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CD2AP/CIN85 Balance Determines Receptor Tyrosine Kinase Signaling Response in Podocytes*

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Defects in podocyte signaling are the basis of many inherited glomerular diseases leading to glomerulosclerosis. CD2-associated protein (CD2AP) is highly expressed in podocytes and is considered to play an important role in the maintenance of the glomerular slit diaphragm. Mice deficient for CD2AP ($CD2AP^{-/-}$) appear normal at birth but develop a rapid onset nephrotic syndrome at 3 weeks of age. We demonstrate that impaired intracellular signaling with subsequent podocyte damage is the reason for this delayed podocyte injury in $CD2AP^{-/-}$ mice. We document that CD2AP deficiency in podocytes leads to diminished signal initiation and termination of signaling pathways mediated by receptor tyrosine kinases (RTKs). In addition, we demonstrate that CIN85, a paralog of CD2AP, is involved in termination of RTK signaling in podocytes. CIN85 protein expression is increased in $CD2AP^{-/-}$ podocytes *in vitro*. Stimulation of $CD2AP^{-/-}$ podocytes with various growth factors, including insulin-like growth factor 1, vascular endothelial growth factor, and fibroblast growth factor, resulted in a significantly decreased phosphatidylinositol 3-kinase/AKT and ERK signaling response. Moreover, increased CIN85 protein is detectable in podocytes in diseased $CD2AP^{-/-}$ mice, leading to decreased base-line activation of ERK and decreased phosphorylation after growth factor stimulation *in vivo*. Because repression of CIN85 protein leads to a restored RTK signaling response, our results support an important role of CD2AP/CIN85 protein balance in the normal signaling response of podocytes.

Phosphatidylinositol 3-kinase (PI3K)[2] and Ras/ERK mitogen-activated protein kinase signaling pathways are key factors for determining the specificity of cellular responses, including cell proliferation, cell differentiation, and cell survival (1, 2). We and others have previously demonstrated that the PI3K/AKT signaling response plays an important role for podocyte survival in particular in the presence of active transforming growth factor β (3, 4). We recently demonstrated that the PI3K/AKT response is directly targeted by cytokine cross-talk and subsequently influences the cellular outcome (5). The adaptor molecules CD2-associated protein (CD2AP) and CIN85 belong to a family of adaptor molecules that selectively control the spatial and temporal assembly of multiprotein complexes that transmit intracellular signals. For both molecules various interaction partners have been described placing them at the center of regulatory pathways involving signaling (6, 7), cytoskeletal arrangement (8, 9), vesicular trafficking (10), and endocytosis (11, 12). In this study we demonstrate that CD2AP and CIN85 contribute to the balance of RTK signaling in podocytes. The anatomical localization of the podocyte exposes this highly specialized cell type to a variety of cellular stressors like stretch force, reactive oxygen species, osmotic milieu changes, cytokines and chemokines, filtrated toxins, and waste products. Therefore, this location requires the cells to have a highly effective cellular response system to counteract proapoptotic cell programs induced by these factors. Deficiency in CD2AP leads to a differentiation-dependent increase in expression of its paralog CIN85 *in vitro* and *in vivo*. This leads subsequently to a severe signaling defect in podocytes. Our results could explain the sudden and uniform disease onset and the rapid progression of the disease in this mouse model. We hypothesize that any inherited or acquired change in the CD2AP/CIN85 protein balance may lead to a debilitation in podocyte survival signaling with subsequent podocyte loss and the development of glomerulosclerosis.

EXPERIMENTAL PROCEDURES

Antibodies and Reagents—Primary antibodies used for coimmunoprecipitation, Western blotting, and immunocytochemical studies were rabbit anti-phospho^{S473}AKT, mouse anti-phospho^{Y204}ERK, mouse anti-AKT1, rabbit anti-ERK, rabbit anti-FLAG, rabbit anti-Myc (Cell Signaling Technology, Beverly, MA), rabbit anti-CD2AP, rabbit anti-Grb2, rabbit anti-β-tubulin (Santa Cruz Biotechnology), mouse anti-SOS1 (Transduction Laboratories), rabbit anti-Cin85 (CT) (gift from Ivan Dikic), mouse anti-Synaptopodin (Progen, Heidelberg,

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[2] The abbreviations used are: PI3K, phosphatidylinositol 3-kinase; ERK, extracellular-regulated kinase; MEKK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; FGF, fibroblast growth factor; VEGF, vascular endothelial growth factor; siRNA, small interference RNA; RTK, receptor tyrosine kinase; GST, glutathione *S*-transferase; SH domain, Src homology domain; SOS, son of sevenless.

Germany). Horseradish peroxidase-conjugated goat anti-rabbit and horseradish peroxidase-conjugated goat anti-mouse as secondary antibodies for Western blotting were purchased from Santa Cruz Biotechnology. Cytokines and inhibitors were purchased from the following vendors: rhIGF-1 (R&D Systems, Minneapolis, MN), rhFGF4, rhVEGF1 (Cell Sciences, Canton, MA), LY294002, PD98059 (Calbiochem), farnesylthiosalicylic acid (Sigma).

Podocyte Culture, Cytokine Stimulation, and Inhibition of MEKK, PI3K, and Ras—Cultivation of conditionally immortalized mouse podocytes was performed as described by Mundel *et al.* (13). To propagate podocytes, cells were cultivated on type I collagen (BD Biosciences) at 33 °C in the presence of 10 units/ml mouse recombinant γ-interferon (Cell Sciences) (permissive conditions) to enhance expression of a thermo-sensitive T antigen. To induce differentiation, podocytes were maintained at 37 °C for 14 days without γ-interferon, resulting in absence of thermo-sensitive T antigen (non-permissive conditions). Differentiated podocytes were stimulated with fibroblast growth factor (FGF) (20 ng/ml) and vascular endothelial growth factor (VEGF) (20 ng/ml). To inhibit AKT and ERK activation, cells were pretreated with 20 μM LY294002, PD98059, or farnesylthiosalicylic acid for 20 min prior to cytokine stimulation.

Western Blot Analysis—To analyze whole cell protein lysates from cultivated podocytes, either untreated or treated cells were lysed on ice in immunoprecipitation buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 0.5% sodium deoxycholate, 1% Nonidet P-40, 0.1% SDS) containing protease inhibitor (complete mini; Roche Applied Sciences), 1 mM sodium orthovanadate, 50 mM NaF, and 200 μg/liter okadaic acid. Lysates were centrifuged at 12,000 rpm, and aliquots of the supernatants were separated by 10% SDS-PAGE and transferred to polyvinylidene difluoride membrane (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.

GST Pulldown Assays—For pulldown assay 500 μg of total cell lysate was incubated with 1 μg of GST Grb2 full-length (gift from S. M. Feller, Oxford, UK) and glutathione-Sepharose beads in pulldown 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) overnight at 4 °C. The pellets were washed in pulldown buffer and separated by SDS-polyacrylamide gel electrophoresis. Western blot was performed using the methods mentioned above.

Animals—Target disruption and generation of CD2AP-deficient homozygous mice on a 129/J background is described elsewhere (14). Kidney tissue was prepared from 2- and 3-week-old mice.

Immunohistochemistry—After dissection, kidneys were washed with phosphate-buffered saline and immediately frozen in tissue molds containing optimal cutting temperature compound. Sections were collected onto slides, blocked with 10% donkey serum, stained with the appropriate primary antibody followed by Cy3-conjugated donkey anti-rabbit and fluorescein isothiocyanate-conjugated donkey anti-mouse secondary antibodies (Jackson Immunoresearch). For light microscopy slides and electron microscopy the tissue was perfusion-fixed and processed following a standard protocol.

Isolation and Processing of Glomeruli—Glomeruli were isolated from kidneys of 3-week-old $CD2AP^{+/+}$ and $CD2AP^{-/-}$ mice using a sequential sieving technique with mesh sizes of 180, 100, and 71 μm. The fraction collected from the 71-μm sieve was maintained in tissue culture dishes at 37 °C for 24 h before stimulation. After stimulation glomeruli were resuspended in immunoprecipitation buffer and disrupted with a dounce homogenizer by hand with 50 up-and-down strokes. For immunohistochemistry, glomeruli were washed with phosphate-buffered saline and the pellets were immediately frozen in optimal cutting temperature. 2-μm cryosections were cut and fluorescence staining was performed using the methods mentioned above.

Design and Cloning of Lentiviral siRNA Vectors—The pLVTHM-deltaH-2K lentivirus transfer vector used in this study was generated by replacing enhanced green fluorescent protein sequences in the pLVTHM vector (kindly provided by Dr. Didier Trono, Department of Genetics and Microbiology, Faculty of Medicine, University of Geneva, Switzerland) on delta H-2K from pMACS K^k.II vector encoding the truncated H-$2K^k$ surface marker (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). The pLVTHM-deltaH-2K lentivirus transfer vector was used as a first step to ligate the Cin85 siRNA oligonucleotides (Cin85si): sense, 5′-CGCGTCCCCgaggcacaga atgatgatgaattTTCAAGAGAaattcatcatcattctgtgcctcTTTTTGGAAAT; antisense, 5′-CGATTTCCAAAAAGaggcacagaatgatgatgaattTCTC TTGAAaattcatcatcattctgtgcctcGGGGA. The hybridized oligonucleotides were ligated into the lentivirus transfer vector between MluI and ClaI restriction sites.

Lentiviral Vector Production and Cell Infection—Lentiviral vectors were produced by transient transfection of 293T cells according to standard protocols. 293T cells were cultured in Dulbecco's modified Eagle's medium, supplemented with 10% fetal calf serum and, when subconfluent, transfected with FuGENE 6 (Roche Diagnostics) using ratio of pCMV-dR8.74:pMD2G:pLVTHM-deltaH-2K-Cin85si or pLVTHM-deltaH-2K as a control = 3:2:1. After 48 h post-transfection cell supernatants, containing viral particles, were filtered using 0.4 μm Steriflip vacuum filtration system (Millipore) and concentrated by ultracentrifugation at 50,000 × *g* for 1.5 h at 4 °C. For lentiviral infection, differentiated $CD2AP^{-/-}$ podocytes were grown until 70–80% confluence and infected in the presence of 8 μg/ml polybrene with viruses and used for experiments 3 days after infection.

Transfection—The day before transfection the podocytes were seeded on 60-mm dishes. On the day of transfection cells were transfected using Effectene transfection reagent (Qiagen, Hilden, Germany) according to the manufacturer's protocol using FLAG-mCIN85, Myc-mCD2AP (gift from T. Huber), and pcDNA3.1 as a control. Cells were cultured in normal growth medium for 72 h after transfection, serum-starved overnight, and stimulated with FGF.

RESULTS

Podocyte Damage in 3-Week-old CD2AP$^{-/-}$ Mice Occurs Simultaneously in All Glomeruli—Previously we described that podocyte apoptosis is an early lesion during the development of the disease in CD2AP$^{-/-}$ mice and that apoptotic cell death is detectable in the endocapillary/mesangial compartments only at later time points (3). It still remains unclear why CD2AP$^{-/-}$ mice are born normal, develop a regular glomerular filtration barrier, and then within a time frame of 1–2 days become severely nephrotic. With detailed morphological analysis of diseased kidneys, we revealed global podocyte damage in a uniform temporal fashion. Glomerular damage was evaluated by periodic acid-Schiff staining at 2 and 3 weeks of age in CD2AP$^{+/+}$ and CD2AP$^{-/-}$ mice ($n = 5$ in each group). We found in CD2AP$^{-/-}$ mice at 2 weeks of age no mesangial matrix accumulation or damage in 221 examined glomerular profiles. However, at 3 weeks of age we found glomerular profiles with mesangial expansion and damage in 288 of 302 (95.4%) examined, compared with 378 normal-appearing glomerular profiles in CD2AP$^{+/+}$ mice. When we examined glomerular profiles of CD2AP$^{+/+}$ and CD2AP$^{-/-}$ mice ($n = 2$ in each group) using 1-μm sections (Fig. 1*a*) we found that in 192 examined glomerular profiles all of them (100%) showed mesangial proliferation in at least one lobule. Next, foot process effacement was evaluated in 10 complete glomerular profiles by transmission electron microscopy. In all of them the majority of loops exhibited complete foot process effacement (Fig. 1*b*), whereas controls were negative. Thus, there is a 100% penetrance of the damage in the CD2AP$^{-/-}$ mice. Within the cytoplasm sheets of podocytes that adhere broadly to the GBM we detected a prominent basal cytoskeletal layer consisting of a dense network of actin filaments (Fig. 1*c*, *insert*). The parietal epithelial cells (PECs) were affected and also developed lesions (*i.e.* vacuolization of the cytoplasm) (Fig. 1*b*). However, the parietal cell damage was not as uniform as in the podocytes. Because epithelial cells, the podocytes, and the PECs were all affected, the tuft adhesions were of an advanced type for the GBM directly attached to the parietal basement membrane over large distances without any interposed cells. Concurrently, we detected mesangial expansion with close meshed mesangiolysis followed by mesangial cell proliferation and matrix deposition in every lobule prominently including the vascular pole, resulting in a conspicuous broadening of the glomerular entrance and stalk. In 171 examined CD2AP$^{-/-}$ profiles and 61 examined CD2AP$^{+/+}$ profiles ($n = 2$ in each group) with a visible stalk broadening, we detected mesangial thickening in the afferent vessel portion in 100% of the CD2AP$^{-/-}$ glomeruli and 0% of the CD2AP$^{+/+}$ glomeruli. Taken together, these results indicate that the disease in CD2AP$^{-/-}$ mice is neither focal nor segmental. The uniformity of the lesions seems highly suggestive for the dysregulation of a physiological process necessary for the maintenance of normal glomerular tuft architecture.

CD2AP$^{-/-}$ Podocytes Show Dysregulated Activation Profiles for PI3K/AKT and ERK1/2 after Growth Factor Stimulation of Receptor Tyrosine Kinases (RTKs)—When we tested the PI3K/AKT and ERK1/2 signaling response after stimulation of RTKs with different growth factors, we detected diminished activation profiles in various CD2AP$^{-/-}$ podocytes clones (see "Podocyte Culture" under "Materials and Methods"). Depending on the growth factors tested we detected a more pronounced effect on signal initiation or on signal termination in the activation profiles of PI3K/AKT or ERK1/2. After stimulation with insulin-like growth factor 1 (100 ng/ml) we could not detect a significant difference in phosphorylation of AKT in CD2AP$^{-/-}$ podocytes compared with CD2AP$^{+/+}$ podocytes. However, in CD2AP$^{-/-}$ podocytes phosphorylation of ERK1/2 was terminated already at 4 h after stimulation, whereas strong phosphorylation was still visible 24 h after stimulation in CD2AP$^{+/+}$ podocytes (Fig. 2, *A* and *B*). In contrast, treatment of the cells with FGF (20 ng/ml) or VEGF (20 ng/ml) resulted in weak AKT phosphorylation (Fig. 2*A*) or ERK1/2 phosphorylation (Fig. 2*B*) in CD2AP$^{-/-}$ podocytes compared with CD2AP$^{+/+}$ cells.

Treatment of the cells with platelet-derived growth factor, hepatocyte growth factor, or epidermal growth factor revealed

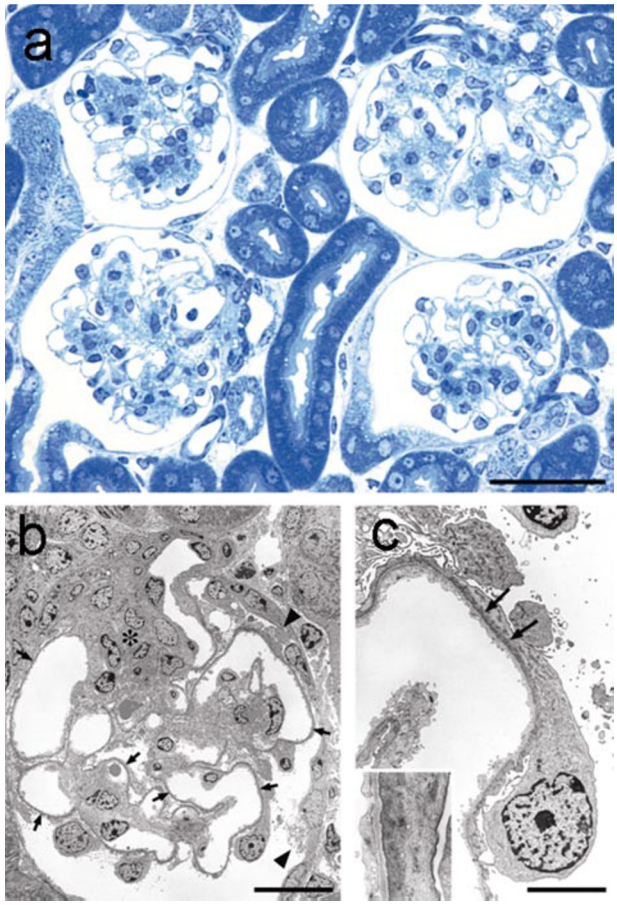

FIGURE 1. **Structural damage in 3-week-old CD2AP$^{-/-}$ mice concerns the podocytes simultaneously in all glomeruli.** *a*, overview of cortical tissue with four glomerular profiles, all of them exhibiting prominent mesangial expansion in the center of each lobule. *b*, low power transmission electron microscopy showing an entire glomerular profile. Foot process effacement is universally encountered as indicated by the continuous dark line (*arrows*) along the entire glomerular basement membrane. As seen by higher magnification in *panel c*, this dark line represents the dense actin-based cytoskeletal mat (*arrows*) that resulted from the fusion of the foot processes. *Insert in panel c* shows part of the area between the two arrows; the actin cytoskeleton including α-actinin densities is clearly visible. Note the broadening of the glomerular stalk (*asterisk*). Already at this stage injuries are also seen at parietal epithelial cells (*arrowheads in panel b*). Bar length, 50 μm (*a*), 20 μm (*b*), and 5 μm (*c*).

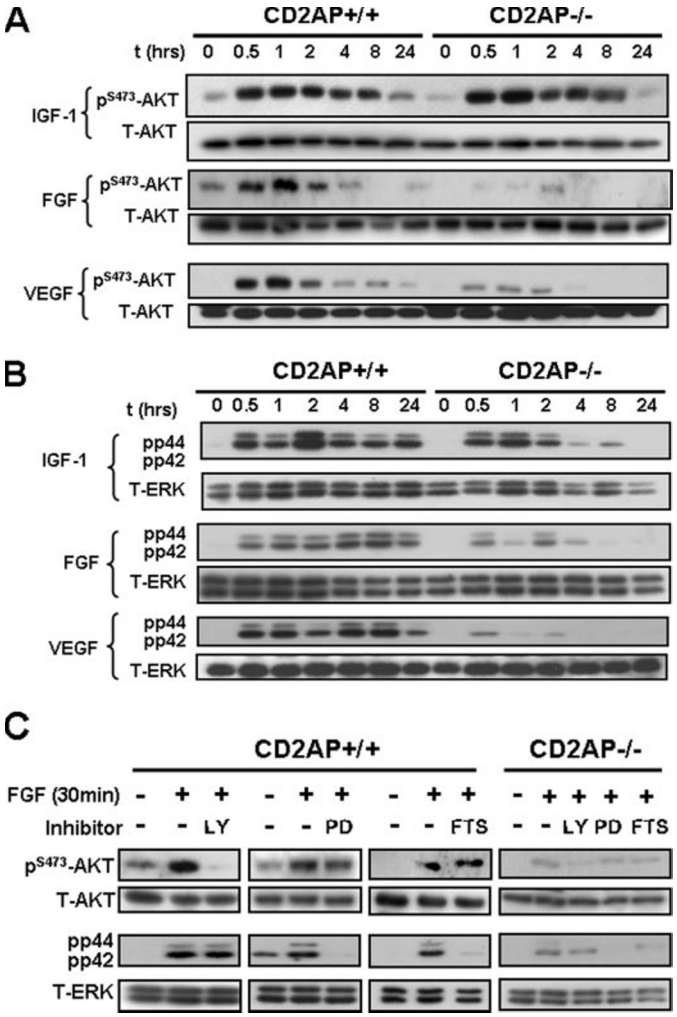

FIGURE 2. **CD2AP deficiency results in disturbed signal initiation and/or signal termination of RTK-induced PI3K/AKT and ERK1/2 signaling in podocytes.** CD2AP$^{+/+}$ and CD2AP$^{-/-}$ podocytes were maintained for 14 days under non-permissive conditions and were treated with insulin-like growth factor 1 (*IGF-1*), FGF, or VEGF for the time points indicated. CD2AP deficiency results in severely disturbed PI3K/AKT (*A*) and ERK1/2 signal (*B*) activation profiles after stimulation with insulin-like growth factor 1, FGF, or VEGF compared with wild-type control cells. *C*, ERK pathway activation in podocytes is independent of PI3K activation but dependent on Ras-Raf-MEK activation. CD2AP$^{+/+}$ and CD2AP$^{-/-}$ podocytes were treated with FGF for 30 min in the absence or presence of PI3K inhibitor LY294002 (*LY*), Ras inhibitor farnesylthiosalicylic acid (*FTS*), or MEK inhibitor PD98059 (*PD*). Inhibition of PI3K/AKT had no effect on ERK activation and inhibition of MEK or Ras had no effect on PI3K/AKT activation in podocytes. Results are representative for three independent experiments in different clones.

activation defects similar to the treatment with insulin-like growth factor 1 with comparable phosphorylation levels for AKT and ERK1/2 but earlier signal termination in CD2AP$^{-/-}$ podocytes (data not shown).

It has been demonstrated that direct interaction of CD2AP with the p85 subunit of PI3K resulted in AKT phosphorylation (6). However, so far only little is known about potential interaction partners of CD2AP resulting in phosphorylation of ERK1/2 (15). To dissect the possible pathway defects we first investigated whether phosphorylation of AKT and ERK1/2 are dependent on each other. In some cell types PI3Ks have been identified as effectors of Ras proteins (16). Therefore, we used chemical inhibitors of PI3K, Ras, and MEK activation to selectively mimic the situation found in CD2AP$^{-/-}$ cells. Using the PI3K inhibitor LY294002 we detected specific inhibition of PI3K/AKT phosphorylation but no effect on ERK1/2 phosphorylation (Fig. 2*C*). Preincubation of the cells with farnesylthiosalicylic acid or PD98059 for specific inhibition of Ras and MEK activation resulted in complete abrogation of ERK1/2 phosphorylation but no effect was detectable on PI3K/AKT signaling. To elucidate whether the signaling defect in CD2AP$^{-/-}$ podocytes is localized upstream of Ras-GTP we examined phospho-Raf in CD2AP$^{+/+}$ and CD2AP$^{-/-}$ cells. In CD2AP$^{-/-}$ podocytes we did not detect phospho-Raf, indicating that the CD2AP-dependent effect is localized upstream of Ras-Raf activation (data not shown). Taken together these results indicate that CD2AP is essential for the normal course of both signaling cascades. Furthermore, in podocytes the activation of both pathways is not dependent on each other, suggesting an autonomous role for CD2AP in the regulation of these two pathways.

CD2AP$^{-/-}$ Podocytes Show an Inducible Disruption of Grb2-SOS Binding Profile and an Inducible Binding of Grb2-CIN85 after Stimulation of RTK with FGF or VEGF—Because Ras activation is directly influenced by Grb2-SOS (son of sevenless) binding, we wanted to examine whether CD2AP deficiency has an influence on Grb2-SOS binding activity in podocytes. Therefore, we performed GST-Grb2 pulldown experiments with cell lysates of CD2AP$^{+/+}$ and CD2AP$^{-/-}$ podocytes. On stimulation of CD2AP$^{-/-}$ podocytes with FGF (20 ng/ml) we detected significant reduction of Grb2-SOS binding after 30 min. Interestingly, we also detected binding of CIN85 to Grb2 after stimulation of CD2AP$^{-/-}$ podocytes with FGF for 30 min (Fig. 3*A*). Investigating a time course in CD2AP$^{+/+}$ cells, we detected a reduction of Grb2-SOS binding 4 h after stimulation with FGF accompanied by binding of CIN85 to Grb2. Stimulation of podocytes with VEGF also resulted in inducible binding of CIN85 and reduced binding of SOS to Grb2. Again reduction of Grb2-SOS binding was detectable at later time points in CD2AP$^{+/+}$ podocytes as well (Fig. 3*B*). To further evaluate our Grb2 pulldown experiments, we examined the base-line expression levels of involved proteins in unstimulated CD2AP$^{+/+}$ and CD2AP$^{-/-}$ podocyte clones. We found no difference in the basic expression levels of SOS or Grb2; however, in lysates of CD2AP$^{-/-}$ clones we detected a marked increase in basic expression of two isoforms of CIN85, a paralog of CD2AP (Fig. 3*C*). In summary, these results demonstrate that CD2AP deficiency leads to a dysregulation of Grb2-SOS binding in podocytes. Moreover, loss of CD2AP results in upregulation of CIN85, a binding partner of Grb2, competing with SOS for Grb2 binding. The inducible binding of CIN85 to Grb2 in CD2AP$^{+/+}$ podocytes at later time points may indicate that this interaction is part of the physiological ERK1/2 signal termination in podocytes.

Dysregulated Expression of CIN85 and Subsequent Signaling Defects Are Dependent on the Differentiation State of Podocytes—Interestingly, examination of the signaling response in undifferentiated, proliferating CD2AP$^{-/-}$ podocytes showed that these cells were not affected in activation of ERK1/2 after stimulation with FGF. In contrast to differentiated cells in undifferentiated, proliferating CD2AP$^{+/+}$ and

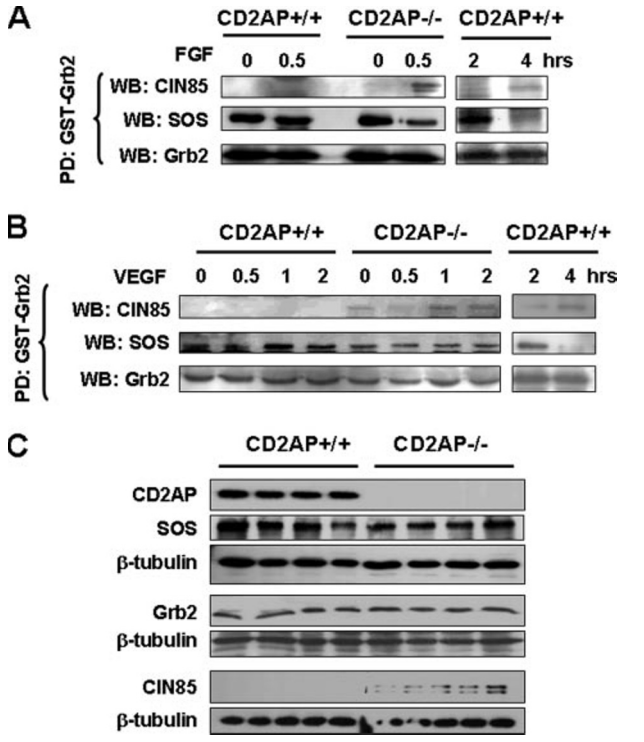

FIGURE 3. **GST-Grb2 pulldown experiments reveal cytokine-inducible disruption of Grb2-SOS binding and inducible interaction of CIN85 with Grb2.** Pulldown experiments with full-length GST-Grb2 were performed in lysates of CD2AP$^{+/+}$ and CD2AP$^{-/-}$ podocytes left untreated or treated with FGF (*A*) or VEGF (*B*) for the time points indicated. Grb2-SOS binding is disrupted earlier in CD2AP$^{-/-}$ podocytes compared with CD2AP$^{+/+}$ cells. Cytokine-inducible binding of CIN85 to Grb2 is detected earlier in CD2AP$^{-/-}$ podocytes. Results are representative for three independent experiments in different clones. *C*, Western blot shows basic expression levels of proteins involved in ERK1/2 activation in CD2AP$^{+/+}$ and CD2AP$^{-/-}$ podocyte clones. Podocytes were maintained for 14 days under non-permissive conditions, and Western blot was performed and developed with antibodies specific for CD2AP, SOS, Grb2, and Cin85. Blots were reincubated with a β-tubulin-specific antibody to normalize for protein content.

CD2AP$^{-/-}$ podocytes the signal initiation and termination profiles of ERK1/2 were identical (Fig. 4*A*). When we examined CIN85 protein expression levels, we detected low expression of CIN85 that was identical to undifferentiated wild-type cells. When we harvested the cells after 2, 6, 8, and 12 days of differentiation, we found in CD2AP$^{-/-}$ podocytes an accumulation of CIN85 protein. In contrast, in CD2AP$^{+/+}$ podocytes the expression level of CIN85 was unchanged (Fig. 4*B*). The degree of differentiation was not different between CD2AP$^{+/+}$ and CD2AP$^{-/-}$ podocytes as indicated by comparable levels of the differentiation marker synaptopodin. However, when we examined the expression of CIN85 mRNA in differentiating cells we did not detect a difference in CIN85 mRNA levels between CD2AP$^{+/+}$ and CD2AP$^{-/-}$ cells over time (data not shown). Next we examined the phosphorylation response of ERK1/2 after stimulation with FGF at different points of differentiation and found that in CD2AP$^{-/-}$ cells ERK1/2 activation decreased with ongoing differentiation in CIN85-expressing cells (Fig. 4*C*). To confirm that the effect on ERK1/2 phosphorylation had been modulated by CIN85 in differentiated CD2AP$^{-/-}$ cells, we selectively down-regulated CIN85 expression using a lentivirus-based CIN85 siRNA expression vector. As expected, ERK1/2 phosphorylation had been restored in dif-

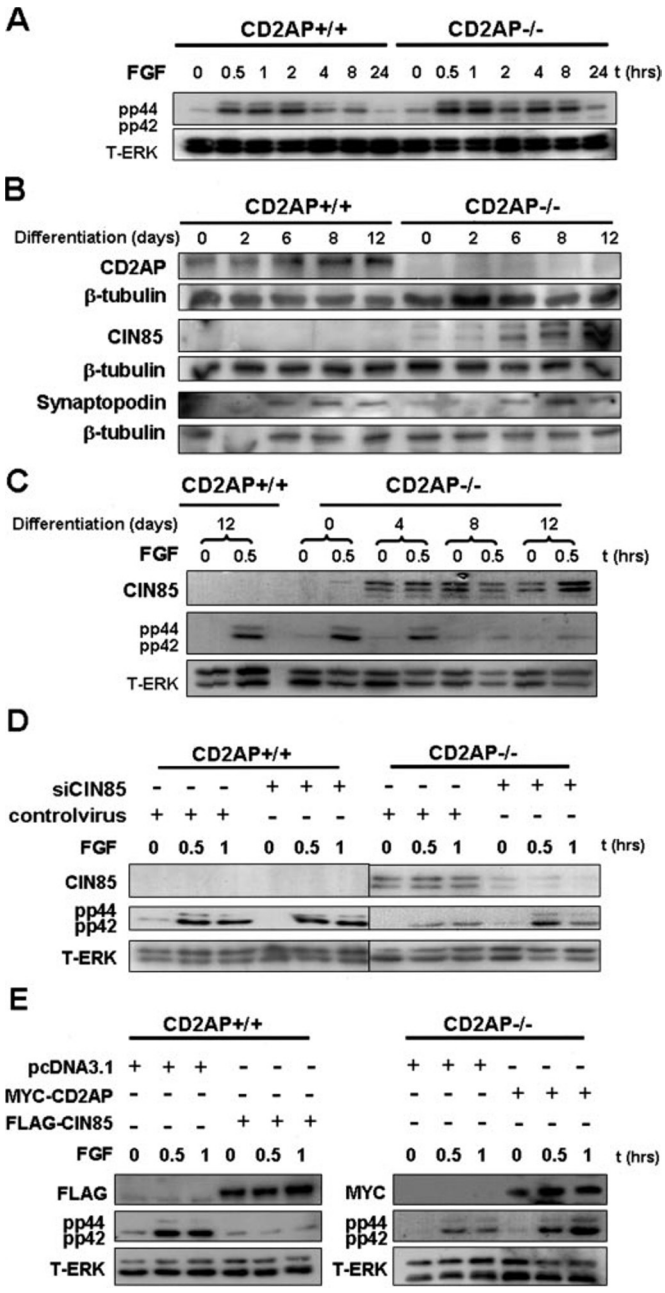

FIGURE 4. **CIN85 expression depends on differentiation state, and the expression level of CIN85 determines the signaling response in podocytes.** *A*, CD2AP$^{+/+}$ and CD2AP$^{-/-}$ podocytes kept under permissive conditions at 33 °C in the proliferating state were treated with FGF for the time points indicated. CD2AP deficiency in undifferentiated cells had no effect on ERK1/2 signal activation profiles after stimulation with FGF compared with CD2AP$^{+/+}$ cells. *B*, Western blot demonstrates increase in CIN85 protein expression levels in CD2AP$^{-/-}$ podocytes compared with CD2AP$^{+/+}$ podocytes with days of culture in non-permissive conditions. *C*, CD2AP$^{+/+}$ cells after 12 days of differentiation and CD2AP$^{-/-}$ cells after 0, 4, 8, and 12 days of differentiation were stimulated with FGF for 0.5 h, and ERK1/2 activation was visualized by Western blot. ERK1/2 activation decreases with increase of CIN85 protein expression. *D*, CIN85 expression was silenced in CD2AP$^{+/+}$ and CD2AP$^{-/-}$ podocytes differentiated for 12 days using a lentivirus-based siRNA vector, and ERK1/2 activation was monitored on a Western blot after stimulation with FGF for the time points indicated. Signaling response is restored after silencing of CIN85 in CD2AP$^{-/-}$ podocytes. *E*, Western blot demonstrates disrupted signaling response in CD2AP$^{+/+}$ podocytes transiently transfected with a FLAG-tagged CIN85 expression construct (*left panel*) and restored signaling response in CD2AP$^{-/-}$ podocytes transiently transfected with a Myc-tagged CD2AP expression construct compared with control pcDNA3.1-transfected cells (*right panel*). Results are representative for three independent experiments in different clones.

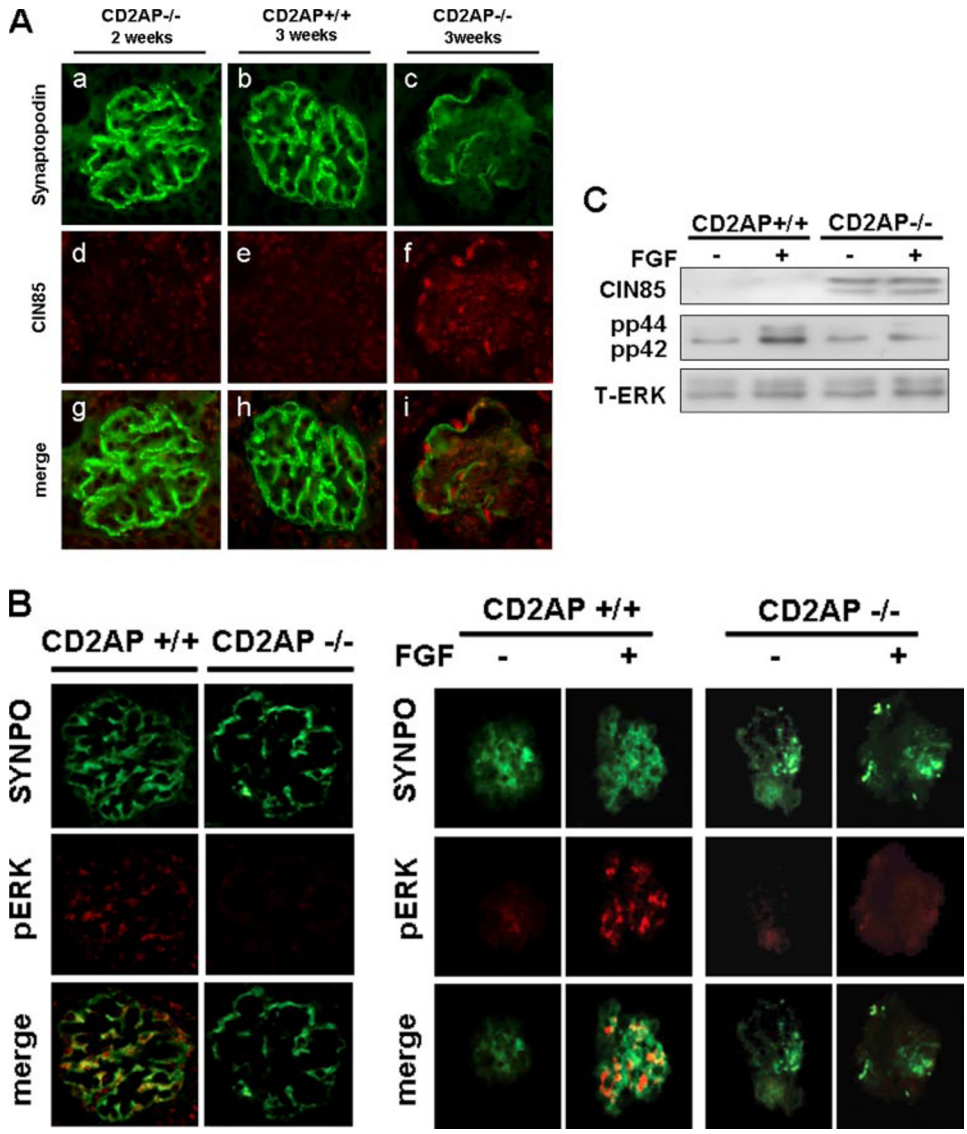

FIGURE 5. **CIN85 is expressed in the majority of podocytes in 3-week-old CD2AP$^{-/-}$ mice and leads to a suppressed glomerular signaling response.** *A*, fluorescence labeling demonstrates expression of CIN85 (*red fluorescence*) and synaptopodin (*green fluorescence*) in renal cortex sections of 2-week-old CD2AP$^{-/-}$ (*a*, *d*, *g*), 3-week-old CD2AP$^{+/+}$ (*b*, *e*, *h*), and 3-week-old CD2AP$^{-/-}$ (*c*, *f*, *i*) mice using anti-CIN85 (*d*, *e*, *f*) and anti-synaptopodin (*a*, *b*, *c*) antibodies, merged in *panels g*, *h*, *i*. *B*, fluorescence labeling demonstrates reduced base-line expression of pERK (*red fluorescence*) in synaptopodin-labeled podocytes (*green fluorescence*) in renal cortex sections of 3-week-old CD2AP$^{-/-}$ mice (*left panel*) and reduced activation in isolated glomeruli after stimulation with FGF (*right panel*). *C*, Western blot demonstrates increase in CIN85 protein expression levels in glomerular extracts of 3-week-old CD2AP$^{-/-}$ mice and attenuated phosphorylation response after stimulation with FGF compared with 3-week-old CD2AP$^{+/+}$ mice.

ferentiated podocytes by suppression of CIN85 expression (Fig. 4*D*). Introduction of the siRNA expression vector in CD2AP$^{+/+}$ cells did not result in a changed signaling response after growth factor stimulation. In contrast, transient overexpression of CIN85 in CD2AP$^{+/+}$ cells resulted in a significant decrease of ERK phosphorylation whereas the transient expression of CD2AP in CD2AP$^{-/-}$ cells could partially rescue the activation response (Fig. 4*E*). Taken together, these data indicate that a changed CD2AP/CIN85 balance leads to a severe dysregulation of RTK-mediated signaling in podocytes *in vitro*.

CIN85 Is Up-regulated in Podocytes in Diseased CD2AP$^{-/-}$ Mice and Leads to a Reduced ERK Signaling Response—CD2AP$^{-/-}$ mice are born normal and develop a nephrotic syndrome only 3 weeks after birth. Before the onset of proteinuria there is complete integrity of podocytes and the slit diaphragm proteins nephrin and podocin, the two well described interaction partners of CD2AP at the glomerular filtration slit, are completely unaffected (14, 17, 18). We examined expression of CIN85 in CD2AP$^{+/+}$ and CD2AP$^{-/-}$ mice and found that CIN85 is up-regulated in glomeruli and tubuli in diseased 3-week-old CD2AP$^{-/-}$ mice. In the glomerulus we observed strong expression of CIN85 in the majority of podocytes throughout the whole kidney in diseased 3-week-old CD2AP$^{-/-}$ mice (Fig. 5*A*, *panels c*, *f*, *i*). In contrast, in 2-week-old non-proteinuric CD2AP$^{-/-}$ mice no CIN85 expression was detected (Fig. 5*A*, *a*, *d*, *g*), similar to CD2AP$^{+/+}$ control animals (Fig. 5*A*, *panels b*, *e*, *h*). Interestingly, this corresponds to a lower base-line expression level of pERK in podocytes *in vivo* (Fig. 5*B*, *left panel*). To link increased CIN85 expression with impaired ERK signaling response *in vivo* we isolated glomeruli of CD2AP$^{+/+}$ and CD2AP$^{-/-}$ mice, treated them in a culture dish with FGF, and examined pERK levels in cryosections and by Western blot analysis. As expected, we detected a reduced ERK signaling response in podocytes in CD2AP$^{-/-}$ glomeruli after FGF stimulation (Fig. 5*B*, *right panel*). These data are supported by a reduced pERK response detected in protein lysates of CD2AP$^{-/-}$ glomeruli (Fig. 5*C*). Consistently, we detected a high level of CIN85 protein in the examined glomerular extracts. Taken together, these data indicate that CD2AP deficiency leads to a dysregulated expression of CIN85 in podocytes *in vivo* resulting in a changed signaling response.

DISCUSSION

PI3K and Ras/ERK MAPK are two of the most important signaling pathways in eukaryotic cells. Recruitment of PI3K to the plasma membrane requires the regulatory subunit p85. The SH2 domain of p85 binds to phosphorylated tyrosine residues in specific docking sites of the intracellular domain of RTKs or adaptor proteins (1). MAPK pathways convey signals via phosphorylation events and form a complex with their cognate MAPKKs utilizing their common docking domain, and a vari-

ety of scaffolding proteins interact with several components of the MAPK cascades to tether both enzymes and substrates specifically to achieve accurate signal transduction (19). CD2AP and CIN85 are ubiquitously expressed adaptor molecules containing three SH3 domains, a proline-rich region, and a coiled-coil domain (20). In this study we report the regulatory involvement of the CD2AP/CIN85 balance in the orchestrated signal transduction response induced by RTKs in podocytes. We hypothesize that in podocytes CD2AP and CIN85 are the key components influencing initiation, maintenance, and termination of RTK-transduced signals. Further on, dysregulation in this balance, as induced by the CD2AP knock out, affects all signaling networks influenced by RTKs. As a consequence, important differentiation and survival signaling cascades necessary for the maintenance of a normal glomerular tuft are disturbed.

To support our hypothesis we provide the following lines of evidence:

First, we showed that CD2AP deficiency in mice results in a synchronized podocyte damage with global foot process effacement simultaneously in all glomeruli. Moreover, our results from CD2AP-deficient podocytes indicate a profound defect in AKT and ERK1/2 signaling response after stimulation of RTKs with various growth factors. Therefore, we presume that CD2AP acts in podocytes as a necessary cofactor for early and continuous activation of PI3K/AKT and ERK1/2 signaling response after stimulation of RTKs. Particularly in the case of VEGF this is of great interest because this would directly affect paracrine signaling activity via VEGF in podocytes. Depending on recent data, this mechanism is discussed as important for podocyte survival *in vitro* (21, 22). Because these signaling effects were demonstrated for the activation of the PI3K/AKT and the Ras-ERK-MAPK pathway, we confirmed that these two pathways are not dependent on each other. We demonstrated that selective inhibition of MEK or Ras in wild-type podocytes did not affect PI3K/AKT activation and, in turn, inhibition of PI3K/AKT did not affect ERK1/2 phosphorylation. Depending on these data, we suggest that in podocytes the activation of these two distinct pathways is clearly not dependent on each other.

Second, when we examined Grb2-SOS binding we detected a dynamic binding using GST-Grb2 pulldown experiments. Grb2-SOS binding is reduced in $CD2AP^{-/-}$ cells after growth factor stimulation, and there is inducible binding of CIN85 to Grb2 already 30 min after growth factor stimulation. When we performed extended time course experiments growth factor-inducible reduction of Grb2-SOS and inducible Grb2-CIN85 binding was also detectable in wild-type cells, but at later time points. Interestingly, Grb2 was identified earlier in association with CIN85 complexes in other cells; however, a time-dependent dynamic of this interaction was not previously described (23). In other epithelial cells, CIN85 is a well known mediator of ligand-dependent ubiquitination and down-regulation of RTKs (12, 24, 25), and is also described as a regulator of MEKK activity (26). Therefore we assume that the dysregulated expression of CIN85 in podocytes accounts for the detected signaling effects on different levels. To further confirm our results we also examined the potential role of disabled-2 (Dab-2) in our system. Dab-2 is a tumor suppressor gene with a C-terminal proline-rich SH3-binding domain. Binding of Dab-2 to Grb2 is described as disrupting SOS binding and suppressing downstream ERK phosphorylation in other cells (27). When we examined Grb2-Dab-2 interaction in $CD2AP^{+/+}$ and $CD2AP^{-/-}$ podocytes, we did not detect a difference in binding of Grb2-Dab-2 in the presence or absence of growth factors (data not shown).

Third, our *in vitro* data confirm that the expression of CIN85 and the associated signaling effects are dependent on the differentiation state of the podocytes. We demonstrated in $CD2AP^{-/-}$ podocytes that with ongoing degree of differentiation the expression of CIN85 protein increased. Depending on the differentiation a diminished ERK1/2 phosphorylation coincided with up-regulated CIN85 expression in the $CD2AP^{-/-}$ podocytes. Silencing of CIN85 can restore the signaling answer in fully differentiated $CD2AP^{-/-}$ podocytes. Transient overexpression of CIN85 in $CD2AP^{+/+}$ cells leads to a similar disruption of ERK signaling, whereas transient expression of CD2AP in $CD2AP^{-/-}$ cells can partially restore the signaling response.

Fourth, we detected in podocytes of 3-week-old $CD2AP^{-/-}$ mice a dramatic increase of CIN85 protein expression. In the diseased mice expression of CIN85 protein is detectable in the majority of podocytes. In contrast, in 2-week-old $CD2AP^{-/-}$ mice and $CD2AP^{+/+}$ mice we did not detect CIN85 expression in podocytes. We detected decreased base-line ERK activity in podocytes of 3-week-old $CD2AP^{-/-}$ mice and demonstrated a diminished response to growth factor stimulation on isolated glomeruli. Finally, we take our findings of synchronized podocyte damage and the uniformity of the lesions as additional evidence that we elucidated a dysregulated physiological mechanism in podocytes. We conclude from our results that CD2AP and CIN85 are responsible for a very delicate physiological balance that is important for RTK response in podocytes. The up-regulation of CIN85 *in vivo* in podocytes might have devastating effects on the normal physiological paracrine and exocrine signals that are necessary to maintain functional podocytes. Secondary to that, the regulatory axis involving a cross-talk of podocytes to endothelial cells and/or mesangial cells is severely deteriorated, leading to the observed phenotype in $CD2AP^{-/-}$ mice. Because the podocyte-specific rescue of CD2AP in the mouse model system leads to a normal phenotype with no other abnormality detected (28), we presume that this mechanism is specific for podocytes. In other cell types CIN85 function might compensate for CD2AP deficiency. The podocyte-specific rescue model also demonstrates that the concomitant and prominent mesangiolysis and mesangial proliferation, as well as the parietal cell damage, are secondary to the podocyte damage. So far, work from various groups has demonstrated similar functions for CD2AP and CIN85 in receptor down-regulation (12, 29). Our data demonstrate for the first time that these molecules might have antagonizing functions.

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