PDZ domain-mediated interaction of rabbit podocalyxin and Na\(^+/\)H\(^+\) exchange regulatory factor-2

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PODOCALYXIN IS A SULFATED sialoglycoprotein expressed on the apical surface of the glomerular podocytes, the luminal surface of vascular endothelial cells, hematopoietic stem cells, and platelets (2, 3, 10, 14, 15). On the podocyte, podocalyxin is thought to maintain the fine interdigitating foot process structure by the charge-repulsive effects of its highly anionic extracellular domain (21). Takeda et al. (29) have recently shown direct evidence for a charge-repulsive effect of podocalyxin on the surface of cultured cells whereby expression of podocalyxin inhibits cell-cell adhesion. In addition, expression of podocalyxin in Madin-Darby canine kidney (MDCK) cell monolayers decreases transepithelial resistance (TER) and causes a redistribution of junctional proteins, suggesting that podocalyxin may interact with the actin cytoskeleton to regulate cell junctions (29).

The intracellular domain of podocalyxin is highly conserved among species, with 96% amino acid identity between human and rabbit podocalyxin (also known as rabbit podocalyxin-like protein 1) (11, 12). The COOH-terminal amino acids [Asp-Thr-His-Leu (DTHL)] of podocalyxin resemble the consensus sequence (X-S/T-V/I) for COOH-terminal motifs of proteins that interact with PSD95-Dlg-zona occludens-1 (PDZ-1) protein interaction domains (25). Proteins that contain PDZ domains can act to link transmembrane proteins in multiprotein complexes that may include regulatory enzymes and the actin cytoskeleton. The interaction of transmembrane proteins with PDZ domain-containing proteins has been shown to be important in the localization, function, and regulation of transmembrane proteins (4, 23). To define the protein-protein interactions of the intracellular domain of podocalyxin, a podocalyxin COOH-terminal peptide probe was used to screen a rabbit glomerular cDNA phage library and clones coding for a PDZ domain-containing protein, sodium hydrogen exchange regulatory factor-2 (NHERF-2), were obtained. This study characterizes the interaction of podocalyxin with NHERF-2.

EXPERIMENTAL PROCEDURES

Peptides and primary antibodies. Peptides corresponding to the COOH-terminal 20 amino acid residues of human nephrin (GDLTLPEDSLPLFELRGLHV), rabbit podocalyxin (WIVPLDLTKDLDDEEDTHL), or a mutant podocalyxin missing the COOH-terminal leucine (WIVPLDLTKDDLDEEDTH), were synthesized at the University of Michigan.

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gan Peptide Synthesis Facility (nephrin peptide) or the W. M. Keck Foundation Biotechnology Resource Laboratory at Yale University (podocalyxin peptides). Peptides were coupled to an NH2-terminal biotin and purified by high-pressure liquid chromatography.

Chickens were immunized with a purified NHERF-2 glutathione S-transferase (GST) fusion protein containing amino acids 53–316 using the custom antibody service of Lampire Biological Laboratories. Chicken IgY was purified from egg yolks with the EGGstract IgY purification system (Promega). IgY was affinity purified using full-length NHERF-2 fusion protein covalently linked to cyanogen bromo mide beads (Sigma). Rabbit serum against human NHERF-2, which recognizes rabbit NHERF-2, was kindly provided by C. Chris Yun (34). Mouse anti-podocalyxin monoclonal antibody (Mab) 4B3 and affinity-purified rabbit anti-mLin-7 have been previously described (12, 27). Anti-Myc Mab (clone 9E10) was used as a control Mab. Anti-uvomorulin (E-cadherin) antibody was from Sigma.

**Screening of phage libraries.** A phage library made with purified poly-A mRNA from isolated rabbit glomeruli was prepared for expression screening at 20,000–40,000 plaque-forming units per plate as described previously (12). Nitrocellulose filters were treated with isopropyl-β-d-thiogalactopyranoside and incubated on top of the plates at 37°C for 4 h. Filters were washed five times in PBS with 0.1% Triton X-100, blocked for 1 h in 2% bovine serum albumin in PBS. Biotinylated probe was prepared, and screening was performed essentially as described by Sparks et al. (26). For each filter, 25 pmol of biotinylated peptide were incubated with 1 μg of streptavidin-alkaline phosphatase (Sigma). Excess biotin-binding sites were blocked by the addition of 500 pmol d-biotin (Sigma), and the biotinylated peptide/streptavidin-alkaline phosphatase complex was incubated with the filters in fresh blocking solution overnight at 4°C. The filters were washed four times for 15 min in PBS with 0.1% Triton X-100, and plaques binding the biotinylated peptides were detected as blue spots after incubation with an alkaline phosphatase color reagent (nitroblue-tetrazolium-chloride and 5-bromo-4-chloro-3-indolyl-phosphate-toluidine salt in 0.1 M Tris-HCl, pH 9.4, 0.1 M NaCl, 50 mM MgCl2). The library was also screened with rabbit NHERF-2 clone cDNA to obtain a full-length NHERF-2 cDNA using techniques previously described (11).

**Bacterial expression and purification of fusion proteins.** GST fusion protein constructs of full-length NHERF-2 (amino acids 1–316, primers cgtagagtcatcagccggcggacggcgct/tgcatactggctcaagttgatgaaga; PDZ-1 (amino acids 1–118, primers cgtagagtcatcagccggcggacggcggt/tgcatactggctcaagttgatgaaga; PDZ-2 (amino acids 149–257, primers tgcatactggctcaagttgatgatgatgatgcaggtg) were produced by PCR amplification of full-length rabbit NHERF-2 cDNA using the primers listed containing unique restriction sites. Digested PCR products were ligated to pGEX4-T3 vector (Amersham Pharmacia Biotech), and clones were sequenced. A cDNA clone coding for amino acids 53–316 of NHERF-2 was excised from pBluescript and ligated in frame to pGEX4-T3. This construct was used to make a fusion protein for antibody production. Fusion proteins were expressed in *Escherichia coli* DH5α as described by Smith and Johnson (24). Fusion protein purification was performed as described by Guan and Dixon (6), and quantitation was done by a modified Bradford assay (Bio-Rad). A GST fusion protein containing the fourth PDZ domain of MAGI-1 was a gift from Benjamin Margolis.

**Glomerular isolation and Western and Far-Western blot analyses.** Rabbit glomeruli were isolated from New Zealand White rabbits (2.0–2.5 kg) by iron oxide magnetization as described previously (12). For glomerular extraction, 5 × 104 glomeruli were suspended in 1 ml of either PBS containing 1% Triton X-100, 0.1% SDS (PBS extract buffer), or 150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholic acid, and 0.1% SDS in 50 mM Tris (RIPA extract buffer). Glomerular extract (GE) prepared in this fashion contains ~1 μg/μl of protein. Complete protease inhibitor (Roche Molecular Biochemicals) was added to all extracts. They were then sonicated in six short bursts of 10 s each, and insoluble material was removed by centrifugation at 12,000 g. SDS sample buffer (100 mM Tris-HCl (pH 6.8), 4% SDS, 0.2% bromphenol blue, 20% glycerol with 5% β-mercaptoethanol) was added, and extracts were analyzed by SDS-PAGE and transferred to nitrocellulose (12). Secondary antibodies were a peroxidase-conjugated rabbit anti-chicken IgY (Sigma), a peroxidase-conjugated goat anti-mouse IgG (Bio-Rad), and a peroxidase-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories) for use blocking of chicken anti-NHERF-2 antibody. 50 μg of either GST fusion protein or NHERF-2 GST fusion protein were preincubated with affinity-purified chicken anti-NHERF-2 antibody (1:500) overnight at 4°C before membranes were probed. Far-Western blotting was performed by blocking the blots in Tris-buffered saline (TBS) with 1% Triton X-100 and 3% nonfat dried milk for 1 h. Blots were incubated overnight in 0.01 μg of biotinylated peptide complexed with 1 μl streptavidin-horseradish peroxidase (Life Technologies) in 10 ml TBS with 1% Triton X-100 with 0.5% bovine serum on a rocker at 4°C. After washing four times in TBS with 1% Triton X-100, they were developed using the enhanced chemiluminescence reagent (Amersham).

**Immunoprecipitation and affinity purification experiments.** Immunoprecipitations were carried out using a modification of published protocols (8, 11). One hundred twenty microliters of rabbit GE in PBS extract buffer were preabsorbed with protein G-agarose beads (Sigma). The Mabs (4B3 and control IgG, 9E10) were incubated with 50 μg of protein G-agarose beads, washed four times with TBS, and incubated overnight with the preabsorbed rabbit GE in PBS extract buffer at 4°C on a rotor. Beads were washed six times with TBS, and samples were prepared for Western blot analysis. “Pull-down” experiments were performed with peptides or fusion proteins. For biotinylated peptide experiments, 200 μl of GE in RIPA extract buffer with protease inhibitors were precleared by incubation with 5 μl of streptavidin-agarose beads (Sigma) for 30 min at 4°C. After centrifugation, the supernatant was incubated with 3 μg of biotinylated peptide at 4°C on a rocker for 1 h. Twenty microliters of streptavidin-agarose beads were added, and incubation continued for 30 min. The beads were washed three times with ice-cold PBS and prepared for Western blot analysis. Purified GST fusion proteins (10 μg) complexed with glutathione-agarose beads (Sigma) were washed in ice-cold PBS. Twenty-five microliters of GE in PBS extract buffer were added, and the beads were incubated overnight on a rocker at 4°C. Beads were washed four times with PBS and used for Western blot analysis. For peptide inhibition experiments, 10 μg of NHERF-2 GST fusion protein complexed to glutathione-agarose beads were incubated with biotinylated peptides for 30 min at 4°C on a rocker. Twenty-five microliters of GE in PBS buffer were added and incubated overnight at 4°C. Samples were processed as described above for pull-down experiments.

**Immunostaining and culture of MDCK cells.** Rabbit podocalyxin cDNA (GenBank accession no. U352399) was PCR amplified with a 3’ primer designed to result in a deletion of...
the four COOH-terminal amino acids (DTHL) or mutate the terminal leucine to glycine (tttagctttagctgtagcgag and tttagctttagctgtagcgag) and a 5′ primer (tttagctttagctttagcgag) and a 5′ primer (tttagctttagctttagcgag), PCR products were subcloned and sequenced. The mutant intracellular domain region was excised with HinClII and XhoI and inserted into a rabbit podocalyxin construct digested with HindIII and XhoI to give a full-length mutant podocalyxin cDNA. Constructs were placed into pcDNA3 (Invitrogen) and sequenced to ensure that only the designed mutation was present and used to transfect MDCK cells with the Fugene reagent (Roche Molecular Biochemicals). Cells were grown in the presence of 800 µg/ml G418 (Life Technologies), and clones of stable transfectants were isolated by limiting dilution in 96-well plates.

Transfected MDCK cells were seeded at high density (10^5 cells/well) onto Transwell Clear membrane filters (0.4 µm pore size; Corning Costar, Cambridge, MA). The cells were allowed to grow to confluence to form a polarized monolayer. After being washed with PBS, the cells were fixed with 4% formaldehyde in PBS and were permeabilized with 0.1% Triton X-100 in PBS. After being blocked for 1 h with 10% goat serum in PBS, the cells were incubated with primary antibodies diluted in 2% goat serum in PBS in a humidified chamber for 12–16 h (affinity-purified anti-mLin-7 at 1:100; anti-E-cadherin at 1:1,000; and anti-podocalyxin antibody 4B3 at 1:2). After being washed three times with 2% goat serum in PBS, the cells were incubated with appropriate secondary antibodies coupled to FITC (Jackson ImmunoResearch Laboratories, or Texas red (diluted at 1:500 in 2% goat serum in PBS; Molecular Probes, Eugene, OR) for 2 h in a humidified chamber. Membrane filters were cut from their plastic casing with a scalpel and mounted with ProLong antifade reagent (Molecular Probes). Confocal laser scanning microscopy was performed on a Nikon Diaphot 200 microscope paired with a Noran laser and InterVision software (Noran Instruments, Middleton, WI) at the Morphology and Image Analysis Core of the University of Michigan Diabetes Research and Training Center.

Cryostat sections (2 µm) of rabbit renal cortex were fixed in methanol and blocked in 10% goat serum in PBS. Slides were incubated with the Mab 4B3 (1:1) and rabbit anti-NHERF-2 serum (1:200) described above or controls (Mab 9E10 or serum). Secondary antibodies were Texas red-conjugated horse anti-mouse IgG at 1:400 (Vector Laboratories) and an FITC-conjugated goat anti-rabbit IgG at 1:300 (Sigma). A Nikon Diaphot microscope and a Hamamatsu digital camera were used to obtain the dual-labeled immunofluorescence images.

Measurement of transepithelial resistance and surface biotinylation of MDCK cells. MDCK cells (1.5 × 10^5 cells) were split and placed on 12-mm Transwell filters in calcium-free DMEM (Invitrogen) for 3 h. Media were changed to DMEM with calcium, and the cells were grown to confluence. The transepithelial resistance (TER) was measured (Millipore, Millipore) in culture media. The TER was calculated by subtracting the measured background TER of a blank filter and multiplying by the surface area of the filter per the manufacturer’s instructions. For biotinylation experiments, cells were selectively biotinylated at either the apical or basolateral cell surface (5, 13). Briefly, membranes were washed on both sides with PBS containing 1 mM MgCl2 and 1.3 mM CaCl2 (PBS-CM) at 4°C and then incubated with freshly dissolved sulfo-NHS-LC-biotin (500 µg/ml; Pierce, Rockford, IL) added to the apical or basolateral surface for 30 min on ice. The biotinylation reaction was quenched by extensively washing with PBS-CM containing 50 mM NH4Cl.

Filters were excised, and cells were lysed in 400 µl of RIPA extract buffer with protease inhibitors. Cell lysates contained between 0.52 and 0.63 µg/µl of protein. Two hundred microliters of the lysate were incubated overnight with 100 µl of streptavidin-agarose beads (Sigma). After centrifugation, the post bead lysate was removed. Beads were washed three times with PBS at 4°C. Proteins bound to the streptavidin-agarose beads were eluted by boiling in 80 µl of SDS sample buffer. Samples were prepared for SDS-PAGE as described above with 20 µl of lysate, streptavidin bead pull-down, or post bead lysate loaded per lane.

The stability of podocalyxin at the apical surface of MDCK cells was determined by biotinylation of the apical surface of confluent MDCK cell monolayers, followed by selective immunoprecipitation of apically expressed podocalyxin (see the time points in Fig. 8). After biotinylation was performed as outlined above, the apical surface was washed with PBS-CM, and culture media were replaced. At the times indicated, the media were removed, the apical surface was washed with PBS-CM, and 300 µl of Mab 4B3 were then placed on the apical surface for 20 min. Filters were then washed with PBS-CM to remove unbound antibody, and monolayers were excised and lysed in PBS with 0.1% Triton X-100 with Complete protease inhibitor. Lysates were incubated with 40 µl of protein G beads/well to bind the Mab 4B3/podocalyxin complexes, and immunoprecipitation was completed as described above. Samples were prepared for Western blot analysis, and detection was performed with either streptavidin-horseradish peroxidase or Mab 4B3. Densitometry was captured using an EDAS 120 system (Kodak) and analyzed with Kodak 1D software V.3.5.3. Statistical analysis of TER and densimetric data was performed by ANOVA with post hoc comparisons using the Statview software program (Abacus Concepts, Berkeley, CA).

RESULTS

Screening of a rabbit glomerular cDNA library with podocalyxin COOH-terminal peptide probe. To find potential proteins that interact with the COOH-terminal amino acids of podocalyxin, we screened a cDNA library made from isolated rabbit glomeruli. The probe for expression screening was an NH2-terminal biotinylated peptide that contained the COOH-terminal 20 amino acids of rabbit podocalyxin. Approximately 600,000 plaque-forming units were screened, and 7 positive clones were obtained and purified (Fig. 1B). All of the clones overlapped and were unique. We obtained full-length cDNA by screening this library with a rabbit NHERF-2 cDNA clone. The 1,648-bp cDNA contains a predicted 948-bp open reading frame coding for a 316-amino acid protein (GenBank accession no. AF358433). Analysis of the amino acid sequence with the SMART program predicted two PDZ domains (Fig. 1A) with 64% amino acid identity (22). This amino acid sequence showed 81% overall identity with human NHERF-2 (GenBank accession no. AAC63061) by BLAST analysis (1). NHERF-2 (also known as E3KARP) is a linker protein containing two PDZ domains and a COOH-terminal ezrin-binding domain that is known to have multiple splice isoforms (18, 23, 34). The isoform of NHERF-2 we cloned has an open reading frame of 316 amino acids and lacks amino acids 265–285 of the 337-amino acid human NHERF-2 sequence. Comparison of our amino acid sequence and...
human NHERF-2 missing amino acids 265–285 showed 93% overall identity. This is the same region that undergoes alternative splicing in SRY-interacting protein-1, a 326-amino acid splice variant of NHERF-2 (18). Notably, the common overlapping region of all the clones obtained by expression screening contained the sequence coding for the second PDZ domain of NHERF-2 and none of these clones coded for the complete first PDZ domain (Fig. 1B). Thus we conclude that our screening detected an interaction of the second PDZ domain of NHERF-2 with the podocalyxin peptide probe.

Podocalyxin peptide interacts with both PDZ domains of NHERF-2. We then produced and purified GST fusion proteins of the full-length NHERF-2, the PDZ-1 domain, and the PDZ-2 domain of NHERF-2 (Fig. 1C). These fusion proteins underwent SDS-PAGE and were transferred to nitrocellulose membranes. To determine the domain(s) of NHERF-2 with which podocalyxin interacts, biotinylated peptides were used to probe these membranes by Far-Western blot analysis (Fig. 2). The podocalyxin peptide bound to GST fusion proteins containing the first or second PDZ domain of NHERF-2, as well as binding to full-length NHERF-2. No binding was seen in lanes of a control GST fusion protein or a control GST fusion protein containing the fourth PDZ of MAGI-1 (Fig. 2A). Control biotinylated peptide showed no binding (Fig. 2C). Thus the podocalyxin peptide probe is capable of binding to either PDZ domain of NHERF-2 by Far-Western blot analysis.

The interaction of podocalyxin peptide and NHERF-2 requires the COOH-terminal leucine of podocalyxin. To test whether the interaction of podocalyxin and NHERF-2 occurs via the podocalyxin COOH-terminal DTHL motif, we probed the NHERF-2 GST fusion protein blots with a podocalyxin biotinylated peptide missing the COOH-terminal leucine. This mutant peptide was unable to bind to the NHERF-2 GST fusion protein (Fig. 2B). To determine whether the podocalyxin peptide could affinity purify NHERF-2 from rabbit glomeruli, we performed pull-down experiments of rabbit GE in RIPA extract buffer using immobilized biotinylated peptides. A rabbit polyclonal rabbit anti-human NHERF-2 was used to detect rabbit NHERF-2, which appears as a 38-kDa band on a Western blot (Fig. 3A). The podocalyxin biotinylated peptide was able to pull down NHERF-2 from GE (Fig. 3A) and bind to a band at the appropriate size of NHERF-2 on Far-Western blots (Fig. 2A). The control peptide and the podocalyxin peptide missing the COOH-terminal leucine did not pull down NHERF-2 or bind to any proteins on Far-Western blots (Figs. 2, B and C, and
The results of the experiments shown in Figs. 2 and 3 indicate that the COOH terminus of podocalyxin can bind to both PDZ domains of NHERF-2 in vitro and that the interaction of NHERF-2 and podocalyxin requires the COOH-terminal leucine of podocalyxin.

**Podocalyxin interacts with NHERF-2 in vivo.** To determine whether the interaction of podocalyxin and NHERF-2 occurs in vivo, we performed immunoprecipitation experiments. The anti-podocalyxin MAb 4B3 was used to immunoprecipitate podocalyxin from GE in PBS extract buffer (Fig. 3B). To detect coprecipitated NHERF-2, we used a chicken IgY against amino acids 53–316 of rabbit NHERF that binds to a 38-kDa NHERF-2 band on Western blots of GE and that can be blocked by incubation with NHERF-2 GST fusion protein (Fig. 3C). MAb 4B3 (Fig. 3B, top) coprecipitated NHERF-2 with podocalyxin from rabbit GE. No NHERF-2 was coprecipitated with the control MAb. The coprecipitation of these proteins from GE strongly supports the suggestion that podocalyxin and NHERF-2 interact in vivo.

**Affinity purification of podocalyxin from glomerular extract with NHERF-2 GST fusion proteins and inhibition of the interaction of NHERF-2 and podocalyxin by podocalyxin COOH-terminal peptide.** The experiments shown thus far demonstrate that both PDZ-1 and PDZ-2 can interact with a podocalyxin biotinylated peptide and that podocalyxin and NHERF-2 interact in glomerular extracts. To test whether the PDZ domains of NHERF-2 can independently interact with podocalyxin protein produced in vivo, we performed pull-down experiments of rabbit GE in PBS extract buffer with GST fusion proteins. The GST fusion proteins were bound to glutathione-agarose beads and incubated with GE overnight. After the beads were washed, samples were prepared for Western blot analysis with MAb 4B3 to detect podocalyxin pull down by the fusion proteins. Both the PDZ-1 and PDZ-2 GST fusion proteins independently pulled down podocalyxin from GE (Fig. 4A). In these experiments, the PDZ-1 fusion protein was able to pull down podocalyxin slightly more efficiently than the PDZ-2 fusion protein. The most efficient pull down was seen with the full-length NHERF-2 fusion protein. To show that the interaction of the NHERF-2 fusion protein with podocalyxin was dependent on the COOH terminus of podocalyxin, we used podocalyxin peptides to try to block the interaction. Podocalyxin peptide was able to inhibit the interaction of podocalyxin with full-length NHERF-2 fusion protein (Fig. 4B). The mutant podocalyxin peptide missing the COOH-terminal leucine showed minimal ability to block the interaction of NHERF-2 fusion protein with podocalyxin (Fig. 4B), consistent with the results by Far-Western blot analysis and pull-down experiments (Figs. 2B and 3A).
Figure 4. Interaction of podocalyxin and NHERF-2 GST fusion proteins and inhibition of this interaction with podocalyxin COOH-terminal peptides. A: GE in PBS extract buffer was incubated with the GST-fusion proteins (10 μg each) immobilized on glutathione-agarose beads. After being washed, samples were prepared for Western blotting under reduced conditions. The NHERF-2 GST fusion proteins used are shown in Fig. 1. Control fusion proteins include GST alone (GST) and a GST fusion protein containing the fourth PDZ domain of MAGI-1 (GST-PDZ). MAb 4B3 binds to podocalyxin in the GE lane and podocalyxin is pulled down by PDZ-1, PDZ-2, and NHERF-2 GST fusion proteins. No signal is seen in lanes with control fusion proteins or in blots with control antibodies. B: NHERF-2 fusion protein was incubated with podocalyxin COOH-terminal peptide (B-PODO) or podocalyxin COOH-terminal peptide missing the terminal leucine (B-PODO-L) for 30 min at 4°C. GE was added and incubated overnight at 4°C with the final concentration of blocking peptide shown. After being washed, samples were prepared for Western blot analysis under reduced conditions. The podocalyxin COOH-terminal peptide inhibits the interaction of NHERF-2 GST fusion protein with podocalyxin, and this inhibition is concentration dependent. Podocalyxin peptide missing the COOH-terminal leucine does not effectively inhibit the interaction of podocalyxin with NHERF-2 at the highest concentration used.

Colocalization of NHERF-2 and podocalyxin in the glomerulus in vivo. In the kidney, podocalyxin is expressed on the apical surface of the podocytes and to a lesser extent on the luminal surface of the vascular endothelium. The isolated rabbit glomeruli used to make the cDNA library that was screened and the glomerular extracts used in the experiments above had some contamination with renal tubular elements. To determine whether NHERF-2 expression in the kidney colocalizes with glomerular podocalyxin expression, dual-labeled immunofluorescence of rabbit kidney cortex with antibodies to NHERF-2 and podocalyxin was performed. Indirect immunofluorescence of rabbit kidney cortex with MAb 4B3 (Texas red-conjugated secondary antibody) showed strong staining of the glomeruli with some staining of the peritubular capillaries (Fig. 5A). The same section stained with rabbit anti-NHERF-2 serum (FITC-conjugated secondary antibody) showed strong staining of the glomeruli with some vascular staining of the interstitium (Fig. 5B), similar to that described in rat renal cortex (31). The merged image of podocalyxin (red) and NHERF-2 (green) showed intense orange staining of the peripheral capillary loops (Fig. 5C, arrows), consistent with colocalization of podocalyxin and NHERF-2 in the podocyte and in peritubular capillaries. That podocalyxin and NHERF-2 colocalize at the apical membrane of the podocyte cannot be determined at the resolution of immunofluorescent microscopy. Staining with control antibodies are shown (Fig. 5, D–F).

Role of PDZ interaction motif in apical localization of podocalyxin in MDCK cells. Podocalyxin is localized to the apical surface of the podocyte and the luminal surface of vascular endothelial cells in vivo. To determine the role of the COOH-terminal PDZ interaction motif in the membrane localization of podocalyxin, MDCK cell lines expressing podocalyxin, podocalyxin missing the COOH-terminal DTHL motif, or podocalyxin containing a COOH-terminal leucine-to-glycine mutation were made. Stable transfectants were allowed to grow on permeable membrane supports to form polarized monolayers. Confocal microscopy of MDCK cells expressing podocalyxin stained with antibody 4B3 showed intense apical labeling when these cells were colabeled with the adherens junction marker E-cadherin or the basolateral marker mLin-7 (Fig. 6, A and B). In contrast, mutant podocalyxin, either missing the COOH-terminal DTHL motif or containing a COOH-terminal leucine-to-glycine mutation, was distributed in the cytoplasm as well as the apical membrane (Fig. 6, C–F). The experiments shown are representative of two independent clones for each construct.

To confirm these results, surface biotinylation of MDCK cells was performed. MDCK cell monolayers expressing wild-type podocalyxin showed strong apical biotinylation of podocalyxin (BP in Fig. 7A) with little nonbiotinylated podocalyxin detected (NP in Fig. 7A). Dot-blot experiments have confirmed a lack of biotinylated proteins in the NP fractions (data not shown). Surface biotinylation experiments of MDCK cells expressing mutant podocalyxin lacking the COOH-terminal motif or containing a COOH-terminal leucine-to-glycine mutation (Fig. 7, B and C) showed reduced apical biotinylation of podocalyxin with a relative increase in the nonbiotinylated fraction, consistent with increased cytoplasmic expression of podocalyxin. No labeling of podocalyxin occurred in either wild-type or mutant cell lines when the basal surface was labeled (Fig. 7, A–C). Thus the COOH-terminal DTHL motif of podocalyxin is required for efficient apical localization of podocalyxin in MDCK cells.

To determine whether the DTHL motif may function to stabilize podocalyxin at the apical membrane, we biotinylated the apical surface of transfected MDCK cells and assayed the apically expressed biotinylated podocalyxin over time (Fig. 8). Biotinylated wild-type podocalyxin expressed at apical membrane persisted longer than apically expressed podocalyxin missing the DTHL motif or containing a terminal leucine-to-glycine mutation (Fig. 8, A and B). Overall, the amount of podocalyxin expressed on the apical surface did not change after biotinylation (Fig. 8A). Compared with wild-type podocalyxin, both mutant podocalyxins
showed reduced retention at the apical surface of MDCK cells (Fig. 8B). These results suggest that one function of the DTHL motif is to stabilize podocalyxin at the apical surface.

**PDZ interaction motif is not required to modify TER of MDCK monolayers.** The expression of podocalyxin by monolayers of MDCK cells has been previously shown to modify the tight junctional structure and reduce TER (29). This effect was shown to be dependent on the presence of the highly sialylated extracellular domain of podocalyxin. To determine whether the modification of TER in MDCK monolayers by podocalyxin requires an intact PDZ interaction motif, the TER of stably transfected MDCK monolayers was measured (Fig. 9). Consistent with what has been previously reported, the stable expression of podocalyxin lowered the TER of MDCK monolayers compared with the control cell line. In addition, MDCK monolayers with stable expression of mutant podocalyxin (missing an intact PDZ interaction motif) also had a lower TER than the control cell line. This suggests that an intact PDZ interaction motif is not required for podocalyxin to modify the TER of MDCK cell monolayers. However, monolayers expressing wild-type podocalyxin showed a trend toward slightly lower TER compared with the monolayers expressing podocalyxin with a nonfunctional PDZ interaction motif at most time points (Fig. 9).

**DISCUSSION**

The COOH-terminal DTHL motif of podocalyxin was expected to interact with a PDZ domain containing protein based on the strong sequence similarity of this motif to the PDZ interaction domains of other transmembrane proteins (25). The expression cloning of NHERF-2 was mediated by an interaction of the peptide probe with the second PDZ of NHERF-2 on the basis of the overlapping sequence of the expression clones. The finding that both PDZ domains of NHERF-2 interact with the PDZ interaction motif of podocalyxin was unexpected because these PDZ domains are not highly conserved (64% amino acid identity). Dual PDZ interaction has not been reported for transmembrane proteins interacting with NHERF-2 (23). Despite our demonstration of an interaction of podocalyxin with the first PDZ domain of NHERF-2, we did not obtain any clones containing the first PDZ domain by expression screening. We have previously had difficulty cloning 5’ transcripts from the library that we used, and the failure to expression clone PDZ-1-containing cDNAs may have been due to the relatively low number of phage plaques screened (600,000) or relative lack of complete cDNAs in the library (12).

The interaction of podocalyxin with both PDZ domains of NHERF-2 shows similarities to the interaction of NHERF with the cystic fibrosis transmembrane conductance regulator (CFTR). CFTR is a transmembrane chloride channel localized on the apical surface of epithelial cells. CFTR contains a COOH-terminal PDZ interaction motif (DTRL) that has been proposed to interact with the first PDZ domain of NHERF (7, 32). Recently, however, it has been demonstrated that both PDZ domains of NHERF can interact with the
COOH terminus of CFTR (9, 19). This binding can occur in a bivalent fashion, and the binding of NHERF to CFTR has been shown to regulate chloride channel activity (19). In our experiments, we did find more podocalyxin binding to the full-length NHERF-2 than to either PDZ domain alone (Figs. 2 and 4). This may reflect NHERF-2 simultaneously binding two podocalyxin molecules. However, the interaction of NHERF PDZ domains and its ligands is sensitive to the sequence adjacent to the putative PDZ domains (19), and the increased binding of podocalyxin to full-length NHERF-2 relative to PDZ-1 or PDZ-2 alone may be due to the influence of the regions flanking the PDZ domains.

The interaction of NHERF-2 and podocalyxin requires an intact podocalyxin COOH terminus (DTHL). Podocalyxin expressed in MDCK cells is localized to the apical surface (Figs. 6 and 7) (29), and mutant podocalyxin lacking this interaction motif shows a decrease in apical expression and an increase in cytoplasmic expression in MDCK cells (Figs. 6 and 7). This could be due to mistargeting of podocalyxin; however, no podocalyxin was detected at the basolateral surface in wild-type or mutant cell lines (Figs. 6 and 7). Podocalyxin is heavily glycosylated (~50% of its apparent molecular weight), and podocalyxin produced by the wild-type and mutant cells migrates at the same molecular weight on SDS-PAGE. This would suggest that the PDZ interaction motif is not required for the bulk of the posttranslational processing of podocalyxin. Our confocal data suggest that the intact COOH terminus of podocalyxin is required for the efficient expression of podocalyxin at the apical membrane (Fig. 6). This could involve an interaction with a PDZ domain-containing protein in the delivery or retention of podocalyxin at the apical surface. Our data are consistent with the hypothesis that this motif acts to improve the retention of podocalyxin at the apical surface (Fig. 8). This is similar to the reported role of a COOH-terminal PDZ binding motif (ETHL) in the retention of the γ-amino butyric acid transporter at the basolateral cell sur-

Fig. 6. Requirement of PDZ interaction motif for apical stability of podocalyxin in Madin-Darby canine kidney (MDCK) cells. MDCK cells stably transfected with full-length podocalyxin (WT), podocalyxin missing the COOH-terminal PDZ interaction motif [− Asp-Thr-His-Leu (−DTHL)], or podocalyxin with the COOH-terminal leucine-to-glycine mutation (LΔG) were grown as polarized monolayers on permeable membrane supports. Cells were stained with podocalyxin antibody 4B3 (FITC-labeled secondary antibody) and antibodies to either the adherens junction marker E-cadherin (B, D, and F) or the basolateral membrane marker mammalian Lin-7 (mLin-7; A, C, and E) and a Texas red-labeled secondary antibody. Full-length podocalyxin predominately localizes to the apical surface of transfected MDCK cells (A and B). Mutant podocalyxins (−DTHL and LΔG) were also localized apically, as well as showing a relative increase in cytoplasmic localization compared with full-length podocalyxin (C–F). A–F: digital micrographs of XY section (top) of immunostained cells and Z section of a Z series (bottom) taken with a confocal laser-scanning microscope. ap, Apical; bl, basolateral. Bars, 20 μm.

Fig. 7. Surface biotinylation of MDCK cells expressing mutant and wild-type podocalyxin. MDCK cells grown on permeable supports were surface biotinylated on the apical (AP) or basolateral surface (BL). Filters were excised, and cells were lysed in RIPA buffer. Biotinylated proteins were bound to streptavidin beads, and the biotinylated proteins (BP) were eluted by boiling in SDS sample buffer. Twenty microliters of biotinylated proteins were loaded/lane. The loading of lane AP reflects the biotinylated proteins purified from 50 μl of CL. Results from WT, −DTHL, and L delta G cell lines, respectively, probed with anti-podocalyxin antibody 4B3 are shown (A–C). In WT cells, podocalyxin is biotinylated at the apical surface (BP) with faint signal in lane NP, representing nonapical nonbiotinylated podocalyxin (A). Apical biotinylation of mutant cell lines shows an increase in the relative amount of podocalyxin in the NP fraction and a decrease in the BP fraction compared with the WT cell line (B and C). No podocalyxin was biotinylated on the basolateral surface in any cell line (BP in A–C).
A

and 4B3 (lyzed by Western blotting with streptavidin-horseradish peroxidase precipitate apical surface podocalyxin bound to MAb 4B3 and ana-

excised. Lysates were incubated with protein G-agarose to immuno-

MAb 4B3 to bind apical podocalyxin and washed, and

media for the times indicated. The apical surface was incubated with

were biotinylated, and the monolayers were incubated in culture

from the extracellular domain of podocalyxin. In our

studies, the stable expression of podocalyxin on the

surface compared with wild-type. Values are means

lacking the PDZ binding motif had reduced retention at the apical

remaining at the time points indicated. The podocalyxin constructs

were unable to detect endogenous NHERF-2 in

isoform we cloned contains the region homologous to

terminal 30 amino acids are suf-

main of NHERF-2 has not been determined but ap-

displays to require the COOH-terminal 23 amino acids

Since the initial submission of this paper, Takeda et

et al. (30) have reported that rat podocalyxin inter-

and this interaction has been shown to link NHE3 to

effect of sialylated extracellular domain of podocalyxin on TER.

TER was not dependent on a functional PDZ interaction motif because mon-

expressing podocalyxin missing the DTHL motif or with the key terminal leucine mutated also had

significant reductions in TER. The effect of podocalyxin on the TER of MDCK monolayers did tend to be somewhat less for cell lines expressing podocalyxin with a mutated/missing PDZ interaction motif than for wild-

for MAb 4B3 and analyzed by Western blotting with streptavidin-horseradish peroxidase

and 4B3 (A). B: fraction of biotinylated apical surface podocalyxin bound to MAb 4B3 remaining at the time points indicated. The podocalyxin constructs lacking the PDZ binding motif had reduced retention at the apical surface compared with wild-type. Values are means ± SE; n = 3 independent experiments. P < 0.05 at 4 and 8 h for L delta G mutant. P < 0.05 at 4 h for −DTHL mutant.

B

Fig. 8. Stability of podocalyxin at the apical surface of MDCK cells. The apical surface of confluent MDCK monolayers grown on filters were biotinylated, and the monolayers were incubated in culture media for the times indicated. The apical surface was incubated with MAb 4B3 to bind apical podocalyxin and washed, and filters were excised. Lysates were incubated with protein G-agarose to immunoprecipitate apical surface podocalyxin bound to MAb 4B3 and analyzed by Western blotting with streptavidin-horseradish peroxidase and 4B3 (A). B: fraction of biotinylated apical surface podocalyxin remaining at the time points indicated. The podocalyxin constructs lacking the PDZ binding motif had reduced retention at the apical surface compared with wild-type. Values are means ± SE; n = 3 independent experiments. P < 0.05 at 4 and 8 h for L delta G mutant. P < 0.05 at 4 h for −DTHL mutant.

Fig. 9. Transepithelial resistance (TER) of MDCK monolayers transfected with podocalyxin. The transepithelial resistance of MDCK monolayers grown on Transwell filters was measured at times indicated. Values are means ± SE from WT, −DTHL, L delta G, and pcDNAIII stably transfected cell lines; n = 12 wells/cell line except for n = 6 wells for pcDNAIII. The TER from the podocalyxin-transfected lines (WT, −DTHL, and L delta G) differed from control at all time points (P < 0.01). The WT podocalyxin cell line had a reduced TER compared with the L delta G cell line at 48, 96, and 120 h (P < 0.05) and the −DTHL cell line at 48 and 120 h (P < 0.05).
REFERENCES


29. Wang S, Raab RW, Schatz PJ, Guggino WB, and Li M. Peptide binding consensus of the NHE-RF-PDZ1 domain
