J Am Soc Nephrol 13: 1766–1772, 2002

Interaction of Endogenous Nephrin and CD2-Associated Protein in Mouse Epithelial M-1 Cell Line

TUULA PALMÉN,* SANNA LEHTONEN,† ARI ORA,† DONTSCHO KERJASCHKI,‡ CORINNE ANTIGNAC,§ EERO LEHTONEN,† and HARRY HOLTHÖFER*

*Departments of *Bacteriology and Immunology and †Pathology, Helsinki University and Helsinki University Central Hospital, Helsinki, Finland; ‡Institute for Clinical Pathology, University of Vienna, Vienna, Austria; and §INSERM U423, Université René Descartes, Hôpital Necker-Enfants Malades, Paris, France.*

Abstract. The interpodocyte slit diaphragm is an essential structure for maintaining the functional glomerular filtration barrier. The slit diaphragm is proposed to consist of an interacting meshwork of nephrin molecules. Earlier studies with tagged proteins have suggested that the intracellular part of nephrin interacts with CD2-associated protein (CD2AP). This study was addressed to show by coimmunoprecipitation and pulldown assays an interaction of endogenously expressed nephrin and CD2AP in the kidney-derived mouse epithelial M-1 cell line, to provide evidence of the domain(s) of CD2AP involved in the interaction, and to show the localization of the respective proteins by immunoelectron microscopy in kidney cortex. In addition, the localization of CD2AP, podocin, α-actinin 4, and nephrin was studied in human kidney glomeruli and in M-1 cells by immunofluorescence microscopy. The results indicate an endogenous interaction between nephrin and CD2AP in M-1 cells and suggest that this interaction is mediated by the third Src homology 3 (SH3) domain of CD2AP. We also show by immunoelectron microscopy that nephrin and CD2AP are detected at the slit diaphragm area, supporting their interaction in the glomeruli *in vivo*. In addition, nephrin was found to partially colocalize with CD2AP and podocin in double immunofluorescence microscopy, confirming the close proximity of these proteins and proposing that these proteins may belong to nephrin-associated protein complex in glomeruli. The existence of nephrin, CD2AP, podocin, and α-actinin 4 enables further characterization of their relationship in M-1 cells.

The glomerular visceral epithelial cells, podocytes, are the outermost layer of the glomerular filtration barrier (1). Their foot processes form a tight, dynamic web around the basement membrane of glomerular capillaries (1). The foot processes of adjacent podocytes are closely apposed with each other and separated only by the interpodocyte slit diaphragm that prevents proteins and other large molecules from leaking to urine (1,2). The slit diaphragm is ultrastructurally a zipper-like complex that consists of interpodocyte bridges that are connected with a continuous central filament (2). The molecular composition of the slit diaphragm complex is, however, still poorly understood. Until recent years, the only identified protein of the slit diaphragm area was zonula occludens-1 (ZO-1) (3,4). The α-motif lacking isoform of this peripheral membrane protein was shown to be concentrated near insertion sites of the slit diaphragms on the lateral cell membranes of the podocytes (3,4). Besides ZO-1, P-cadherin has been shown to reside near the slit diaphragm area, and it has been proposed to form the core scaffold of the slit diaphragm (5). In this model, the slit diaphragm represents a modified adherens junction consisting of P-cadherin, ZO-1, and α-, β- and γ-catenins (5). The catenins have been shown to associate with cadherins, linking them via α-actinin to the cytoskeleton (6,7,8,9). Another member of the cadherin superfamily, FAT, was recently found to be located at the slit diaphragm area colocalizing with ZO-1 and showing identical localization to the epitope of a monoclonal antibody 5-1-6 (10,11,12,13).

The cloning of *NPHS1*, the causative gene for the congenital nephrotic syndrome of the Finnish type (CNF) (14), has brought new insight into the pathophysiology of proteinuria and into regulation of the glomerular filtration barrier. The mutations in the coding sequence of the gene have been shown to cause the massive proteinuria of CNF (14), and the protein product of the gene, nephrin, has been localized primarily to the interpodocyte slit diaphragm (15,16,17). We (18,19) and others (20,21) have shown that nephrin can be dislocated from its typical sites in proteinuric diseases. This is also paralleled by reduced levels of nephrin mRNA, as reported in various experimental models of proteinuria (18,19,21) and in acquired human nephrotic syndromes (20,22). All the accumulated data have provided convincing evidence of the crucial role of nephrin in the glomerular filtration as a major component of the slit diaphragm (23).

To elucidate the precise function of nephrin, it is important to search for nephrin-binding proteins and thereby characterize

*Drs. Palmén and Lehtonen contributed equally to this article.

Received July 19, 2001. Accepted March 13, 2002.

Correspondence to Dr. Harry Holthöfer, Department of Bacteriology and Immunology, Helsinki University and Helsinki University Central Hospital, PB 63 (Haartmaninkatu 8), FIN-00014 University of Helsinki, Finland. Phone: +358-9-191-25-500; Fax: +358-9-191-25-501; E-mail: Harry.Holthofer@Helsinki.Fi

1046-6673/1307-1766
Journal of the American Society of Nephrology

DOI: 10.1097/01.ASN.0000019842.50870.41

the signaling cascades and the cytoskeletal connections of nephrin. The first indications of the involvement of nephrin in signaling were established by showing the association of nephrin with the lipid rafts of the slit diaphragm in which phosphorylation of nephrin could be induced (24) and by showing that expression of nephrin mRNA was affected by protein kinase C (PKC) (25). It has also been recently demonstrated that nephrin stimulates mitogen-activated protein kinases (26).

CD2-associated protein (CD2AP), an intracellular interaction partner of CD2, is an 80-kD protein that is initially found in T cells where CD2AP has been shown to be essential for CD2 clustering and for inducing T cell polarization at the contact area between T cell and antigen-presenting cell (27). In a differential screening analysis, we simultaneously identified a new gene named METS-1 (mesenchyme-to-epithelium transition protein with SH3 domains) as a gene that is strongly upregulated during kidney development (28). The respective protein was found at low levels in undifferentiated metanephric mesenchyme but was strongly upregulated upon mesenchymal cell aggregation and the following epithelial cell differentiation (28). In the maturing nephron, it was downregulated in a subpopulation of the tubules, whereas strong staining persisted in the glomerular podocytes, where its localization was mainly basolateral (28,29), as the reported localization of nephrin (15,16,17,29). Subsequent sequence comparison showed the identity of CD2AP and METS-1. Interestingly, the knockout mice of CD2AP were reported to have a phenotype closely resembling human CNF with massive proteinuria and effacement of the podocyte foot processes (30,31,32), and coimmunoprecipitation experiments of transiently overexpressed CD2AP and the intracellular part of nephrin in HeLa cells suggested an interaction between nephrin and CD2AP (30). This suggested interaction could not be confirmed with HEK 293T cells transfected with the human nephrin (amino acids 1087–1244) and CD2AP sequences (26), but it was detected in HeLa cells as well as in cultured podocytes (33).

Two other genes involved in proteinuria have recently been discovered. Of these, *NPHS2*, the causative gene for autosomal recessive steroid-resistant nephritic syndrome, encodes an integral membrane protein of the stomatin protein family, podocin (34). It has been recently reported to localize to the membrane of the podocyte foot processes (35), shown to interact with CD2AP (35) and nephrin (26,35), and proposed to function as a scaffolding protein (35). In addition, podocin has been shown to facilitate nephrin signaling (26). The other gene, *ACTN4*, has been shown to cause progressive kidney damage in patients with familial focal segmental glomerulosclerosis (FSGS) (36). The encoded protein, α-actinin 4, is an actin-crosslinking protein (36).

In this study, we show by immunofluorescence microscopy that CD2AP and podocin partially colocalize with nephrin in human kidney glomeruli and show that these proteins are expressed in M-1 kidney epithelial cells. In addition, we show for first time an interaction of endogenous nephrin and endogenous CD2AP by reciprocal coimmunoprecipitation. Nephrin pulldown experiments reveal that the third SH3 domain of CD2AP mediates the interaction. Furthermore, we show by immunoelectron microscopy that both nephrin and CD2AP localize in the slit diaphragm region in glomerular podocytes.

Materials and Methods

Immunofluorescence

Successive 5-μm-thick cryostat sections of human kidney cortex were fixed in ice-cold acetone for 10 min before washes with phosphate-buffered saline (PBS). For stainings with CD2AP antibodies, the sections were fixed with 3.5% paraformaldehyde (PFA) for 2 min before the acetone fixation. The following antibody pairs were used in double immunofluorescence: (*1*) mouse monoclonal antibody against an extracellular part of rat nephrin (clone 053; Glomega Inc., Helsinki, Finland; 1:150) recognizing the EMFSWE epitope (amino acids 771–776) of nephrin (GenBank accession no. AF125521) and rabbit polyclonal antibodies to CD2AP (H-290; Santa Cruz Biotechnology Inc., Santa Cruz, CA; 1:100); (*2*) nephrin (clone 053) and rabbit polyclonal antibodies to podocin (amino acids 237–383) (Dr. C. Antignac, Université René Descartes, Hôpital Necker-Enfants Malades, Paris, France; 1:400); (*3*) nephrin (clone 053) and rabbit affinity-purified polyclonal antibodies to α-actinin 4 (1:200) (37). After washes with PBS, the sections were incubated with secondary antibodies, tetramethylrhodamine (TRITC)-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA; 1:200) and FITC-conjugated goat anti-mouse IgG+A+M (Zymed Laboratories Inc., San Francisco, CA; 1:50). Microscopy was performed with a Zeiss Axiophot2 microscope (Carl Zeiss Jena GmbH, Jena, Germany).

Mouse epithelial M-1 cortical collecting duct cells (ATCC, Rockville, MD) were grown on the coverslips to confluence in the culture medium (1:1 mix of Ham F12 medium and Dulbecco modified Eagle medium containing 5 μM dexamethasone and 5% fetal bovine serum). The cells were fixed with ice-cold acetone for 10 min for staining with antibodies to nephrin, podocin, and α-actinin 4 and with 3.5% PFA for 20 min for staining with antibodies to CD2AP. As primary antibodies, rabbit affinity-purified antibodies to an intracellular peptide of nephrin (1:20) (15) and anti-rabbit antiserum to CD2AP (R1774) (1:100) (28) were used. These polyclonal antibodies were used further in coimmunoprecipitation assay. Podocin and α-actinin 4 antibodies were the same as described above. As secondary antibody TRITC-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories, Inc.; 1:150) was used.

Coimmunoprecipitation

M-1 cells endogenously expressing nephrin and CD2AP were lyzed in 20 mM HEPES, 150 mM NaCl, 1 mM $CaCl_2$, 1% NP-40, 0.5% saponin, pH 7.6, supplemented with EDTA-free proteinase inhibitor cocktail (Roche Molecular BioChemicals, Mannheim, Germany). Equal volumes of the cell lysate were incubated with rabbit anti-nephrin (15), rabbit anti-CD2AP (28) antibodies, or rabbit preimmune serum with rotation for 1 h at 4°C before incubation with protein A-sepharose beads (Zymed Laboratories Inc.). The immunocomplexes were collected by centrifugation at 2400 $\times$ *g* for 60 s, washed three times with the lysis buffer, suspended in Laemmli sample buffer (Bio-Rad, Richmond, CA), and heated at 95°C. Proteins were resolved with SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto nitrocellulose filters (Amersham Life-Science, Amersham International, Buckinghamshire, England). The presence of CD2AP in the sample precipitated with the nephrin antibodies was monitored by immunoblotting with the CD2AP antibody (28). The presence of nephrin in the sample precipitated with the CD2AP antibodies was monitored by immunoblotting with the nephrin antibody (15). Immunoblotting was performed as described earlier

(38), except that the secondary antibody was peroxidase-conjugated goat anti-rabbit IgG (Jackson ImmunoReseach Laboratories, Inc.; 1:45,000), and the detection was performed with SuperSignal ECL substrate (Pierce, Rockford, IL).

Pulldown Assay

The three SH3 domains of CD2AP (GenBank accession no. AF149092), covering amino acids 1–59, 108–169, and 269–330, respectively, and COOH-terminus (amino acids 331–637) of CD2AP were subcloned into pGEX-4T-1 vector (Amersham Pharmacia Biotech, Uppsala, Sweden) containing a glutathione-S-transferase (GST) tag. The proteins were produced in *Escherichia coli* and purified according to the manufacturer's instructions (Amersham Pharmacia Biotech).

M-1 cells endogenously expressing nephrin were lysed as described above. The GST-fusion proteins of CD2AP or GST alone as a negative control were added to the lysates and incubated with rotation at 4°C for 1 h. The glutathione sepharose matrix–bound proteins were collected by centrifugation at 1000 × *g* at 4°C for 3 min, washed three times with the lysis buffer, suspended in Laemmli sample buffer, and immunoblotted with nephrin antibodies (15) (as above) to reveal which fragment(s) of CD2AP have the ability to precipitate nephrin.

Immunoelectron Microscopy

Immunoelectron microscopy using indirect immunogold labeling on ultrathin frozen sections of cortical kidney biopsies fixed in freshly prepared 4% PFA was performed as described earlier (39). The sections were incubated with rabbit anti-nephrin antibody (15) or with affinity-purified rabbit anti-CD2AP antibody (clone R211, manuscript in preparation; Lehtonen S, Tienari J, Ora A, Lehtonen E) (1:5) before incubation with 10-nm gold-conjugated goat anti-rabbit IgG (Amersham LifeScience; 1:50).

Results

Nephrin Partially Colocalizes with CD2AP and Podocin in Human Kidney Cortex

To get evidence of the proteins in the close proximity of nephrin, we performed indirect double immunofluorescence microscopy on the same glomeruli of successive human kidney cortical sections. The double immunofluorescence with the monoclonal nephrin antibody and the polyclonal CD2AP, podocin, or α-actinin 4 antibodies was repeated five times with two different kidney cortex biopsies.

Nephrin exhibited a typical epithelial-type linear patchy staining pattern along the peripheral capillary loops (Figure 1, A, D, and G). In single labeling, the staining pattern of CD2AP resembled that of nephrin (not shown); however, in double staining, the fluorescence intensity of CD2AP was always clearly weaker (Figure 1B), probably resulting from different optimal fixation conditions of nephrin and CD2AP. The merged image revealed, however, a partial colocalizion of nephrin and CD2AP as indicated by yellow color (Figure 1C). Podocin staining (Figure 1E) was also found to overlap with nephrin staining (Figure 1, D through F). In contrast, nephrin (Figure 1G) and α-actinin 4 (Figure 1H) did not typically colocalize. The signals rather resided next to each other (Figure 1I). As a negative control, secondary antibodies alone did not stain glomeruli (not shown).

Localization of Nephrin, Podocin, and CD2AP Show Similarities in the M-1 Cells

The expression and localization of nephrin, CD2AP, podocin, and α-actinin 4 was studied by immunofluorescence in M-1 cells before proceeding to coimmunoprecipitation studies.

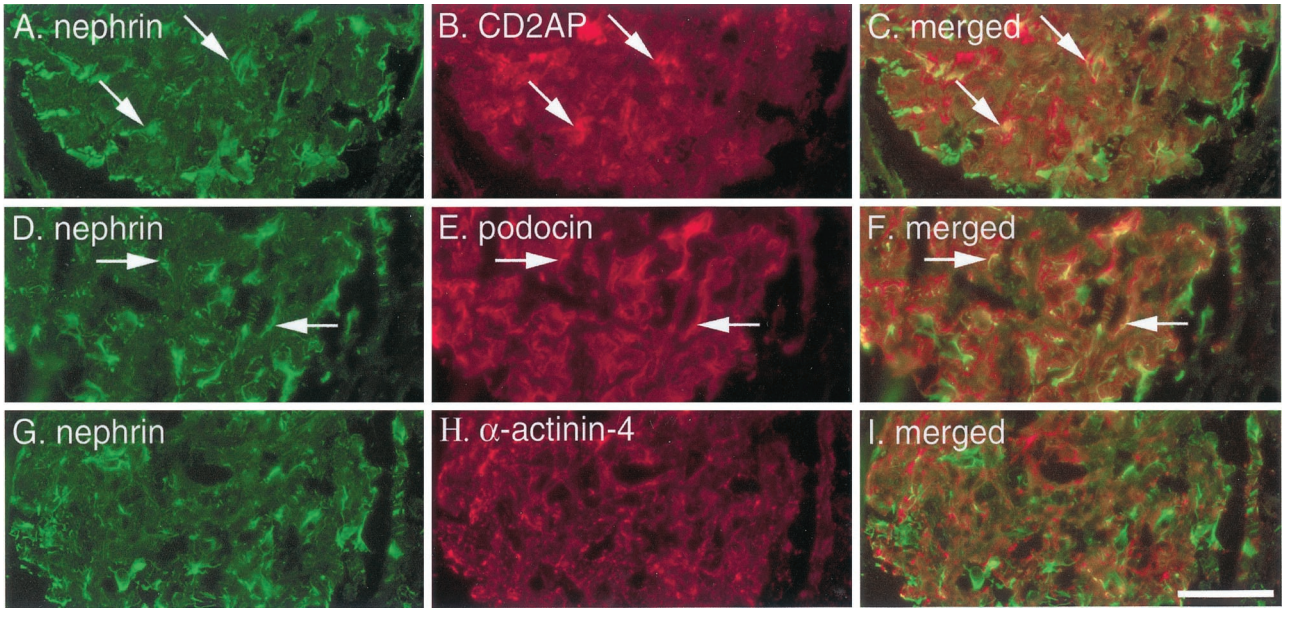

Figure 1. Double immunofluorescence stainings of nephrin, CD2-associated protein (CD2AP), podocin, and α-actinin-4 in human kidney cortex. Double staining of nephrin (A) and CD2AP (B) showed a partial colocalization of the proteins, as indicated by the yellow color in the merged image (C) (arrows). Nephrin (D) and podocin (E) were also detected to partially overlap (F) (arrows). In contrast, nephrin (G) and α-actinin-4 (H) were not typically found to colocalize (I). Scale bar: 50 μm.

The expression of nephrin was evident but rather weak. Nephrin showed nearly continuous staining along the plasma membranes of the cells (Figure 2A). Occasionally, nearby cell membrane nephrin showed concentrations resembling those seen in CD2AP staining (insets in Figures 2A and 2B). CD2AP antibody showed staining at the close vicinity of cell membranes and very prominent cytoplasmic staining (Figure 2B). Podocin antibody also gave a staining that showed a clear similarity to nephrin staining (Figure 2C). The α-actinin 4 showed a cytoplasmic filamentous staining pattern reaching the peripherium of the cells and paralleling cell membranes (Figure 2D).

Endogenously Expressed Nephrin and CD2AP Interact in the M-1 Cell Line

Earlier studies using tagged proteins have suggested that the intracellular part of nephrin can associate with CD2AP (30). Attempts to verify this interaction have resulted in differing data (26,33). Here we have analyzed by reciprocal coimmunoprecipitations whether nephrin and CD2AP interact in wild-type M-1 cells.

The immunoblot stained with the nephrin antibody (Figure 3A) showed an approximately 180-kD band in glomerular lysate, which corresponds the size of nephrin (A1). The immunoblot stained with the CD2AP antibody (Figure 3B) detected an 80 kD-band corresponding to the size of CD2AP (B1). The M-1 precipitates by nephrin antibody immunoblotted with nephrin antibody revealed the 180-kD band of nephrin (A2) and revealed the coprecipitated 80-kD band of CD2AP (B2) when immunoblotted with the CD2AP antibody. Conversely, the M-1 precipitates by CD2AP antibody immunoblotted with nephrin antibody showed the coprecipitated nephrin band (A3), and the 80-kD of CD2AP (B3) when immunoblotted with the CD2AP antibody. Instead, the M-1 precipitates by preimmune serum precipitates showed no bands (A4 and B4), confirming the specificity of the reciprocal coimmunoprecipitation.

The Third SH3 Domain of CD2AP Mediates the Interaction with Nephrin

To analyze which domain(s) of CD2AP are required for the interaction with nephrin, we performed experiments to pull down endogenous nephrin with the different SH3 domains of CD2AP with the carboxy terminus (COOH) of CD2AP and with GST as a negative control (Figure 3C). The pulldowns were resolved by SDS-PAGE, and the immunoblot was stained with anti-nephrin antibody.

The nephrin pulldown assay with first, second, and third SH3 domains (lanes 1 to 3, respectively) as well as with the COOH-terminus of CD2AP revealed that the third SH3 domain of CD2AP mediates the interaction with nephrin (lane 3) either directly or indirectly. In high stringency conditions, no inter-

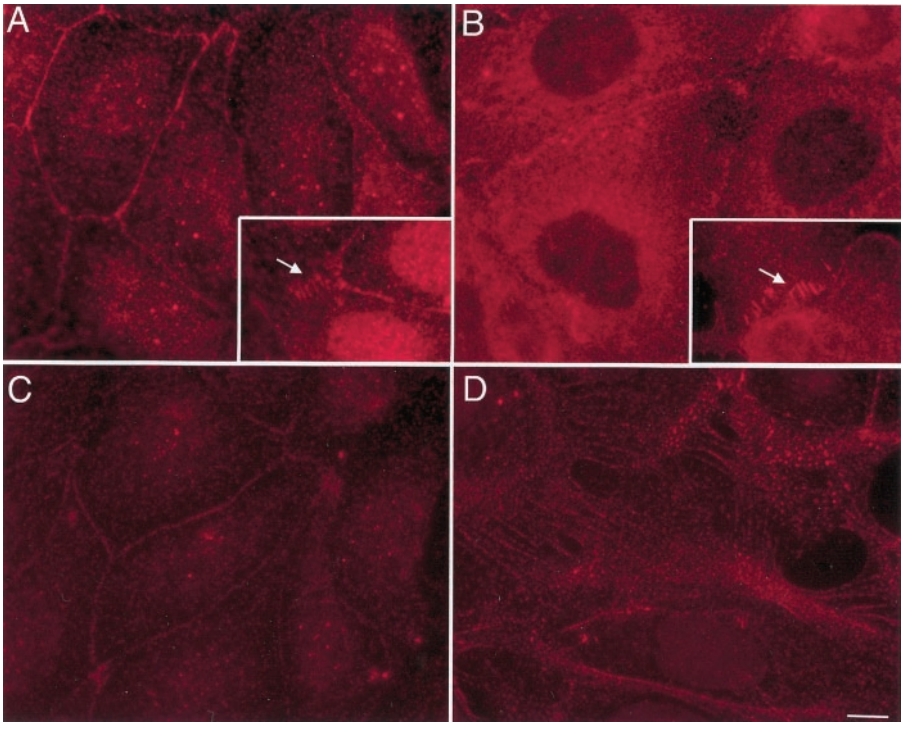

Figure 2. Immunofluorescence staining of nephrin, CD2AP, podocin, and α-actinin-4 in wild-type mouse epithelial M-1 cortical collecting duct cells. Nephrin (A) and podocin (C) showed similar continuous membranous staining. CD2AP staining was very strong in the cytoplasm of M-1 cells, but it was also present in the close vicinity of the cell membrane (B). In addition, both nephrin and CD2AP staining showed concentrations near cell membrane (arrows in A and B insets). The α-actinin-4 staining pattern was clearly different from the staining pattern of nephrin and CD2AP. α-actinin-4 paralleled the cytoplasmic surface of the cells and formed pearl-like chains toward to peripheriсum of cells or toward cell projections (D). Scale bar: 8 μm.

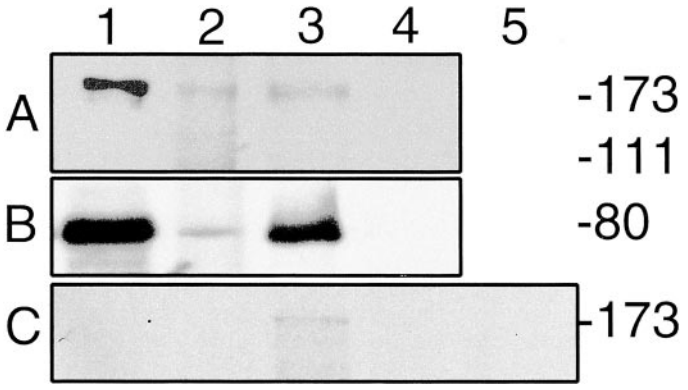

Figure 3. A reciprocal coimmunoprecipitation of nephrin and CD2AP in M-1 cells (panels A and B): human glomerular lysate showed a doublet band at approximately 180-kDa when immunoblotted with nephrin antibody (A1). The M-1 cell lysate immunoprecipitated with nephrin antibody showed a band with the size comparable to the size of nephrin in glomerular lysate when immunostained with nephrin antibody (A2) and a coprecipitated 80-kDa band when immunostained with CD2AP antibody (B2). The 80-kDa band corresponds to the size of CD2AP (B1). Conversely, the M-1 cell lysate immunoprecipitated with CD2AP antibody revealed a coprecipitated 180-kDa band when immunoblotted with nephrin antibody (A3) and an expected 80-kDa band when immunoblotted with CD2AP antibody (B3). The M-1 cell lysate immunoprecipitated with preimmune rabbit serum showed no bands when immunostained with nephrin (A4) or CD2AP antibodies (B4), confirming the specificity of the coimmunoprecipitations. The coprecipitation experiment was performed twice. Nephrin pulldown by GST-fusion proteins of CD2AP (panel C): The GST fusions of the first (lane 1), the second (lane 2), the third SH3 domain (lane 3), the COOH-terminus of CD2AP (lane 4) and GST alone (lane 5) were used in the nephrin pulldown. At high stringency, the third SH3 domain of CD2AP mediated the pulldown of nephrin. At lower stringency, the third and the first SH3 domain of CD2AP pulled nephrin down (not shown). The pulldown experiment was performed four times.

action was observed between nephrin and the first SH3 domain (lane 1), the second SH3 domain (lane 2), the COOH-terminus (lane 4), or the negative control GST (lane 5).

In Immunoelectron Microscopy, Nephrin and CD2AP Were Detected at the Slit Diaphragm Area of Podocytes

To further confirm the association of nephrin and CD2AP in the glomeruli *in vivo*, we performed immunogold electron microscopy on kidney sections.

Nephrin localized preferentially at the filtration slit area, and some reactivity was found at the lateral membranes of the podocytes (Figure 4A). CD2AP was also detected at the cytosolic side of the filtration slits (Figure 4B).

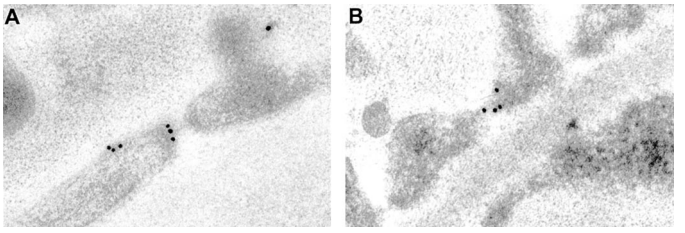

Figure 4. Subcellular localization of nephrin and CD2AP in the kidney cortex by immunogold electron microscopy. Both nephrin (A) and CD2AP (B) can be found at the filtration slit area.

Discussion

In this study, we show by immunofluorescence the expression of nephrin and CD2AP in wild-type mouse epithelial M-1 cell line. We provide evidence for the association of CD2-associated protein with nephrin in these cells. The interaction is shown by reciprocal coimmunoprecipitations and by pulldown assays, also giving indications of the interaction site. The partial colocalization of nephrin and CD2AP in human glomeruli is shown at the light microscopic level and further confirmed by immunogold electron microscopy, which shows reactivity at the same cellular site at the slit diaphragm area, supporting their interaction in glomerular podocytes *in vivo*.

The first indication of a possible interaction between nephrin and CD2AP arose from the knockout mice of CD2AP shown to exhibit severe proteinuria and effacement of the podocyte foot processes (30), a phenotype resembling human CNF (31,32) in which nephrin has been found to be mutated (14). Overexpression studies with a VSV/G-chimeric intracellular domain of nephrin and myc-tagged CD2AP further suggested an interaction (30). Later interaction studies with overexpressed nephrin and CD2AP constructs have yielded differing data. Podocin was found to interact with nephrin, but neither nephrin nor podocin were found to interact with CD2AP (26). A CD2AP antibody was recently shown to coprecipitate nephrin in an immortalized wild-type podocyte cell line with nephrin staining mainly at the intracellular membranes (33). In this study, we confirm the interaction between nephrin and CD2AP in M-1 cells expressing both proteins endogenously—first time by reciprocal coimmunoprecipitations. The immunofluorescence staining of nephrin and CD2AP supports the possibility for the interaction: nephrin was detected in the cell membranes, and stainings with the CD2AP antiserum showed a signal at the vicinity of the plasma membrane in addition to a strong perinuclear staining. We found by nephrin pulldown assays that the third SH3 domain of CD2AP is apparently the major mediator of the interaction. A recent report using transfected HeLa cells showed that the intracellular domain of nephrin fused with GST failed to pulldown myc-tagged CD2AP lacking the COOH-terminal part of the protein (33). This suggests that the COOH-terminus of CD2AP would mediate the interaction with nephrin. The differing results may originate from different experimental set-ups used in these studies. First, endogenously expressed nephrin was pulled down in our study. In the other study (33), GST-tagged intracellular domain of nephrin was used to pull down different deletion mutants of CD2AP. Second, the deletion mutants of CD2AP lack only a certain sequence but contain the rest of the sequence (33). In our study, individual domains of CD2AP containing a certain sequence (*e.g.*, the third SH3 domain) but lacking the rest were

used in the pulldown of nephrin. This difference affects the availability of other interaction sites. It has been reported that a CD2AP homolog has the ability to homodimerize via its COOH-terminus (40). Due to the ubiquity of CD2AP (present also in HeLa cells [17]), a natural CD2AP via its COOH-terminus may play a role in pulldown assays. An involvement of other interacting proteins cannot be excluded.

The interaction between nephrin and CD2AP in glomeruli was evaluated by indirect immunofluorescence microscopy. Nephrin and CD2AP were found to partially colocalize in human kidney glomeruli, supporting the possibility for the interaction. Furthermore, in immunoelectron microscopy, CD2AP was found at the slit diaphragm area in the close proximity of nephrin, supporting the results at the light microscopic level. In a recent study, CD2AP was found randomly distributed in podocyte foot processes but a signal was also found close the slit diaphragm (33,41).

The immunofluorescence data in kidney cortex and in M-1 cells suggest that podocin could belong to nehprin-associated proteins. This was recently verified by showing that podocin interacts with both CD2AP and nephrin in lipid-rafts of the glomerular slit diaphragm (42).

α-actinin 4 was not typically found to colocalize with nephrin. In the stainings, an occasional colocalization with nephrin was found in some sections but the orientation of the section may influence the result in immunofluorescence microscopy. The immunostaining in M-1 cells, however, showed a differential staining for α-actinin 4 compared with nephrin.

Tyrosine-rich intracellular domain of nephrin suggests a role in cell signaling (14). The first evidence of the signaling was recently reported by showing phosphorylation of nephrin (24). CD2AP, an interaction partner of nephrin, has been implicated in the regulation of the spatial and temporal assembly of signaling complexes linking transmembrane proteins to the cytoskeleton (40). Both nephrin and CD2AP were very recently shown to associate with the actin cytoskeleton (43). However, whether the association of nephrin to actin is mediated via CD2AP or via another adaptor protein remains unknown. Podocin, proposed to be an organizer of the slit diaphragm (26), could be involved in the cytokeletal interaction as well.

Taken together, endogenous nephrin and CD2AP were found to interact. On the basis of the pulldown results, the interaction is mediated by the third SH3 domain of CD2AP. Podocin was found to be a potential candidate for interacting with nephrin or CD2AP or both, as verified recently. The epithelial M-1 cells appear useful in examining the relationships of the proteins of the slit diaphragm area.

Acknowledgments

We thank Dr. T. Yamada for kindly providing the α-actinin 4 antibody. We are grateful to Ms. Liisa Pirinen, Ms. Eeva Häyri, and Ms. Ulla Kiiski for excellent technical assistance. The European Union (grant QLRT-1999-30619), the Paulo Foundation, the Finnish Kidney Foundation, the Emil Aaltonen Foundation, the Finnish Cultural Foundation, and the Sigrid Juselius Foundation have financially supported this work.

References

1. Spargo BH, Haas M: The kidney. In: *Pathology*, 2nd ed., edited by Rubin E, Farber JL, Philadelphia, JB Lippincott, 1994, pp. 805–809
2. Rodewald R, Karnovsky MJ: Porous substructure of the glomerular slit diaphragm in the rat and mouse. *J Cell Biol* 60: 423–433, 1974
3. Schnabel E, Anderson JM, Farquhar MG: The tight junction protein ZO-1 is concentrated along slit diaphragms of the glomerular epithelium. *J Cell Biol* 111: 1255–1263, 1990
4. Kurihara H, Anderson JM, Farquhar MG: Diversity among tight junctions in rat kidney: Glomerular slit diaphragms and endothelial junctions express only one isoform of the tight junction protein ZO-1. *Proc Natl Acad Sci USA* 89: 7075–7079, 1992
5. Reiser J, Kriz W, Kretzler M, Mundel P: The glomerular slit diaphragm is a modified adherens junction. *J Am Soc Nephrol* 11: 1–8, 2000
6. Ozawa M, Baribault H, Kemler R: The cytoplasmic domain of the cell adhesion molecule uvomorulin associates with three independent proteins structurally related in different species. *EMBO J* 8: 1711–1717, 1989
7. Ozawa M, Ringwald M, Kemler R: Uvomorulin-catenin complex formation is regulated by a specific domain in the cytoplasmic region of the cell adhesion molecule. *Proc Natl Acad Sci USA* 87: 4246–4250, 1990
8. Ozawa M, Kemler: Molecular organization of the uvomorulin-catenin complex. *J Cell Biol* 116: 989–996, 1992
9. Knudsen KA, Soler AP, Johnson KR, Wheelock MJ: Interaction of α-actinin with cadherin/catenin cell-cell adhesion complex via α-catenin. *J Cell Biol* 130: 67–77, 1995
10. Inoue T, Yaoita E, Kurihara H, Shimizu F, Sakai T, Kobayashi T, Ohshiro K, Kawachi H, Okada H, Suzuki H, Kihara I, Yamamoto T: FAT is a component of glomerular slit diaphragms. *Kidney Int* 59: 1003–1012, 2001
11. Orikasa M, Matsui K, Oite T, Shimizu F: Massive proteinuria induced in rats by a single intravenous injection of a monoclonal antibody. *J Immonol* 141: 807–814, 1988
12. Kawachi H, Abrahamson DR, St John PL, Goldatein DJ, Shia MA, Matsui K, Shimitzu F, Salant D: Developmental expression of the nephritogenic antigen of monoclonal antibody 5–1-6. *Am J Path* 147: 823–833, 1995
13. Topham PS, Kawachi H, Haydar SA, Chugh S, Addona TA, Charron KB, Holzman LB, Shia M, Shimizu F, Salant DJ: Nephritogenic mAb 5–1-6 is directed at the extracellular domain of rat nephrin. *J Clin Invest* 104: 1559–1566, 1999
14. Kestilä M, Lenkkeri U, Männikkö M, Lamerdin J, McCready P, Putaala H, Ruotsalainen V, Morita T, Nissinen M, Herva R, Kashtan C, Peltonen L, Holmberg C, Olsen A, Tryggvason K: Positionally cloned gene for a novel glomerular protein –nephrin –is mutated in congenital nephrotic syndrome. *Molecular Cell* 1: 575–582, 1998
15. Holthöfer H, Ahola H, Solin ML, Wang SX, Palmén T, Luimula P, Miettinen A, Kerjaschki D: Nephrin localizes at the podocyte filtration slit area and is characteristically spliced in the human kidney. *Am J Pathol* 155: 1681–1687, 1999
16. Ruotsalainen V, Ljungberg P, Wartiovaara J, Lenkkeri U, Kestilä M, Jalanko H, Holmberg C, Tryggvason K: Nephrin is specifically located at the slit diaphragm of glomerular podocytes. *Proc Natl Acad Sci USA* 96: 7962–7967, 1999
17. Holzman LB, St John PL, Kovari IA, Verma R, Holthofer H, Abrahamsom DR: Nephrin localizes to the slit pore of the glomerular epithelial cell. *Kidney Int* 56: 1481–1491, 1999

18. Luimula P, Ahola H, Wang S-X, Solin M-L, Aaltonen P, Tikkanen I, Kerjaschki D, Holthöfer H: Nephrin in experimental glomerular disease. *Kidney Int* 58: 1461–1468, 2000
19. Aaltonen P, Luimula P, Åström E, Palmen T, Grönholm T, Palojoki E, Jaakkola I, Ahola H, Tikkanen I, Holthöfer H: Changes in the expression of nephrin gene and protein in experimental diabetic nephropathy. *Lab Invest* 81: 1185–1190, 2001
20. Doublier S, Ruotsalainen V, Salvidio G, Lupia E, Biancone L, Conaldi PG, Reponen P, Tryggvason K, Camussi G: Nephrin redistribution on podocytes is a potential mechanism for proteinuria in patients with primary acquired nephritic syndrome. *Am J Pathol* 158: 1723–1731, 2001
21. Kawachi H, Koike H, Kurihara H, Yaoita E, Orikasa M, Shia MA, Sakai T, Yamamoto T, Salant DJ, Shimizu F: Cloning of rat nephrin: Expression in developing glomeruli and in proteinuric states. *Kidney Int* 57: 1949–1961, 2000
22. Furness PN, Hall LL, Shaw JA, Pringle H: Glomerular expression of nephrin is decreased in acquired human nephritic syndrome. *Nephrol Dial Transplant* 14: 1234–1237, 1999
23. Tryggvason K: Unraveling the mechanisms of glomerular ultrafiltration. Nephrin, a key component of the slit diaphragm. *J Am Soc Nephrol* 10: 2440–2445, 1999
24. Simons M, Kriz W, Miettinen A, Reiser J, Mundel P, Holthöfer H: Involvement of lipid rafts in nephrin phosphorylation and organization of the glomerular slit diaphragm. *Am J Pathol* 159: 1069–1077, 2001
25. Wang S-X, Menè P, Holthöfer H: Nephrin mRNA regulation by protein kinase C. *J Nephrol* 14: 98–103, 2001
26. Huber T, Kottgen M, Schilling B, Walz G, Benzing T: Interaction with podocin facilitates nephrin signalling. *J Biol Chem* 276: 41543–41546, 2001
27. Dustin ML, Olszowy MW, Holdorf AD, Li J, Bromley S, Desai N, Widder P, Rosenberger F, van der Merwe PA, Allen PM, Shaw AS: Novel adaptor protein orchestrates receptor patterning and cytoskeletal polarity in T-cell contacts. *Cell* 94: 667–677, 1998
28. Lehtonen S, Ora A, Olkkonen VM, Geng L, Zerial M, Somlo S, Lehtonen E: In vivo interaction of the adapter protein CD2-associated protein with the type 2 polycystic kidney disease protein, polycystin-2. *J Biol Chem* 275: 32888–32893, 2000
29. Li C, Ruotsalainen V, Tryggvason K, Shaw A, Miner J: CD2AP is expressed with nephrin in developing podocytes and is found widely in mature kidney and elsewhere. *Am J Physiol Renal Physiol* 279: F785–F792, 2000
30. Shih NY, Li J, Karpitskii V, Nguyen A, Dustin ML, Kanagawa O, Miner JH, Shaw AS: Congenital nephrotic syndrome in mice lacking CD2-associated protein. *Science* 286: 312–315, 1999
31. Rapola J: Congenital nephrotic syndrome. *Pediatr Nephrol* 1: 441–446, 1987
32. Holmberg C, Antikainen M, Rönnholm K, Ala-Houhala M, Jalanko H: Management of congenital nephrotic syndrome of the Finnish type. *Pediatr Nephrol* 9: 87–93, 1995
33. Shih NY, Li J, Cotran R, Mundel P, Miner JH, Shaw AS: CD2AP localizes to the slit diaphragm and binds to nephrin via a novel C-terminal domain. *Am J Pathol* 159: 2303–2308, 2001
34. Boute N, Gribouval O, Roselli S, Benessy F, Lee H, Fuchshuber A, Dahan K, Gubler MC, Niaudet P, Antignac C: NPHS2, encoding the glomerular protein podocin, is mutated in autosomal recessive steroid-resistant nephrotic syndrome. *Nat Genet* 24: 349–354, 2000
35. Scwarz K, Simons M, Reiser J, Saleem MA, Faul C, Kriz W, Shaw AS, Holzman LB, Mundel P: Podocin, a raft-associated component of the glomerular slit diaphragm, interacts with CD2AP and nephrin. *J Clin Invest* 108: 1621–1629, 2001
36. Kaplan JM, Kim SH, North KN, Rennke H, Correia LA, Tong HQ, Mathis BJ, Rodriguez-Perez JC, Allen PG, Beggs AH, Pollak MR: Mutations in ACTN4, encoding alpha-actinin-4, cause familial focal segmental glomerulosclerosis. *Nat Genet* 24: 251–256, 2000
37. Honda K, Yamada T, Endo R, Ino Y, Gotoh M, Tsuda H, Yamada Y, Chiba H, Hirohashi S: Actinin-4, a novel actin bundling protein associated with cell motility and cancer invasion. *J Cell Biol* 140: 1383–1393, 1998
38. Palmén T, Ahola H, Palgi J, Aaltonen P, Luimula P, Wang S-X, Jaakkola I, Knip M, Otonkoski T, Holthöfer H: Nephrin is expressed in the pancreatic beta cells. *Diabetologia* 44: 1274–1280, 2001
39. Breitenedeer-Geleff S, Matsui K, Soleiman A, Meraner P, Poczewski H, Kalt R, Schaffner G, Kerjaschki D: Podoplanin, novel 43-kd membrane protein of glomerular epithelial cells, is downregulated in puromycin nephrosis. *Am J Pathol* 151: 1141–1152, 1997
40. Kirsch KH, Georgescu M-M, Ishimaru S, Hanafusa H: CMS: An adapter molecule involved in cytoskeletal rearrangements. *Proc Natl Acad Sci USA* 96: 6211–6216, 1999
41. Welsch T, Endlich N, Kriz W, Endlich K: CD2AP and pCAS130 localize to different F-actin structures in podocytes. *Am J Pathol* 281: F769–F777, 2001
42. Schwarz K, Simons M, Reiser J, Saleem Ma, Faul C, Kriz W, Shaw AS, Holzman, Mundel P: Podocin, a raft-associated component of the glomerular slit diaphragm, interacts with CD2AP and nephrin. *J Clin Invest* 108: 1621–1629, 2001
43. Yuan H, Takeuchi E, Salant D: Podocyte slit-diaphragm protein nephrin is linked to the actin cytoskeleton. *Am J Physiol Renal Physiol* 282: F585–F591, 2002