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

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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). Besides ZO-1, P-cadherin has been 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...
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 comminunoprecipitation. 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+G+A+M (Zymed Laboratories Inc., San Francisco, CA; 1:50). Microscopy was performed with a Zeiss Axioskop2 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 antiseraum 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 lysed in 20 mM HEPES, 150 mM NaCl, 1 mM CaCl2, 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 × 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.
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 colocalization 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-

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**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 periphericum of cells or toward cell projections (D). Scale bar: 8 μm.
associated protein with nephrin in these cells. The interaction is
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 Immunelectron 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 cyto-
solic side of the filtration slits (Figure 4B).

Discussion
In this study, we show by immunofluorescence the expres-
sion 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 glo-
eruli 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). Overexpres-
sion studies with a VSV/G-chimeric intracellular domain of
nephrin and myc-tagged CD2AP further suggested an interac-
tion (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 stain-
ning 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 immuno-
fluorescence staining of nephrin and CD2AP supports the possi-
bility 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 pe-
rinuclear 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 inter-
action 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

![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.](image)
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 nephrin-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 cytoskeletal 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.

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