Published in final edited form as:
Arterioscler Thromb Vasc Biol. 2012 February ; 32(2): . doi:10.1161/ATVBAHA.111.241018.

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

Ying Liu, **Frank Echtermeyer**, **Florian Thilo**, **Gregor Theilmeier**, **Antje Schmidt**, **Ralf Schülein**, **Boye L. Jensen**, **Christoph Loddenkemper**, **Vera Jankowski**, **Niels Marcussen**, **Maik Gollasch**, **William J. Arendshorst**, and **Martin Tepel**
Odense University Hospital, Department of Nephrology, and University of Southern Denmark, Institute for Molecular Medicine, Cardiovascular and Renal Research, Institute of Clinical Research, Odense, Denmark (Y.L., B.L.J., M.T.); Department of Medicine, Division of Nephrology, Charité Campus Benjamin Franklin, Berlin, Germany (Y.L., F.T., V.J., M.T.); Department of Urology, Tenth People's Hospital, Tongji University of Shanghai, People's Republic of China (Y.L.); Department of Anesthesiology and Intensive Care Medicine, Medical University Hannover, Hannover, Germany (F.E., G.T.); Max-Delbrück-Center of Molecular Medicine, Berlin, Germany (A.S.); Leibniz-Institute for Molecular Pharmacology, Berlin, Germany (R.S.); Institute of Pathology, Technical University Munich, Munich, Germany (C.L.); Institute of Pathology, Charité Campus Benjamin Franklin, Berlin, Germany (C.L.); Department of Clinical Pathology, Odense University Hospital, Odense, Denmark (N.M.); Medical Clinic for Nephrology and Intensive Care Medicine, Charité Campus Virchow, Berlin, Germany (M.G.); Experimental and Clinical Research Center, Berlin, Germany (M.G.); Cell and Molecular Physiology, University of North Carolina at Chapel Hill, Chapel Hill, NC (W.J.A.)

Abstract

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 knockdown in cultured podocytes reduced TRPC6 gene expression and reduced the association of TRPC6 with plasma membrane and TRPC6-mediated calcium influx and currents. Sdc4 knockdown 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,

Correspondence to Martin Tepel, Odense University Hospital and University of Southern Denmark, Institute for Molecular Medicine, Cardiovascular and Renal Research, Institute of Clinical Research, Winsløwparken 21.3, DK-5000 Odense C, Denmark. mtepel@health.sdu.dk.
Drs Liu and Echtermeyer contributed equally to this work.

Disclosures
None.

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.

Keywords

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 disease 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.[14 –17] The visualization of green fluorescent protein (GFP)–or yellow fluorescent protein (YFP)-tagged-TRPC6 in transfected cells, intra-cellular 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$^{-/-}$ mice has been reported previously,[26] and the Animal Use Committee for the Hannover Medical School (Niedersaechsisches Landesamt für

Verbraucherschutz und Lebensmittelsicherheit, approval number 33.9-42502-04-08/1517) approved all procedures.

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.

Results

Sdc4$^{-/-}$ Mice Show Reduced TRPC6 Transcripts, Proteins, and Urinary Albumin Excretion

In the present study, we compared TRPC6 expression in glomeruli of Sdc4$^{-/-}$ mice[26] and wild-type (Sdc4$^{+/+}$) 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$^{-/-}$ compared with Sdc4$^{+/+}$ mice (Supplemental Figure IA). Immunoblotting confirmed a significant reduction of TRPC6 protein by 82% in renal cortex of Sdc4$^{-/-}$ vs Sdc4$^{+/+}$ 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$^{-/-}$ mice (Figure 1C), providing further evidence for selective regulation of TRPC6 by Sdc4. Most importantly, under baseline conditions Sdc4$^{-/-}$ 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$^{-/-}$ mice. Bovine serum albumin overload enhanced urinary albumin excretion 2.5±0.4-fold in Sdc4$^{-/-}$ mice, whereas it enhanced urinary albumin excretion 13.4±5.2-fold in Sdc4$^{+/+}$ littermates (P<0.05; Figure 1E). Sdc4$^{-/-}$ showed increased glomerular filtration rate, indicating healthy kidney function. Glomerular filtration rate was higher in Sdc4$^{-/-}$ compared with Sdc4$^{+/+}$ 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$^{-/-}$ mice whereas Sdc3, Sdc2, and Sdc1 transcripts were not different between the 2 groups (Supplemental Figure IB). The kidney weight to body weight was 6.4±0.3 mg/g in Sdc4$^{-/-}$ mice and 6.6±0.5 mg/g in wild-type littermates, respectively (n=5 each; P>0.05). The ultrastructure of glomerular filtration barrier, including the foot process, slit diaphragm, basement membrane, and endothelium, were structurally normal in Sdc4$^{-/-}$ mice, as examined by electron microscopy (Supplemental Figure IC and ID). Collectively, our results link parallel reductions in Sdc4 and TRPC6 with reduced urinary protein excretion. These results are in line with findings showing the association of TRPC6 with proteinuria in kidney diseases.[8–12]

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.

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 (Supplemental Figure IID), quantitative fluorescence assays of green fluorescent protein-tagged TRPC6 (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 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 59% ($P<0.01$). In contrast, increased TRPC6 channel density after transfection with Sdc4FL enhanced cation currents by 47% ($P<0.05$). 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, 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±0.1; controls, 1.7±0.0; 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 obtained 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.[8,10,11] 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 knockdown in podocytes decreased TRPC6 transcripts, protein abundance, and TRPC6-mediated cation influx; and (3) Sdc4 knockdown 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[38] reported similar blood pressure in conscious Sdc4$^{-/-}$ mice and wild-type littermates, and Partovian 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 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.[29] 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 tissue 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Sources of Funding

This study was supported by the Deutsche Forschungsgemeinschaft, Else Kröner-Fresenius Stiftung, Sonnenfeld-Stiftung, Ingeniør K.A. Rohde og Hustrus Legat, Tømrermester Alfred Andersen og Hustrus Fond, European Union Grant FP7-HEALTH-2009-2.4.5-2 SYSKID, European Union Grant INTERREG 4A, number 62-1.2-10, and the Danish Council for Independent Research (Det Frie Forskningsråd, 10-084667).

References

1. Bass MD, Humphries MJ. Cytoplasmic interactions of syndecan-4 orchestrate adhesion receptor and growth factor receptor signaling. Biochem J. 2002; 368:1–15. [PubMed: 12241528]
2. Partovian C, Ju R, Zhuang ZW, Martin KA, Simons M. Syndecan-4 regulates subcellular localization of mTOR Complex2 and Akt activation in a PKCα-dependent manner in endothelial cells. Mol Cell. 2008; 32:140–149. [PubMed: 18851840]
3. Kim J, Lee JH, Park HS, Hwang J, Han IO, Bae YS, Oh ES. Syndecan-4 regulates platelet-derived growth factor-mediated MAP kinase activation by altering intracellular reactive oxygen species. FEBS Lett. 2008; 582:2725–2730. [PubMed: 18619965]
4. Fan Q, Shike T, Shigihara T, Tanimoto M, Gohda T, Makita Y, Wang LN, Horikoshi S, Tomino Y. Gene expression profile in diabetic KK/Ta mice. Kidney Int. 2003; 64:1978–1985. [PubMed: 14633120]
5. Yung S, Woods A, Chan TM, Davies M, Williams JD, Couchman JR. Syndecan-4 up-regulation in proliferative renal disease is related to microfilament organization. FASEB J. 2001; 15:1631–1633. [PubMed: 11427509]
6. Chen S, Wassenhove-McCarthy D, Yamaguchi Y, Holzman L, van Kuppevelt TH, Orr AW, Funk S, Woods A, McCarthy K. Podocytes require the engagement of cell surface heparan sulfate proteoglycans for adhesion to extracellular matrices. Kidney Int. 2010; 78:1088–1099. [PubMed: 20463653]
7. Johnstone DB, Holzman LB. Clinical impact of research on the podocyte slit diaphragm. Nat Clin Pract Nephrol. 2006; 2:271–282. [PubMed: 16932440]
8. Winn MP, Conlon PJ, Lynn KL, Farrington MK, Creazzo T, Hawkins AF, Daskalakis N, Kwan SY, Ebersviller S, Burchette JL, Pericak-Vance MA, Howell DN, Vance JM, Rosenberg PB. A mutation in the TRPC6 cation channel causes familial focal segmental glomerulosclerosis. Science. 2005; 308:1801–1804. [PubMed: 15879175]
9. Wu LJ, Sweet TB, Clapham DE. International Union of Basic and Clinical Pharmacology: LXXVI: current progress in the mammalian TRP ion channel family. Pharmacol Rev. 2010; 62:381– 404. [PubMed: 20716668]
10. Nilius B, Owsianik G, Voets T, Peters JA. Transient receptor potential cation channels in disease. Physiol Rev. 2007; 87:165–217. [PubMed: 17237345]
11. Möller CC, Wei C, Altintas MM, Li J, Greka A, Ohse T, Pippin JW, Rastaldi MP, Wawersik S, Schiavi S, Henger A, Kretzler M, Shankland SJ, Reiser J. Induction of TRPC6 channel in acquired forms of proteinuric kidney disease. J Am Soc Nephrol. 2007; 18:29–36. [PubMed: 17167110]
12. Krall P, Canales CP, Kairath P, Carmona-Mora P, Molina J, Carpio JD, Ruiz P, Mezzano SA, Li J, Wei C, Reiser J, Young JI, Walz K. Podocyte-specific overexpression of wild type or mutant trpc6 in mice is sufficient to cause glomerular disease. PLoS One. 2010; 5:e12859. [PubMed: 20877463]
13. Schiwek D, Endlich N, Holzman L, Holthöfer H, Kriz W, Endlich K. Stable expression of nephrin and localization to cell-cell contacts in novel murine podocyte cell lines. Kidney Int. 2004; 66:91–101. [PubMed: 15200416]
14. Thilo F, Baumunk D, Krause H, Schrader M, Miller K, Loddenkemper C, Zakrzewicz A, Krueger K, Zidek W, Tepel M. Transient receptor potential canonical type 3 channels and blood pressure in humans. J Hypertens. 2009; 27:1217–1223. [PubMed: 19417689]
15. Semplicini A, Lenzini L, Sartori M, Papparella I, Calò LA, Pagnin E, Strapazzon G, Benna C, Costa R, Avogaro A, Ceolotto G, Pessina AC. Reduced expression of regulator of G-protein signaling 2 (RGS2) in hypertensive patients increases calcium mobilization and ERK1/2 phosphorylation induced by angiotensin II. J Hypertens. 2006; 24:1115–1124. [PubMed: 16685212]
16. Liu Y, Thilo F, Kreutz R, Schulz A, Wendt N, Loddenkemper C, Jankowski V, Tepel M. Tissue expression of TRPC3 and TRPC6 in hypertensive Munich Wistar Frömter rats showing proteinuria. Am J Nephrol. 2010; 31:36– 44. [PubMed: 19887786]
17. Liu D, Maier A, Scholze A, Rauch U, Boltzen U, Zhao Z, Zhu Z, Tepel M. High glucose enhances transient receptor potential channel canonical type 6-dependent calcium influx in human platelets

via phosphatidylinositol 3-kinase-dependent pathway. Arterioscler Thromb Vasc Biol. 2008; 28:746–751. [PubMed: 18258814]

18. Schmidt A, Wiesner B, Weisshart K, Schulz K, Furkert J, Lamprecht B, Rosenthal W, Schülein R. Use of Kaede fusions to visualize recycling of G protein-coupled receptors. Traffic. 2009; 10:2–15. [PubMed: 18939954]
19. Essin K, Welling A, Hofmann F, Luft FC, Gollasch M, Moosmang S. Indirect coupling between Cav1.2 channels and ryanodine receptors to generate Ca2+ sparks in murine arterial smooth muscle cells. J Physiol. 2007; 584:205–219. [PubMed: 17673505]
20. Schordan S, Schordan E, Endlich N, Lindenmeyer MT, Meyer-Schwesinger C, Meyer TN, Giebel J, Cohen CD, Endlich K, Maurer MH. Alterations of the podocyte proteome in response to high glucose concentrations. Proteomics. 2009; 9:4519– 4528. [PubMed: 19688724]
21. Ma HT, Venkatachalam K, Li HS, Montell C, Kurosaki T, Patterson RL, Gill DL. Assessment of the role of the inositol 1,4,5-trisphosphate receptor in the activation of transient receptor potential channels and store-operated Ca^{2+} entry channels. J Biol Chem. 2001; 276:18888–18896. [PubMed: 11259416]
22. Jankowski V, Vanholder R, van der Giet M, Tölle M, Karadogan S, Gobom J, Furkert J, Oksche A, Krause E, Tran TN, Tepel M, Schuchardt M, Schlüter H, Wiedon A, Beyermann M, Bader M, Todiras M, Zidek W, Jankowski J. Mass-spectrometric identification of a novel angiotensin peptide in human plasma. Arterioscler Thromb Vasc Biol. 2007; 27:297–302. [PubMed: 17138938]
23. Woods A, Beier F. RhoA/ROCK signaling regulates chondrogenesis in a context-dependent manner. J Biol Chem. 2006; 281:13134–13140. [PubMed: 16565087]
24. Leuner K, Kazanski V, Müller M, Essin K, Henke B, Gollasch M, Harteneck C, Müller WE. Hyperforin: a key constituent of St. John's wort specifically activates TRPC6 channels. FASEB J. 2007; 21:4101– 4111. [PubMed: 17666455]
25. Lee EY, Chung CH, Khoury CC, Yeo TK, Pyagay PE, Wang A, Chen S. The monocyte chemoattractant protein-1/CCR2 loop, inducible by TGF-β, increases podocyte motility and albumin permeability. Am J Physiol Renal Physiol. 2009; 297:F85–F94. [PubMed: 19420107]
26. Echtermeyer F, Streit M, Wilcox-Adelman S, Saoncella S, Denhez F, Detmar M, Goetinck P. Delayed wound repair and impaired angiogenesis in mice lacking syndecan-4. J Clin Invest. 2001; 107:R9–R14. [PubMed: 11160142]
27. Bass MD, Morgan MR, Roach KA, Settleman J, Goryachev AB, Humphries MJ. p190RhoGAP is the convergence point of adhesion signals from $\alpha_5\beta_1$ integrin and syndecan-4. J Cell Biol. 2008; 181:1013–1026. [PubMed: 18541700]
28. Bradley WD, Hernández SE, Settleman J, Koleske AJ. Integrin signaling through Arg activates p190RhoGAP by promoting its binding to p120RasGAP and recruitment to the membrane. Mol Biol Cell. 2006; 17:4827– 4836. [PubMed: 16971514]
29. Granés F, Urena JM, Rocamora N, Vilaró S. Ezrin links syndecan-2 to the cytoskeleton. J Cell Sci. 2000; 113:1267–1276. [PubMed: 10704377]
30. Eckel J, Lavin PJ, Finch EA, Mukerji N, Burch J, Gbadegesin R, Wu G, Bowling B, Byrd A, Hall G, Sparks M, Zhang ZS, Homstad A, Barisoni L, Birbaumer L, Rosenberg P, Winn MP. TRPC6 enhances angiotensin II-induced albuminuria. J Am Soc Nephrol. 2011; 22:526–535. [PubMed: 21258036]
31. Foster RR, Zadeh MA, Welsh GI, Satchell SC, Ye Y, Mathieson PW, Bates DO, Saleem MA. Flufenamic acid is a tool for investigating TRPC6-mediated calcium signaling in human conditionally immortalised podocytes and HEK293 cells. Cell Calcium. 2009; 45:384–390. [PubMed: 19232718]
32. Jung S, Strotmann R, Schultz G, Plant TD. TRPC6 is a candidate channel involved in receptor-stimulated cation currents in A7r5 smooth muscle cells. Am J Physiol Cell Physiol. 2002; 282:C347–C359. [PubMed: 11788346]
33. Reiser J, Polu KR, Möller CC, Kenlan P, Altintas MM, Wei C, Faul C, Herbert S, Villegas I, Avila-Casado C, McGee M, Sugimoto H, Brown D, Kalluri R, Mundel P, Smith PL, Clapham DE, Pollak MR. TRPC6 is a glomerular slit diaphragm-associated channel required for normal renal function. Nat Genet. 2005; 37:739–744. [PubMed: 15924139]

34. Stirling L, Williams MR, Morielli AD. Dual roles for RHOA/RHO-kinase in the regulated trafficking of a voltage-sensitive potassium channel. Mol Biol Cell. 2009; 20:2991–3002. [PubMed: 19403695]
35. Dietrich A, Mederos Y, Schnitzler M, Gollasch M, Gross V, Storch U, Dubrovska G, Obst M, Yildirim E, Salanova B, Kalwa H, Essin K, Pinkenburg O, Luft FC, Gudermann T, Birnbaumer L. Increased vascular smooth muscle contractility in TRPC6−/− mice. Mol Cell Biol. 2005; 25:6980–6989. [PubMed: 16055711]
36. Vannier B, Zhu X, Brown D, Birnbaumer L. The membrane topology of human transient receptor potential 3 as inferred from glycosylation-scanning mutagenesis and epitope immunocytochemistry. J Biol Chem. 1998; 273:8675–8679. [PubMed: 9535843]
37. Hofmann T, Obukhov AG, Schaefer M, Harteneck C, Gudermann T, Schultz G. Direct activation of human TRPC6 and TRPC3 channels by diacylglycerol. Nature. 1999; 397:259–263. [PubMed: 9930701]
38. Ishiguro K, Kadomatsu K, Kojima T, Muramatsu H, Iwase M, Yoshikai Y, Yanada M, Yamamoto K, Matsushita T, Nishimura M, Kusugami K, Saito H, Muramatsu T. Syndecan-4 deficiency leads to high mortality of lipopolysaccharide-injected mice. J Biol Chem. 2001; 276:47483–47488. [PubMed: 11585825]
39. Peitsch WK, Hofmann I, Endlich N, Prätzel S, Kuhn C, Spring H, Gröne HJ, Kriz W, Franke WW. Cell biological and biochemical characterization of drebrin complexes in mesangial cells and podocytes of renal glomeruli. J Am Soc Nephrol. 2003; 14:1452–1463. [PubMed: 12761245]
40. Ridley AJ. Rho proteins: linking signaling with membrane trafficking. Traffic. 2001; 2:303–310. [PubMed: 11350626]
41. Symons M, Rusk N. Control of vesicular trafficking by Rho GTPases. Curr Biol. 2003; 13:R409–R418. [PubMed: 12747855]
42. Dovas A, Yoneda A, Couchman JR. PKCβ-dependent activation of RhoA by syndecan-4 during focal adhesion formation. J Cell Sci. 2006; 119:2837–2846. [PubMed: 16787950]
43. Ren XD, Kiosses WB, Schwartz MA. Regulation of the small GTP-binding protein Rho by cell adhesion and the cytoskeleton. EMBO J. 1999; 18:578–585. [PubMed: 9927417]
44. Kawase K, Nakamura T, Takaya A, Aoki K, Namikawa K, Kiyama H, Inagaki S, Takemoto H, Saltiel AR, Matsuda M. GTP hydrolysis by the Rho family GTPase TC10 promotes exocytic vesicle fusion. Dev Cell. 2006; 11:411–421. [PubMed: 16950130]
45. Cayouette S, Lussier MP, Mathieu EL, Bousquet SM, Boulay G. Exocytotic insertion of TRPC6 channel into the plasma membrane upon Gq protein-coupled receptor activation. J Biol Chem. 2004; 279:7241–7246. [PubMed: 14662757]

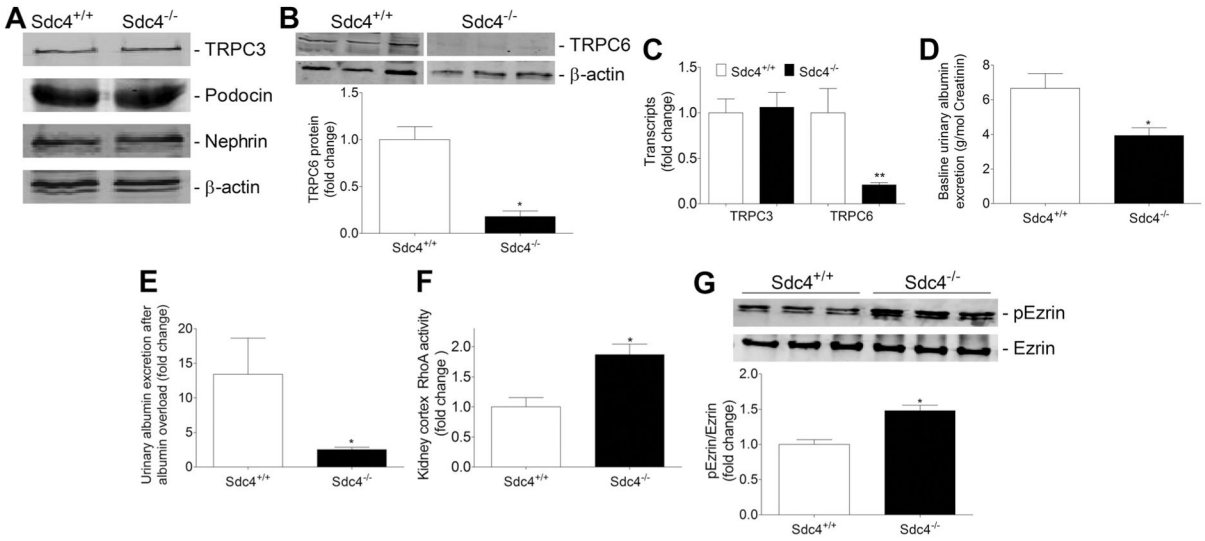

Figure 1.

Characteristics of wild-type (syndecan 4 [Sdc4]$^{+/+}$) and Sdc4$^{-/-}$ 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$^{-/-}$ mice. Sdc4$^{-/-}$ 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$^{+/+}$ littermates (**E**) (n=4–5; **P*<0.05). Sdc4$^{-/-}$ mice showed increased RhoA activity (**F**) (n=5; **P*<0.05) and phosphorylation of ezrin (pEzrin), indicated by immunoblotting (**G**).

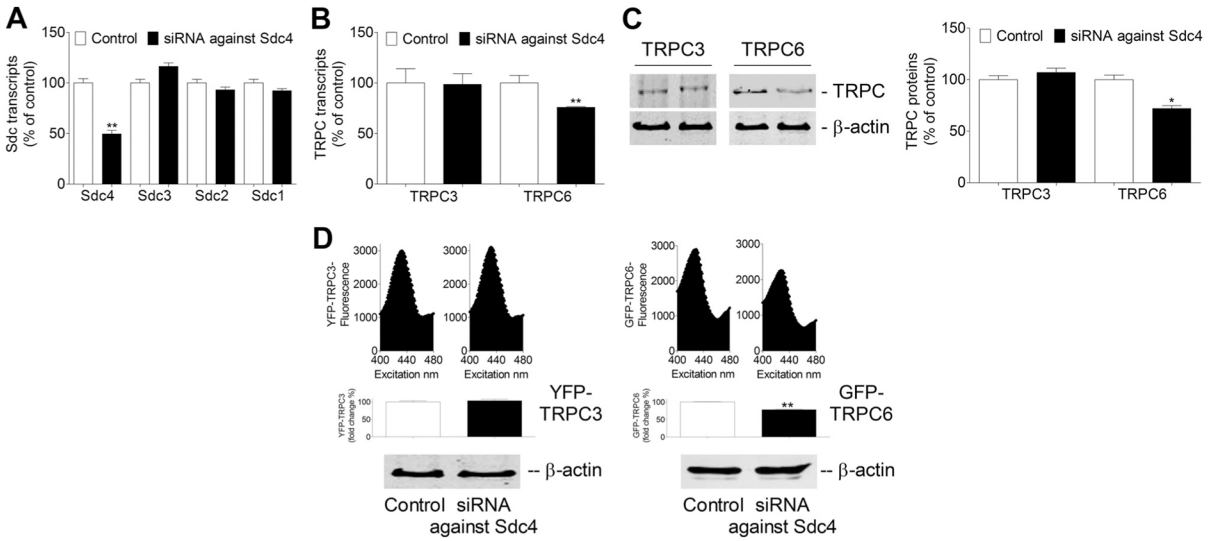

Figure 2.

Syndecan 4 (Sdc4) regulates transient receptor potential canonical 6 (TRPC6) expression in podocytes. Podocytes transfected with small interfering RNA (siRNA) against Sdc4 showed reduced transcripts for Sdc4 (**A**) and TRPC6 (**B**), but not Sdc3, Sdc2, Sdc1, or TRPC3 (quantitative reverse transcription–polymerase chain reaction; n=5– 8; ***P*<0.01). Podocytes transfected with siRNA against Sdc4 showed reduced TRPC6 protein expression (**C**) (n=4; **P*<0.05). Sdc4 knockdown specifically reduced TRPC6 protein expression in podocytes, as measured by quantitative fluorescence assays of green fluorescent protein (GFP)–tagged TRPC6 (**D**) (n=4; ***P*<0.01).

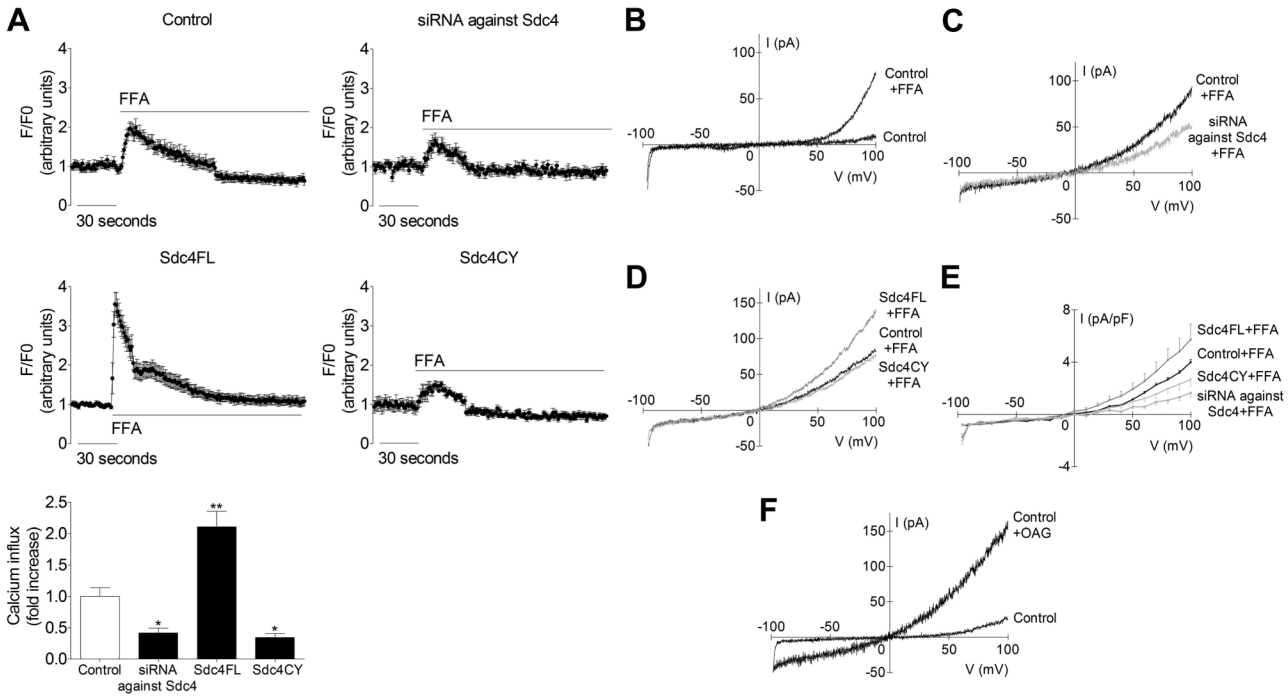

Figure 3.

Syndecan 4 (Sdc4) regulates transient receptor potential canonical 6 (TRPC6)–mediated calcium influx and membrane currents in podocytes. **A**, The cytosolic Ca^{2+} concentration in response to 100 μmol/L flufenamic acid (FFA) was determined by Fluo-4. Pictures at baseline, at peak calcium, and after recovery are shown, as well as summary data of peak calcium influx. n=5; **P*<0.05, ***P*<0.01. FFA-induced, TRPC6-mediated membrane currents in podocytes (**B**), after Sdc4 knockdown (**C**), and Sdc4 overexpression (**D**) are shown. siRNA indicates small interfering RNA. **E**, Normalization of currents (n=4). 1-Oleoyl-2-acetyl-*sn*-glycerol (OAG), a diacylglycerol (DAG) analog, can trigger the TRPC-mediated currents in podocytes (F).

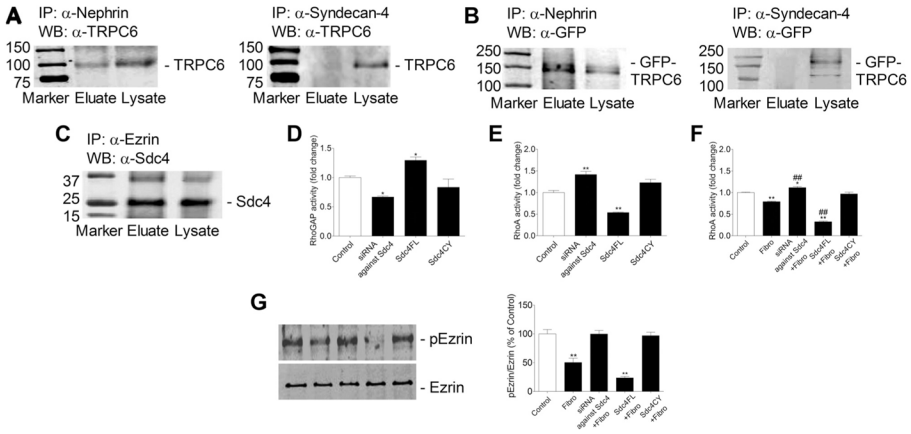

Figure 4.

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.

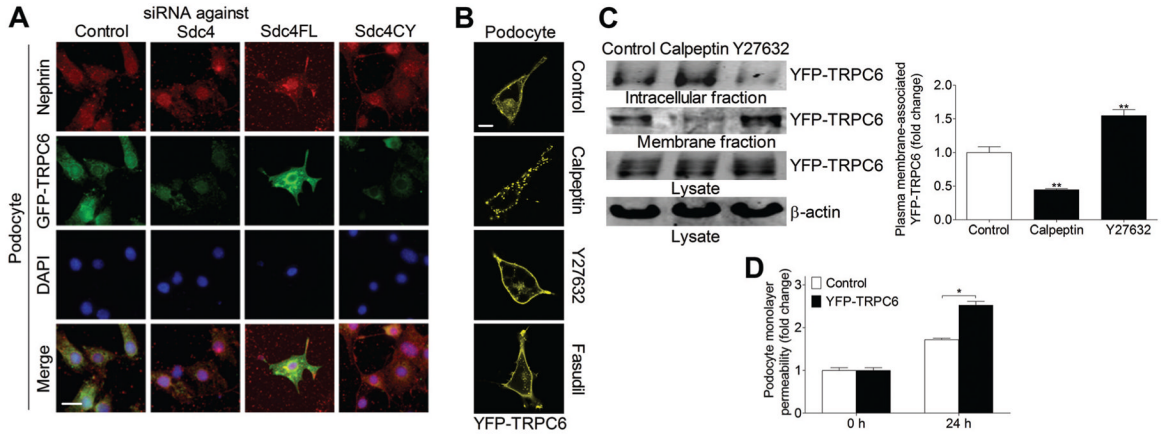

Figure 5.

Syndecan 4 (Sdc4) enhances the density of transient receptor potential canonical 6 (TRPC6) channels in the plasma membrane of podocytes via 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).