figure IVC and IVD). In support of these results, insertion of other ion channels by the RhoA pathway has been reported previously.\(^{34}\)

### Discussion

We identified a major role of Sdc4 in glomeruli. In particular, we observed that Sdc4 regulates glomerular permeability and podocytes’ functions by affecting RhoA/ROCK activity and TRPC6 gene expression. Recent studies implicate that Sdc4 could participate in kidney diseases.\(^{4, 6}\) Furthermore, increased expression of TRPC6 in the podocyte slit diaphragm and gain-of-function mutations in TRPC6 have been identified to cause podocyte injury and human kidney disease.\(^{5, 7, 8}\)

It still remains unknown whether Sdc4 is responsible for the regulation of glomerular filtration. Now, we provide experimental evidence that Sdc4 regulates glomerular permeability by affecting TRPC6 expression in podocytes. We found that (1) Sdc4\(^{-/-}\) mice showed reduced TRPC6 mRNA, TRPC6 channel protein, and albuminuria, both under baseline conditions and after bovine serum albumin overload, and increased RhoA/ROCK activity in kidney cortex compared with wild-type littermates; (2) Sdc4 knockout in podocytes decreased TRPC6 transcripts, protein abundance, and TRPC6-mediated cation influx; and (3) Sdc4 knockout increased baseline podocyte RhoA activity and phosphorylation of the cytoskeleton-related protein ezrin, thereby reducing the association of TRPC6 channel proteins with plasma membrane. By contrast, overexpression of functionally intact Sdc4 reversed these effects. Experiments on TRPC6\(^{-/-}\) mice revealed that lack of TRPC6 expression cannot be functionally replaced by TRPC3 in vivo.\(^{35}\) Our results demonstrate a specific Sdc4-TRPC6 interaction, which underscores TRPC subtype-specific characteristics of TRPC6 compared with TRPC3. First, TRPC6 channels carry 2 extracellular N-linked glycosylation sites, whereas TRPC3 channel is a monoglycosylated protein.\(^{36}\) Second, TRPC6 and TRPC3 show different electrophysiological characteristics. TRPC6 is a tightly receptor-regulated store-independent cation channel, whereas TRPC3 displays considerable basal activity.\(^{37}\) Third, FFA seems to be a specific activator of TRPC6 but not other TRPC channel proteins.\(^{31, 32}\) In line with this, we found an increased FFA-induced calcium influx after overexpression of Sdc4FL and consecutively increased TRPC6 channel protein expression. Similarly, inward currents showing characteristic TRPC6 features\(^{24, 32}\) were increased after overexpression of Sdc4 using the Sdc4FL construct, but not after overexpression using the Sdc4CY construct. This corroborates that Sdc4 is a specific regulator in podocytes mediating plasma membrane association of TRPC6.

The features of Sdc4\(^{-/-}\) mice have been reported previously. Ishiguro et al\(^{18}\) reported similar blood pressure in conscious Sdc4\(^{-/-}\) mice and wild-type littermates, and Patrovian et al\(^2\) showed slightly increased blood pressure in anesthetized animals. Notably, the finding of increased glomerular filtration rate in Sdc4\(^{-/-}\) mice should be interpreted together with our observation that Sdc4\(^{-/-}\) mice showed reduced albuminuria and reduced TRPC6 expression in podocytes. These findings are important for several human kidney diseases where reduced glomerular filtration rate, increased proteinuria and increased TRPC6 expression have been reported.\(^{11}\) Indeed, overexpression of functional TRPC6 is sufficient to cause increased permeability in vitro.

To evaluate the role of Sdc4 for regulation of the TRPC6 gene expression, we studied Sdc4 and TRPC6 in cultured podocytes. Using reverse transcription–polymerase chain reaction and immunoblotting, we identified their unique characteristics, which are indistinguishable from those of native podocytes.\(^{13, 20, 39}\) We found that siRNA against Sdc4 reduced Sdc4 mRNA, TRPC6 mRNA abundance by 51% and TRPC6 by 25% but did not affect Sdc3, Sdc2, Sdc1, TRPC3, nephrin, or podocin mRNA expression. These data rule out the involvement of nonspecific silencing during knockdown of Sdc4 using siRNA. Instead, these results strongly suggest that Sdc4 or Sdc4-dependent signaling affects TRPC6 gene transcription. We used 4 different techniques to further characterize Sdc4-TRPC6 interactions, including immunoblotting, quantitative fluorescence assay of fluorescent tagged TRPC channels, immunofluorescence, and quantitative in-cell Western assay. Together, the results clearly show that Sdc4 selectively affects TRPC6 channel protein expression and membrane association in podocytes.

We further uncovered a mechanistic link between Sdc4 and TRPC6. Small GTPases have been reported to act downstream of syndecans regulating several cellular functions.\(^{40, 41}\) In the present study, we found that overexpression of Sdc4FL reduced baseline RhoA activity in podocytes. We also observed that overexpression of Sdc4FL inhibited RhoA/ROCK activity, as confirmed by reduced phosphorylation of ezrin. Ezrin, which binds to the syndecan cytoplasmic domain, is known to link RhoA/ROCK activity with actin cytoskeleton rearrangements.\(^{39}\) To the best of our knowledge the effects of Sdc4 on RhoA activity have not been reported in podocytes. However in fibroblasts, Sdc4 showed variable effects dependent on cell culture conditions, cooperative interactions between integrins and syndecans with the extracellular matrix, and exposure time to different Sdc4-binding substrates, including fibronectin. In accordance with our data in podocytes, Bass et al showed that Sdc4 reduces RhoA activity in fibroblasts via activation of p190RhoGAP-A, which is a negative regulator of active Rho GTPase.\(^{27}\) However, in rat
embryo fibroblasts, other groups reported conflicting results, showing that fibronectin may activate RhoA in a Sdc4-dependent manner.42

Because the effects of Sdc4 on RhoA activity may be affected by culture conditions, it should be noted that in the present study podocytes were seeded on plates without precoated fibronectin, thereby avoiding premature Sdc4 activation. Moreover, exposure time may be important. Hence, in the RhoA activity assay, podocytes were treated with fibronectin for less than 30 minutes. In accordance with our findings, Ren et al showed that short-term exposure inhibits RhoA activity in Swiss 3T3 cells.43

Rho-GTPases have a role in cytoskeletal rearrangement, but they also regulate vesicular trafficking.40,41 Previous studies indicate that the RhoGAP, a negative regulator of RhoA, promotes fusion of intracellular vesicles to the plasma membrane.44 TRPC6 channels are localized in the plasma membrane and in caveolae-related microdomain vesicles subjacent to the plasma membrane.45 Because Sdc4 affects RhoA/ROCK activity via RhoGAP, we further investigated whether Sdc4/RhoGAP/RhoA/ROCK may affect TRPC6 channels in cultured podocytes. Using confocal laser scanning microscopy and biotinylation assays, we observed that the activation of RhoA/ROCK signaling, via inactivation of RhoGAP by Sdc4 knockdown or via treatment with RhoA/ROCK activator, triggered the translocation of TRPC6 channels from the plasma membrane into the cytoplasm. On the other hand, the inhibition of RhoA/ROCK signaling, via activation of RhoGAP by overexpression of functional Sdc4 or via treatment with RhoA/ROCK inhibitors, increased the plasma membrane pool of TRPC6 channels. Our results established a previously unknown function of Sdc4 for regulation of TRPC6 channels and supported a major role of Sdc4 for the regulation of glomerular permeability. The wide distribution of Sdc4 and TRPC6 channels in vasculature and our confirmation of these events in HEK293 cells strongly suggest that the regulation of TRPC6 channels by proteoglycan Sdc4 via RhoA/ROCK signaling may be a general process in vascular biology. The current studies suggest a direct casual link between Sdc4 and TRPC6 in podocytes. However, we cannot rule out the involvement of other glomerular components. A podocyte-specific knockout of Sdc4 may be needed to confirm the current findings.

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Disclosures

None.

References


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The Proteoglycan Syndecan 4 Regulates Transient Receptor Potential Canonical 6 Channels via RhoA/Rho-associated Protein Kinase Signaling: Correction

In the article by Liu et al, which appeared in the February issue of the journal (Arterioscler Thromb Vasc Biol. 2012;32:378–385. DOI: 10.1161/ATVBAHA.111.241018, the primary data in Figure 5A and Supplemental Figure IID are the same but have been oriented differently by the authors to give the clearest presentation in both figures. To clarify this, the legend for Supplemental Figure IID has been changed to, “Immunofluorescence showing that Sdc4 knockdown specifically reduces TRPC6 protein expression (lower panels, please note that these panels have been oriented differently compared to Figure 5A), but not TRPC3 expression (upper panels) in podocytes (scale bar=5 μm).”

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Supplemental Material

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The Proteoglycan Syndecan 4 Regulates TRPC6 Channels via RhoA/ROCK Signaling

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Supplement Material

Contains extended Material and Methods, related References, IV Supplemental Figures and Figure Legends.

Materials and Methods

Cell culture

Conditionally immortalized mouse podocytes (podocyte cell line E11) cloned from the outgrowth of glomeruli isolated from H-2kb-tsA58 transgenic mice were purchased from Cell Lines Service (Eppelheim, Germany). The podocytes were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 units/mL penicillin, and 100 µg/mL streptomycin (BIOCHROM AG, Berlin, Germany). Cells were cultivated at 33°C (permissive condition) for propagation and at 38°C (non-permissive condition resulting in the inactivation of the SV40 large T-antigen) for differentiation. Podocytes were seeded on plates without pre-coated collagen or fibronectin and were subject to treatments when cells reached 80% confluence. We determined markers of differentiated in vivo podocytes showing several unique podocyte proteins, including nephrin, podocin, Wilms-Tumor-Protein 1 (WT1), and synaptopodin to verify podocyte integrity as reported for these cells previously.¹ Human
endothelial cell line EA.hy926, human umbilical vein endothelial cells (HUVEC) and human embryonic kidney cells (HEK293) were obtained from German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany) and were cultured at 37°C and 5% CO₂ in Dulbecco’s modified Eagle’s medium containing 10% heat-inactivated fetal calf serum, 100 units/mL penicillin, and 100 µg/mL streptomycin as described previously.⁵²

**siRNA knockdown of Sdc4 or TRPC6**

Podocytes or HEK293 cells were transfected with siRNA specific for Sdc4 or with siRNA specific for TRPC6 using the silencer siRNA transfection kit (Ambion, Cambridgeshire, UK).

The target sequence for Sdc4 in murine podocytes

was 5’-AGAAUGAGGUGCAUCCAAAtt-3’ (sense)

and 5’-UUAGGAAUGACCUCUUCUt-3’ (antisense).

The target sequence for Sdc4 in HEK293 cells

was 5’-CUACUGCUCAUGUACCGuAtt-3’ (sense)

and 5’-UACGGUACAGACGUAGGa-3’ (antisense).

The target sequence for TRPC6 in murine podocytes

was 5’-CAGAAUAGCUUACAUUUuAtt-3’ (sense)

and 5’-UAAAAUAGCUAUAUCUGGa-3’ (antisense).

In control experiments negative control siRNA (Ambion) that has no significant homology to any known human gene sequence did not affect Sdc4 expression.

**Isolation of RNA and cDNA synthesis**
Cells or tissue from mouse kidney cortex were added to 1 mL TRIzol Reagent (Invitrogen) and homogenized for 30 s with an Ultraturrax (IKA Werke, Staufen, Germany). After centrifugation at 10,000 g for 5 min RNA was extracted from the supernatant using the RNeasy Midi Kit (Qiagen, Hilden, Germany). RNA was used to synthesize first-strand cDNA using the Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics, Mannheim, Germany). PCR was performed using reverse transcription mixture consisting of total RNA template, anchored-oligo (dT)$_{18}$ primer, and transcriptor reverse transcriptase and incubated according to the following procedure: denaturation at 65°C for 10 min, followed by 50°C for 60 min, and heating at 85°C for 5 min.

**Quantitative real-time RT-PCR**

Quantitative real-time RT-PCR was performed as described by our group.$^{52-54}$ The primers were as follows:

TRPC6 (NM_013838.2)

forward CAGTGCTTGCCAACATTGAG, 
reverse TGCTGACAGTTTGGATGAGC;

TRPC3 (NM_019510.2)

forward AGCCGAGCCCTGGGAAAGACAC, 
reverse CCGATGGCGAGGAATGGAAGAC;

genphrin (NM_019459.2)

forward AGGGTCCGGGAGGAGATCGAA, 
reverse GGGAAGCTGGGGACTGAAGT;
podocin (NM_130456.3)
forward TGAGGATGGCGGCTGAGAT,
reverse GGTTTGGAGGAACTTGGGT;
Sdc1 (NM_011519.2)
forward CGCTCGAGAGAGCAGCGAGC,
reverse GCTCTGGAGCTGTGGGCGTG;
Sdc2 (NM_008304.2)
forward GAGGACCCAGGAGGAGGCGG,
reverse ACTCCGGTGTCTGGGTCGCA;
Sdc3 (NM_011520.3)
forward GGCCAAGCCGTCACCTCCAC,
reverse CCGGGCTGGAGGGAATGGGA;
Sdc4 (NM_011521.2)
forward AACCACATCCCTGAGAATGC,
reverse AGGAAAACGGCAAAGAGGAT;
GAPDH (NM_008084.2)
forward ACCTCAACTACATGGTCTAC,
reverse TTGTCATTGAGAGCAATGCC.

Quantitative real-time PCR was performed using LightCycler 2.0 Instrument with LightCycler Software Version 4.0 (Roche Diagnostics). Normalized ratios of gene expressions were calculated as relative expressions of TRPCs normalized to the GAPDH gene, respectively. PCR products were size fractionated on agarose gels and visualized by ethidium bromide staining.
using an imaging analyzer (GelDoc 2000; BioRad Laboratories, Munich, Germany).

**Overexpression of Sdc4**

For Sdc4 overexpression podocytes were transfected either with the full-length Sdc4 sequence (Sdc4FL) or a cytoplasmic deletion construct (Sdc4CY) cloned into the eukaryotic expression vector pEF4 (Invitrogen, Carlsbad, CA, USA) containing the promoter of the elongation factor 4. Constructs were verified by sequencing.

**Immunoblotting of proteins and co-immunoprecipitation**

Immunoblotting was performed as previously described by our group.\(^{52-54}\) For Sdc4 detection, cell lysates were digested for 4 h at 37°C with 5 U/mL of heparinase III (Sigma). Protein samples were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to Hybond-ECL nitrocellulose membranes (NEN Life Science, Boston, MA, USA). Membranes were incubated with primary antibodies and were subsequently incubated with secondary IRDye800CW-infrared fluorescent dye-labeled sheep anti-rabbit antibodies (1:1000, Biomol, Hamburg, Germany) and Alexa Fluor 680-allophyco cyanin-fluorescence-labeled donkey anti-goat antibodies (1:1000; Invitrogen). Imaging was performed using the Odyssey infrared imaging system (Licor Biosciences, Bad Homburg, Germany). For co-immunoprecipitation experiments, podocytes lysates were incubated with appropriate antibody precipitated the target protein, followed by adding protein G-Sepharose slurry (Sigma). Immune complexes were examined by immunoblotting.
Quantitative in cell Western assay of proteins

Quantitative in-cell Western assays of proteins were performed using the Odyssey infrared imaging system (Licor biosciences) as described by our group. S4,S5 Cells were grown on 96-well plates and fixed by 3.7% formaldehyde. Same primary and secondary antibodies were used as described in the immunoblotting of proteins section. Measurements were performed in quadruplicate and averaged.

Immunofluorescence

The expression of TRPC6 channel protein in glomeruli from kidneys of Sdc4+/+ and Sdc4-/- mice was analyzed by immunofluorescence. Sections were incubated with different primary antibodies, and were subsequently incubated with secondary Alexa Fluor 555-conjugated anti-rabbit antibody (1:100, Invitrogen, Carlsbad, CA, USA) or Alexa Fluor 488-conjugated anti-goat antibody (1:100, Invitrogen). Nuclei were counterstained with DAPI (Roche, Mannheim, Germany, 1:1500). Images were acquired using a fluorescence microscope (AxioImager Z1) and processed with Axiovision software (Carl Zeiss MicroImaging, Inc.).

Visualization of green fluorescent protein (GFP)-tagged-TRPC6 or yellow fluorescent protein (YFP)-tagged-TRPC6 in podocytes and HEK cells using confocal laser scanning microscopy

The visualization of GFP- or YFP- tagged-TRPC6 in transfected cells was performed as described previously by our group. S6 Transient transfection of podocytes or HEK293 cells was carried out using lipofectamine (Invitrogen) according to the supplier’s recommendations.
Podocytes or HEK293 cells grown for 24 h in a 35 mm diameter dish containing a poly-L-lysine-coated coverslip were transfected with 800 ng of plasmid DNA. GFP, YFP, or trypan blue fluorescence signals were visualized on a Zeiss LSM 510 META inverted confocal laser scanning microscope. The overlay of signals was computed using Zeiss LSM 510 acquisition software (Release 3.2 SP2). For quantification of plasma membrane GFP or YFP fluorescence intensities, the ratio of cell membrane to intracellular fluorescence signal intensities was calculated from a minimum of 50 separate cells and statistically analyzed.

**Intracellular calcium measurements using laser scanning confocal microscopy**

Podocytes were seeded onto glass coverslips and incubated with the Ca\(^{2+}\) indicator fluo-4-AM (10 μmol/L) for 60 min at room temperature. Podocytes were imaged using a Bio-Rad (Munich, Germany) laser scanning confocal microscope attached to a Nikon Diaphot microscope. The amplitudes were expressed as fractional fluorescence increase (F/F₀) as described by our group.\(^{57}\)

**Intracellular cation measurements using fluorescence spectrophotometry**

For ratiometric imaging experiments cells were loaded with 2 μmol/L of the calcium-sensitive, cell-permeable, intracellular fluorescence dye fura2 AM (Merck Biosciences).\(^{55}\) Fluorescence was measured using a fluorescent plate reader (Fluoroskan Ascent Fluorometer, Thermo LabSystems Oy, Helsinki, Finland). TRPC was activated by membrane-permeable 1-oleoyl-2-acetyl-sn-glycerol (OAG, final concentration 100 μmol/L) and calcium influx was measured.\(^{58}\) Measurements were performed in the presence of membrane-permeable TRPC
blockers, 2-aminoethoxydiphenylborane (2-APB; final concentration 10 µmol/L) or 1-[β-[3-(4-
methoxyphenyl) propoxy]-4-methoxy phenethyl]-1H-imidazole (SKF-96365; final
concentration 10 µmol/L; Merck Biosciences).^9

Matrix assisted laser desorption/ionisation time of flight mass spectrometry of isolated
TRPC6 and Sdc4

The isolated TRPC6 and Sdc4 were identified using matrix assisted laser desorption/ionisation
time of flight mass-spectrometry (MALDI-TOF/TOF mass-spectrometry) as recently
described.^10 The annotated spectra were subjected to a database search (Swiss-Prot, Zurich,
Switzerland) utilizing Bruker-Daltonic Bio-Tools (vers. 3.1) and the Mascot search engine
(vers. 2.2), which compares the experimental MALDI-MS data set with the calculated peptide
masses for each entry in the sequence database (www.matrixscience.com).

Rho GTPase activating protein (RhoGAP) activity assay

RhoGAP activity was measured in cultured podocytes using the RhoGAP assay Biochem Kit
(Cytoskeleton, Inc., Denver, CO, USA). Protein samples were processed rapidly on ice and
snap-frozen until the time of assaying. RhoGAP activity was measured with absorbance set at
630 nm.

RhoA activation assay

RhoA activity was measured in kidney cortex tissues and cultured podocytes using the G-LISA
RhoA activation assay biochemistry kit (Cytoskeleton, Inc., Denver, CO, USA). Protein
samples were processed rapidly on ice and snap-frozen until the time of assaying. RhoA activity was measured with absorbance set at 490 nm.\textsuperset{511}

**Patch clamp measurements**

Membrane currents were recorded using the whole-cell configuration of the patch-clamp technique at room temperature (22–24°C) as described by our group.\textsuperset{512} The pipettes’ resistance varied between 3 and 5 MΩ in whole cell recordings. Whole cell currents were elicited by voltage ramps from –100 to +100 mV (400 ms duration) applied every 15 s from a holding potential of –40 mV. Currents through the pipette were recorded by an Axopatch 200B amplifier (AxonInstruments; MDS Analytical Technologies Inc, Sunnyvale, CA, USA), filtered at 5 or 10 kHz (Besselfilter), analyzed and adjusted using pCLAMP software (version10.1; AxonInstruments). Pipettes for whole cell recordings were filled with a solution composed of 130 mmol/L CsCH$_3$O$_3$, 10 mmol/L CsCl, 2 mmol/L MgCl$_2$, and 10 mmol/L HEPES (pH 7.2 with CsOH). The standard bath solution contained 134 mmol/L NaCl, 6 mmol/L KCl, 2 mmol/L CaCl$_2$, 1 mmol/L MgCl$_2$, 10 mmol/L glucose, and 10 mmol/L HEPES (pH 7.4 with NaOH).

**Podocytes membrane protein biotinylation and isolation**

Podocytes membrane proteins were biotinylated and isolated using a commercially available cell membrane protein isolation kit (Pierce Biotechnology, Rockford, IL, USA) according to the manufacturer’s recommendations. The isolated proteins were identified by immunoblotting as described.
**Transwell permeability assay**

Cultured podocytes were seeded on equilibrated 3.0 μm-pore Polycarbonate Transwell inserts (Corning, Lowell, MA) in a 12 well plate following the manufacturer’s instructions. Transient transfection of podocytes with YFP-tagged-TRPC6 plasmid DNA was performed using lipofectamine (Invitrogen) according to the supplier’s recommendations. To obtain podocyte monolayer, cells were incubated at 38°C for 24 h - 48 h. Evans blue-conjugated albumin (EBA; final concentration 4 mg/mL) was prepared by diluting a stock solution of 2% Evans blue in 100-fold excess of 4% bovine serum albumin as previously described with minor modifications. To measure the permeability of a podocyte monolayer to albumin, EBA was added to the lower compartment and allowed to diffuse into the upper compartment for 24 h at 38°C. The medium levels in both lower and upper compartments were adjusted to the same height. After incubation aliquots of medium from upper compartments were collected and the EBA was assayed by measuring the absorbance at 630 nm using a plate reader.

**Animal treatments**

We created Sdc4−/− mice by homologous recombination in 129SvJae embryonic stem cells and backcrossed founder mice for ten generations on C57BL/6 mice. We generated all data from males and age-matched littermates. The Animal Use Committee for the Hannover Medical School (Niedersaechsisches Landesamt für Verbraucherschutz und Lebensmittelsicherheit, approval number 33.9-42502-04-08/1517) approved all procedures. To set up the protein overload model in mice, both Sdc4−/− and Sdc4+/+ mice received intraperitoneal injections of endotoxin-free bovine serum albumin (BSA, Sigma-Aldrich, Steinheim, Germany) for 5 days.
on a stepwise incremental dosage regimen. We gradually increased the BSA dosage, i.e. beginning with 2 mg per g body weight on the first day and increasing the daily dose by 2 mg per day per g body weight until to the maximum dose of 10 mg per g body weight. Urinary albumin excretion was measured using metabolic cages.

**Isolation of glomeruli**

Glomeruli were isolated using sieves (Retsch Inc, Haan, Germany) with pore sizes of 125 µm and 75 µm, respectively. The kidneys were removed, minced into 1 mm³ pieces, and digested in collagenase (1 mg/mL collagenase A in PBS) at 37°C for 30 min with gentle agitation. The collagenase-digested tissue was gently pressed through a 125-µm sieve using a flattened pestle and the sieve was washed with 5 ml of PBS. The filtered glomeruli tissues were further purified through a 75-µm sieve. The glomeruli suspension was then centrifuged at 300 × g for 5 min. The supernatant was discarded and the glomeruli pellet was lysed and subjected to immunoblotting.

**Electron microscopy**

For electron microscopy, tissue fragments were fixed in 2% glutaraldehyde in 0.1 mol/L sodium cacodylate buffer, pH 7.4, for 120 min at 4 °C. Ultra-thin sections were then cut with a diamond knife and collected on 100 mesh copper grids. Sections were stained with 2% uranyl acetate and lead citrate and examined by a transmission electron microscope using imaging plates.

**Statistical analysis**
Data were expressed as the mean ± SEM. Comparisons between groups were analyzed using t-test or ANOVA and Bonferroni's multiple comparison test as appropriate. A two-tailed probability value less than 0.05 was considered to indicate statistical significance.
References of Supplemental Materials and Methods


between Cav1.2 channels and ryanodine receptors to generate Ca2+ sparks in murine arterial smooth muscle cells. *J Physiol* 2007; 584: 205-219.


**Suppl. Figure I**

Phenotype of Sdc4^{-/-} mice and specificity of nephrin antibodies. (A) Sdc4^{-/-} mice show reduced TRPC6 protein expression compared to Sdc4^{+/+} littermates. Merge of immunofluorescences confirmed co-localization of TRPC6 and nephrin in podocytes of freshly isolated renal cortex (arrow heads). Sdc transcripts (B) of Sdc4^{-/-} and wild type littermates, kidney shape (C; scale bar = 1 cm), ultrastructure of glomerular filtration barrier (D; scale bar = 500 nm; no ultrastructural changes were noted) were shown. The specificity of the nephrin immunostaining was confirmed by Western blotting (E).

**Suppl. Figure II**

Characterization of podocytes and regulation of TRPC6 by Sdc4. Transcripts (A) and proteins (B) in cultured podocytes. We identified transcripts for TRPC6 and Sdc4, TRPC6 protein, and Sdc4 protein in podocytes and endothelial cells, i.e. EA.hy926 and HUVEC. On the other hand, podocyte-specific proteins, i.e. nephrin, podocin, WT1, synaptopodin were solely observed in podocytes, but not in endothelial cells. (C) Mass spectrometry verified TRPC6 (Mascot-Score 183) and Sdc4 (Mascot-Score 84). (D) Immunofluorescence showing that Sdc4 knockdown specifically reduces TRPC6 protein expression (lower panels, please note that these panels have been oriented differently compared to Figure 5A) but not TRPC3 expression (upper panels) in podocytes (scale bar = 5µm). The primary data in some parts of figures 5A and IID are the same but have been oriented differently by the authors to give the clearest presentation in both figures.
Suppl. Figure III

Sdc4 knockdown reduces whereas Sdc4 overexpression specifically increases TRPC6 protein expression in podocytes measured by quantitative in-cell Western assay. Sdc4FL, Sdc4 full-length construct; Sdc4CY, Sdc4 construct lacking cytoplasmic domain (n = 4; **p < 0.01).

Suppl. Figure IV

Sdc4 facilitates the translocation of TRPC6 channels into the plasma membrane via RhoA dependent pathway. Confocal laser scanning microscopy (A) and immunoblotting (B) of podocytes transiently transfected with GFP-tagged-TRPC6 construct (+) or control (-). Trypan blue (red) indicates plasma membrane staining. Scale bar denotes 5 µm. (C) HEK293 cells, transiently transfected with YFP-tagged-TRPC6, were analyzed by confocal laser scanning microscopy. Sdc4 knockdown reduced, whereas Sdc4 overexpression increased TRPC6 protein localized in the plasma membrane. Sdc4FL, Sdc4 full-length construct; Sdc4CY, Sdc4 construct lacking cytoplasmic domain. (D) Rho kinase agonist calpeptin reduced, whereas Rho-kinase inhibitors, Y27632 or fasudil, increased TRPC6 protein localized in the plasma membrane of HEK293 cells transfected with YFP-tagged-TRPC6. n = 50; *p < 0.05 compared to control.
Suppl Fig I

A

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B

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Sdc transcripts (arbitrary units)

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Sdc4  Sdc3  Sdc2  Sdc1
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C

![Image](image9)

D

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- Nephrin
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Suppl Fig II
Suppl Fig III
Suppl Fig IV

A: GFP-TRPC6, Trypan blue, Merge

B: - GFP-TRPC6
- β-actin
+ - GFP-TRPC6

C: Control, Sdc4, Sdc4FL, Sdc4CY

D: Plasma membrane-associated YFP-TRPC6 (fold change)

Suppl Fig IV