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CD2AP Regulates SUMOylation of CIN85 in Podocytes

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Podocytes are highly differentiated and polarized epithelial cells located on the visceral side of the glomerulus. They form an indispensable component of the glomerular filter, the slit diaphragm, formed by several transmembrane proteins and adaptor molecules. Disruption of the slit diaphragm can lead to massive proteinuria and nephrotic syndrome in mice and humans. CD2AP is an adaptor protein that is important for the maintenance of the slit diaphragm. Together with its paralogue, CIN85, CD2AP belongs to a family of adaptor proteins that are primarily described as being involved in endocytosis and downregulation of receptor tyrosine kinase activity. We have shown that full-length CIN85 is upregulated in podocytes in the absence of CD2AP, whereas in wild-type cells, full-length CIN85 is not detectable. In this study, we show that full-length CIN85 is posttranslationally modified by SUMOylation in wild-type podocytes. We can demonstrate that CIN85 is SUMOylated by SUMO-1, -2, and -3 and that SUMOylation is enhanced in the presence of CD2AP. Conversion of lysine 598 to arginine completely abolishes SUMOylation and leads to increased binding of CIN85 to nephrin. Our results indicate a novel role for CD2AP in regulating posttranslational modification of CIN85.

The adaptor molecules CD2-associated protein (CD2AP) and Cbl-interacting protein of 85 kDa (CIN85) belong to a ubiquitously expressed protein family of adaptor molecules that are involved in a variety of cellular processes, like cell signaling (12, 18, 52), cytoskeletal arrangement (2, 16, 29, 50), and degradative trafficking and endocytosis of receptors (15, 24, 26, 43, 45, 49, 57). The two proteins show high sequence and structural similarities, and they both contain three SH3 domains, a proline-rich region, and a coiled-coil domain (7). However, they appear to have completely different functional roles. While CD2AP is solely expressed in its full-length form, multiple CIN85/Ruk isoforms were identified in various tissues and cell lines, due to alternative splicing and different promoters (3, 31).

In podocytes CD2AP is expressed at the slit diaphragm, a specialized intercellular junction between neighboring podocytes covering the outer surface of the glomerular tuft. CD2AP interacts with several proteins at the slit diaphragm. One of the major components is nephrin, a transmembrane adhesion protein of the Ig superfamily. Humans and mice lacking nephrin are born without intact slit diaphragms and develop massive proteinuria *in utero* (22, 40). Mice deficient in CD2AP are born healthy but develop a rapid-onset nephrotic syndrome at 3 weeks of age and die of renal failure 6 weeks after birth (44). We have previously demonstrated that deficiency of CD2AP leads to a differentiation-dependent increase of full-length CIN85 expression, which correlates with a loss of expression of the slit diaphragm protein nephrin in podocytes. Furthermore, we found that CIN85 is a binding partner of nephrin and that overexpression of CIN85 leads to increased endocytosis of nephrin after growth factor stimulation (48, 49).

Here, we present evidence that CD2AP has a direct influence on posttranslational modification of full-length CIN85. Small ubiquitin-related modifier (SUMO) is a transient and reversible posttranslational protein modifier that plays an important role in many cellular pathways, including subcellular localization, protein-protein interaction, transcriptional regulation, activation of ion channels, and intracellular localization (11, 35, 38, 56). Vertebrates contain four ~100-amino-acid SUMO proteins, SUMO-1, -2, -3, and -4. Of these, SUMO-1 to -3 are ubiquitously expressed whereas the recently reported SUMO-4 seems to be expressed mainly in the kidney, lymph node, and spleen. SUMO-2 and -3 are nearly identical, whereas SUMO-1 has only 56% identity with SUMO-2 and -3. SUMOs are similar to ubiquitin in their three-dimensional structure, and the steps involved in the SUMO pathway resemble those of the ubiquitin pathway (11, 19). In contrast to ubiquitination, SUMOs attach to lysines that are often found within a small consensus motif, ψKXE (where ψ is a large hydrophobic amino acid and X can be any amino acid) (41). SUMO modification occurs through an enzymatic pathway consisting of an E1 activation enzyme (SAE-2/1), an E2-conjugating enzyme (Ubc9), and a number of E3 ligases. Ubc9 is capable of directly modifying substrates through interaction with the SUMO conjugation motif ψKXE (11, 21). This type of posttranslational modification is an efficient and rapid way of controlling the activity of a protein. It is well known that posttranslational modifications, such as phosphorylation and ubiquitination, modulate protein interactions (8, 46). There is no simple way to predict what the functional consequence of a SUMOylated target will be. One molecular consequence of SUMOylation is the inhibition of protein-protein interactions. An example of this is SUMOylation of C-terminal binding protein (CtBP), which loses its interaction with the PDZ domain of nNos (28). SUMOylation can also alter the localization, stabil-

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ity, and activity of a protein (11, 35, 38, 56). The ability of CIN85 to bind to other proteins has been attributed to the phosphorylation status of its binding partners (20, 25, 42). The fact that CIN85 is ubiquitinated (mono-, poly-, and multiubiquitinated) but not degraded by the proteasome has been extensively studied (14, 51). Ubiquitination is not always associated with the degradation of modified proteins but could also be involved in regulating the trafficking and enzymatic activities of a protein (39). SUMOylation and ubiquitination have also been reported to act either sequentially or in concert to regulate the function of the substrate protein (4, 17). Until now, it was unknown how the activity and binding ability of CIN85 are regulated. In this study, we demonstrate that CIN85 is posttranslationally modified by SUMOylation in the presence of CD2AP and controls its interaction with nephrin. Since we previously demonstrated that CIN85 interaction with nephrin induces nephrin ubiquitination and endocytosis (49), we propose that this mechanism contributes to the integrity of the slit diaphragm.

MATERIALS AND METHODS

Phylogenetic analysis. Representative CD2AP and CIN85 genes from human, mouse, chicken, zebrafinch, lizard, frog, and zebrafish were downloaded from GenBank and used as query sequences to find close relatives among invertebrates, including hydra, fruit fly, daphnia, tribolium, honeybee, leech, sea urchin, acorn worm, amphioxus, and sea squirt. All sequences were aligned using Muscle (http://www.ebi.ac.uk/Tools/msa/muscle/) (the accession numbers of all genes and alignment are available upon request) and analyzed with PAUP*: Phylogenetic Analysis Using Parsimony (and other methods) 4.0 beta for Macintosh. Distance analysis used minimum evolution as the optimality criterion (heuristic search with tree-bisection-reconnection and random-addition sequence with 100 replications) and mean character difference as the distance measure. Bootstrap analysis used 1,000 replicates.

Skate sequences. Skate (*Leucoraja erinacea*) CIN85 and CD2AP protein sequences were translated from contiguous sequences generated from a transcriptome assembly of 50.4 million 76-bp single-end RNA-sequencing reads from an Illumina Genome Analyzer II. The Illumina RNA-sequencing library was made following the manufacturer's protocol (Illumina, San Diego, CA) using RNA isolated from six pooled skate stage 20 to 29 embryos (Marine Biological Laboratory, Woods Hole, MA) using Tri Reagent (Ambion). The transcriptome assembly was made using CLCBio Genome Workbench version 4.

Antibodies and chemicals. The antibodies that were used for Western blotting and immunofluorescence studies were as follows: rabbit anti-CD2AP, rabbit anti-GAPDH (anti-glyceraldehyde-3-phosphate dehydrogenase), rabbit anti-histone H1, rabbit antinephrin, normal rabbit IgG, normal mouse IgG, and Protein A/G Plus Agarose beads (Santa Cruz, CA); rabbit anti-green fluorescent protein (anti-GFP), rabbit anti-myc, rabbit anti-Flag, rabbit anti-hemagglutinin (anti-HA), mouse anti-ubiquitin, rabbit anti-SUMO-1, rabbit anti-SUMO-2/3, and rabbit anti-Ubc9 (Cell Signaling Technology, Beverly, MA); mouse anti-CIN85 and rabbit anti-CIN85 (targeting the C terminus; kindly provided by I. Dikic, Frankfurt, Germany); and Cy3 labeled goat anti-rabbit and donkey anti-mouse antibodies and fluorescein isothiocyanate (FITC)-labeled donkey anti-mouse antibodies (Jackson ImmunoResearch Laboratories, Suffolk, United Kingdom).

Cell lines and culture. Culture of conditionally immortalized mouse $CD2AP^{+/+}$ and $CD2AP^{-/-}$ podocytes was performed as described in reference 48. In brief, to enhance expression of the thermosensitive large T antigen, cells were cultured at 33°C in the presence of 10 U/ml gamma interferon (permissive conditions). To induce differentiation, podocytes were maintained at 37°C for 14 days without gamma interferon, resulting in the absence of thermosensitive T antigen (nonpermissive conditions). The medium consisted of RPMI 1640 (Biochrom, Berlin, Germany) containing 10% fetal calf serum (FCS) (PAA, Pasching, Austria), 1% penicillin-streptomycin (Invitrogen, Carlsbad, CA), and 10 U/ml recombinant mouse gamma interferon (Cell Sciences, Canton, MA). Every experimental setup and result was confirmed in 3 different clones of $CD2AP^{+/+}$ and $CD2AP^{-/-}$ podocytes. Human embryonic kidney (HEK) 293 cells (DSMZ, Braunschweig, Germany) were cultured in medium consisting of Dulbecco's modified Eagle's medium (MEM) high glucose with L-glutamine (PAA, Pasching, Austria) containing 10% fetal bovine serum (FBS) (PAA, Pasching, Austria) and 1% antibiotic-antimycotic solution (PAA, Pasching, Austria). The cells were inoculated every 3 or 4 days into new medium.

Animals. Targeted disruption and generation of CD2AP-deficient homozygous mice on a 129/J background is described elsewhere (44). Kidney tissue and glomeruli were harvested from 4-week-old $CD2AP^{+/+}$ and $CD2AP^{-/-}$ mice.

Western blot analysis. To analyze whole-cell protein lysates from cultured podocytes, 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, Mannheim, Germany), 1 mM sodium orthovanadate, 50 mM NaF, and 200 μg/liter okadaic acid. The lysates were centrifuged at 12,000 rpm, and aliquots of the supernatants were separated by 10% SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) 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, Rockford, IL) according to the manufacturer's protocol. For subcellular fractionation, differentiated podocytes were trypsinized with trypsin-EDTA (Biochrom, Berlin, Germany) from 10-cm dishes, and fractionation of the membrane, cytosol, and nuclear compartments was performed with a subcellular fractionation kit (Thermo Sciences, Braunschweig, Germany) according to the manufacturer's protocol.

Isolation and processing of glomeruli. Glomeruli were isolated from kidneys of 4-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 identified as glomeruli, resuspended in lysis buffer, and disrupted with a Dounce homogenizer by hand with 50 up and down strokes, and immunoprecipitation (IP) was performed with the lysates.

RT-PCR analysis. Total RNA was prepared from cultivated murine podocytes using the RNeasy Mini Kit (Qiagen, Hilden, Germany) following the protocol recommended by the manufacturer with an additional step of DNase digestion (RNase-Free DNase Set; Qiagen). One microgram of total RNA was reverse transcribed using oligo(dT) 15, random primers, and Moloney murine leukemia virus (M-MLV) reverse transcriptase (Promega, Mannheim, Germany). The N terminus and C terminus of full-length CIN85 were amplified by standard PCR using 2 μl cDNA as a template. The mouse CIN85 primers m1 (5′-TTCCGCCAAC TTTCACTCTG-3′, mmn (5′-GGCAGGAAGTCATTTTCCAC-3′), and mc (5′-TTCACTTCCATCTGCAACCG-3′) were described by Buchman et al. (3), and m6 (5′-GGCATCCTGGATAAGGACCTC-3′) was described by Gaidos et al. (10).

Transfection with siRNAs. Podocytes were subcultured on 10-cm dishes and cultured under nonpermissive conditions at 33°C. One day before transfection, the medium was replaced with growth medium (RPMI medium with 10% FCS) without antibiotics so that the cultures would be 30 to 50% confluent at the time of transfection. Transfection with CD2AP small interfering RNA (siRNA) (Santa Cruz, CA) was performed with Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. After being kept in an incubator for 6 h at 33°C, the medium was replaced with normal growth medium. The cells were incubated at 37°C in a CO_2 incubator for 72 h and used for experiments.

Plasmid construction. The coding sequences for the mature human SUMO-1, SUMO-2, and SUMO-3 were amplified by PCR, cleaved with BamHI and XhoI, and cloned into BamHI/XhoI-cleaved pcDNA3. pRc/CMV-HA-Ubc9 was a gift from R. Bernards (Netherlands Cancer Institute). Mutant Flag-CIN85^{K598R} and GFP-CIN85^{K598R} were generated by site-directed mutagenesis using PfuUltra II Fusion HS DNA Polymerase (Agilent, Santa Clara, CA) and the following primers: forward, 5′-CAGC ACAGAAGGAAAGCCAAGGATGGAGCCAGTGAGC-3′; reverse, 5′-G CTCACTGCTGGCTCCATCCTTGGCTTTCCTTCTGTGCTG-3′. All constructs were confirmed by sequencing.

Transfection. The day before transfection, the podocytes were seeded on cover slides (5,000 cells/slide) for immunofluorescence or on plates and cultured under permissive conditions. The cells were transfected with Fugene transfection reagent (Roche, Grenzach, Germany) according to the manufacturer's protocol using mouse GFP-CIN85, mouse GFP-CIN85^{K598R}, and nephrin-SV5. After transfection, the cells were cultured under nonpermissive conditions in normal growth medium for 72 h. For transfection of HEK 293 cells, cells were seeded on plates the day before transfection. After transfection with Flag-tagged mouse CIN85 and CIN85^{K598R}; mouse myc-CD2AP; untagged SUMO-1,- 2, or -3; enhanced green fluorescent protein (EGFP)–SUMO-1; and HA-Ubc9, the cells were cultured in normal growth medium for 48 h.

Immunoprecipitation. HEK 293T cells were transfected as mentioned above. Then, the cells were washed carefully with ice-cold phosphate-buffered saline (PBS) on ice. For lysis, 900 μl RIPA buffer (50 mM Tris-HCl, pH 7.5, 200 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton, and 0.25% deoxycholate plus protease inhibitors) was added to the cells. The lysate was incubated for 15 min on ice and centrifuged at 14,000 rpm for 15 min at 4°C, and 50 μl Flag-tagged beads (50% slurry in Triton buffer; Sigma, St. Louis, MO) was added to the supernatant and rotated overhead at 4°C for 1 h to overnight. For IgG control, agarose G beads and normal mouse IgG were added to the supernatant. After that, the beads were centrifuged at 3,000 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 blotting was performed using the methods mentioned above. For immunoprecipitation from glomerular and podocyte protein lysates, 500 μg of total cell lysate was incubated with 2 μg anti-SUMO-1 or anti-SUMO-2/3 and agarose A beads in IP buffer (25 mM Tris-HCl, pH 7.5, 1 mM dithiothreitol [DTT], 30 mM $MgCl_2$, 40 mM NaCl, 0.5% NP-40, and protease inhibitors) and rotated overnight at 4°C. The pellets were washed 3 times in IP buffer and separated by SDS polyacrylamide gel electrophoresis. For the isotype-matched control, immunoprecipitation was performed with normal rabbit IgG.

Immunofluorescence. For fluorescence images, cultured podocytes were fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton X-100, blocked with 10% donkey serum, and stained with the appropriate primary antibody or phalloidin-Alexa Fluor 546 overnight. The next day, the appropriate secondary antibody was incubated for 1 h, and cover slides were embedded with DAPI (4′,6-diamidino-2-phenylindole) on slides. For live-cell staining on SV5-nephrin-transfected podocytes, cells were stained unfixed at 4°C for 1 h with the appropriate primary antibody. For cryostaining, 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 or Cy3-conjugated donkey anti-rabbit and fluorescein isothiocyanate-conjugated donkey anti-guinea pig antibodies (Jackson ImmunoResearch). The cells were analyzed using a Zeiss Axioplan-2 imaging microscope with the scientific image-processing software AxioVision 4.6 (Zeiss, Jena, Germany).

Statistics. Data are shown as mean ± SD and were compared by Student's *t* test. Densitometric analysis was performed with Quantity One software. Data analysis was performed using Excel statistical software. Significant differences were accepted when P was <0.05.

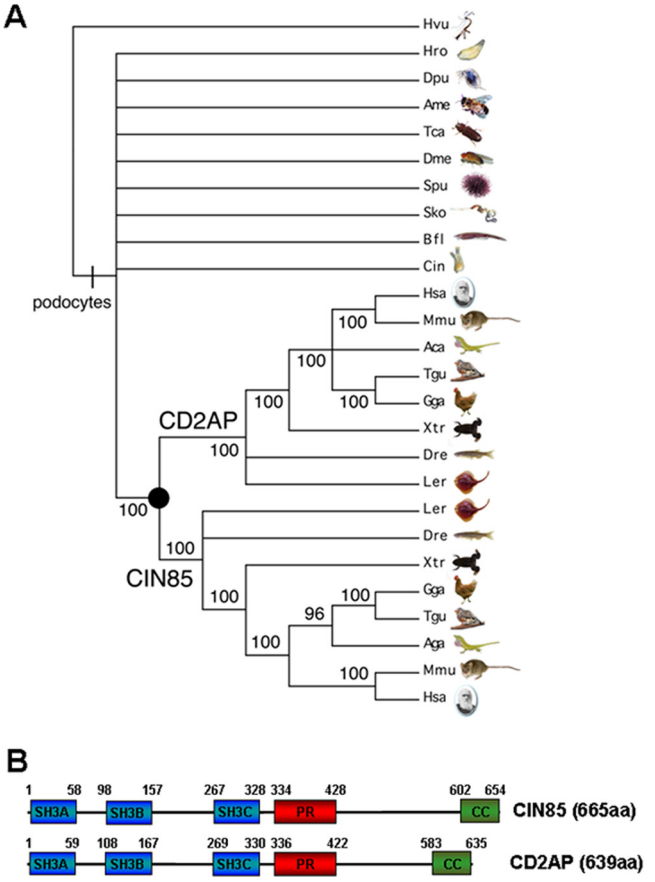

FIG 1 Phylogenetic and sequence analyses of CIN85 and CD2AP. (A) Phylogenetic tree of CD2AP and CIN85 genes in animals. A minimum evolution analysis rooted on the gene sequence from *Hydra vulgaris* (Hvu) strongly supports the hypothesis (bootstrap values are indicated at the respective nodes; nodes with values below 95% are collapsed into polytomies) that these two genes are the result of a gene duplication event (circle) early in the evolutionary history of vertebrates, before the split between chondrichthyians (Ler, the skate *L. erinacea*) and the bony fish. These two genes, presumably the result of a genome duplication events(s), are related to single-copy genes found throughout the invertebrates, including the freshwater cnidarian *Hydra*. Note that according to this phylogenetic analysis, this gene evolved before the evolution of podocytes, a cell type that characterizes the protostomes and deuterostomes, including vertebrates, but is not found in more basal animals, including cnidarians. Aca, the lizard *Anolis carolinensis*; Ame, the honeybee, *Apis melifera*; Bfl, the amphioxus *Branchiostoma floridae*; Cin, the sea squirt *Ciona intestinalis*; Dre, the zebrafish, *Danio rerio*; Dpu, the waterflea *Daphnia pulex*; Gga, the chicken, *Gallus gallus*; Hro, the leech *Helobdella robusta*; Hsa, *Homo sapiens*; Mmu, the mouse, *Mus musculus*; Sko, the hemichordate worm *Saccoglossus kowalevskii*; Spu, the purple sea urchin, *Strongylocentrotus purpuratus*; Tca, the beetle *Tribolium castaneum*; Tgu, the zebrafinch, *Taeniopygia guttata*; Xtr, the frog *Xenopus laevis*. (B) Protein domains and regions annotated according to human CIN85 (top) and CD2AP (bottom), including three SH3 domains (blue), a proline-rich region (red), and a predicted coiled-coil region (green).

RESULTS

Phylogenetic analysis of CIN85 and CD2AP. Because the two molecules have very similar domain structures, we wanted to unravel the evolutionary history of CIN85 and CD2AP in relation to the origin of podocytes and the vertebrate kidney. To accomplish this, we conducted a phylogenetic analysis with a representative sample of vertebrate CIN85 and CD2AP genes and invertebrate relatives. A minimum evolution analysis (Fig. 1A) strongly supports the idea that CIN85 and CD2AP are the products of a gene

duplication event before the split between chondrichthyes (sharks, rays, and skates) and osteichthyes (bony fish) but after the split from invertebrates and presumably associated with the 2-fold genome duplication event early in vertebrate evolution (6) (Fig. 1A, circle). Single-copy relatives of this ancestral gene were found in all major groups of invertebrates, including arthropods, annelids, echinoderms, hemichordates, and invertebrate chordates, and in no case was more than one gene found in GenBank's nucleotide repository. Interestingly, a relative of this single-copy gene was found in hydra, the freshwater cnidarians. Because podocytes appear to be an evolutionary invention of the bilaterians (i.e., protostomes and deuterostomes) and are not known in more basal animals, including cnidarians (37) (Fig. 1A), it appears that the gene evolved before the invention of the podocyte and was coopted into a role in kidney function later in evolution.

In the human genome, *CIN85* (*SH3KBP1*) and *CD2AP* have different gene structures and multiple alternatively spliced transcripts. According to Ensembl (release 63), human *CIN85* (*SH3KBP1*) spans 354 kb of chromosome X (Xp22.1 to -p21.3, positions 19552083 to 19905719 of GRCh37) with 58 exons and has 12 alternatively spliced transcripts, 10 of which encode proteins. The longest protein is 665 amino acids (aa) with a molecular mass of 73.126 kDa. *CD2AP* spans 149 kb of chromosome 6 (6p12, positions 47445525 to 47594999) with 28 exons and five alternatively spliced transcripts, with the longest one encoding the 639-aa (71.451-kDa) protein. No microRNA genes lie within the introns of either *CIN85* or *CD2AP*. The longest isoforms of the human CIN85 and CD2AP proteins both contain three SH3 domains (Fig. 1B). While CD2AP protein is only detectable as a single band in cell lysates, all different splice variants of CIN85/Ruk are detectable at the protein level in various tissues (3, 31).

The expression profile of the CIN85 protein differs in CD2AP$^{+/+}$ and CD2AP$^{-/-}$ podocytes. Previously, we have shown that full-length CIN85 is strongly expressed in CD2AP$^{-/-}$ podocytes, which has detrimental effects on podocyte survival, leading to decreased antiapoptotic signaling and enhanced endocytosis of nephrin (48, 49). Immunoblotting of total cell lysates of CD2AP$^{+/+}$ and CD2AP$^{-/-}$ podocytes revealed multiple molecular forms of CIN85 identified by a C-terminal antibody (kindly provided by I. Dikic). The main bands detected have molecular masses of 130, 85, 70, 56, 34, and 16 kDa. The 85- to 16-kDa isoforms result from splicing of pre-mRNA (an 85-kDa full-length form, a 70-kDa form without the first SH3A domain, a 56-kDa form without two SH3 domains, and a 16-kDa form containing only the C-terminal coiled-coil region) (3, 31) (Fig. 2A). Interestingly, full-length CIN85 with a molecular mass of 85 kDa is not detectable in CD2AP$^{+/+}$ podocytes, in contrast to CD2AP$^{-/-}$ podocytes, where it is strongly expressed. As depicted by Western blotting, the full-length CIN85 generally migrates in doublet form. Furthermore, an immunoreactive band of approximately 130 kDa, which also migrates as a doublet, is strongly expressed in CD2AP$^{+/+}$ podocytes, which was previously described as posttranslational modification of CIN85 through ubiquitination in other cells (51). Compared to its expression in wild-type cells, this band is only weakly detectable in CD2AP$^{-/-}$ podocytes. A closer analysis revealed that there are several bands migrating beneath the 130-kDa form (Fig. 2A). Analysis of ubiquitin expression revealed in CD2AP$^{+/+}$ and CD2AP$^{-/-}$ podocytes an accumulation of ubiquitinated proteins with a high molecular mass, but in contrast to CD2AP$^{-/-}$ podocytes, CD2AP$^{+/+}$ podocytes express several bands migrating with a molecular mass that corresponds to the 130-kDa band. Moreover, a CIN85 form with a molecular mass of approximately 200 kDa is detectable in CD2AP$^{+/+}$ podocytes. However, densitometric analysis revealed that this band is not consistently expressed at significant levels in every probe. One explanation could be that it is an unstable modified form of CIN85. Expression analysis of CIN85 mRNA by reverse transcription (RT)-PCR established previously (3, 10) that full-length CIN85 mRNA is expressed in both CD2AP$^{+/+}$ and CD2AP$^{-/-}$ podocytes (Fig. 2B). Taken together, these data indicate that full-length CIN85 is posttranslationally modified in CD2AP$^{+/+}$ podocytes.

Full-length CIN85 is posttranslationally modified in podocytes in the presence of CD2AP. To confirm that modification of full-length CIN85 occurs in the presence of CD2AP, we overexpressed mCD2AP-myc in CD2AP$^{-/-}$ podocytes. Immunoblot analysis revealed decreased expression of the 85-kDa isoform and increased expression of the 130-kDa form compared to cells transfected with an empty vector (Fig. 3A). Previously, we showed differentiation-dependent expression of full-length CIN85 in CD2AP$^{-/-}$ podocytes (48). To demonstrate that expression of the 85-kDa full-length form of CIN85 depends on CD2AP expression, we inhibited the expression of CD2AP by siRNA in CD2AP$^{+/+}$ podocytes. By immunoblotting, we analyzed total cell lysates of cells treated with a CD2AP siRNA or control siRNA differentiated for 0, 2, 4, and 8 days. After 4 days of differentiation, we detected upregulated expression of the 85-kDa form of CIN85 in the absence of CD2AP expression, which is further enhanced after 8 days of differentiation. In contrast, cells treated with a control siRNA show differentiation-dependent upregulated expression of the 130-kDa form of CIN85, whereas the 85-kDa full-length form is only weakly expressed (Fig. 3B). When we performed RT-PCR, we found that full-length CIN85 mRNA is upregulated in both CD2AP$^{+/+}$ and CD2AP$^{-/-}$ podocytes in a differentiation-dependent manner (Fig. 3C). These results indicate that the 130-kDa form of CIN85 is posttranslationally modified in podocytes, which is also dependent on the presence of CD2AP.

CIN85 is modified by SUMO-1, -2, and -3 *in vivo*. Posttranslational modification of CIN85 via ubiquitination has been described previously. Interestingly, CIN85 is ubiquitinated but not degraded by the proteasome (51). The mass shift of approximately 45 to 50 kDa indicates that ubiquitination cannot be the only posttranslational modification. Thus, to evaluate whether CIN85 is also modified by SUMO *in vivo*, we performed immunofluorescence staining of frozen kidney cortex sections, which revealed strong expression of CIN85 in glomeruli of 4-week-old diseased CD2AP$^{-/-}$ mice in contrast to CD2AP$^{+/+}$ mice. Furthermore, expression of SUMO-1 and Ubc9 is not restricted to the nucleus compared to SUMO-2/3, which mostly show a strong nuclear pattern. Interestingly, colocalization of CIN85 with SUMO-1 and Ubc9 is very strong in CD2AP$^{+/+}$ mice. Although expression of CIN85 is increased in glomeruli of CD2AP$^{-/-}$ mice, colocalization of SUMO-1 and Ubc9 is weaker. Ubc9 expression in CD2AP$^{-/-}$ mice appears to even be decreased in regions of the glomeruli where CIN85 is strongly expressed (Fig. 4A). Immunoprecipitation experiments from isolated glomeruli show an association of SUMO-1 and SUMO-2/3 with the 130-kDa form of CIN85 in glomeruli of CD2AP$^{+/+}$ mice, whereas glomeruli of CD2AP$^{-/-}$ mice show no association of CIN85 with SUMO-1 and SUMO-2/3 (Fig. 4B). In contrast to immunofluorescence stain-

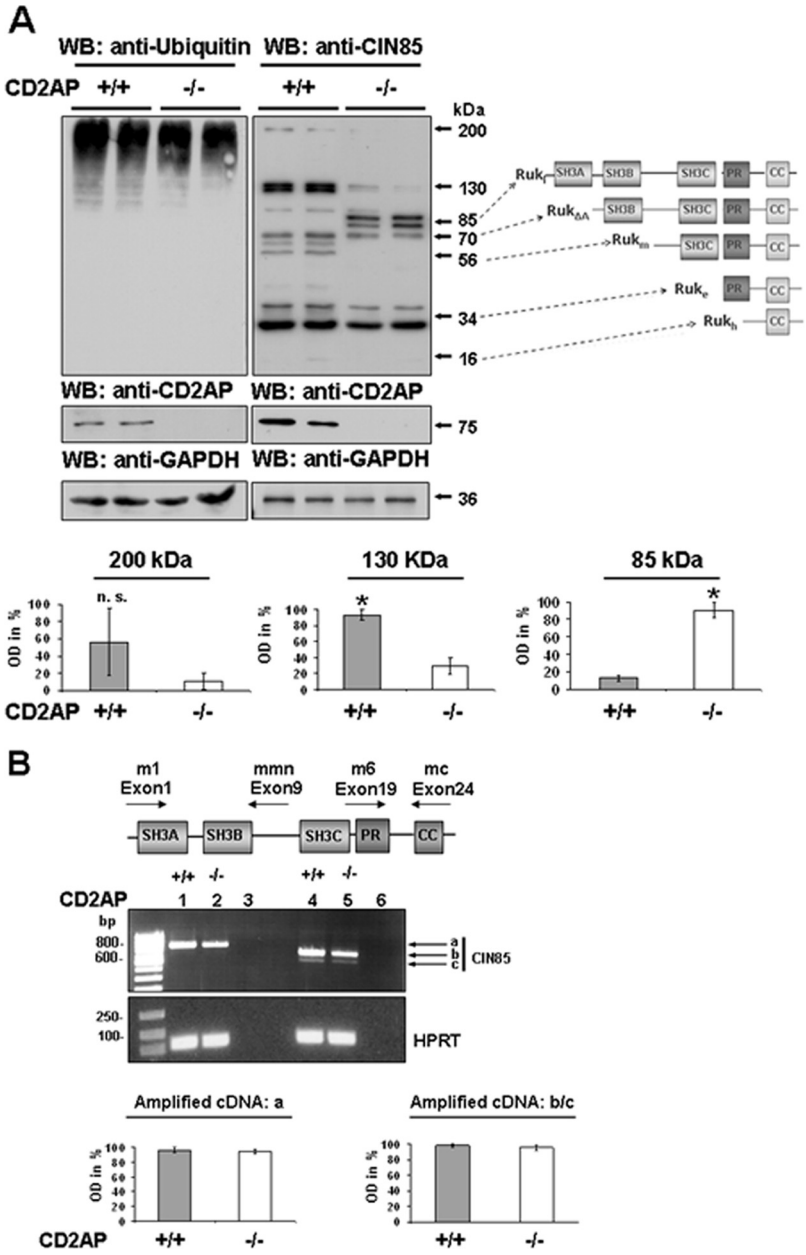

FIG 2 Full-length CIN85 is posttranslationally modified in CD2AP$^{+/+}$ podocytes. (A) Protein lysates from two different clones of cultured differentiated CD2AP$^{+/+}$ and CD2AP$^{-/-}$ podocytes were analyzed for CIN85, ubiquitin, CD2AP, and GAPDH expression by Western blotting (WB). The arrows show the masses of proteins and specific isoforms of CIN85. (Below) Densitometry summarizing the means and standard deviations of 200-, 130-, and 85-kDa CIN85/GAPDH obtained from three independent experiments. OD, optical density. *, $P < 0.0005$ compared to the wild type/knockout (WT/KO) by Student's *t* test; n.s., not significant. (B) PCR was performed on cDNA generated from cultured differentiated CD2AP$^{+/+}$ and CD2AP$^{-/-}$ podocytes. Mouse-specific primers m1 (positioned in exon 1) and mmn (positioned in exon 9) were used for amplification of the 5′ region of CIN85 (lanes 1 and 2). Primer m6 (positioned in exon 19) and mc (positioned in exon 24) were used for amplification of the 3′ region of CIN85, including the coiled-coil (CC) domain (lanes 4 and 5). PCR primers m1, mmn, mc, and m6 were used as described previously (3, 10). Lanes 3 and 6, negative control. The arrows show amplified cDNAs (a, b, and c): a, 5′ region of CIN85; b, full-length 3′ region of CIN85; c, 3′ region of CIN85Δexon21. (Bottom) Hypoxanthin-phosphoribosyl transferase 1 (HPRT-1) as a loading control. (Below) Densitometry summarizing the means and standard deviations of amplified cDNAs a and b/c obtained from three independent experiments.

ing, there is no difference in the binding of SUMO-1 and SUMO-2/3 with CIN85. Endogenous staining of CIN85 in cultured, differentiated CD2AP$^{+/+}$ and CD2AP$^{-/-}$ podocytes revealed a vesicle-like distribution of CIN85 in the cytoplasm and a weak nuclear expression in CD2AP$^{+/+}$ podocytes. In CD2AP$^{-/-}$ podocytes, CIN85 was found at the plasma membrane and in the perinuclear and nuclear regions (Fig. 4C). SUMO-1, SUMO-2/3, and Ubc9 show nuclear expression. Furthermore, SUMO-1 and Ubc9 exhibited a cytoplasmic, vesicle-related distribution pattern which partially colocalized with CIN85 in CD2AP$^{+/+}$ podocytes. Immunoprecipitation of CD2AP$^{+/+}$ and CD2AP$^{-/-}$ podocytes uncovered an association of SUMO-1 and a weak association of

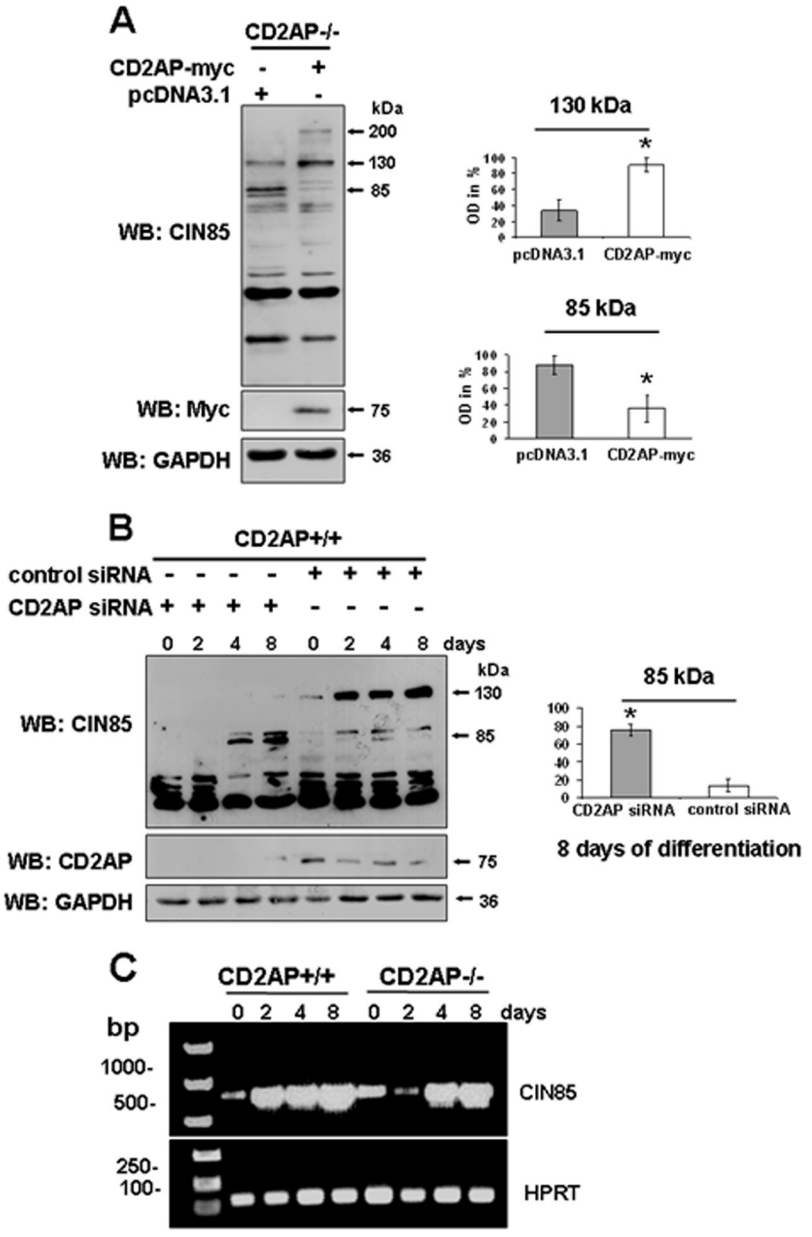

FIG 3 Modification of full-length CIN85 depends on expression of CD2AP. (A) pcDNA3.1, CD2AP$^{-/-}$ podocytes were transiently transfected with myc-CD2AP or empty vector pcDNA3.1, and whole-cell lysates were immunoblotted for CIN85, Myc, and GAPDH. Densitometry summarizing the means and standard deviations of 130- and 85-kDa CIN85/GAPDH obtained from three independent experiments. *, $P < 0.008$ compared to pcDNA3.1/myc-CD2AP by Student's *t* test. (B) Undifferentiated CD2AP$^{+/+}$ podocytes were treated with 20 pmol of CD2AP siRNA or control siRNA (scrambled sequence); cultured for 0, 2, 4, and 8 days at 37°C for differentiation; and analyzed for CIN85, CD2AP, and GAPDH. (Right) Densitometry summarizing the means and standard deviations of 85-kDa CIN85/GAPDH obtained from three independent experiments. *, $P < 0.0003$ compared to CD2AP siRNA/control siRNA by Student's *t* test. (C) PCR was performed on cDNA generated from CD2AP$^{+/+}$ and CD2AP$^{-/-}$ podocytes differentiated for 0, 2, 4, and 8 days. Amplified cDNAs show the full-length 3′ region of CIN85 (top) and HPRT-1 as a loading control.

SUMO-2/3 with the 130-kDa form of CIN85 in CD2AP$^{+/+}$ podocytes, whereas CD2AP$^{-/-}$ podocytes showed no binding (Fig. 4D). Previously, we demonstrated that expression of the 85-kDa full-length form of CIN85 leads to enhanced endocytosis of the slit diaphragm protein nephrin in the absence of CD2AP (49). Subcellular fractionation showed that the 130-kDa form of CIN85 is mostly expressed in the cytosol and nucleus of CD2AP$^{+/+}$ podocytes, whereas the 85-kDa form of CIN85 is expressed in the membrane and nuclear fractions of CD2AP$^{-/-}$ podocytes. Expression of nephrin is restricted to the membrane fraction of CD2AP$^{+/+}$ podocytes, whereas in CD2AP$^{-/-}$ podocytes, expression of nephrin in the membrane fraction is significantly decreased (Fig. 4E).

CIN85 is modified by SUMO-1, -2, and -3 *in vitro*. To evaluate in detail whether CIN85 is modified by SUMO, we cotransfected HEK 293 cells with CIN85-Flag; SUMO-1, -2, or -3; or EGFP–SUMO-1 and HA-Ubc9. Immunoblotting of total cell lysates revealed that CIN85 is SUMOylated by SUMO-1, -2, and -3 and EGFP–SUMO-1 in the coexpression of the SUMO E2-conjugating

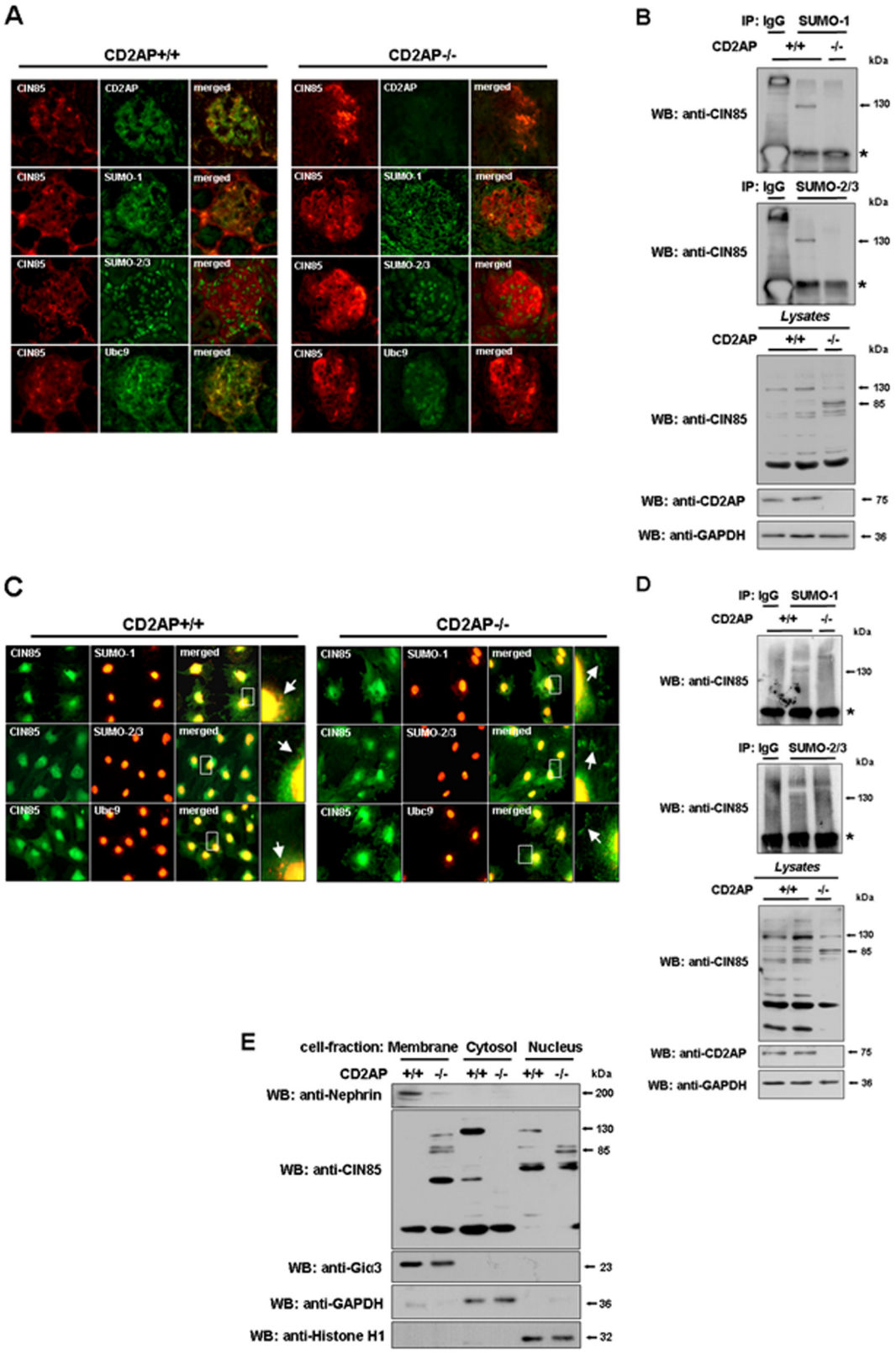

FIG 4 SUMOylation of CIN85 in CD2AP$^{+/+}$ podocytes *in vivo*. (A) Fluorescence labeling demonstrates CIN85 (red) and CD2AP, SUMO-1 and -2/3, and Ubc9 (green) in renal cortex sections of 4-week-old CD2AP$^{+/+}$ and CD2AP$^{-/-}$ mice. Colocalization of the indicated proteins resulted in yellow fluorescence in the merged pictures. (B) Whole lysates from isolated CD2AP$^{+/+}$ and CD2AP$^{-/-}$ glomeruli were precipitated (IP) with SUMO-1 and -2/3 antibodies. The probes were blotted and analyzed for CIN85. For IgG control, glomerular lysates from CD2AP$^{+/+}$ mice were incubated with normal rabbit IgG instead of specific antibody. The lysates were analyzed for CIN85, CD2AP, and GAPDH as a control. (C) Fluorescence labeling of differentiated CD2AP$^{+/+}$ and CD2AP$^{-/-}$

enzyme Ubc9 (Fig. 5A). As depicted, a slowly migrating band was observed with an apparent size that was compatible with a single SUMO conjugated to CIN85-Flag. Interestingly, SUMOylation through SUMO-3 is stronger than through SUMO-1 and -2. No band of that size was observed in the absence of Ubc9. Furthermore, a possible SUMO chain formation is apparent for SUMO-1 and SUMO-2 and strongly for SUMO-3. All three SUMO family members are able to multimerize *in vitro* (9, 47). An EGFP-tagged SUMO-1 construct leads to a band with a higher molecular mass for SUMOylated CIN85, which proves that CIN85 is SUMOylated. Interestingly, when we analyzed SUMOylation of CD2AP, we also found that CD2AP is modified by SUMO-1, -2, and -3 whereas SUMOylation via SUMO-3 is stronger, similar to our results with CIN85. Compared to CIN85, we can also show SUMOylation of CD2AP and, furthermore, SUMO chain formations that are also apparent for CD2AP (Fig. 5B). Additionally, in some experiments, the SUMOylation of CIN85 by SUMO-3 seemed to be enhanced in the presence of CD2AP (Fig. 5C). These data, together with the analysis of the CD2AP$^{+/+}$ and CD2AP$^{-/-}$ mice and cultured podocytes, suggest that CIN85 and CD2AP can be SUMOylated by SUMO-1 and -2 and more clearly by SUMO-3 and that SUMOylation of CIN85 is enhanced in the presence of CD2AP. However, even though densitometric analysis in some experiments revealed a clear trend for enhanced SUMOylation of CIN85 in the presence of overexpressed CD2AP, these results did not reach statistical significance in HEK 293 cells. This could be explained by the fact that HEK 293 cells contain endogenous CD2AP levels leading to a "baseline" SUMOylation effect that is only weakly enhanceable by overexpression of CD2AP.

SUMO-1, -2, and -3 modify CIN85 on lysine 598. To map the site on CIN85 that is modified by SUMOylation, we analyzed the potential SUMOylation motifs using "SUMOplot prediction" (55). There is one consensus SUMOylation sequence motif, PKME, in mouse CIN85 where the lysine in position K598 is the predicted acceptor site for SUMOylation. This motif is also conserved in human CIN85 (Fig. 6A). The lysine is positioned at the C terminus of CIN85 right before the coiled-coil domain starts. To explore whether this site is modified by SUMOylation, we generated a mutant of CIN85, CIN85^{K598R}, where the lysine in position 598 was converted to arginine. CIN85-Flag or CIN85^{K598R}-Flag was cotransfected with SUMO-1, -2, or -3 and HA-Ubc9 in HEK 293 cells. We detected a slowly migrating band that was only observed for wild-type CIN85 and, additionally, SUMO chain formation for SUMO-2 and -3 (Fig. 6B). In contrast, this band was absent when CIN85^{K598R} was used as a substrate, suggesting that conversion of lysine 598 to arginine abrogates SUMOylation of CIN85. To assess if SUMOylation of CIN85 influences binding to nephrin, we cotransfected nephrin-GFP with wild-type or mutant Flag-CIN85. As demonstrated in Fig. 6C, binding of nephrin to mutant CIN85 is stronger, suggesting that SUMOylation of CIN85 decreases binding to nephrin. To explore whether enhanced binding of nephrin to the K598R mutant is related to a changed localization pattern of CIN85, we transfected podocytes with wild-type or K598R GFP-CIN85 and examined the distribution by using immunofluorescence microscopy. We found that wild-type CIN85-GFP displayed a strictly vesicular intracellular distribution pattern in large perinuclear vesicles. In contrast, K598R CIN85 mutant expression was found in smaller vesicles, and some of these vesicles were detectable in a plasma membrane-associated localization pattern. Moreover, a partial overlap with nephrin could be detected in some of the K598R CIN85 mutant vesicles (Fig. 6D). These data indicate that SUMOylation of CIN85 can directly influence the intracellular expression pattern of CIN85 and its interaction with nephrin at the plasma membrane.

DISCUSSION

CD2AP and CIN85 are gene duplicates of one another (paralogues) whose origin can be traced to the early stages of vertebrate evolution, before the separation between cartilaginous and bony fish. As expected of gene duplicates, they contain very similar domain structures with similar promiscuous protein-protein binding motifs. Nevertheless, they have been shown to have completely different functions, particularly in podocytes. CD2AP is expressed as a single protein band and is perceived as a "stabilizing" adaptor in podocytes that connects the slit diaphragm to the actin cytoskeleton. In contrast, CIN85 is an adaptor protein that is known to influence multiple processes. This is most likely due to a specific spatial and temporal pattern of expression, a complex pattern of pre-mRNA splicing, protein interactions, and posttranslational modifications (7, 26, 45, 53). Interestingly, Cindr, the singleton orthologue of CD2AP and CIN85 in *Drosophila melanogaster*, displays four splice variants. In comparison, CIN85 displays 10 splice variants, which encode proteins, indicating a highly specific evolved role for each variant in different tissues and cell lines (3, 31). Our previous work suggests that expression of full-length CIN85 protein has a negative impact on podocyte survival signaling and slit diaphragm integrity. Thus, we wanted to analyze whether these closely related proteins influence each other's expression in the cell to understand how this protein balance is regulated. It is known that regulatory networks that evolved, at least in part, through gene duplication events are not exclusively regulated at transcription initiation but instead are regulated posttranslationally via protein-protein interactions (30). For example, the activity of AIB1, a member of the steroid receptor coactivator (SRC) family, is inhibited by SUMOylation, which is in contrast to SUMOylation of its parologues GRIP1 and SRC-1 (54), where SUMOylation leads to an enhancement of function. In this report, we present evidence that CIN85 is modified by SUMO-1, -2, and -3 in the presence of CD2AP, and we analyze the functional consequences of SUMOylation. When we compared the protein expression of CIN85 in CD2AP$^{+/+}$ and CD2AP$^{-/-}$ podocytes we observed several CIN85 isoforms in both cell types, but full-length CIN85 was expressed only in CD2AP$^{-/-}$ podocytes. Instead,

podocytes demonstrates CIN85 (green) and SUMO-1 and -2/3 and Ubc9 (red). Colocalization resulted in yellow fluorescence in the merged pictures. The arrows indicate colocalization in CD2AP$^{+/+}$ podocytes and membrane distribution of CIN85 in CD2AP$^{-/-}$ podocytes. (D) Whole lysates from differentiated CD2AP$^{+/+}$ and CD2AP$^{-/-}$ podocytes were precipitated (IP) with SUMO-1 and -2/3. The probes were blotted and analyzed for CIN85. For IgG control, lysates from CD2AP$^{+/+}$ podocytes were incubated with normal rabbit IgG instead of antibody. The lysates were analyzed for CIN85, CD2AP, and GAPDH as a control. The asterisks in panels B and D mark nonspecific IgG heavy-chain bands. (E) Subcellular fractionation of CD2AP$^{+/+}$ and CD2AP$^{-/-}$ podocytes into membrane, cytosolic, and nuclear fractions showing expression of CIN85 and nephrin. As a control, membrane fractions were analyzed for Giα3, cytosolic fractions were analyzed for GAPDH, and nuclear fractions were analyzed for histone H1.

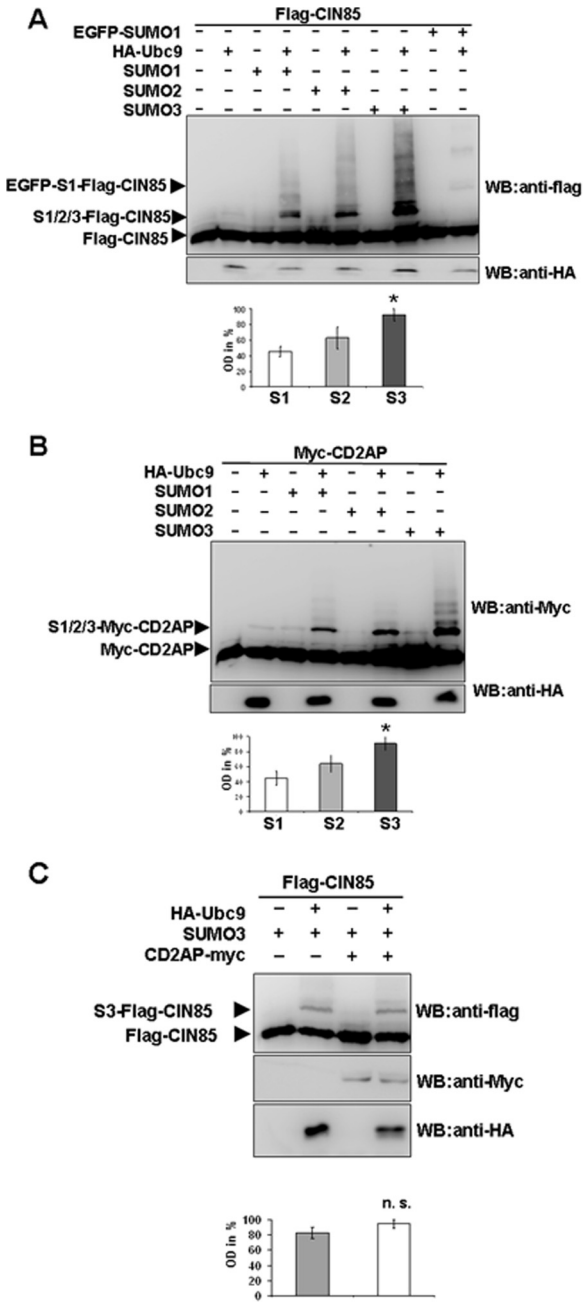

FIG 5 SUMOylation of CIN85 and of CD2AP *in vitro*. HEK 293 cells were cotransfected with plasmids expressing Flag-CIN85 (A) or Myc-CD2AP (B); untagged Sumo-1, -2, and -3; HA-Ubc9; and EGFP-tagged-SUMO-1. Whole-cell lysates were immunoblotted with anti-Flag, anti-Myc, or anti-HA antibody. (Below) Densitometry summarizing the means and standard deviations of SUMO-1-, -2-, and -3-modified/unmodified CIN85 or CD2AP (S1, SUMO-1; S2, SUMO-2; S3, SUMO-3) obtained from three independent experiments. *, $P < 0.04$ compared to S2/S3 by Student's *t* test. (C) HEK 293 cells were cotransfected with Flag-CIN85, Myc-CD2AP, untagged SUMO-3, and HA-Ubc9. Whole-cell lysates were immunoblotted with anti-Flag (top) or anti-Myc and anti-HA antibody (bottom). (Below) Densitometry summarizing the means and standard deviations of SUMO-3-modified/unmodified CIN85 (gray bars, without myc-CD2AP; white bars, with myc-CD2AP) obtained from three independent experiments. *, $P < 0.1$ by Student's *t* test; n.s., not significant. The arrows show expression of un-SUMOylated and SUMOylated protein.

$CD2AP^{+/+}$ podocytes show a strong band of approximately 130 kDa. Expression analysis of mRNA showed no differences between $CD2AP^{+/+}$ and $CD2AP^{-/-}$ podocytes, suggesting that the differences are caused by posttranslational modification influenced by the presence of CD2AP. This doublet protein band was also observed by other groups and was described as ubiquitinated full-length CIN85 (31, 51). Interestingly, several protein bands were observed migrating beneath the 130-kDa form, suggesting that there could be a "step-by-step" modification. However, overexpression of CD2AP in CD2AP-deficient podocytes enhanced the formation of modified CIN85, underlining the fact that CD2AP is involved in regulation of CIN85 modification.

Previously, we demonstrated that full-length CIN85 expression is differentiation dependently upregulated in $CD2AP^{-/-}$ podocytes (48). Consistent with the previous results, inhibition of CD2AP protein expression by siRNA in $CD2AP^{+/+}$ podocytes led to differentiation-dependent upregulation of the 85-kDa full-length form of CIN85. Interestingly, $CD2AP^{+/+}$ podocytes treated with a control siRNA show differentiation-dependent upregulation of the 130-kDa form of CIN85, confirming that the presence of CD2AP leads to posttranslational modification of full-length CIN85 in podocytes.

Numerous proteins have been shown to associate with both CIN85 and CD2AP, which can be explained by the sequence and structural similarities (7). Many of these proteins, including p130 Cas, Crk, and p85 phosphatidylinositol-3 kinase (PI3K), are adaptor molecules linked to protein kinase pathways (1, 18, 23, 31, 53). Given these facts, Grunkemeyer et al. proposed that CIN85 may compensate for the loss of CD2AP in $CD2AP^{-/-}$ podocytes (13). Our previous findings underline the fact that expression of full-length CIN85 leads to decreased survival signaling in $CD2AP^{-/-}$ podocytes (48).

It is known that CIN85 is ubiquitinated but not degraded by the proteasome, suggesting that ubiquitination may regulate endocytotic or interaction activity of CIN85 (15, 51). Due to the mass shift of 45 kDa, a single monoubiquitination probably is not the only possible posttranslational modification of CIN85. We report in this study that CIN85 is SUMOylated *in vivo* by SUMO-1 and SUMO-2/3 in glomeruli from $CD2AP^{+/+}$ mice and *in vitro* in cultured $CD2AP^{+/+}$ podocytes of mice, and we identified the 130-kDa form as SUMOylated CIN85. Glomeruli of $CD2AP^{-/-}$ mice display increased expression of CIN85 compared to $CD2AP^{+/+}$ mice that is not evenly distributed and might contribute to glomerular injury. Cultured $CD2AP^{+/+}$ and $CD2AP^{-/-}$ podocytes show no quantitative difference in CIN85 expression but a difference in its distribution. CIN85 is expressed in $CD2AP^{+/+}$ podocytes mainly in the cytosol and nucleus, whereas $CD2AP^{-/-}$ podocytes reveal a membrane and nuclear expression pattern. Previously, we demonstrated that CIN85 is a binding partner of nephrin and thereby enhances endocytosis of nephrin in $CD2AP^{-/-}$ podocytes (49). We showed in this study that expression of nephrin is strongly decreased in the membrane fraction of $CD2AP^{-/-}$ podocytes and that 85-kDa full-length CIN85 is simultaneously strongly expressed. These observations underline our previous studies showing that full-length CIN85 leads to downregulation of nephrin. Modified CIN85, expressed as a 130-kDa band, cannot be found in the membranes of $CD2AP^{+/+}$ podocytes but only in the cytosol. This indicates that SUMOylation of CIN85 leads to stable localization of CIN85 in the cytosol, where it cannot bind to nephrin. Additionally, CIN85

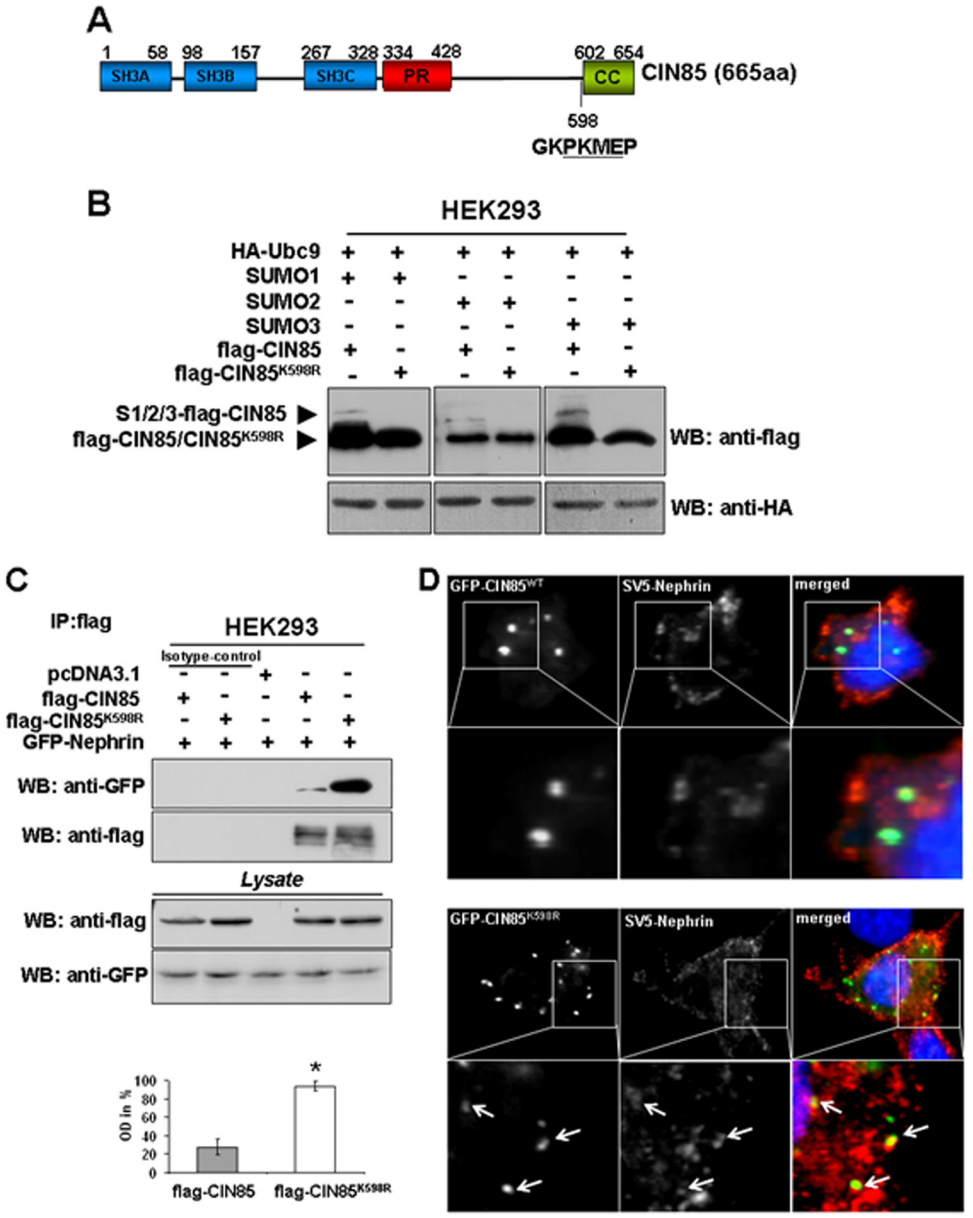

FIG 6 Identification of the SUMO modification site in mouse CIN85. (A) Schematic representation of the putative SUMO site in murine CIN85, where lysine in position 598 is the putative acceptor amino acid. The consensus SUMOylation sequence is PKME, where P is a large hydrophobic amino acid, K is the SUMO-modified lysine residue, and E is an acidic residue. (B) HEK 293 cells were transfected with Flag-CIN85; Flag-CIN85^{K598R}; or untagged SUMO-1, -2, and -3 and were immunoblotted with anti-Flag antibody. The arrows show un-SUMOylated and SUMOylated protein. The immunoblot represents the results of three independent experiments. (C) HEK 293 cells were transfected with Flag-CIN85, Flag-CIN85^{K598R}, or pcDNA3.1 empty vector and GFP-nephrin. Whole-cell lysates were subjected to immunoprecipitation (IP) with Flag-coupled beads or, for the isotope-matched control, with normal mouse IgG and agarose beads and immunoblotted with anti-GFP or anti-Flag antibody. As a control, the lysates were immunoblotted with anti-Flag or anti-GFP. (Below) Densitometry summarizing the means and standard deviations of immunoprecipitated nephrin/nephrin in control lysates obtained from three independent experiments. *, $P < 0.001$ compared to Flag-CIN85/Flag-CIN85^{K598R} by Student's *t* test. (D) Podocytes were transfected with plasmids expressing GFP-tagged wild-type or mutant CIN85^{K598R} (green) and SV5-nephrin (red). After 48 h, the transfected cells were analyzed by fluorescence microscopy. The nucleus was stained with DAPI (blue). Overlap of CIN85^{K598R} and SV5-nephrin is indicated by the arrows.

is expressed in CD2AP$^{+/+}$ and CD2AP$^{-/-}$ podocytes in the nuclear fraction. The localization of CIN85 in the nucleus was first observed by our group, but the function of nuclear localization is not known. Furthermore, SUMO-1 and Ubc9 are expressed in glomeruli and in cultured podocytes in the nucleus, but also in the cytosol, which was also shown for MDCK cells (5), underlining the fact that SUMOylation of CIN85 could also occur in the cytosol. Overexpression studies in HEK 293T cells demonstrated that CIN85 is a SUMO target with preferential binding to SUMO-3 compared to SUMO-1 and -2. Although endogenous binding assays show no preference of CIN85 for binding to SUMO-2/3 and most data of other groups suggest that the SUMO conjugation apparatus is incapable of discriminating between SUMO isoforms, a preferred interaction with SUMO-3 compared to

SUMO-1 was shown for USP25, a ubiquitin-specific protease (32–34). Furthermore, CD2AP could be identified as a SUMO target. Together with the finding of CIN85 SUMOylation in CD2AP$^{+/+}$ podocytes in contrast to non-SUMOylated CIN85 in CD2AP$^{-/-}$ podocytes, this led us to the hypothesis that SUMOylation of CD2AP could probably enhance SUMOylation of CIN85. A similar phenomenon has also been described for other proteins, like SF2/ASF (36). Interestingly, it has been shown that CD2AP and CIN85 homodimerized via their coiled-coil domains (23).

When we performed mutagenesis analysis, we demonstrated that conversion of lysine 598 to arginine, which is part of a SUMO consensus sequence, completely blocked SUMOylation of CIN85. Remarkably, this SUMOylation motif lies within a serine-rich part of the C terminus. Serine residues are suggested to regulate SUMO binding by phosphorylation, as a negative charge strengthens SUMO (Ubc9) binding (11). The complete blocking of SUMOylation indicates that lysine 598 is the only acceptor site of CIN85.

Furthermore, our findings revealed that mutation of CIN85 on lysine 598 led to increased plasma membrane-associated localization and increased binding to nephrin. These findings are also consistent with endogenously expressed CIN85, whose modified form is expressed in the cytosol but not in the membrane. Thus, the effect of CIN85 SUMOylation could be due to a changed localization pattern. SUMOylation and ubiquitination have also been reported to act either sequentially or in concert to regulate the substrate protein (4, 17), suggesting that both also participate in regulating CIN85 activity. Given the fact that the presence of CD2AP led to enhanced posttranslational modification of CIN85 and abolished SUMOylation-enhanced binding to nephrin, we hypothesize that the activity of CIN85 is decreased in podocytes expressing CD2AP. Although SUMOylation and de-SUMOylation are dynamic processes, CD2AP$^{+/+}$ podocytes show constant SUMOylation of CIN85. Lin et al. reported constant SUMOylation of Smad4 that protects it from ubiquitin-dependent degradation and controls subcellular localization (27). Constant SUMOylation could control the stable cytosolic localization of CIN85 in CD2AP$^{+/+}$ podocytes and may simultaneously prevent ubiquitin-dependent degradation. For the tumor suppressor p53, it has been demonstrated that ubiquitination simultaneously enhances SUMOylation, thus driving its nuclear export (4, 17).

We conclude from our experiments that SUMOylation of CIN85 could be an important molecular switch regulating the binding activity of CIN85 to nephrin by determining its subcellular localization. Thus, SUMOylation of CIN85 could regulate slit diaphragm stability and turnover of slit diaphragm molecules.

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