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CIN85/Ruk$_L$ Is a Novel Binding Partner of Nephrin and Podocin and Mediates Slit Diaphragm Turnover in Podocytes*

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Podocyte damage is the basis of many glomerular diseases with ultrastructural changes and decreased expression of components of the slit diaphragm such as nephrin and podocin. Under physiological conditions it is likely that the slit diaphragm underlies permanent renewal processes to indemnify its stability in response to changes in filtration pressure. This would require constant reorganization of the podocyte foot process and the renewal of slit diaphragm components. Thus far, the mechanisms underlying the turnover of slit diaphragm proteins are largely unknown. In this manuscript we examined a mechanism of nephrin endocytosis via CIN85/Ruk$_L$-mediated ubiquitination. We can demonstrate that the loss of nephrin expression and onset of the proteinuria in CD2AP$^{-/-}$ mice correlates with an increased accumulation of ubiquitinated proteins and expression of CIN85/Ruk$_L$ in podocytes. In cultured murine podocytes CD2AP deficiency leads to an early ubiquitination of nephrin and podocin after stimulation with fibroblast growth factor-4. Binding assays with different CIN85/Ruk isoforms and mutants showed that nephrin and podocin are binding to the coiled-coil domain of CIN85/Ruk$_L$. We found that in the presence of CIN85/Ruk$_L$, which is involved in down-regulation of receptor-tyrosine kinases, nephrin is internalized after stimulation with fibroblast growth factor-4. Interestingly, coexpression of CIN85/Ruk$_L$ with CD2AP led to a decreased binding of CIN85/Ruk$_L$ to nephrin and podocin, which indicates a functional competition between CD2AP and CIN85/Ruk$_L$. Our results support a novel role for CIN85/Ruk$_L$ in slit diaphragm turnover and proteinuria.

Podocytes are highly specialized epithelial cells that cover the outer surface of the glomerular capillary tuft. They distend primary processes that further subdivide in numerous interdigitating foot processes. These foot processes form a specialized intercellular junction called "slit diaphragm." Many studies have emphasized the critical role of the slit diaphragm for maintaining the selective filtration barrier of the glomerulus (1, 2). Several proteins of the slit diaphragm have been identified that participate in common signaling pathways (3–5). One of the major components is nephrin. Nephrin is a transmembrane adhesion protein of the Ig superfamily, encoded by NPHS1. Humans and mice lacking nephrin are born without intact slit diaphragms and develop massive proteinuria already *in utero* (6, 7). There is cumulating evidence that nephrin is a signaling receptor molecule; nephrin forms with podocin and Neph1 a protein complex within the lipid raft that structurally functions as a transmembrane receptor (8). The intracellular human nephrin C terminus has several putative tyrosine phosphorylation sites that can be phosphorylated by the Src kinase Fyn. This receptor complex has been shown to interact with several protein kinases including Fyn, Yes, and phosphatidylinositol 3-kinase as well as with several adaptor proteins like Nck, Grb2,[2] and Crk (9, 10). A scaffolding protein that interacts with the nephrin-receptor complex is the cytoplasmic adaptor protein CD2AP, which is considered to play an important role in the maintenance of the slit diaphragm. Mice deficient for CD2AP are born healthy but develop a rapid onset nephrotic syndrome at 3 weeks of age and die of renal failure at 6 weeks after birth (11). Up to now it is completely unclear why the CD2AP$^{-/-}$ mice are born healthy with intact slit diaphragms and develop a significant proteinuria within a few days. Moreover, we previously described the unusual phenotype in these mice that the damage occurs synchronized and concerns all podocytes at the same time (12). A different member of the same adaptor protein family known as CIN85/Ruk$_L$ displays high sequence (54%) and structural similarities to CD2AP (13). Similar to CD2AP, CIN85/Ruk$_L$ contains three SH3 domains and a coiled-coil domain but is missing the actin binding sites of CD2AP. Due to alternative splicing and different promoters, multiple CIN85/Ruk isoforms were identified in cell lines of various tissue origins (14). We could previously show that the balance of CD2AP and CIN85/Ruk$_L$ determines receptor-tyrosine kinase signaling response in podocytes and that this leads to a proapoptotic shift in the intracellular signaling signatures in response to growth factor stimulations (12). These data give an explanation why CD2AP$^{-/-}$ podocytes are more susceptible to cell stress and

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[2] The abbreviations used are: Grb2, growth factor receptor-bound protein; FGF-4, fibroblast growth factor-4; SH3 domain, Src homology domain; EGF, epidermal growth factor; GFP, green fluorescent protein; FCS, fetal calf serum; PBS, phosphate-buffered saline; siRNA, small interfering RNA; RIPA, radioimmune precipitation assay buffer.

have a significantly increased rate of apoptosis after stimulation with transforming growth factor-β (15); however, this would only partially explain the rapid synchronized onset of proteinuria that concerns all glomeruli. In this manuscript we hypothesized that the up-regulation of CIN85/Ruk$_L$ that we detected previously *in vitro* in differentiating CD2AP$^{-/-}$ podocytes as well as *in vivo* in CD2AP$^{-/-}$ glomeruli (12) contributes to destabilization of the slit diaphragm complex. CIN85/Ruk$_L$ function was previously linked to endophilin-dependent and ubiquitin-mediated internalization of the epidermal growth factor (EGF) receptor (16). Ubiquitination is an emerging mechanism implicated in a variety of cellular functions like intracellular trafficking, gene transcription, DNA repair, and replication (17, 18). A selective reduction of extracellular nephrin in IgA nephropathy and also for podocin in other renal damages was observed (19, 20). Recently, it was shown that expression of ubiquitin and ubiquitin C-terminal hydrolase L1 correlates with an internalization and down-regulation of nephrin (21). In this study we demonstrate that CIN85/Ruk$_L$ is a novel binding partner of nephrin and podocin and regulates the internalization of the slit diaphragm complex. Our findings support a molecular competition between CIN85/Ruk$_L$ and CD2AP for binding to nephrin and podocin and lead us to hypothesize that a dynamic interplay between nephrin and podocin with these two adaptor molecules orchestrates the stability and turnover of the slit diaphragm.

EXPERIMENTAL PROCEDURES

Antibodies and Cytokines—Primary antibodies that were used for Western blotting, immunohistochemical, and immunofluorescence studies were rabbit anti-nephrin (targeting for the extracellular domain), rabbit anti-podocin, rabbit anti-FLAG (Santa Cruz Biotechnology, Santa Cruz, CA), guinea pig anti-nephrin (Progen, Heidelberg, Germany), mouse anti-ubiquitin (for Western blot) (Imgenex, San Diego, CA), rabbit anti-ubiquitin (Chemicon, Billerica, MA), rabbit anti-GFP, rabbit anti-myc (Cell Signaling Technology, Beverly, MA), rabbit anti-CIN85 (targeting for the C terminus) (kindly provided by I. Dikic, Frankfurt, Germany), rabbit IgG (Jackson ImmunoResearch Laboratories), and mouse IgG (Santa Cruz, CA). FGF-4 was purchased from Cell Sciences (Canton, MA).

Podocyte Culture—Cultivation of conditionally immortalized mouse CD2AP$^{+/+}$ and CD2AP$^{-/-}$ podocytes was performed as previously described (12). In brief, to enhance expression of the thermosensitive large T antigen, cells were cultured at 33 °C in the presence of 10 units/ml γ-interferon (permissive conditions). To induce differentiation, podocytes were maintained at 37 °C for 14 days without γ-interferon, resulting in the absence of thermosensitive T antigen (nonpermissive conditions). The medium consists of RPMI 1640 (Biochrom, Berlin, Germany) containing 10% FCS (PAA, Pasching, Austria), 1% penicillin-streptomycin (Invitrogen), and 10 units/ml recombinant mouse γ-interferon (Cell Sciences, Canton, MA). Every experimental setup and result was confirmed in three different clones of CD2AP$^{+/+}$ and CD2AP$^{-/-}$ podocytes.

Western Blot Analysis—To analyze whole cell protein lysates from cultured podocytes, either untreated or treated cells were lysed on ice in RIPA buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 0.5% sodium deoxycholate, 1% Nonidet P-40, 0.1% SDS) containing protease inhibitors (Complete mini, Roche Applied Science), 1 mM sodium orthovanadate, 50 mM NaF, and 200 μg/liter okadaic acid. Lysates were centrifuged at 12000 rpm, and aliquots of the supernatants were separated by 10% SDS-PAGE and transferred to polyvinylidene difluoride membranes (Immobilon-P, Millipore, Bedford, MA). After probing with primary antibodies, antigen-antibody complexes were detected with horseradish peroxidase-labeled anti-rabbit and anti-mouse antibodies, respectively, and visualized using enhanced chemiluminescence reagents (Pierce) according to the manufacturer's protocol.

Transfection—The day before transfection the podocytes were seeded on coverslides (5000 cells/slide) and cultured under permissive conditions. Cells were transfected using FuGENE transfection reagent (Roche Applied Science) according to the manufacturer's protocol using CIN85-GFP, Nephrin-SV5, CD2AP-GFP, pdsRed-Monomer-Golgi (Clontech), and empty vector GFPN1. After transfection cells were cultured under nonpermissive conditions in normal growth medium for 48 h. For transfection of HEK 293T cells, cells were seeded on plates the day before transfection. After transfection with FLAG-tagged CIN85/Ruk isoforms and mutants (kindly provided by Prof. V. Buchman and described in Ref. 14), CD2AP-myc, Nephrin-GFP, podocin-GFP, and p85-myc the cells were cultured in normal growth medium for 48 h.

Transfection with Small Interfering RNAs—Podocytes were subcultured in a 24-well plate (for enzyme-linked immunosorbent assay) or in 10-cm dishes (for immunoprecipitation) and allowed to differentiate for 4 days. One day before transfection, the medium was replaced with growth medium (RPMI medium with 10% FCS) without antibiotics so that they would be 30–50% confluent at the time of transfection. Transfection with CIN85/Ruk$_L$ or control siRNA (Santa Cruz) was performed with Lipofectamine^{TM} 2000 (Invitrogen) according to the manufacturer's protocol. After 6 h in the incubator the medium was replaced with normal growth medium. The cells were incubated at 37 °C in a CO_2 incubator for 72 h.

Immunoprecipitation—HEK 293T cells were transfected as mentioned above. Then the cells were washed carefully with ice-cold PBS on ice. For lysis, 900 μl of RIPA buffer (50 mM Tris-HCl, pH 7.5, 200 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton, and 0.25% deoxycholate + protease inhibitors) were added to cells. The lysate was incubated for 15 min on ice and centrifuged at 14,000 rpm for 15 min at 4 °C. 50 μl of FLAG beads (50% slurry in Triton buffer) (Sigma) were added to the supernatant and rotated overhead at 4 °C for 1 h (up to overnight). After that the beads were centrifuged at 3000 rpm for 1 min at 4 °C and washed with RIPA buffer 5 times. Proteins were eluted by boiling the beads in Laemmli buffer and separated by SDS-polyacrylamide gel electrophoresis. Western blots were performed using the methods mentioned above. For the ubiquitination assay of nephrin and podocin, 500 μg of total cell lysate was incubated with 2 μg of anti-nephrin or anti-podocin antibody/1 ml immunoprecipitation volume and Sepharose-A beads (40 μl) in immunoprecipitation-buffer (25 mM

Tris-HCL, pH 7.5, 1 mM dithiothreitol, 30 mM $MgCl_2$, 40 mM NaCl, 0,5% Nonidet P-40 and protease inhibitors) and rotated overnight at 4 °C. The pellets were washed three times in immunoprecipitation buffer and separated by SDS-polyacrylamide gel electrophoresis.

Immunofluorescence-based Endocytosis Assay—For internalization studies, podocytes were plated on glass slides. Cells were then transfected as mentioned above. The day before stimulation the cells were serum-starved overnight. The next day cells were simultaneously incubated with an ectodomain anti-nephrin antibody, Cy3-conjugated secondary antibody, and stimulated for 1 h with 20 ng/ml FGF-4 in RPMI without FCS. After that the cells were washed 5 times with PBS and fixed with 4% paraformaldehyde. The pictures were taken with an Inverted-2 Confocal Microscope and Leica Application Suite Software (Leica, Bonn, Germany). Post-processing was done with Photo Shop 6.0.

Endocytosis Assay (Enzyme-linked Immunosorbent Assay-based)—HEK 293T cells were transfected with constructs SV5-Nephrin (a gift from T. Huber, Freiburg, Germany), GFPN1, CD2AP-GFP, podocin-GFP, CIN85-GFP, and FLAG-tagged Ruk mutants as described above. 24-Well plates were coated with poly-L-lysine 1:1 diluted with H_2O (Sigma P4707 0,01% solution) and incubated overnight at 37 °C. The next day the plate was washed once with PBS, and transfected HEK 293T cells were seeded at a density of 6×10^5/well. About 30 h after the transfection cells were cooled on ice for 10–15 min. The medium was replaced with DFH medium (1% FCS and 20 mM HEPES in RPMI 1640) containing 1:750 mouse anti-V5 antibody (Serotec, MCA-1360). The cells were incubated for 30–60 min at 4 °C and then washed 3 times with cold DFHI-medium (1% FCS and 25 mM HEPES in RPMI 1640). To induce internalization, cells were incubated with warm DFHII medium (1% FCS and 5 mM HEPES in RPMI 1640) for 1 h at 37 °C. The cells were fixed with 3.7% paraformaldehyde for 15 min, washed twice with PBS, and kept overnight at 4 °C. For blocking, 2% normal goat serum (Jackson ImmunoResearch) in PBS was used for at least 30 min. After washing once with PBS, the cells were incubated in RPMI 1640 medium with alkaline phosphatase-coupled anti-mouse antibody (dilution 1:7500) for 1 h and washed 3 times with PBS, each wash 5–10 min. The cells were then incubated with *p*-nitrophenyl phosphate (Sigma, N2765; one tablet was resuspended in 20 ml of 0.1 M glycine, 1 mM $MgCl_2$, 1 mM $ZnCl_2$, pH 10.4) for about 1 h at 37 °C or until the solution turned yellow. One aliquot (100 μl) from the reaction was transferred in a 96-well plate, and the extinction was measured at 405 nm in a microplate reader. $CD2AP^{+/+}$ and $CD2AP^{-/-}$ podocytes were treated for inhibition of CIN85/Ruk$_L$ expression as mentioned above, and nephrin endocytosis was measured by the same method.

Biotinylation of Nephrin—$CD2AP^{+/+}$ and $CD2AP^{-/-}$ podocytes were seeded on plates and differentiated for 14 days. For biotinylation, cells were cooled at 4 °C for 30 min to stop endocytosis. After that cells were incubated for 60 min at 37 °C for endocytosis of nephrin or remained at 4 °C as control. Cells were then washed 3 times with ice-cold PBS containing 1 mM $MgCl_2$ and 0.1 mM $CaCl_2$ to remove soluble proteins. 80 μl of 10 mM Sulfo-NHS-SS-Biotin (Pierce) per ml of PBS were added to the cells and incubated at room temperature for 30 min. After incubation cells were washed once with ice-cold PBS, and non-reacting biotinylation reagent was quenched by incubation with 50 mM Tris, pH 8.0, followed by three washes in ice-cold PBS. Cells were lysed in RIPA buffer and centrifuged at 14,000 rpm for 15 min at 4 °C, and the resulting supernatant was incubated with 50 μl of 50% streptavidin-agarose (Thermo Scientific, Waltham, MA) and kept rotating overnight at 4 °C. After that the beads were washed five times with RIPA buffer, bound proteins were eluted with sample buffer by boiling for 5 min, and supernatant was loaded on SDS-gels. Biotinylated nephrin was analyzed by immunoblotting.

Immunofluorescence and Immunohistochemistry—After dissection, kidneys were flushed with PBS and immediately frozen in tissue molds containing optimal cutting temperature compound. Sections were blocked with 10% normal donkey serum and stained with the appropriate primary antibody followed by Cy3-conjugated donkey anti-mouse, Cy3-conjugated donkey anti-rabbit, or fluorescein isothiocyanate-conjugated donkey anti guinea pig antibodies (Jackson ImmunoResearch). For light microscopy the tissue was perfusion-fixed. One-micrometer paraffin sections were deparaffinized, and antigen retrieval was performed by microwaving (10 mM citrate buffer, pH 6.1) or by protease digestion (protease XXIV, 5 mg/ml or protease XIV, 10 μg/ml) (Sigma). Unspecific binding was blocked (5% human serum, 30 min, room temperature). Primary antibody incubations (5% human serum, overnight, 4 °C) were followed by incubation with biotinylated secondary antibody (1:400, 30 min, room temperature). Color development was performed with the ABC-AP kit (Vector, Burlingame, CA) The immunohistochemical protocol was performed as described earlier (21). For IgG controls, sections were incubated with rabbit or mouse IgG instead of the primary antibody.

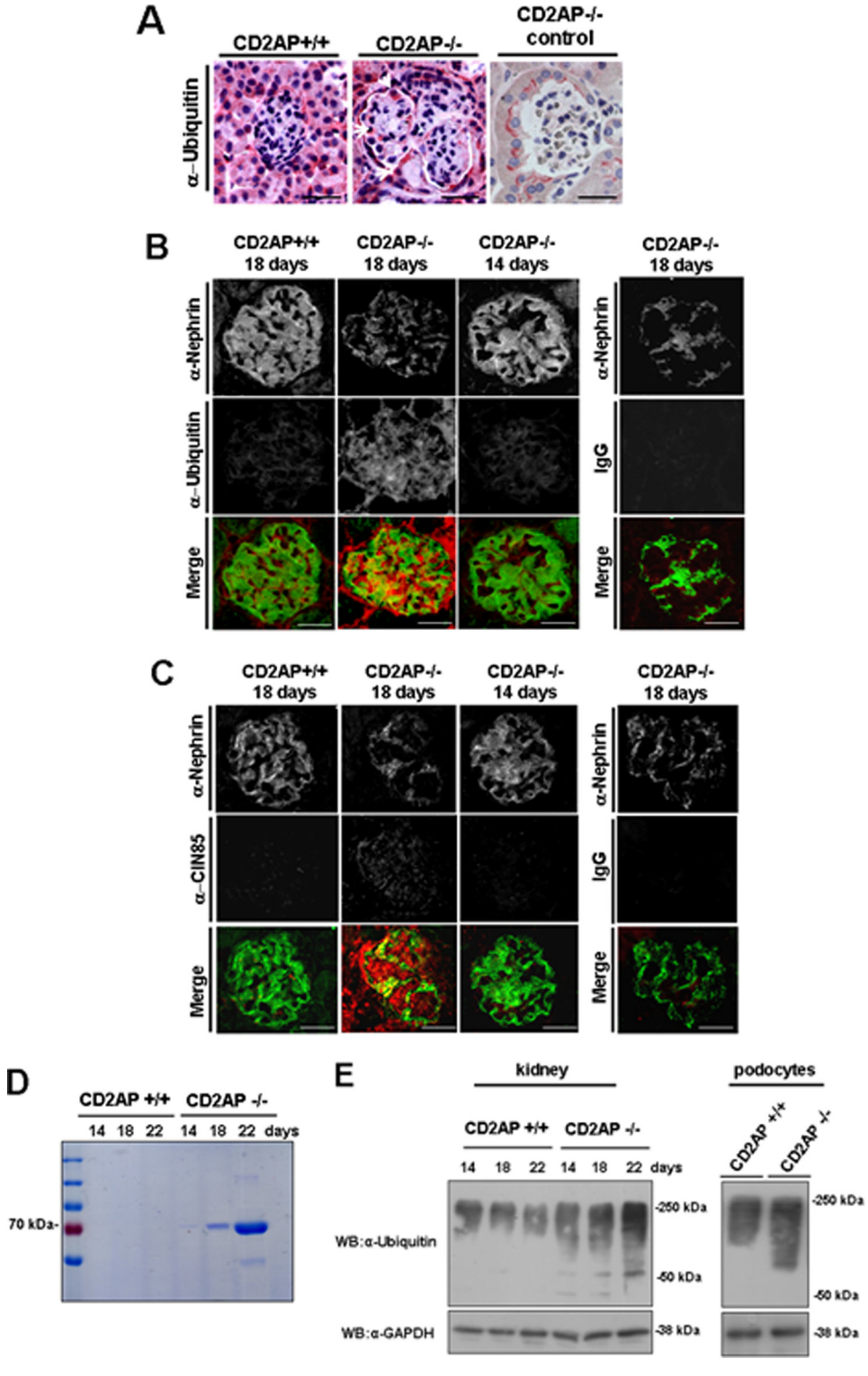
Statistics—Data are shown as the mean ± S.D. and were compared by Student's *t* test. Data analysis was performed using Excel statistical software. Significant differences were accepted when $p < 0.05$.

RESULTS

The Onset of Proteinuria in $CD2AP^{-/-}$ Mice Correlates with an Increased Accumulation of Ubiquitinated Proteins and CIN85/Ruk$_L$ Expression in Podocytes—A recent study revealed that the ubiquitin system is involved in nephrin internalization in podocytes in various diseases with podocyte injury (21). To test whether ubiquitination would play a role in the CD2AP-deficient mice, we performed immunohistochemical staining against ubiquitin that revealed an increase of ubiquitin-positive podocytes in diseased $CD2AP^{-/-}$ mice compared with $CD2AP^{+/+}$ mice. To exclude nonspecific binding, the primary antibody was replaced by rabbit IgG (Fig. 1*A*). Immunofluorescence staining of frozen kidney cortex sections shows an increased number of ubiquitin- and CIN85/Ruk$_L$-positive podocytes in 18-day-old $CD2AP^{-/-}$ mice that partially colocalized with nephrin (Fig. 1*B*). Nephrin staining was significantly reduced and displayed a typical speckled expression pattern, which is characteristic in a variety of proteinuric diseases and usually coincides with foot process effacement. In contrast to

that, we could not detect ubiquitin- and CIN85/Ruk$_L$-positive podocytes in age-matched CD2AP$^{+/+}$ or healthy (non-proteinuric) 14-day-old CD2AP$^{-/-}$ mice. To exclude nonspecific binding of the primary antibody, the staining was performed with rabbit or with mouse IgG (Fig. 1, *B* and *C*). Interestingly, when we examined spot urine samples of CD2AP$^{+/+}$ and CD2AP$^{-/-}$ mice over time we found that the increase of ubiquitin-positive expressing cells coincides with the onset of proteinuria in CD2AP$^{-/-}$ mice 18 (+/−1) days after birth ($n = 5$) (Fig. 1*D*). Moreover, by Western blot we could show an increased accumulation of ubiquitinated proteins in kidneys of diseased CD2AP$^{-/-}$ mice as well as in cultured CD2AP$^{-/-}$ podocytes (Fig. 1*E*). These data indicate that podocyte injury and proteinuria in this model system are accompanied by an increased accumulation of ubiquitinated proteins and CIN85/Ruk$_L$ expression and in parallel reduced nephrin expression coinciding with the onset of proteinuria.

Nephrin and Podocin Are Early-ubiquitinated in CD2AP$^{-/-}$ Podocytes in Vitro in Response to FGF—Because CIN85/Ruk$_L$, which we found up-regulated in CD2AP$^{-/-}$ podocytes as early as 4 days after differentiation (12), is involved in ubiquitin-mediated endocytosis and down-regulation of receptor-tyrosine kinases, we wanted to analyze the possible ubiquitination of nephrin and podocin in CD2AP$^{+/+}$ and CD2AP$^{-/-}$ podocytes. Since we did not detect a significant amount of ubiquitination of nephrin and podocin at base line (data not shown), we examined the ubiquitination profiles of nephrin and podocin after growth factor stimulation. Because it was shown that phosphorylated nephrin associates with adaptor proteins like Grb2, Nck, and phospholipase C-γ1, which are recruited after FGF treatment to the activated receptor (10, 22, 23), we treated CD2AP$^{+/+}$ and CD2AP$^{-/-}$ podocytes with FGF-4 for 0, 0.5, 1, and 2 h and performed immunoprecipitation of endogenous nephrin and podocin. Interestingly, when we analyzed the precipitates for ubiquitin by Western blotting, we found ubiquitination of nephrin as early as after 1 h (Fig. 2*A*) and ubiquitination of podocin as early as 30 min after FGF stimulation in CD2AP$^{-/-}$ podocytes compared with CD2AP$^{+/+}$ podocytes (Fig. 2*B*). In CD2AP$^{+/+}$ podocytes we found no ubiquitination of nephrin, and we could only detect a weak ubiquitination response of podocin after 2 h (Fig. 2, *A* and *B*). Similarly, we could demonstrate an inducible recruitment of CIN85/Ruk$_L$ to nephrin and podocin after 30 min until 1 h of stimulation with FGF-4 (Fig. 2, *A* and *B*). To confirm that ubiq-



uitination of nephrin and podocin is dependent on CIN85/Ruk$_L$ expression, we inhibited expression of CIN85/Ruk$_L$ protein by siRNA in CD2AP$^{-/-}$ podocytes. When we analyzed the precipitates for ubiquitin by Western blot, we found no ubiquitination of nephrin and a weak ubiquitination of podocin after 2 h of stimulation with FGF-4 in CD2AP$^{-/-}$ podocytes treated with a CIN85/Ruk$_L$ siRNA, whereas treatment of the cells with a control siRNA showed strong ubiquitination of nephrin after 1 h and for podocin after 30 min of stimulation (Fig. 2*C*). These results suggest that ubiquitination of both slit diaphragm proteins occurs accelerated in differentiated CD2AP$^{-/-}$ podocytes and is dependent on CIN85/Ruk$_L$ expression.

CIN85/RUK$_L$ Is a Novel Binding Partner of Nephrin and Podocin and Binds to the Same Region as CD2AP—CD2AP interacts with nephrin and podocin and forms a complex that participates in common signaling pathways like phosphatidylinositol 3-kinase activation (4, 5). Because CIN85/Ruk$_L$ contains, except for the actin binding sites, identical binding domains, we wanted to examine if CIN85/Ruk$_L$ would bind to nephrin and podocin as well. To dissect the possible interaction of CIN85/Ruk$_L$ with nephrin and podocin, we coexpressed different isoforms and deletion mutants of CIN85/Ruk$_L$ with the p85 subunit of phosphatidylinositol 3-kinase, CD2AP, nephrin, and podocin in HEK 293T cells. We used expression plasmids encoding various CIN85/Ruk isoforms and deletion mutants with FLAG tag: Ruk$_L$ (full-length), Ruk$_{\Delta A}$ (lacks SH3A), Ruk$_m$ (lacks SH3A-B), Ruk$_e$ (lacks SH3A-C and a specific part of the proline-rich region), Ruk$_h$ (lacks SH3A-C and the whole proline-rich region), Ruk$_{\Delta C\text{-}T}$ (lacks the C-terminal end) and Ruk$_{\Delta CC}$ (lacks the coiled-coil domain). To test the binding integrity of our constructs we used the known interaction of CIN85/Ruk$_L$ and p85 as a test system (Fig. 3*a*). We can detect, as previously described, that p85 binds to the proline-rich region of CIN85/Ruk$_L$ (24), which is lacking in the Ruk$_e$ and Ruk$_h$ constructs. When we looked for the known interaction of CD2AP and CIN85/Ruk$_L$ (25) we found that the coiled-coil domain that is missing in Ruk$_{\Delta C\text{-}T}$ and Ruk$_{\Delta CC}$ is required for CD2AP and CIN85/Ruk$_L$ interaction (Fig. 3*b*). When we performed coexpression and co-immunoprecipitation experiments with nephrin and podocin, we can demonstrate that both interact with CIN85/Ruk$_L$ and that the integrity of the coiled-coil domains is essential for this association (Fig. 3, *c* and *d*). There is no binding of nephrin and podocin to CIN85/Ruk mutants lacking the coiled-coil domain or the whole C terminus of CIN85/Ruk$_L$. To support our data that CIN85/Ruk$_L$ binding is required for ubiquitination of nephrin and podocin in podocytes, we overexpressed Ruk$_L$ and Ruk$_{\Delta CT}$ in CD2AP$^{+/+}$ podocytes, and we can demonstrate that Ruk$_L$ overexpression leads to a strong and early ubiquitination response of nephrin and podocin. In contrast, in the presence of the C-terminal mutant, which is unable to bind to nephrin and podocin, we detect a weaker ubiquitination of nephrin at 2 h and no ubiquitination response of podocin (Fig. 3, *e* and *f*). The weak ubiquitination response of nephrin in the presence of Ruk$_{\Delta CT}$ is particularly interesting. There are two potential explanations for this finding. It either indicates an effect that is mediated by endogenous CIN85/Ruk$_L$ in wild type cells or a partial binding of the Ruk$_{\Delta CT}$ to endogenous nephrin that is still sufficient to induce ubiquitination. This binding could be directly or mediated by a different component of the slit diaphragm multiprotein complex.

In summary, these results define CIN85/Ruk$_L$ as a novel binding partner of the slit diaphragm proteins nephrin and podocin. The site of interaction with both molecules is identical to the interaction site of CD2AP. Furthermore, ubiquitination of nephrin and podocin is dependent on CIN85/Ruk$_L$ binding.

CIN85/Ruk$_L$ Enhances Endocytosis of Nephrin and Podocin—To study endocytosis of nephrin in the presence of CIN85/Ruk$_L$ on a quantitative level, we performed an enzyme-linked immunosorbent assay-based endocytosis assay. To accomplish that, we transiently transfected HEK 293T cells with Nephrin-SV5 and GFPN1 empty vector, CD2AP-GFP, podocin-GFP, or CIN85-GFP (kindly provided by I. Dikic. We could demonstrate a significant enhancement of nephrin endocytosis in the presence of CIN85/Ruk$_L$. Overexpression of CIN85/Ruk$_L$ resulted in a more than 2-fold increase in nephrin endocytosis compared with cells co-transfected with GFPN1 empty vector, CD2AP-GFP, or podocin-GFP. (Fig. 4*A*, *lower panel*; *, $p < 0.007$ CIN85/Ruk$_L$ *versus* GFPN1, CD2AP, and podocin). Control lysates show equal expression of proteins (Fig. 4*A*, *upper panel*). Overexpression of Ruk$_{\Delta CC}$, Ruk$_{\Delta CT}$, and coexpression of Ruk$_L$ with CD2AP leads to a decreased endocytosis compared with Ruk$_L$ alone (Fig. 4*B*, *lower panel*); *, $p < 0.04$ Ruk$_L$ *versus* Ruk$_{L\Delta CC}$, Ruk$_{\Delta CT}$, Ruk$_L$+CD2AP. Control lysates show equal expression of proteins (Fig. 4*B*, *upper panel*). To see whether endocytosis of endogenous expressed nephrin is equally enhanced in differentiated CD2AP$^{-/-}$ podocytes and depends on CIN85/Ruk$_L$ expression, we treated CD2AP$^{+/+}$ and CD2AP$^{-/-}$ podocytes with a CIN85/Ruk$_L$ siRNA and applied the same assay labeling endogenous nephrin. We could detect a more than 2-fold increase in nephrin endocytosis in CD2AP$^{-/-}$ podocytes treated with a control siRNA compared with CD2AP$^{-/-}$ podocytes treated with a CIN85/Ruk$_L$ siRNA. Endocytosis of nephrin in CD2AP$^{+/+}$ podocytes is significantly lower compared with CD2AP$^{-/-}$ podocytes, whereas there is no difference between CD2AP$^{+/+}$ podocytes treated with a control or CIN85/Ruk$_L$ siRNA. (Fig. 4*C*, *lower panel*; *, $p < 0.05$, CD2AP$^{-/-}$ podocytes treated with control siRNA *versus*

FIGURE 1. **Onset of proteinuria correlates with accumulation of ubiquitinated proteins in podocytes of CD2AP$^{-/-}$ mice.** *A*, immunohistochemistry using an anti-ubiquitin antibody shows ubiquitin-positive podocytes of diseased 3-week-old CD2AP$^{-/-}$ mice (*white arrows*). As a control the primary antibody was replaced by rabbit IgG. *B* and *C*, fluorescence labeling demonstrates ubiquitin (*B*) or CIN85/Ruk$_L$ (*C*) (*red fluorescence*) and nephrin (*green fluorescence*) in renal cortex sections of 18-day-old CD2AP$^{+/+}$ and proteinuric CD2AP$^{-/-}$ mice as well as healthy 14-day-old CD2AP$^{-/-}$ mice as control. Glomerular ubiquitin-positive cells and CIN85/Ruk$_L$ expression is increased, whereas nephrin expression is down-regulated in 18-day-old CD2AP$^{-/-}$ mice. Ubiquitin and CIN85/Ruk partially colocalize with nephrin resulting in *yellow fluorescence* in the merged picture. Control staining was performed with mouse or rabbit IgG instead of the primary antibody. The size of the *scale bars* is 30 μm. *D*, spot urine of 14-, 18-, and 22-day-old CD2AP$^{+/+}$ and CD2AP$^{-/-}$ mice was used for SDS-PAGE and stained with Coomassie Blue. The onset of proteinuria is clearly detectable at 18 days in the CD2AP$^{-/-}$ mice. *E*, whole kidney lysates of 14-, 18-, and 22-day-old CD2AP$^{+/+}$ and CD2AP$^{-/-}$ mice (*left*) and protein lysates from cultured CD2AP$^{+/+}$ and CD2AP$^{-/-}$ podocytes (*right*) were analyzed for ubiquitin by Western blot (*WB*). CD2AP$^{-/-}$ kidneys and cultured podocytes show an accumulation of ubiquitinated proteins. *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase.

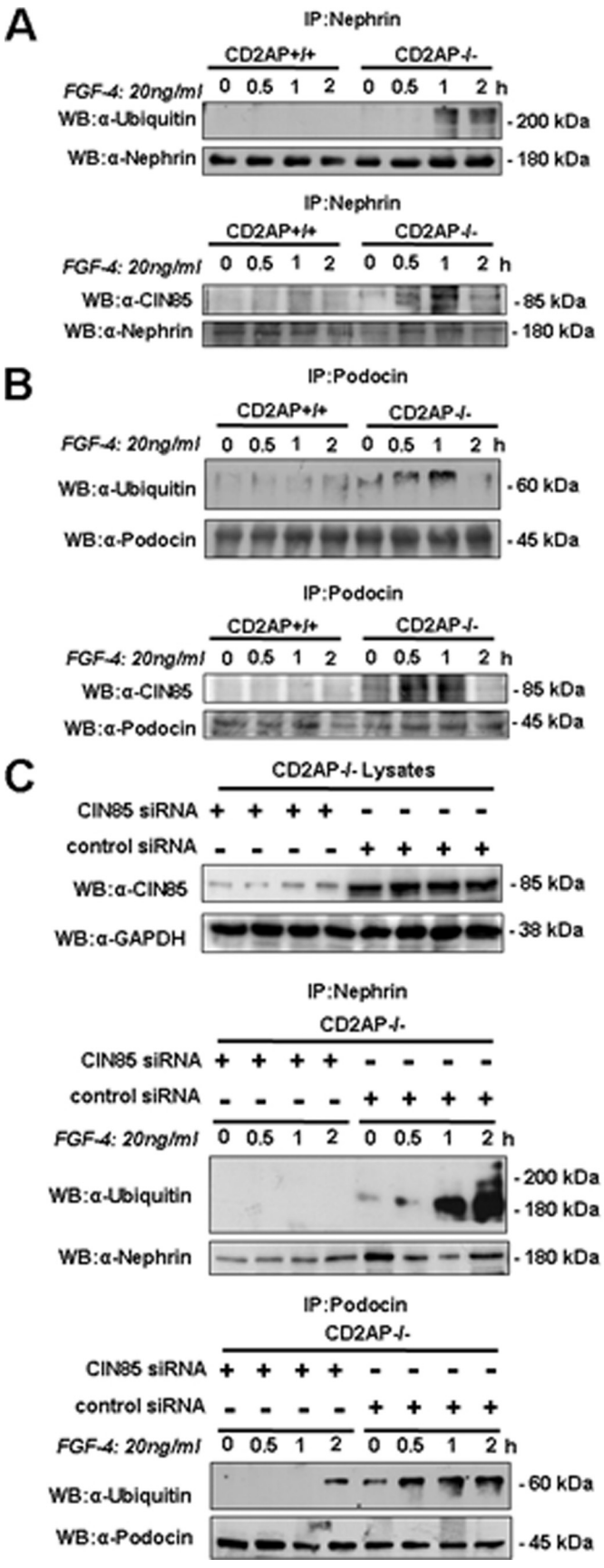


FIGURE 2. **Nephrin and podocin are early ubiquitinated in CD2AP$^{-/-}$ podocytes after growth factor stimulation.** CD2AP$^{+/+}$ and CD2AP$^{-/-}$ podocytes were stimulated for 0, 0.5, 1, and 2 h with FGF-4, and the whole cell lysate was then precipitated (*IP*) with nephrin (*A*) or podocin (*B*). The probes were blotted and analyzed for ubiquitin, CIN85/Ruk$_L$, nephrin, and podocin. *C*, CD2AP$^{-/-}$ podocytes were treated with 20 pmol of CIN85/Ruk$_L$ siRNA or control siRNA (scrambled sequence) and cultured for 72 h. Lysates were then analyzed for CIN85/Ruk$_L$ and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) to show inhibited expression of CIN85/Ruk$_L$ (*upper panel*). Silenced cells were stimulated with FGF-4 for 0, 0.5, 1, and 2 h, and the whole cell lysates were then precipitated with anti-nephrin (*middle*) and anti-podocin (*lower panels*). Precipitates where then analyzed for ubiquitin content. *WB*, Western blot.

CD2AP$^{-/-}$ podocytes treated with CIN85/Ruk$_L$ siRNA and CD2AP$^{+/+}$ podocytes treated with control siRNA). Control lysates show inhibited expression of CIN85/Ruk$_L$ and expression of CD2AP (Fig. 4*C*, *upper panel*). To confirm this result with a different method, we performed a surface biotinylation assay. Again we were able to detect a significant enhancement of nephrin endocytosis in the CD2AP$^{-/-}$ podocytes (*white bar*, *left panel*) (Fig. 4*D*, *left panel*; *, $p < 0.005$, CD2AP$^{-/-}$ podocytes *versus* CD2AP$^{+/+}$ podocytes). To confirm the data that endocytosis of nephrin in CD2AP$^{-/-}$ podocytes is dependent on CIN85/Ruk$_L$ expression, we inhibited expression of CIN85/Ruk$_L$. CD2AP$^{-/-}$ podocytes treated with a CIN85/Ruk$_L$ siRNA (*black bar*, *right panel*) showed a decreased endocytosis of nephrin compared with CD2AP$^{-/-}$ podocytes treated with a control siRNA (*white bar*), whereas CIN85/Ruk$_L$ expression is not inhibited (Fig. 4*D*, *right panel*; *, $p < 0.04$, CD2AP$^{-/-}$ podocytes treated with control siRNA *versus* CD2AP$^{-/-}$ podocytes treated with CIN85/Ruk$_L$ siRNA). These data suggest that in the presence of CIN85/Ruk$_L$ nephrin endocytosis is enhanced.

In the Presence of CIN85/Ruk$_L$ Nephrin and Podocin Are Internalized and Colocalized in Intracellular Vesicles after Growth Factor Stimulation—Former studies indicate that CIN85/Ruk$_L$ can act as a linker between Cbl and endophilin and is recruited upon EGF stimulation to the EGF receptor, which leads to receptor endocytosis (16). Other studies show that CIN85/Ruk$_L$ is associated with the Golgi complex, which takes part in membrane trafficking (26, 27). We overexpressed CIN85-GFP with β1,4-galactotransferase-pDsRed, a Golgi-associated protein, in CD2AP$^{+/+}$ podocyte and stimulated the cells for 60 min with FGF-4. By confocal microscopy we could show vesicle formation and colocalization of CIN85/Ruk$_L$ (*green*) with the Golgi-associated protein (*red*) (Fig. 5*A*), whereas the distribution of CD2AP (*green*) after FGF-4 stimulation remains the same, and no obvious colocalization of CD2AP (*green*) with the Golgi complex (*red*) is detectable (Fig. 5*B*). To visualize that nephrin trafficking from the membrane into the cell after FGF-4 stimulation depends on CIN85/Ruk$_L$ expression, we transiently overexpressed Nephrin-SV5 with CIN85-GFP in CD2AP$^{+/+}$ podocytes. We simultaneously stained the living cells and stimulated them with FGF-4 (20 ng/ml) for 60 min. We found that, in contrast to unstimulated cells where nephrin (*red*) is expressed alongside the cell membrane, stimulated cells displayed a vesicle-like, perinuclear distribution pattern for nephrin that significantly colocalized with CIN85-GFP (Fig. 5*C*). Compared with that, nephrin remained at the cell surface after stimulation with FGF-4 in podocytes transfected with an empty GFP expression plasmid (Fig. 5*D*). These data indicate that expression of CIN85/Ruk$_L$ leads to internalization of nephrin after FGF-4 treatment and enrich-

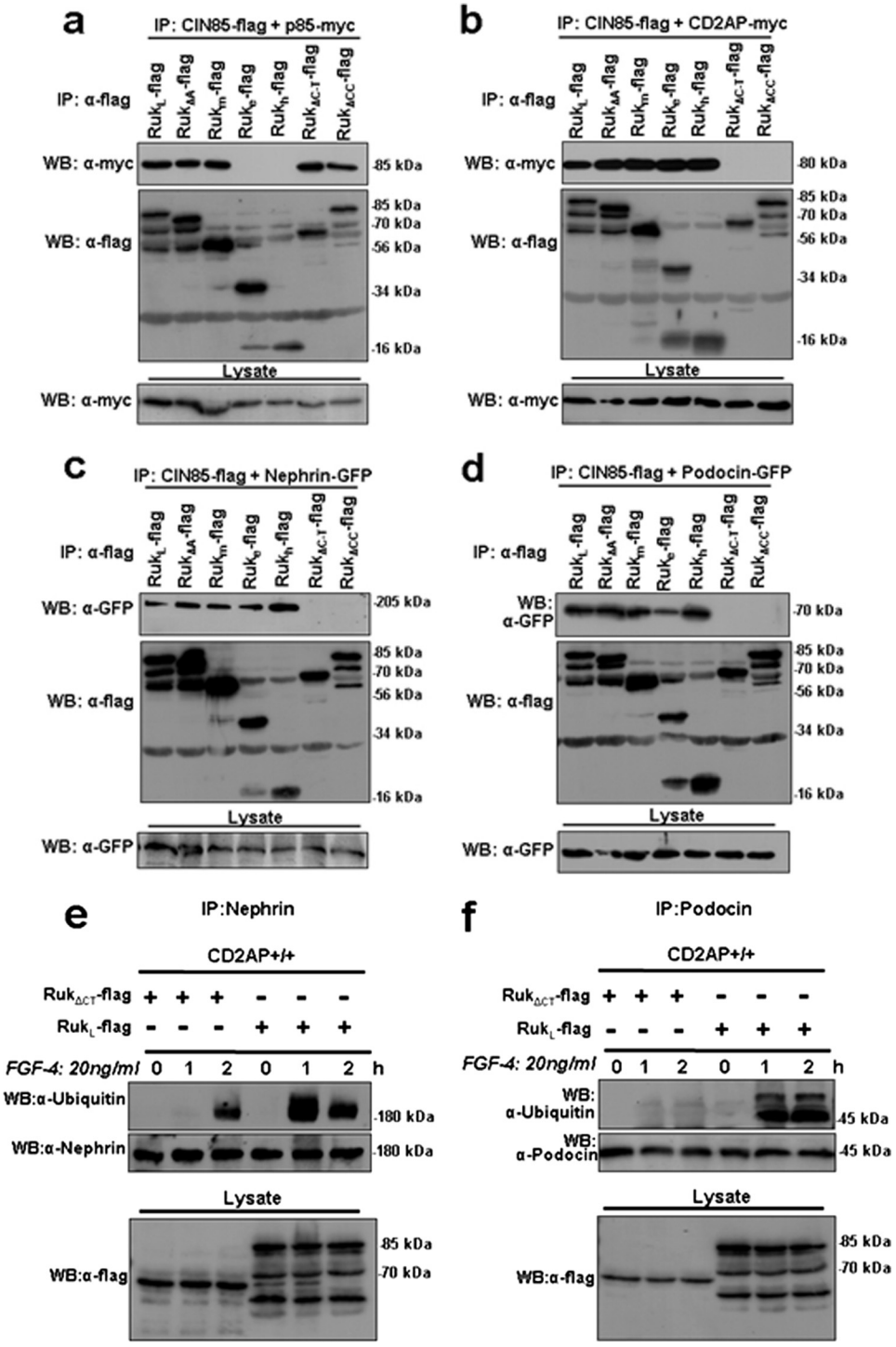


FIGURE 3. **CIN85/Ruk$_L$ interacts with nephrin and podocin at the coiled-coil domain.** HEK 293T cells were transiently transfected with constructs encoding FLAG-tagged isoforms of CIN85/Ruk and Nephrin-GFP, Podocin-GFP, CD2AP-myc, or p85-myc. CIN85/Ruk-FLAG was precipitated (*IP*) from whole cell lysate using FLAG beads. The probes were blotted (*WB*) and analyzed for GFP or myc expression. For control of expression whole cell lysates were blotted and analyzed for FLAG, GFP, or myc expression. The binding of p85 (*a*) and CD2AP (*b*) to the Ruk isoforms was performed as a control for specific binding and proof of integrity of the coiled-coil domain. Nephrin (*c*) and podocin (*d*) interact with the coiled-coil domain of CIN85/Ruk. CD2AP$^{+/+}$ podocytes were transiently transfected with FLAG-Ruk$_{L\Delta CT}$ or FLAG-Ruk$_L$ and stimulated for 0, 1, and 2 h with FGF-4, and the whole cell lysates were precipitated with anti-nephrin (*e*) or anti-podocin (*f*). The probes were then blotted and analyzed for ubiquitin, nephrin or podocin. Lysates were analyzed for FLAG expression.

ment of nephrin in CIN85/Ruk$_L$-containing vesicles. Moreover, the CIN85/Ruk$_L$-dependent trafficking of nephrin takes place at the Golgi complex.

Functional Competition between CD2AP and CIN85/Ruk$_L$ for Binding to Nephrin and Podocin—Since we demonstrate that nephrin, podocin, and CD2AP bind to the same region of CIN85/Ruk$_L$, we wanted to examine if CD2AP and CIN85/Ruk$_L$ compete for binding to nephrin and podocin. To test that, we coexpressed CD2AP with CIN85/Ruk$_L$ and nephrin or podocin in HEK 293T cells. When we precipitated CIN85/Ruk$_L$, we could detect a strong binding of CIN85/Ruk$_L$ with nephrin; however, in the presence of CD2AP this binding was significantly reduced (Fig. 6*a*). As a control, overexpression of p85 had no effect on nephrin binding. Similarly, when we precipitated for overexpressed nephrin, we could clearly detect binding of CIN85/Ruk$_L$ in the absence of CD2AP; however, when we coexpressed CD2AP, the binding of CIN85/Ruk$_L$ to nephrin was significantly reduced (Fig. 6*b*). Similarly, when we performed the same series of experiments for podocin, we detected a strong binding of podocin and CIN85/Ruk$_L$ in the absence of CD2AP; however, in the presence of CD2AP, this binding was significantly reduced (Fig. 6, *c* and *d*). These results indicate functional competition of CD2AP and CIN85/Ruk$_L$ for binding to nephrin and podocin. When both proteins are coexpressed, nephrin and podocin bind preferentially to CD2AP.

DISCUSSION

The podocyte slit diaphragm is a delicate extracellular protein structure that has to withstand blood pressure-associated changes in the glomerular perfusion pressure and, thus, requires constant renewal. Podocytes can retract and reform their foot processes in response to several cellular stresses (28). These dynamics require a tightly regulated interplay of signaling adaptors that orchestrate the tight regulation of protein trafficking and turnover. The nephrin-podocin receptor complex is the backbone of the zipper-like structure of the slit diaphragm (3). Given its structure and its localization, it is not surprising that this complex has signal

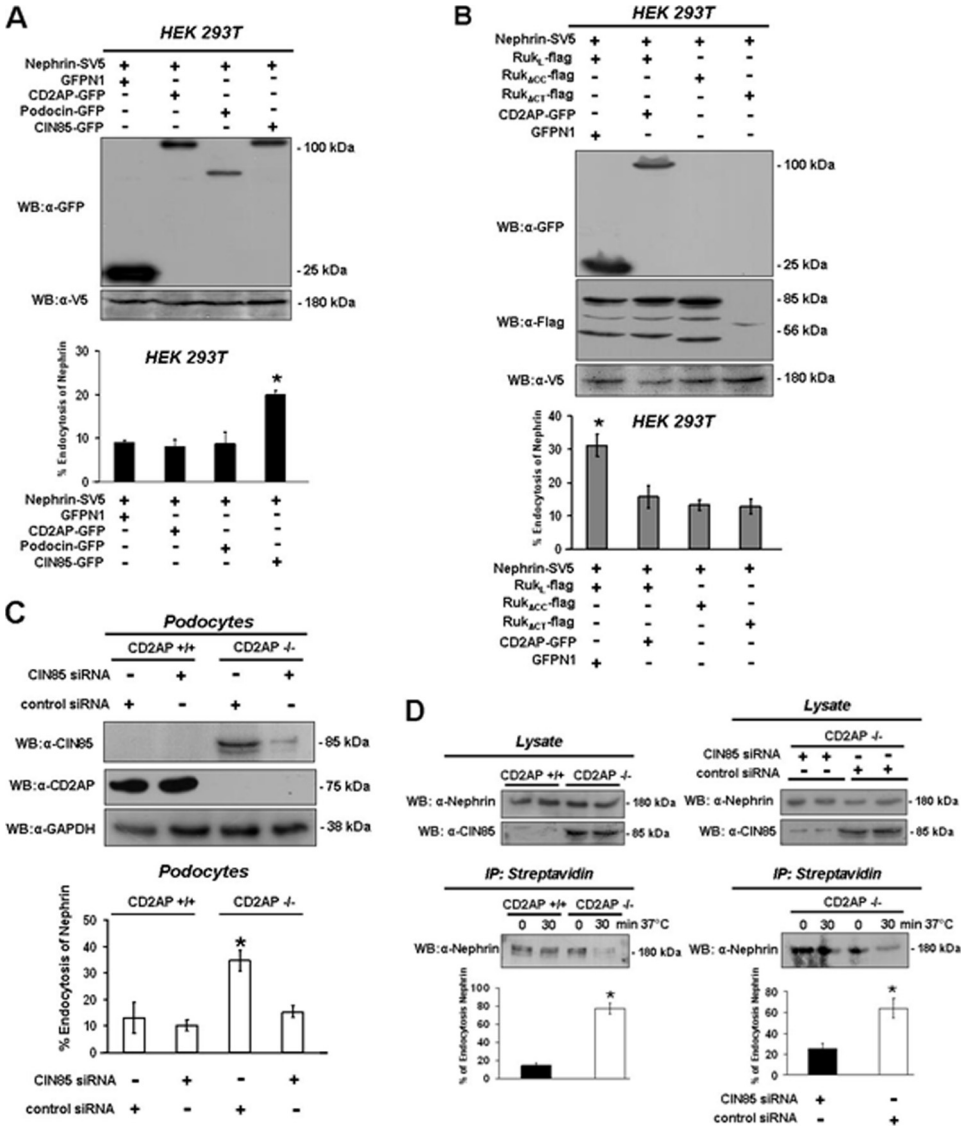


FIGURE 4. **Endocytosis of nephrin is enhanced in the presence of CIN85/Ruk$_L$.** HEK 293T cells were cotransfected with Nephrin-SV5 and GFPN1, CD2AP-GFP, Podocin-GFP, or CIN85-GFP (*A*) or with Nephrin-SV5 and GFPN1, Ruk$_L$-FLAG, Ruk$_L$-FLAG with CD2AP-GFP, Ruk$_{\Delta CC}$-FLAG, or Ruk$_{\Delta CT}$-FLAG (*B*). *WB*, Western blot. Nephrin-SV5 expressed at the surface of the cells was labeled with an anti-V5-antibody. The cells were labeled at 4 °C followed by incubation at 37 °C for 60 min to induce endocytosis. The controls remained at 4 °C. The extinction was measured at 405 nm. *A*, *, $p < 0.007$ CIN85/Ruk$_L$ *versus* GFPN1, CD2AP, and Podocin. *B*, *, $p < 0.04$ Ruk$_L$ *versus* Ruk$_L$ + CD2AP, Ruk$_{\Delta CC}$, and Ruk$_{\Delta CT}$. The *upper panels* show expression of proteins analyzed by Western blot. *C*, CD2AP$^{+/+}$ and CD2AP$^{-/-}$ were treated with a CIN85/Ruk$_L$ siRNA or control siRNA and cultured for 72 h. Surface-expressed nephrin was labeled with an anti-nephrin antibody. Endocytosis of endogenous nephrin was measured by the same method. *. $p < 0.05$, CD2AP$^{-/-}$ podocytes treated with control siRNA *versus* CD2AP$^{-/-}$ podocytes treated with CIN85/Ruk$_L$ siRNA and CD2AP$^{+/+}$ podocytes treated with control siRNA. The *upper panels* show expression of proteins analyzed by Western blot. *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase. *D*, podocyte surface proteins were biotinylated in CD2AP$^{+/+}$ and CD2AP$^{-/-}$ podocytes, and CD2AP$^{-/-}$ podocytes were treated with a CIN85/Ruk$_L$ siRNA and control siRNA. Lysates were precipitated (*IP*) using streptavidin-coupled beads, and nephrin expression was analyzed using a Western blot. *Left panel*, *, $p < 0.005$, CD2AP$^{-/-}$ *versus* CD2AP$^{+/+}$ podocytes; *right panel*, *, $p < 0.04$ CD2AP$^{-/-}$ podocytes treated with control siRNA *versus* CD2AP$^{-/-}$ podocytes treated with CIN85/Ruk$_L$ siRNA. The *upper panels* show expression of nephrin and CIN85/Ruk$_L$ in the lysates.

transduction properties that could underlie the same mechanisms of receptor desensitization as receptor-tyrosine kinases. The scaffolding molecule CD2AP has an important role for intracellular receptor-mediated signaling (29). In addition to its described signaling functions in combination with the slit diaphragm complex and growth factor receptors (5, 30, 31) it serves as a linker to the cytoskeleton. The deficiency of CD2AP in mice causes a nephrotic syndrome similar to nephrin deficiency. However, in contrast to nephrin-deficient mice, CD2AP-deficient mice are born healthy and die at 6 weeks of age (11), whereas nephrin deficiency in mice is fatal in their first days of life (6). We and others observed that normal foot processes and slit diaphragms are formed independently of CD2AP (6, 11, 12). Therefore, CD2AP seems not to be an indispensible component for slit diaphragm development but apparently for maintaining the filtration slit. Because we could show that in diseased CD2AP$^{-/-}$ mice, CIN85/Ruk$_L$, a protein that belongs to the same adaptor protein family as CD2AP, is up-regulated in podocytes (12), we wanted to investigate if the dysregulated CIN85/Ruk$_L$ expression is the factor that destabilizes the slit diaphragm by enhanced endocytosis of the nephrin-podocin receptor-complex.

First, we detected an increase of ubiquitin- and CIN85/Ruk$_L$-positive podocytes in diseased CD2AP$^{-/-}$ mice that correlates with the beginning of proteinuria. Furthermore, we could show by Western blot an accumulation of ubiquitinated proteins in diseased kidneys of CD2AP$^{-/-}$ mice and in cultured CD2AP$^{-/-}$ podocytes (Fig. 1). These findings are in line with a recently published work where an increase in podocytic ubiquitin C-terminal hydrolase (UCH-L1) and ubiquitin content was demonstrated in a subset of glomerulopathies that correlated with an internalization of nephrin and podocin (21). Therefore, an activated ubiquitination system is a relevant process in human diseases as well. Our *in vitro* data confirmed that nephrin and podocin are early ubiquitinated after growth factor stimulation in CD2AP$^{-/-}$ compared with CD2AP$^{+/+}$ podocytes. To strengthen the data that ubiquitination of nephrin and podocin depends on CIN85/Ruk$_L$ we inhibited CIN85/Ruk$_L$ expression in CD2AP$^{-/-}$ podocytes, and we could show a decreased ubiquitination of nephrin and podocin when CIN85/Ruk$_L$ expression is inhibited (Fig. 2). Cell surface transmembrane molecules are primarily ubiquitinated, which alters their subcellular localization or tar-

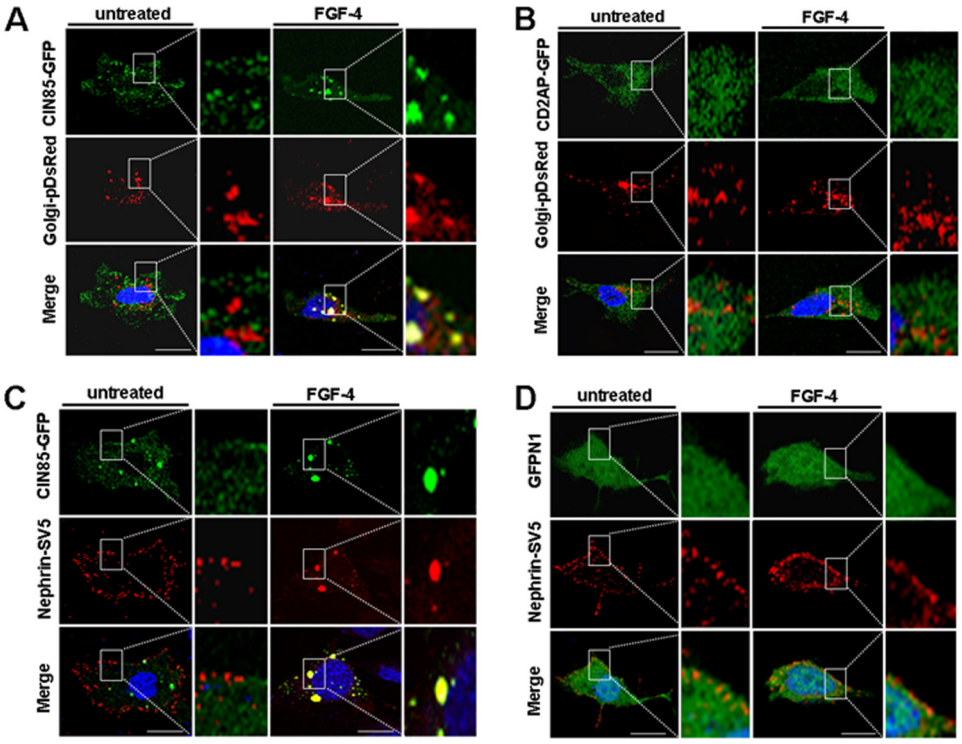


FIGURE 5. **Nephrin is internalized after growth factor stimulation in the presence of CIN85/Ruk$_L$.** *A* and *B*, CD2AP$^{+/+}$ podocytes were co-transfected with CIN85-GFP (*A*) or CD2AP-GFP (*B*) and β-1,4-galactotransferase-pDsRed (Golgi-associated protein). The cells were left untreated or stimulated for 60 min with FGF-4 (20 ng/ml). CIN85/Ruk$_L$ (*green*) colocalizes with β-1, 4-galactotransferase (*red*) after treatment with FGF-4. *C* and *D*, CD2AP$^{+/+}$ podocytes were co-transfected with Nephrin-SV5 and with CIN85-GFP or GFPN1 empty vector. Nephrin was visualized by staining the living cells with an anti-SV5 antibody and a Cy3-labeled secondary antibody. The cells were left untreated or stimulated for 60 min with FGF-4 (20 ng/ml). Nephrin (*red*) is expressed alongside the cell membrane in unstimulated cells. After stimulation, nephrin (*red*) is distributed perinuclear in the presence of CIN85/Ruk$_L$ (*green*). Colocalization results in *yellow fluorescence* in the merged pictures. Nuclei were stained with 4′,6-diamidino-2-phenylindole for visualization. Size of scale bars is 10 μm.

gets them for degradation. We hypothesize that because of increased ubiquitination of nephrin and podocin the slit diaphragm in CD2AP$^{-/-}$ is destabilized. Because CIN85/Ruk$_L$ is described as the mediator of ubiquitin-mediated endocytosis of the EGF receptor (16), we had to explore first whether CIN85/Ruk$_L$ interacts with nephrin and podocin. To test that, we overexpressed nephrin and podocin with several isoforms and deletion constructs of CIN85/Ruk$_L$, and we detected a specific binding of both nephrin and podocin to CIN85/Ruk$_L$ (Fig. 3). Because no association of nephrin and podocin can be detected with the ΔC-T and ΔCC constructs, we postulate that the coiled-coil domain of CIN85/Ruk$_L$ is required for binding to nephrin and podocin. It is shown by various groups that the coiled-coil domain participates in endocytosis and is essential for the whole process (32–36). Interestingly, as previously described, CD2AP binds to the same region of CIN85/Ruk$_L$, and coexpression of both molecules leads to formation of heterotypic complexes (37). Whether this binding would also influence the abundance of free CIN85/Ruk$_L$ in the presence of CD2AP remains unclear. Interestingly, transient transfection of the C-terminal deletion mutant of Ruk$_L$ leads to a decreased ubiquitination of nephrin and podocin compared with full-length Ruk$_L$ (Fig. 3). This is in line with other studies where it is shown that the coiled-coil domain is needed for down-regulation of the EGF receptor (38, 39). To further investigate a possible CIN85/Ruk$_L$-dependent endocytosis of nephrin in a quantitative way, we performed an enzyme-linked immunosorbent assay-based endocytosis assay that measured nephrin surface expression. Overexpression of nephrin with CIN85/Ruk$_L$ led to enhanced endocytosis of nephrin compared with coexpression of nephrin with CD2AP, podocin, Ruk$_{\Delta CT}$, and Ruk$_{\Delta CC}$ and coexpression of Ruk$_L$ with CD2AP in HEK 293T cells. Furthermore, endocytosis of endogenous expressed nephrin at the surface of CD2AP$^{-/-}$ podocytes is enhanced compared with CD2AP$^{+/+}$ and CD2AP$^{-/-}$ podocytes treated with a CIN85/Ruk$_L$ siRNA. Additionally, a biotinylation of surface-expressed nephrin confirmed that the remaining nephrin at the surface of podocytes is dramatically decreased in CD2AP$^{-/-}$ compared with CD2AP$^{+/+}$ and CD2AP$^{-/-}$ podocytes treated with a CIN85/Ruk$_L$ siRNA (Fig. 4). Immunofluorescence assays by confocal microscopy revealed that nephrin is internalized in the presence of CIN85/Ruk$_L$ after growth factor stimulation. Interestingly, we observed colocalization of nephrin with CIN85/Ruk$_L$ and prominent vesicle formations at the perinuclear area (Fig. 5), which is characteristic for activated receptors that are down-regulated by endocytosis and subsequently degraded in lysosomes (40). Interestingly, we can show a colocalization of CIN85/Ruk$_L$ with the Golgi complex after stimulation with FGF-4. Other studies showed that CIN85/Ruk$_L$ associated with the Golgi complex and could, therefore, take part in trafficking of receptors (27). Furthermore, it has been demonstrated that ubiquitin mediates sorting of proteins from the trans-Golgi network to the endosomes, thereby preventing their appearance on the cell surface and hastening their degradation in the lysosome vacuole (41). Further experiments to elucidate the candidate machinery in nephrin trafficking are ongoing studies in our laboratory. We further hypothesized that CD2AP and CIN85/Ruk$_L$ compete for binding to nephrin and podocin as it is known that nephrin also interacts with the C-terminal ending of CD2AP (42). To demonstrate a competition of CD2AP and CIN85/Ruk$_L$ for binding to nephrin and podocin, we performed coexpression experiments. When we examined nephrin and podocin binding after co-immunoprecipitation, we detected that both molecules would preferentially bind to CD2AP when CD2AP is coexpressed with CIN85/Ruk$_L$ (Fig. 6). The domain organization of CIN85/Ruk$_L$ is identical to that of CD2AP (25). The similarity is not only restricted to the general organization. CIN85/Ruk$_L$ is the only known protein that has amino acid homology to CD2AP outside the SH3

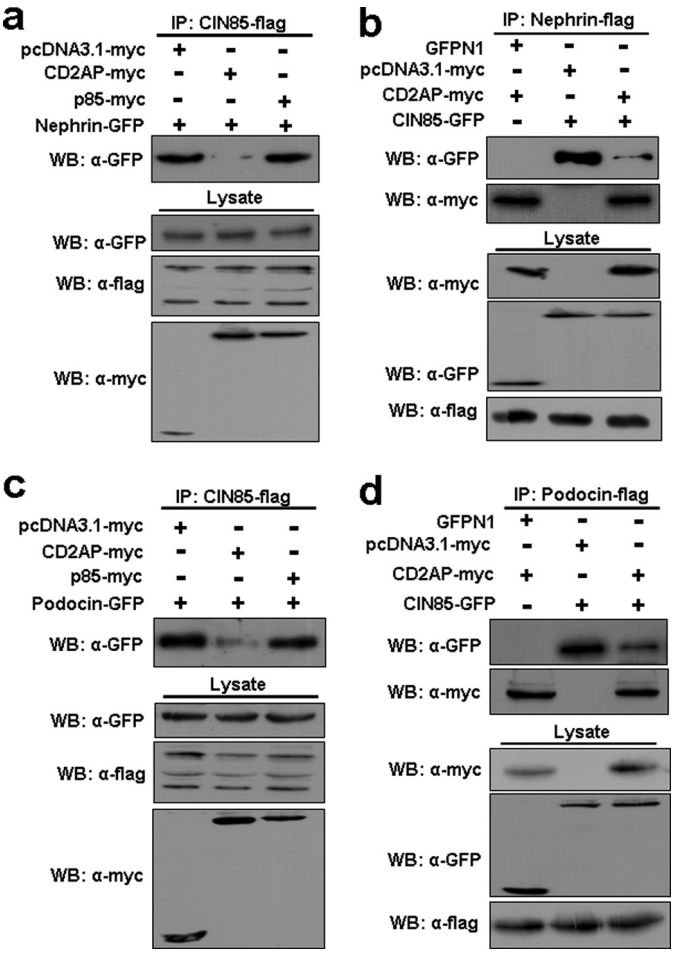


FIGURE 6. **Nephrin and podocin bind preferentially to CD2AP.** HEK 293T cells were transiently transfected with CD2AP, CIN85/Ruk$_L$, nephrin, and podocin. CIN85/Ruk$_L$-FLAG, Nephrin-FLAG, or Podocin-FLAG was precipitated (*IP*) from whole cell lysates using anti-FLAG beads. The probes were analyzed for GFP or myc expression. Lysates were blotted (*WB*) and analyzed for protein content using anti-GFP, FLAG, or myc antibodies. In the presence of CD2AP, binding of CIN85/Ruk$_L$ to nephrin is significantly reduced (*a* and *b*). Similarly, the binding of podocin to CIN85/Ruk$_L$ is significantly impaired in the presence of CD2AP (*c* and *d*).

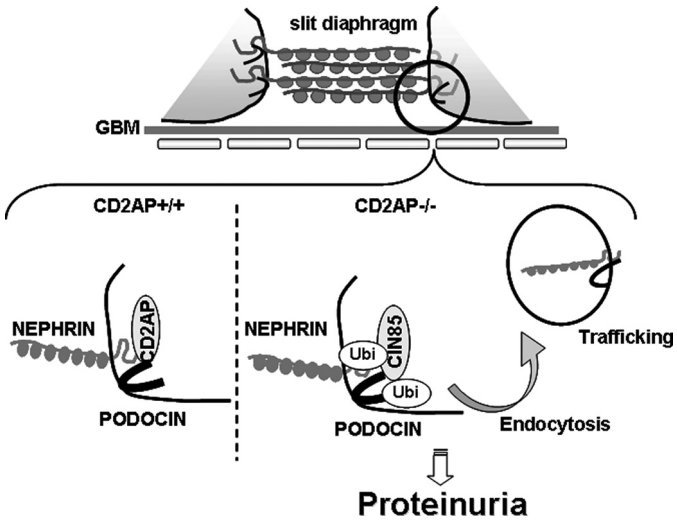


FIGURE 7. **Working model of slit diaphragm turnover and trafficking of nephrin and podocin.** In the presence of CD2AP the slit diaphragm complexes are stabilized. If CD2AP dissociates or is absent, CIN85 can bind to both molecules and induce trafficking of nephrin and podocin. *Ubi*, ubiquitin; *GBM*, glomerular basement membrane.

and proline-rich domains, namely in the C-terminal coiled-coil domain. Interestingly, it was shown by others that interaction of CIN85/Ruk$_L$ with phosphatidylinositol 3-kinase leads to inhibition (24) and binding of CD2AP to phosphatidylinositol 3-kinase leads to enhancement of the lipid kinase activity (4).

In summary, our presented data define a previously undescribed dynamic concept of the glomerular filtration slit. In the absence of CD2AP the abundance of CIN85/Ruk$_L$ increases, and the intracellular domain of nephrin associates with CIN85/Ruk$_L$. Previously, it was hypothesized by others that CIN85/Ruk$_L$ can compensate the absence of CD2AP (43). Our data demonstrate for the first time that binding of CIN85/Ruk$_L$ to nephrin and podocin leads to ubiquitination of both, which results in heavy proteinuria and demarcates the beginning of disease in the CD2AP$^{-/-}$ mouse (Fig. 7). It is not surprising that a receptor-like complex as the nephrin-podocin complex can undergo the same fate as other activated membrane receptors, as the slit diaphragm is constantly exposed to a number of cytokines and toxins. It is possible that association of the nephrin-podocin complex with activated growth factors occurs and that this whole complex is internalized. This question will be addressed in further studies in our laboratory. We presume that the enhanced and constant binding of CIN85/Ruk$_L$ to the nephrin-podocin receptor complex leads to endocytosis and destabilization of the slit diaphragm. How much this mechanism contributes to the normal turnover of slit diaphragm complex under physiological conditions remains unclear. Posttranslational modifications and phosphorylation of CD2AP and CIN85/Ruk$_L$ or cleavage of CD2AP by intracellular enzymes are possible mechanisms that are also under current investigations in our laboratory. Those mechanisms could lead to changes in protein binding and, thus, contribute to the orchestrated trafficking of the slit diaphragm.

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