Nephrin binds to the COOH terminus of a large-conductance $\text{Ca}^{2+}$-activated $K^+$ channel isoform and regulates its expression on the cell surface

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Kim EY, Choi K-J, Dryer SE. Nephrin binds to the COOH terminus of a large-conductance $\text{Ca}^{2+}$-activated $K^+$ channel isoform and regulates its expression on the cell surface. Am J Physiol Renal Physiol 295: F235–F246, 2008. First published May 14, 2008; doi:10.1152/ajprenal.00140.2008.—We carried out a yeast two-hybrid screen to identify proteins that interact with large-conductance $\text{Ca}^{2+}$-activated $K^+$ (BK$_{\text{Ca}}$) channels encoded by the Slo1 gene. Nephrin, an essential adhesion and scaffolding molecule expressed in podocytes, emerged in this screen. The Slo1-nephrin interaction was confirmed by coimmunoprecipitation from the brain and kidney, from HEK-293T cells expressing both proteins, and by glutathione S-transferase pull-down assays. We detected nephrin binding to the Slo1$\text{VEDEC}$ splice variant, which is typically retained in intracellular stores, and to the $\beta$-subunit. However, we did not detect significant binding of nephrin to the Slo1$\text{QEERL}$ or Slo1$\text{EMVYR}$ splice variants. Coexpression of nephrin with Slo1$\text{VEDEC}$ increased expression of functional BK$_{\text{Ca}}$ channels on the surface of HEK-293T cells but did not affect steady-state surface expression of the other COOH-terminal Slo1 variants. Nephrin did not affect the kinetics or voltage dependence of channel activation in HEK-293T cells expressing Slo1. Stimulation of Slo1$\text{VEDEC}$ surface expression in HEK-293T cells was also observed by coexpressing a small construct encoding only the COOH-terminal domains of nephrin that interact with Slo1. Reduction of endogenous nephrin expression by application of small interfering RNA to differentiated cells of an immortalized podocyte cell line markedly reduced the steady-state surface expression of Slo1 as assessed by electrophysiology and cell-surface biotinylation assays. Nephrin therefore plays a role in organizing the surface expression of ion channel proteins in podocytes and may play a role in outside-in signaling to allow podocytes to adapt to mechanical or neurohumoral stimuli originating in neighboring cells.

podocyte; Slo1; calcium; trafficking

The initial steps in renal function entail filtration of blood in the glomerulus, which acts as a sieve to exclude macromolecules from the urinary space in the lumen of Bowman’s capsule. Normal glomerular filtration requires a population of specialized cells called podocytes, whose interdigitating foot processes attach to the glomerular basement membrane and form rectangular zipper-shaped structures known as slit diaphragms (38). Glomerular slit diaphragms are permeable to small inorganic and organic solutes but normally inhibit passage of macromolecules such as albumin and other plasma proteins into the filtrate. Mutations in several genes encoding podocyte proteins lead to congenital nephrotic syndromes. One form, originally described in Finnish patients, is associated with mutations in the gene encoding nephrin, a key structural and signaling protein expressed in podocyte foot processes (12, 21, 33). Nephrin interacts with a number of proteins in podocytes, and deletion of nephrin in mice results in a massive and lethal neonatal proteinuria (36).

Surprisingly little is known about the cellular physiology of podocytes, but there is evidence that these cells play a dynamic role in the regulation of glomerular filtration. The arrangement of F-actin, myosin, and $\alpha$-actinin within foot processes allows podocytes to generate a contractile force that is postulated to facilitate adaptation of the glomerulus to changes in intraluminal hydrostatic pressure or to modify the surface area for filtration (5, 18, 31). Proteins of the slit diaphragm complex, including nephrin, are coupled to underlying contractile elements by a variety of adaptor proteins (7, 13, 35) that are postulated to allow for outside-in signaling from the nephrin ectodomains. Moreover, the size-selective barrier properties of podocyte monolayers are regulated by Ca$^{2+}$-dependent changes in proteins that regulate the cytoskeleton and the slit diaphragm (11). Notably, congenital proteinurias have been associated with at least three different gain-of-function mutations in the gene encoding the Ca$^{2+}$-permeable cation channel TRPC6, which is heavily expressed in podocytes (27, 37, 50), and overexpression of wild-type TRPC6 channels causes marked changes in podocyte actin dynamics and function (27).

TRPC6 channels have complex permeability properties. At more negative membrane potentials, they are permeable to Ca$^{2+}$, but moderate depolarization causes them to function essentially as monovalent cation channels (6). For this reason, TRPC6 cannot be an efficient pathway for Ca$^{2+}$ influx in most nonexcitable cells unless there is a mechanism in place to limit the amount of depolarization that occurs as a result of their own activation. Large-conductance Ca$^{2+}$-activated K$^+$ (BK$_{\text{Ca}}$) channels are ideally situated to play that role. BK$_{\text{Ca}}$ channels are widely expressed in excitable and nonexcitable cells, including podocytes (29). Their gating is markedly voltage dependent, and binding of Ca$^{2+}$ to sites on the large cytosolic COOH-terminal portion of the molecule shifts the activation voltage dependence into the physiological range. BK$_{\text{Ca}}$ channels in podocytes (29) and in other cell types (8, 45) also can be activated by membrane stretch. The pore-forming subunits of BK$_{\text{Ca}}$ channels are encoded by the Slo1 gene (also known as KCNMA1), which is expressed in a large number (≥20) of splice variants. The large COOH-terminal domains of Slo1 channels contain multiple high- and low-affinity sites for Ca$^{2+}$ binding, as well as many phosphorylation and protein-protein interaction sites (24). In addition, at least four genes encode $\beta$-subunits that modulate BK$_{\text{Ca}}$ gating and trafficking but that are not essential to make functional channels (24). Among these, the $\beta$- and $\beta$4-subunits are expressed in podocytes (29).

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Alternative splicing in the extreme COOH terminus of Slo1 gives rise to channels with markedly different patterns of trafficking to the plasma membrane (15, 16, 26). For example, one variant, known as VEDEC (Slo1VEDEC), after the last five residues at the COOH terminus, tends to be retained in intracellular compartments but can be translocated to the plasma membrane by treating cells with appropriate growth factors (15, 16). Two other COOH-terminal splice variants, referred to as Slo1QEREL and Slo1EMVYR, show much higher levels of steady-state expression in the plasma membrane (15, 16, 26). Coexpression of at least some BKCa β-subunits also can stimulate trafficking of Slo1 to the plasma membrane (15). For this reason, we have carried out a series of yeast two-hybrid screens in search of proteins that exhibit isoform-specific binding to Slo1 subunits. Nephrin emerged in this screen. In the present study we have confirmed using several methods that nephrin binds to the Slo1VEDEC isoform but not to Slo1QEREL or Slo1EMVYR, and we also have shown that it colocalizes with endogenous Slo1 channels in differentiated cells of an immortalized podocyte cell line. Moreover, we have shown that coexpression of nephrin stimulates steady-state surface expression of Slo1VEDEC channels in a heterologous expression system, whereas small interfering (si)RNA-mediated nephrin knockdown reduces surface expression of functional Slo1 channels in cultured podocytes.

MATERIALS AND METHODS

Yeast two-hybrid screen. Yeast two-hybrid analysis was carried out using the Matchmaker system (BD Biosciences, San Jose, CA) according to the manufacturer’s instructions, as described in detail previously (17). We screened a cDNA library of the embryonic day 9 (E9) chick ciliary ganglion transcriptome homologously recombined into the pGADT7-rec plasmid. The resulting library was transformed into the AH109 yeast strain and selected on a SD/Ade- single dropout medium. We obtained nephrin using a bait construct encoding amino acids 175-200 of the chick β1-subunit of BKCa channels (15), cloned in-frame into the pGBK7 bait vector in the Matchmaker system. The bait vectors were transformed into the Y187 strain, which were selected on SD/Tp- single dropout medium. The transformed AH109 cDNA library cells were then mated with the transformed Y187 bait cells. Positive colonies expressing putative interacting proteins were selected by blue-white selection carried out on a quadruple dropout medium. Positive colonies expressing putative interacting proteins were cotransfected with other constructs to allow identification of trans-acting proteins identical except at the COOH terminus (16, 26). Constructs encoding green fluorescent protein (GFP), tagged truncated nephrin protein (GFP-nephrin-CT2) was generated by subcloning the COOH-terminal fragment-encoding residues (R1160–V1241) into NT-GFP-TOPO vector (Invitrogen). The fidelity of all constructs was confirmed by sequencing. We obtained siRNAs directed against mouse nephrin from Santa Cruz Biotechnology (Santa Cruz, CA). We obtained a negative control siRNA composed of a scrambled sequence from the same vendor.

Cell culture and transfection. HEK-293T (human embryonic kidney) cells were grown in Dulbecco’s modified Eagle’s medium (Sigma, St. Louis, MO) containing 10% heat-inactivated fetal bovine serum at 37°C in a 5% CO2 incubator. These cells were transiently transfected using Lipofectamine-2000 (Invitrogen) in serum-reduced medium (Opti-MEM; Invitrogen) following the manufacturer’s instructions. The DNA concentration in the transfection medium was 1 µg/ml of each plasmid. Cells were used for physiology or biochemistry 24–48 h after transfection. A podocyte cell line was obtained from Dr. Peter Mundel (Mount Sinai School of Medicine, New York, NY). It was maintained in RPMI 1640 medium (GIBCO) supplemented with 10% fetal bovine serum and 100 U/ml penicillin-streptomycin, with or without recombinant mouse γ-interferon (Sigma), in humidified 5% CO2 incubators. Podocytes were propagated on collagen I-coated plates at 33°C in the presence of recombinant mouse γ-interferon (10 U/ml). Removal of γ-interferon and a temperature switch to 37°C inactivated the SV40T antigen and induced podocytes to differentiate, a process that was complete in 2 wk. In experiments using siRNA, differentiated podocytes were transiently transfected in 6- (for biochemistry) or 24-well plates (for electrophysiology) with siRNA targeting nephrin, or control siRNA, using Oligofectamine (Invitrogen) in Opti-MEM medium following the manufacturer’s instructions. To do this, Oligofectamine was combined with Opti-MEM for 5 min before being combined with a solution containing 20 µM siRNA in Opti-MEM. This was left to form complexes for 20 min, and a portion of this complex was then added to the wells in a final concentration of 1:5 relative to the amount of medium in the wells. After 4 h, a saturating volume of growth medium containing 30% fetal bovine serum concentration was added to the transfection mixture to stop the reaction. Cells were maintained in this medium and used 48–72 h after transfection.

Electrophysiology. Whole cell recordings were made from HEK-293T cells as described previously (15). Plasmids encoding GFP were cotransfected with other constructs to allow identification of transfected cells in the recording chamber. The bathing solution contained (in mM) 150 NaCl, 0.08 KCl, 0.8 MgCl2, 5.4 CaCl2, 10 glucose, and 10 HEPES, and the pH was adjusted to 7.4 with NaOH. The pipette solution contained (in mM) 145 NaCl, 2 KCl, 6.2 MgCl2, 10 HEPES, and 0.5 H-EDTA, pH 7.2. The free Ca2+ concentration in this solution was titrated to a concentration of 5 µM by addition of CaCl2 using an Orion 97-20 calcium electrode (Thermo Fisher Scientific, Waltham, MA) calibrated using solution standards obtained from World Precision Instruments (Sarasota, FL). HEK-293T cells do not express endogenous voltage-activated Ca2+ currents, and these ionic conditions were chosen to provide sufficient intracellular Ca2+ for activation of BKCa channels by depolarizing step pulses while at the same time keeping the resulting macroscopic currents sufficiently small to avoid saturation of the patch-clamp amplifier or significant series resistance errors (15). In the absence of Ca2+ in the pipette, no whole cell currents were seen. For podocytes, the bath solution contained 150 mM NaCl, 5.4 mM KCl, 0.8 mM MgCl2, 5.4 mM CaCl2, and 10 mM HEPES, pH 7.4. Pipette solution contained 10 mM NaCl, 125 mM KCl, 6.2 mM MgCl2, 10 mM HEPES, pH 7.2, and 5 µM free Ca2+ buffered with 10 mM H-EDTA, as determined with the calcium electrode. Voltage-activated whole cell currents in podocytes were not detectable with those ionic gradients when recording pipettes contained no added CaCl2 and 10 mM EGTA (data not shown). The recording electrodes had resistances of 3–4 MΩ, and it was possible to compensate up to 85% of this without introducing oscillations into...
the current output of the patch-clamp amplifier (Axopatch 1D; Axon Instruments). In podocytes and HEK-293T cells, whole cell currents were evoked by a series of eight 450-ms depolarizing steps (from −25 to +80 mV in 15-mV increments) from a holding potential of −60 mV.

Cell-surface biotinylation assays. Cell-surface biotinylation assays were carried out as described in detail previously (15, 16, 17). Briefly, intact cells were treated with a membrane-impermeable biotinylation reagent, sulfo-N-hydroxy-succinimidobiotin (1 mg/ml in PBS buffer; Pierce Biotechnology, Rockford, IL) for 1 h on ice with gentle shaking. The reaction was stopped, cells were lysed, and biotinylated proteins from the cell surface were recovered from lysates by incubation with immobilized streptavidin-agarose beads (Pierce Biotechnology). A sample of the initial cell lysate also was retained for analysis of total proteins. These samples were separated on SDS-PAGE, and proteins were quantified by immunoblot analysis. Protein bands in immunoblots were quantified by densitometry using ImageJ software (National Institutes of Health, Bethesda, MD). For Slo1, we analyzed the most intense band associated with Slo1 monomers (−135 kDa). Higher molecular mass bands represent multimers of Slo1 (48). These and all other biochemical experiments were repeated at least three times.

Coimmunoprecipitation and immunoblot analysis. For coimmunoprecipitation, NH2-terminal Myc-tagged Slo1VEDEC, Slo1GPERL, and Slo1EMVYR were expressed in HEK-293T cells. Cells were lysed in 50 mM Tris-Cl, pH 7.6, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 2 mM EDTA, 1 mM PMSF, and protease inhibitor mixture (Sigma). Cells extracts (500–700 μg of protein) were incubated in the presence of primary antibodies anti-Myc (Cell Signaling) or IgG (1–2 μg) for 4 h at 4°C, followed by the addition of 20 μl of protein A/G-agarose (Santa Cruz Biotechnology) for 12 h. Pellets were washed four times, boiled for 5 min in SDS sample buffer, and subjected to SDS-PAGE on 10% gels. Cell-extracted protein (50–100 μg) was used as control in each experiment. Blots were blocked with 5% nonfat dry milk dissolved in TBST buffer (10 mM Tris, 150 mM NaCl, and 0.1% Tween 20) for 1 h at room temperature, washed three times with TBST, incubated with the primary antibody overnight at 4°C, and washed again with TBST, and the membrane was incubated with horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. The proteins were visualized using a chemiluminescent substrate (SuperSignal West Pico; Pierce Biotechnology). Mouse kidney and brain were lysed, and the soluble cell extracts (300 μg of protein) were incubated in the presence of primary antibodies anti-Slo1VEDEC (16), anti-β4 (Alomone, Jerusalem, Israel), or IgG (1–2 μg) for 4 h at 4°C, followed by the addition of 20 μl of protein A/G-agarose (12 h at 4°C with gentle rotation. Pellets were washed and boiled in sample buffer, separated on 10% SDS-PAGE, and analyzed by immunoblot as described above. Anti-nephin and anti-NEPH1 used in immunoblot assays were obtained from Santa Cruz Biotechnology.

Confocal microscopy. For immunofluorescent labeling, HEK-293T cells were transfected with Myc-tagged Slo1VEDEC, GFP-nephin-CT2, or full-length nephin. These cells, or differentiated podocytes, were fixed in 4% paraformaldehyde, blocked, permeabilized in PBST, and exposed to the anti-mSlo1 (NeuroMAB, Davis, CA) and anti-nephin (Santa Cruz Biotechnology). To examine the role of nephin in surface expression of BKCa channels, HEK-293T cells were transiently transfected with NH2-terminal Myc-tagged Slo1VEDEC or Slo1GPERL constructs, either alone or together with constructs encoding full-length nephin as described above. Cells were subsequently exposed to fluorescein-conjugated goat anti-Myc (1:500; Abcam, Cambridge, MA) in Opti-MEM medium for 1 h at 37°C to label surface Slo1 channels. Cells were then washed in PBS, fixed by 30 min of exposure to 4% paraformaldehyde in PBS, rinsed in PBS, blocked with 10% normal goat serum, and then permeabilized in PBS containing 0.5% Triton X-100. They were then incubated with mouse anti-Myc antibody (1:1,000; antibody 9B11) for 1 h and exposed to Alexa568-conjugated anti-mouse IgG (1:1,000; Molecular Probes) for 1 h to label intracellular Slo1 channels. The cells were then rinsed in PBS and mounted using Vectashield (Vector Laboratories, Burlingame, CA). Control samples were treated with species-appropriate IgG instead of primary antibody. All images were collected on an Olympus FV-1,000 inverted stage confocal microscope using a Plan Apo N ×60 1.42-NA oil-immersion objective. Green fluorescence (from FITC) was evoked using an excitation wavelength of 495 nm while monitoring emission at 519 nm. Red fluorescence (from Alexa568) was evoked by excitation at 580 nm, and emission was monitored at 620 nm.

RESULTS

Nephin emerged in a yeast two-hybrid screen of a neuronal (chick ciliary ganglion) transcriptome that was probed with a bait derived from the COOH-terminal domain (amino acids 175–200) of the chicken BKCa channel β1-subunit (15). Because yeast two-hybrid screens can yield false positives, we confirmed that nephin binds to Slo1 channel subunits by several independent experimental approaches. Initially, we observed that nephin could be detected in immunoprecipitates of mouse kidney and mouse brain that were prepared using commercially available antibodies against either Slo1 or the β4-subunits of BKCa channels (Fig. 1A). We chose to use anti-β4 for this experiment because nephin is a podocyte protein, and β4-subunits are expressed in both podocytes (29) and brain (1). This result suggests that nephin can interact with endogenously expressed BKCa complexes in multiple tissues. However, the Slo1 antibody used in those experiments does not discriminate between different Slo1 COOH-terminal splice variants. Therefore, we carried out coimmunoprecipitation experiments in HEK-293T cells transiently overexpressing nephin together with one of three different Slo1 splice variants: Slo1VEDEC, Slo1GPERL, or Slo1EMVYR. It was previously reported that these Slo1 isoforms diverge at the extreme COOH terminus and exhibit markedly different patterns of intracellular-
lar trafficking (15, 16, 26). These expression constructs encode full-length Slo1 channels with an NH2-terminal (ectofacial) Myc tag, which allows us to use the same antibody to detect the different splice variants in a variety of experimental conditions. In these experiments, immunoprecipitation was carried out with anti-nephrin, and Slo1 interactions were detected by probing the resulting samples with an antibody against the Myc tags. We obtained a much more intense signal from HEK-293T cells expressing Slo1VEDEC channels than from cells expressing either Slo1QEERL (which could not be detected in immunoprecipitates) or Slo1EMVYR (which was only present at trace levels) (Fig. 1B, top). Together with the data on native tissues, this result shows that Slo1-nephrin immunoprecipitation can be detected regardless of which protein is used for the initial precipitation, and it can be seen with multiple antibodies. In control experiments we observed that the Myc-tagged Slo1 channels and nephrin were expressed at comparable levels in the HEK-293T cells (Fig. 1B, bottom), and therefore, the differences in the amount of channel protein that coimmunoprecipitated with anti-nephrin probably reflect differential affinity of various Slo1 isoforms for nephrin. This was addressed more directly by means of GST pull-down assays. To perform these assays, we prepared a series of GST-Slo1 fusion proteins that contained the unique COOH-terminal portions of Slo1VEDEC, Slo1QEERL, and Slo1EMVYR, as described elsewhere (17), and used them to probe lysates of HEK-293T cells overexpressing nephrin. We observed that the GST-Slo1VEDEC fusion protein was able to pull nephrin out of the lysates, whereas GST, GST-Slo1QEERL, and GST-Slo1EMVYR were ineffective (Fig. 1C). Thus the Slo1-nephrin interaction appears to be isoform specific. In addition, we observed using confocal microscopy that endogenous Slo1 channels and nephrin were extensively

Fig. 1. Nephrin binds to large-conductance Ca2⁺-activated K⁺ (BKCa) channel subunits. A: coimmunoprecipitation of nephrin observed by immunoblot analysis after immunoprecipitation using antibodies against Slo1 or the β4-subunit of BKCa channels from mouse kidney or mouse brain, as indicated. The “input” lane contained a diluted sample of cell extract used to illustrate electrophoretic mobility; it was not designed for quantification of signal intensity. B: coimmunoprecipitation of Slo1 observed by immunoblot analysis after immunoprecipitation with an antibody against nephrin (top). This procedure was carried out on HEK-293T cells transiently expressing Myc-tagged isoforms Slo1VEDEC, Slo1QEERL, or Slo1EMVYR, as indicated, and channels were detected using anti-Myc. Note that a strong signal was only detected from cells expressing the Slo1VEDEC isoform. Interaction with Slo1EMVYR was detectable but much weaker. IB analysis shows that the various Slo1 variants (middle) and nephrin (bottom) were expressed at comparable levels throughout the assay. C: glutathione S-transferase (GST) pull-down assay showing that a GST fusion protein containing the COOH-terminal sequences of Slo1VEDEC could bind to nephrin, whereas GST and a GST fusion protein containing the COOH-terminal sequences of Slo1QEERL did not. The interaction with Slo1EMVYR with nephrin was very weak. D: confocal microscopy showing colocalization of endogenous Slo1 and nephrin in differentiated cells of a podocyte cell line (top) and in HEK-293T cells transiently expressing nephrin and Slo1VEDEC (bottom). Middle, control images from podocytes in which the primary antibody was a species-appropriate IgG. E: immunoblot using the same commercial antibody against nephrin on extracts of differentiated and undifferentiated cells from a podocyte cell line. IB, immunoblot; IP, immunoprecipitation.
colocalized in differentiated cells of an immortalized podocyte cell line (Fig. 1D, top), as well as in HEK-293T cells coexpressing nephrin and Slo1VEDEC (Fig. 1D, bottom). The characteristics of the nephrin antibody are shown by immunoblot analysis of differentiated and undifferentiated cells of the podocyte cell line (Fig. 1E).

Is the nephrin-Slo1 interaction functionally significant? To address this question, we transiently expressed Slo1 channels in HEK-293T cells in the presence or absence of nephrin and examined the resulting distribution of Slo1 channels using confocal microscopy (Fig. 2A) and cell surface biotinylation assays (Fig. 2, B and C). We previously established that nontransfected HEK-293T cells do not express detectable levels of endogenous nephrin (data not shown). Before the use of confocal microscopy, Slo1 channels were labeled with a fluorescein-conjugated anti-Myc antibody. The cells were then fixed, permeabilized, and labeled with a nonconjugated anti-Myc raised in a different species, which allowed us to obtain a distinct fluorescence signal from tagged Slo1 channels in intracellular pools. Note that we used identical processing procedures, Myc antibodies, and laser excitation intensities to determine the distribution of the Slo1 isoforms. As with our previous studies (15, 16, 17), we observed that Slo1VEDEC channels expressed by themselves in HEK-293T cells were almost exclusively in intracellular locations (red fluorescence) and exhibited comparatively low constitutive expression on the plasma membrane (green fluorescence). However, enhanced surface expression was observed in HEK-293T cells transiently coexpressing nephrin with Slo1VEDEC (Fig. 2A). Nephrin coexpression had no obvious effect on the distribution of fluorescence in HEK-293T cells expressing either the Slo1QEERL or Slo1EMVYR isoforms (data not shown).

A similar pattern was observed using cell surface biotinylation assays (Fig. 2B). We observed that biotinylated Slo1VEDEC channels on the cell surface represent a small fraction of the total signal, especially compared with what is observed in HEK-293T cells expressing either Slo1QEERL or Slo1EMVYR. However, coexpression of nephrin caused a large increase in the relative surface expression of Slo1VEDEC such that its steady-state expression on the surface was indistinguishable from that of the other isoforms. Coexpression of nephrin had no discernible effect on the surface expression of Slo1QEERL or Slo1EMVYR. These were consistent observations, as can be seen by the results of densitometric quantification of three repetitions of this experiment (Fig. 2C).

Nephrin-induced stimulation of Slo1VEDEC surface expression could also be observed using whole cell recordings from HEK-293T cells. In these experiments, we examined whole cell currents using methods described previously (15, 16). The recording pipettes contained 5 μM free Ca2+ in a solution buffered with H-EDTA to allow activation of BKCa channels by step pulses from a holding potential of -60 mV, and the concentrations of permeant ions were reduced to keep current amplitudes in a range that did not saturate our recording amplifier. Currents evoked by voltage steps to +80 mV were used for quantification of surface expression. We have previously shown that these currents are not detectable in nontransfected HEK 293T cells or when cells overexpressing Slo1 are examined using recording electrodes filled with Ca2+-free solutions and EGTA buffer. We observed that HEK-293T cells expressing nephrin-Slo1VEDEC increases steady-state expression on the surface of HEK-293T cells. A: confocal immunofluorescence using antibodies against the Myc tags of transiently expressed Slo1VEDEC channels. Cell surface channels in cells expressing Myc-tagged Slo1VEDEC were labeled with an FITC-conjugated goat anti-Myc applied to intact cells (green). After fixation and permeabilization, intracellular channels were stained using a mouse anti-Myc revealed using Alexa568-conjugated anti-mouse IgG (red). Identical laser excitation intensities and detection sensitivities were used for image collection from all of these samples. B: representative cell surface biotinylation assay shows that nephrin coexpression caused marked increase in surface expression of Slo1VEDEC but had no effect on Slo1QEERL or Slo1EMVYR, which had high levels of constitutive surface expression even in the absence of nephrin. C: densitometric quantification of 3 repetitions of the experiment shown in B. Data are means ± SE of relative Slo1 expression in the absence (C) or presence (N) of nephrin.
expressing Slo1\textsubscript{VEDEC} had much smaller macroscopic outward currents than cells expressing Slo1\textsubscript{QEERL} or Slo1\textsubscript{EMVYR} (Fig. 3A, top), consistent with previous reports (16, 26). However, these differences were not seen in cells coexpressing nephrin with the channel constructs, which can be attributed to a fivefold increase in mean currents observed in cells expressing Slo1\textsubscript{VEDEC}. Coexpression of nephrin had no effect on the mean current amplitudes in cells expressing Slo1\textsubscript{QEERL} or Slo1\textsubscript{EMVYR} (Fig. 3B). Moreover, nephrin coexpression had no discernible effect on the activation kinetics or voltage dependence measured in HEK-293T cells expressing any of the Slo1 splice variants (Fig. 4). This can be seen when amplitude-normalized traces are superimposed (Fig. 4A), and data from groups of cells are summarized in Table 1. These were obtained by fitting the rising phase of macroscopic currents with a single exponential, as described previously (15), and comparing the resulting time constants. Coexpression of nephrin had no significant effect on the mean time constant for any of the three Slo1 splice variants. Conductance-voltage curves show that nephrin had no effect on the voltage-dependence of Slo1\textsubscript{VEDEC} activation (Fig. 4B). Nephrin coexpression caused an increase in mean current at all membrane potentials in cells expressing Slo1\textsubscript{VEDEC}. Collectively, these data suggest that nephrin can stimulate the steady-state surface expression of Slo1\textsubscript{VEDEC} channels on the cell surface.

That hypothesis predicts that knocking down endogenous nephrin expression should reduce surface expression of Slo1\textsubscript{VEDEC} channels in cells that endogenously express that isoform. Differentiated cells derived from a conditionally immortalized podocyte cell line (32) are a useful model to test that prediction, since we observed that the Slo1\textsubscript{VEDEC} and Slo1\textsubscript{QEERL} isoforms were expressed in a differentiated podocyte cell line by using immunoblot analysis (Fig. 5A) and confocal microscopy (Fig. 5B). The isoform-specific antibodies used for this analysis were described previously (16). Antibodies selective for Slo1\textsubscript{EMVYR} are not available. In addition, we observed that voltage-evoked outward currents with the characteristics of Slo1 channels could be quantified in whole cell recordings from differentiated podocytes. In these experiments, the recording pipettes contained 5 mM free Ca\textsuperscript{2+} in a solution buffered with H-EDTA, K\textsuperscript{+} gradients were physiological, and the resulting voltage-evoked outward currents were partially inhibited by iberiotoxin and completely blocked by paxilline (Fig. 5C). These outward currents could not be detected when

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**Fig. 3.** Effects of nephrin coexpression on whole cell currents recorded from HEK-293T cells expressing various Slo1 splice variants. Recording electrodes contained 5 mM free Ca\textsuperscript{2+} to allow for activation of BK\textsubscript{Ca} channels by families of depolarizing voltage steps (shown in A) from a holding potential of −60 mV. A: representative traces illustrating a marked increase of currents in cells coexpressing nephrin with Slo1\textsubscript{VEDEC}. Nephrin had no effect in cells expressing the other isoforms, which exhibited large currents even in the absence nephrin. B: quantification of results from many cells. Data are means ± SE (n > 15 cells in each group). The only significant effect of nephrin was in cells expressing Slo1\textsubscript{VEDEC} (P < 0.05).
the recordings were made with electrodes containing Ca\(^{2+}\)-free solutions (data not shown). These data indicate that all of the voltage-evoked outward current under our experimental conditions is carried by Slo1 channels. The activation kinetics of these macroscopic currents are quite slow, at least compared with what is typically seen in neuronal preparations (2) or in HEK-293T cells expressing Slo1 variants recorded under similar conditions (Fig. 4), and this observation is consistent with earlier observations made in inside-out patches (29). The slow activation kinetics and the incomplete blockade by iberiotoxin may reflect the presence of the \(b_{4}\)-subunit in podocytes (1, 29, 49). In any case, it is clear that Slo1\(_{VEDEC}\) and nephrin are both endogenously expressed in podocytes, which are therefore a useful cell line for testing the functional significance of the nephrin interaction.

To test the hypothesis more directly, we used siRNA to knock down nephrin expression in differentiated podocytes. The effectiveness of the knockdown strategy was ascertained using immunoblot analysis, which showed that nephrin expression was substantially reduced in podocytes treated with control siRNA but not in cells treated with nephrin siRNA but not in cells treated with control siRNA (Fig. 6A). Total expression of a closely related protein, NEPH1, was not altered by these procedures. We observed using cell surface biotinylation assays that surface expression of Slo1 was markedly attenuated in podocytes with reduced nephrin expression (Fig. 6B), and macroscopic outward currents were reduced by an order of magnitude (Fig. 6, C and D). These data therefore demonstrate that nephrin is required for the normal steady-state expression of functional BK\(_{Ca}\) channels and Slo1 subunits on the surface of differentiated cells in a conditionally immortalized cell line derived from podocytes.

One question that emerged from these results pertains to the portions of nephrin that bind to the COOH-terminal domains of Slo1\(_{VEDEC}\) and that stimulate its expression on the cell surface. Nephrin is a 180-kDa transmembrane protein that has structural features in common with many other adhesion molecules (14). The extracellular portion contains eight Ig-like motifs and one type III-fibronectin domain that are involved in cell adhesion and formation of the glomerular slit diaphragm (39), whereas the cytoplasmic COOH terminus appears to play a role in cell signaling (10). One could be concerned that the stickiness of the extracellular domains in some way caused an artifactual alteration in Slo1\(_{VEDEC}\) trafficking. To address this, we prepared a series of GST fusion-proteins that comprise portions of nephrin, including each of the extracellular Ig-like and fibronectin domains, as well as two portions of the COOH terminus, and we used these in pull-down assays to determine and identify regions that bind to Slo1 (Fig. 7). We observed that only the fusion protein containing the most distal COOH-terminal portions of nephrin (nephrin-CT2, composed of R1160–V1241) was able to bind to Slo1 in this assay. Interestingly, this relatively small portion of nephrin was biologically active by itself, and its coexpression was sufficient to cause a significant increase in Slo1 on the cell surface (Fig. 8), although it was not as effective as full-length nephrin (data not shown). Specifically, we observed that a GFP-nephrin-CT2 fusion protein colocalized with Slo1\(_{VEDEC}\) when they were coexpressed in HEK-293T cells (Fig. 8A). The signal appeared especially concentrated in paranuclear regions. Nephrin-CT2 coexpression in HEK-293T cells caused an increase in cell

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**Table 1. Nephrin coexpression and voltage dependence in HEK-293T cells expressing Slo1 splice variants**

<table>
<thead>
<tr>
<th>Splice Variant</th>
<th>Activation Time Constant, ms</th>
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<tbody>
<tr>
<td>Slo1(_{VEDEC})</td>
<td>Control: 6.41 ± 0.31</td>
</tr>
<tr>
<td>Slo1(_{QEERL})</td>
<td>7.63 ± 0.46</td>
</tr>
<tr>
<td>Slo1(_{EMVYR})</td>
<td>6.25 ± 0.54</td>
</tr>
</tbody>
</table>

Coexpression of nephrin did not affect the activation kinetics of currents evoked by a depolarizing step pulse in HEK-293T cells expressing various Slo1 splice variants. Time constants were calculated from single-exponential fits to the rising phase of currents evoked by a depolarizing step to +80 mV from a holding potential of −60 mV. Examples of the types of current traces used for these analyses are shown in Fig. 4A. Data are means ± SE of the time constant derived from 10 cells in each group. None of these data are statistically significant at the 0.05 level as determined using Student’s unpaired t-test.
surface expression of Slo1_{VEDEC} as measured using cell surface biotinylation assays (Fig. 8B) and whole cell recordings (Fig. 8C).

**DISCUSSION**

In the present study we have shown that one of the COOH-terminal variants of BK_{Ca} channels encoded by the Slo1 gene binds to nephrin, a scaffolding and adhesion molecule expressed in podocytes and the brain. This interaction, which occurs at the COOH termini of both proteins, promotes the steady-state surface expression the Slo1_{VEDEC} isoform in a podocyte cell line and in a heterologous expression system. However, coexpression of nephrin does not appear to have significant effects on the gating properties of BK_{Ca} channels, and nephrin does not appear to bind to or alter the functional properties of the Slo1_{QEERL} variant. However, nephrin interacts with the β4-subunit of BK_{Ca} channels, which is expressed in podocytes as well as in the nervous system (29, 49).

Nephrin appears to have multiple functions in podocytes. The extracellular portions are composed of immunoglobulin- and fibronectin-like domains that contribute to the slit diaphragm and that promote adhesive interactions between the podocyte foot processes and the underlying capillary basement membrane (34, 51). The intracellular domains are much smaller but provide a rich substrate for interactions with many other proteins, including podocin (43), ZO-1 (20), P-cadherin (40), Neph1 (23), and synaptopodin (32), as well as adaptor proteins such as CD2-associated protein and Nck that tie the nephrin complex to the underlying cytoskeleton (13). Importantly, nephrin interactions with proteins such as podocin and CD2AP (9) cause recruitment of signaling proteins such as phosphoinositide 3-OH kinase (PI3K) to the plasma membrane, resulting in stimulation of PI3K-dependent Akt signaling in podocytes (10). Nephrin also has been reported to interact with TRPC6, a Ca^{2+}-permeable stretch-sensitive cation channel known to play a role in regulation of glomerular filtration (37).

Because of these interactions, and based on precedents derived from other adhesion molecules (25), nephrin has been suggested to play a role in outside-in signaling, a process by
which mechanical stimuli transduced through the nephrin ectodomains lead to changes in the status of cytosolic and plasma membrane proteins (46). Nephrin interacts with subja-
cent actin filaments of podocyte foot processes via adapter
proteins such as CD2AP (44) and Nck (13). In this way,
mechanical stimuli delivered to nephrin ectodomains could be
transduced to underlying actin filaments. The gating of BKCa
channels in podocytes and several other cell types is stretch
sensitive (29), a process that is mediated by interactions of the
channels with actin-binding proteins (45) and possibly by
direct interactions between Slo1 subunits and actin microfila-
ments (52). In addition, the ability of nephrin to organize a
variety of signaling molecules could provide a mechanism to
organize changes in BKCa gating in response hormones, cyto-
kines, or second messengers. In this regard, we have previously
demonstrated that PI3K and Akt, which can be recruited to
nephrin cytosolic domains (10), are essential for regulated
trafficking of BKCa to the plasma membrane in neurons (3, 22).
The physiological impact of BKCa activation is para-
doxically likely to be an increase in net Ca2+ influx, at least in
podocytes. These cells are electrically compact, so activation
of a relatively small number of BKCa channels would cause

Fig. 6. Nephrin is required for normal surface expression of BKCa channels in differentiated cells of a
podocyte cell line. A: immunoblot analysis showing that treatment with a small interfering (si)RNA directed
against nephrin markedly reduced expression of nephrin but had no effect on total expression of NEPH1 in
podocytes. A control siRNA had no effect. B: application of nephrin siRNA reduced surface expression of
Slo1 channels as determined by cell surface biotinylation assay. Representative blots are shown at top, and
densitometric analyses of 3 repetitions of this experiment are shown at bottom. C: representative traces
illustrating that nephrin siRNA caused marked reduction in BKCa currents measured using whole cell recor-
ddings as Fig. 5. D: means ± SE of BKCa currents (n > 15 cells per group). The means are significantly
different (P < 0.05), as determined using Student’s unpaired t-test.

Fig. 7. Evidence that the distal COOH-terminal portion of nephrin binds to Slo1VEDEC. Composition of a series GST-fusion proteins
is indicated above a representative GST pull-down assay showing binding of the nephrin-CT2 construct (composed of residues R1160–
V1241) to Slo1VEDEC. The assay was carried out on lysates of HEK-293T cells expressing this Slo1 splice variant.
marked cell hyperpolarization. One of the key sources of Ca\(^{2+}\) influx in podocytes is through activated TRPC6 channels (37). TRPC6 channels have an unusual and physiologically important biophysical property: their permeability to Ca\(^{2+}\)/relative to Na\(^+\) (\(P_{Na}/P_{Ca}\)) is highly voltage dependent. Thus, at more negative membrane potentials, they are permeable to Ca\(^{2+}\), but moderate depolarization causes them to exclude divalent cations due to a combination of pore blockade and a reduction in driving force (6). Because of this, active TRPC6 channels can be a direct source of steady Ca\(^{2+}\) influx, but only if there is a mechanism in place to limit the amount of depolarization that occurs as a result of their own activation. BKCa channels are ideally suited to counteract TRPC6-mediated depolarization because their gating is Ca\(^{2+}\)-sensitive and because the presence of \(\beta_4\)-subunits will confer a steeper voltage dependence of activation (47). Moreover, TRPC6 channels also bind to nephrin (37), suggesting the existence of a multichannel complex built around nephrin scaffolds in which a subset of BKCa channels provides positive feedback to Ca\(^{2+}\) influx through TRPC6. This arrangement could also provide a mechanism to couple the mechanosensitivity and/or chemosensitivity of BKCa channels to local changes in Ca\(^{2+}\) dynamics. This in turn could trigger changes in the structure of podocyte foot processes and their cytoskeletal elements (11) to allow filtration pathways to adapt to changes in hydrostatic pressure. Note that podocytes also express a splice variant of Slo1 that does not interact with nephrin, and these channels could be targeted to other portions of the plasma membrane in such a way as to allow them to be regulated independently of the ones that bind to nephrin.

The mechanism whereby nephrin controls BKCa trafficking in podocytes or other cell types is not known. However, nephrin was recently shown to be necessary for the translocation of an insulin-sensitive GLUT4 glucose transporter to the plasma membrane of podocytes (4). Those workers also noted that nephrin can bind to a vesicle-associated soluble N-ethylmaleimide-sensitive factor attachment protein receptor (V-SNARE) that is essential for docking and fusion of exocytotic vesicles in a host of cell types. Therefore, it is possible that nephrin plays a more general role in regulation of vesicle trafficking in podocytes. One possibility is that interactions between synaptobrevin and nephrin allow the vesicle to dock close to the plasma membrane; in other words, nephrin could function analogously to a plasma membrane SNARE protein to regulate vesicle trafficking in cells where it is expressed. Although we cannot exclude this hypothesis, it is worth noting that a construct containing only the distal COOH-terminal residues of nephrin is able to cause a statistically significant (\(P < 0.05\)) increase in mean whole cell currents in cells expressing Slo1VEDEC. Control cells expressed GFP.

Fig. 8. Coexpression of a green fluorescent protein (GFP) fusion protein containing only the distal COOH-terminal residues of nephrin (GFP-nephrin-CT2) causes an increase in surface expression of Slo1VEDEC in HEK-293T cells but is not as effective as full-length nephrin. A: colocalization of the GFP-nephrin-CT2 with Myc-tagged Slo1VEDEC (red) as revealed using confocal microscopy. Note expression of both proteins on the cell surface and accumulation in perinuclear regions. B: coexpression of GFP-nephrin-CT2 caused a marked increase in steady-state surface expression of Slo1VEDEC as determined using cell surface biotinylation assays. Representative blots (top) and quantitative densitometric analysis of 3 repetitions of this experiment (bottom) are shown. Control cells expressed GFP. C: coexpression of GFP-nephrin-CT2 caused a statistically significant (\(P < 0.05\)) increase in mean whole cell currents in cells expressing Slo1VEDEC. Control cells coexpressed GFP.
What is the role of nephrin in neuronal function? Our initial yeast two-hybrid screen was carried out on a library representing a neuronal transcriptome, and we have confirmed earlier reports that nephrin is expressed in neurons (36), as are Slo1<sub>VEDEC</sub> channels (16). Therefore, it is reasonable to propose that nephrin plays a role in trafficking of neuronal Slo1 channels, and possibly other membrane proteins, similar to the one it plays in podocytes. In this regard, nephrin can stimulate steady-state expression of Slo1<sub>VEDEC</sub> on the surface of HEK-293T cells, which do not endogenously express other podocyte proteins, suggesting that nephrin can promote trafficking to the plasma membrane in a variety of cellular contexts. Moreover, it is notable that ~10% of Finnish patients bearing nephrin mutations also exhibit congenital neurological symptoms, especially ataxia, athetosis, and hearing defects (19). In many cases, these problems did not resolve after kidney transplantation and did not appear related to the previous nephrotic symptoms. Although these neurological problems could reflect a role for nephrin in regulation of axon guidance or fasciculation (28), it is striking that mice lacking Slo1 proteins or functional BK<sub>Ca</sub> channels also show marked ataxia and alterations in Purkinje neuron firing (42), as well as a progressive loss of hearing (41). This raises the possibility that some of the extrarenal effects of nephrin deficiencies could be related to abnormalities in the function of a subset of BK<sub>Ca</sub> channels.

In summary, we have shown that one of the COOH-terminal splice variants of Slo1 expressed in podocytes binds to cytosolic domains of nephrin. These interactions regulate the steady-state expression of Slo1 proteins on the cell surface of podocytes and possibly in other cell types where nephrin or related molecules are expressed.

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REFERENCES


