

- K. P. Mullane-Robinson, N. L. Lill, P. Douillard, H. Band, *J. Biol. Chem.* **274**, 16619 (1999).
8. G. Levkowitz *et al.*, *Genes Dev.* **12**, 3663 (1998); H. Waterman, G. Levkowitz, I. Alroy, Y. Yarden, *J. Biol. Chem.* **274**, 22151 (1999).
 9. C. H. Yoon, J. Lee, G. D. Jongeward, P. W. Sternberg, *Science* **269**, 1102 (1995).
 10. P. Tan *et al.*, *Mol. Cell* **3**, 527 (1999); T. Ohta, J. J. Michel, A. J. Schottelius, Y. Xiong, *ibid.*, p. 535; T. Kamura *et al.*, *Science* **284**, 657 (1999); W. Zachariae *et al.*, *ibid.* **279**, 1216 (1998).
 11. DNA fragments encoding the RING finger were amplified by polymerase chain reaction (PCR) of c-Cbl WT and 70Z/3 cDNA with primers CAAGACCAT-CAAAGTGACCCAG and CTATGCTCCTTGCCTCAACAG. PCR products were subcloned first into pCRL1, verified by sequencing, and then subcloned into the Eco RI site of pGEX-4T2. Point mutations were generated with QuikChange (Stratagene) with pGEX-4T2-RING as template. pGEX-cbl 1-480 (encoding GST-SH2-RING) was a gift from L. Samelson. GST fusion proteins were produced in *E. coli* BL21 (DE3) by induction with 0.4 mM isopropyl- β -D-thiogalactopyranoside for 3 hours at 30°C. Cells were lysed in 50 mM tris-HCl (pH 8), 120 mM NaCl, 1 mM dithiothreitol (DTT), and protease inhibitors. Proteins bound to glutathione-Sepharose beads were eluted with lysis buffer containing 20 mM glutathione and dialyzed against lysis buffer containing 50 mM NaCl and 10% glycerol.
 12. His-tagged E1 (a gift of F. Yamao) and Ubc4 [a gift of M. Nakao; T. Anan *et al.*, *Genes Cells* **3**, 1 (1998)] were produced as in (11), except that Talon beads (Clontech) and 100 mM imidazole were used for His-tagged protein binding and elution, respectively. Iodination of Ub was as described [R. Oughtred, N. Bedard, A. Vrielink, S. S. Wing, *J. Biol. Chem.* **273**, 18435 (1998)]. Ubiquitination and Ubc4 activation assays: Reactions (10 to 15 μ l) contained His-E1 (50 to 500 nM), His-Ubc4 (0.5 to 5 μ M), GST fusion proteins (10 μ g), ubiquitin (5 μ M), and ATP (2 mM) in Ub buffer [50 mM tris-HCl (pH 7.5), 2.5 mM MgCl₂, and 0.5 mM DTT]. Incubation was at 23°C for 90 min, unless otherwise indicated. Reactions were stopped with 2 \times SDS sample buffer, containing 4% SDS and 5.8 M β -mercaptoethanol. Ub-protein ligation was dependent on the presence of both E1 and Ubc4 (21). Ubc4 was chosen for these experiments because it had been shown to support EGF-R ubiquitination in fractionated cell extracts [S. Mori *et al.*, *Eur. J. Biochem.* **247**, 1190 (1997)]. Monoclonal antibodies used for protein immunoblots were anti-Tetra-His (Qiagen) and anti-GST (Santa Cruz Biotechnology). Prestained molecular weight markers were used to determine approximate protein sizes.
 13. The difference between the distribution of Ub adducts in Fig. 1, A and B, is likely due to the fact that any given poly-ubiquitination product of GST-RING has only one site for anti-GST binding, but multiple labeled Ub moieties.
 14. The species indicated in Figs. 1C, 2C, and 3C as Ub_n-Ubc4 are resistant to cleavage by 2.9 M β -mercaptoethanol and boiling and therefore do not represent E2~Ub thioesters. The inefficiency of the Ubc4 activation reaction is likely due either to a lower propensity of Ubc4 to auto-ubiquitinate [as compared with Cdc34; A. Banerjee, L. Gregori, Y. Xu, V. Chau, *J. Biol. Chem.* **268**, 5668 (1993)] or to the competing reaction, ubiquitination of GST-RING.
 15. C. E. Andoniou, C. B. Thien, W. Y. Langdon, *EMBO J.* **13**, 4515 (1994).
 16. P. S. Freemont, *Ann. N. Y. Acad. Sci.* **684**, 174 (1993).
 17. R. Y. Hampton, R. G. Gardner, J. Rine, *Mol. Biol. Cell* **7**, 2029 (1996).
 18. J. Parkinson, S. P. Lees-Miller, R. D. Everett, *J. Virol.* **73**, 650 (1998).
 19. R. Grossberger *et al.*, *J. Biol. Chem.* **274**, 14500 (1999).
 20. GST pull-down assay: GST fusion protein (5 μ g) and His-Ubc4 (1 μ g) were incubated for 2 hours at 4°C in 1 ml of binding buffer [20 mM tris-HCl (pH 7.5), 150 mM NaCl, 5 mM EDTA, 1% NP-40, bovine serum albumin (25 μ g/ml)], and then with 30 μ l of glutathione-Sepharose beads (Pharmacia) for 30 min. Precipitates were washed four times with binding buffer and subjected to 12% SDS-polyacrylamide gel electrophoresis (PAGE) and immunoblotting with anti-Ubc4 [V. Rajapurohitam *et al.*, *Dev. Biol.* **212**, 217 (1999)].
 21. C. A. P. Joazeiro and T. Hunter, unpublished data.
 22. PDGF-R β cDNA in the pcDNA3 expression vector was transfected into 293T human embryonic kidney cells. Cells were kept in 0.5% serum for 48 hours before lysis with 10 mM tris-HCl (pH 7.5), 1% Triton X-100, 5 mM EDTA, 50 mM NaCl, and protease inhibitors. PDGF-R β was immunoprecipitated from cleared lysates with PR7212 monoclonal antibody (a gift of D. Bowen-Pope). Beads were washed three times with lysis buffer, and twice with 20 mM Hepes (pH 7.4), 100 mM NaCl, and aprotinin (20 μ g/ml). An in vitro kinase reaction with [γ -³²P]ATP was done to label the receptor and to provide phosphotyrosine binding sites for the c-Cbl SH2 domain: Beads were washed twice and resuspended with kinase reaction buffer [20 mM Hepes (pH 7.4), 10 mM MnCl₂, aprotinin (20 μ g/ml), and 20 μ M Na₃VO₄], to which 20 μ Ci of [γ -³²P]ATP was added for 45 min at room temperature. The reaction was chased with 1 mM ATP for 20 min. Beads were washed once with phosphate-buffered saline containing 0.5 mM EDTA, and four times with Ub buffer, and samples were taken for ubiquitination reactions (12).
 23. The tyrosyl residues in RPTKs that mediate direct interaction with the Cbl SH2 domain are unknown. The association of the Cbl SH2 and COOH-terminal domains with RPTKs in vivo has been discussed [L. Smit and J. Borst, *Crit. Rev. Oncog.* **8**, 359 (1997)].
 24. To test whether cullins interact with c-Cbl in vivo to regulate its activity, hemagglutinin A (HA)-tagged c-Cbl or Roc1 and Myc-tagged cullins were overexpressed in 293T cells. Whereas HA-tagged Roc1 efficiently coimmunoprecipitated with cullins 1, 2, 3, 4A, and 5 [3, 4, 70] (gifts of Z.-Q. Pan, J. Michel, and Y. Xiong), c-Cbl, 70Z/3 Cbl, and Cbl 1-480 (L. Samelson) failed to do so. In addition, neither EGF stimulation nor v-Src coexpression promoted c-Cbl-cullin association (21).
 25. F. Ou and A. Varshavsky, personal communication.
 26. S. Miyake *et al.*, *Crit. Rev. Oncog.* **8**, 189 (1997); C. B. Thien and W. Y. Langdon, *Immunol. Cell Biol.* **76**, 473 (1998); Y.-C. Liu and A. Altmann, *Cell Signal.* **10**, 377 (1998).
 27. The intensity of these bands does not correlate with the Ub ligase activity of the protein preparation. In addition, mixing of ubiquitination-defective c-Cbl RING mutants with the WT protein did not reduce the WT Ub ligase activity (21).
 28. Ponceau S staining and antibody cross-reaction with GST revealed similar relative amounts of GST fusion proteins.
 29. L. Claesson-Welsh *et al.*, *Mol. Cell. Biol.* **8**, 3476 (1988).
 30. The efficiency of the reactions in Fig. 4A was determined as follows: Total counts in each lane (Ub_n-R β + R β) were normalized to those in lane 1. Counts in the Ub_n-R β or R β regions were then divided by the respective normalization factor. Counts in the Ub_n-R β portion of each lane were further corrected by subtracting from the background in lane 1. The values in Fig. 4B were calculated as (Ub_n-R β)/(R β + Ub_n-R β). This measure is an underestimate, because oligoubiquitinated precursor R β (160 kD) cannot be resolved from unmodified mature R β (190 kD), and oligoubiquitinated and unmodified mature R β bands are not resolved in 7.5% acrylamide gels.
 31. We thank M. Nakao, L. Samelson, F. Yamao, and A. Kumar for reagents; D. Koepf for advice; A. Altman, W. Langdon, G. A. Kassavetis, J. Noel, M. Verdecia, and the Hunter laboratory for discussions; E. P. Geiduschek for comments on the manuscript; C. Elly, J. Meisenhelder, S. Simon, and A. Young for help, and F. Orzalesi for encouragement. This work was supported by Fellowship 2-41-98 from the American Cancer Society, California Division (C.A.P.), by Medical Research Council of Canada grant MT12121 (S.S.W.), by Fellowship DRG1531 from the Damon Runyon-Walter Winchell Foundation (H.-k.H.), by American Cancer Society grant PF9922801CCG and NIH grant T32CA09523 (J.D.L.), by grant R01 DK56558 from NIH (Y.-C.L.), and by U.S. Public Health Service grants CA39780 and CA82683 from the National Cancer Institute (T.H.). S.S.W. is a recipient of a Clinician-Scientist Award from the Medical Research Council of Canada; T.H. is a Frank and Else Schilling American Cancer Society Professor.

19 August 1999; accepted 9 September 1999

Congenital Nephrotic Syndrome in Mice Lacking CD2-Associated Protein

Neng-Yao Shih,^{1*} Jun Li,^{1*} Vladimir Karpitskii,¹ Ancho Nguyen,¹ Michael L. Dustin,¹ Osami Kanagawa,¹ Jeffrey H. Miner,² Andrey S. Shaw^{1†}

CD2-associated protein (CD2AP) is an 80-kilodalton protein that is critical for stabilizing contacts between T cells and antigen-presenting cells. In CD2AP-deficient mice, immune function was compromised, but the mice died at 6 to 7 weeks of age from renal failure. In the kidney, CD2AP was expressed primarily in glomerular epithelial cells. Knockout mice exhibited defects in epithelial cell foot processes, accompanied by mesangial cell hyperplasia and extracellular matrix deposition. Supporting a role for CD2AP in the specialized cell junction known as the slit diaphragm, CD2AP associated with nephrin, the primary component of the slit diaphragm.

CD2AP is an adapter protein that interacts with the cytoplasmic domain of CD2 (1). CD2, a T cell and natural killer cell membrane protein, facilitates T cell adhesion to antigen-presenting cells. CD2AP enhances CD2 clustering and anchors CD2 at sites of cell contact (1). As CD2AP is widely ex-

pressed, it may play roles in other tissues.

Mice lacking CD2AP were generated by replacing the exon encoding the first SRC homology 3 (SH3) domain of CD2AP with a neomycin-resistance gene (Fig. 1A) (2). Two independent homologous recombinant clones were injected into blastocysts to generate chi-

REPORTS

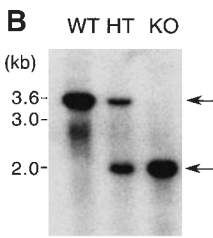
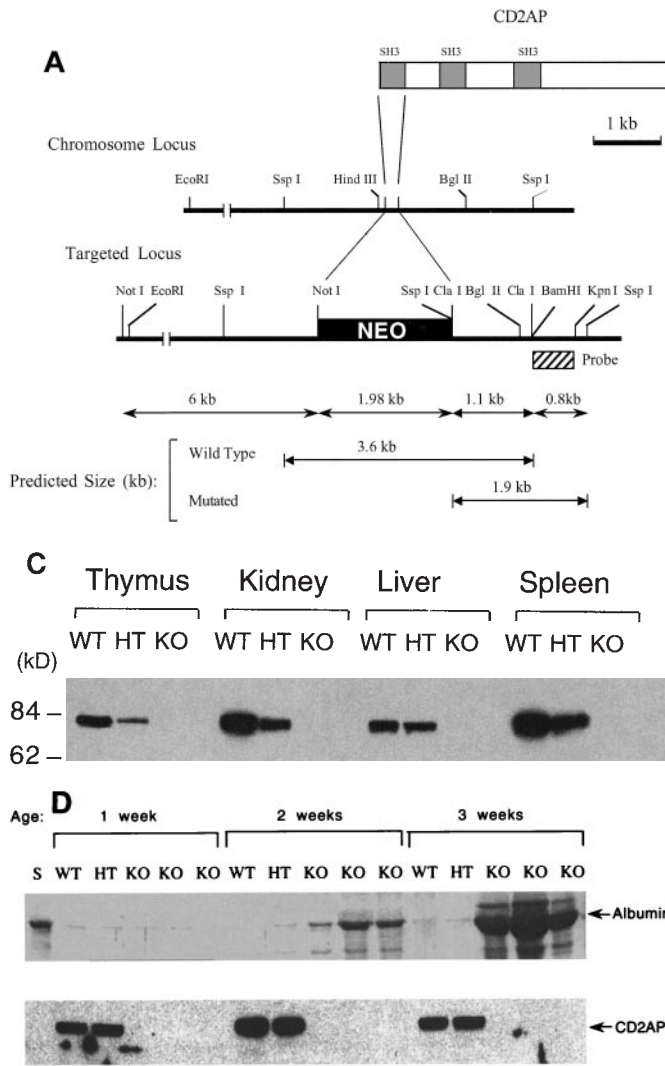


Fig. 1. (A) Strategy for homologous replacement of the first SH3 domain of CD2AP with a neomycin-resistance cassette (NEO). Restriction sites in the flanking introns around the targeted exon and the predicted sizes of the resulting fragments from Ssp I digestion are shown. (B) Southern blot analysis of wild-type (WT), heterozygous (HT), and homozygous CD2AP (KO) mice. (C) CD2AP immunoblotting of thymus, kidney, liver, and spleen from WT, HT, and KO mice. (D) SDS-PAGE analysis of urinary protein in WT, HT, and KO mice. Two microliters of urine from 1-, 2-, and 3-week-old mice was analyzed by SDS-PAGE and Coomassie stained. Genotypes were confirmed by immunoblotting with antibodies to CD2AP (bottom). One microgram of bovine serum albumin was run as a control (lane 1).

meric mice (3). Homozygous CD2AP knockout (KO) mice were generated from heterozygous mice with a normal frequency of 25%. The genotypes of the mice were confirmed by Southern (DNA) blotting analysis (Fig. 1B). Immunoblotting (4) demonstrated loss of CD2AP protein in KO animals and reduced levels of CD2AP in heterozygotes (Fig. 1C).

At about 3 weeks of age, CD2AP KO animals began to exhibit substantial growth retardation, and most KO mice were dead by 6 to 7 weeks of age. Postmortem examination of these mice revealed cardiac hypertrophy, splenic and thymic atrophy, and ascites. Histological examination revealed evidence of severe kidney pathology (Fig. 2D) (5). This pathology correlated with proteinuria, elevated blood urea nitrogen and creatinine concentrations, and reduced serum albumin concentrations, all signs of kidney dysfunction (6). Proteinuria was first detectable around 2 weeks of age (Fig. 1D) (7).

Microscopic examination revealed that the predominant kidney pathology involved the glomerulus, a specialized collection of capillary loops that constitutes the filtration apparatus of the kidney. The filtration barrier consists of the fenestrated capillary endothelial cells, the glomerular basement membrane (GBM), and the foot processes of the glomerular epithelial cells or podocytes (Fig. 3A). The spaces between the podocyte foot processes are regular (~35 nm) and contain a specialized junctional structure called the slit diaphragm.

¹Center for Immunology and Department of Pathology, ²Department of Medicine, Renal Division, Washington University, Saint Louis, MO 63110, USA.
*These authors contributed equally to this work.
†To whom correspondence should be addressed. E-mail: shaw@immunology.wustl.edu

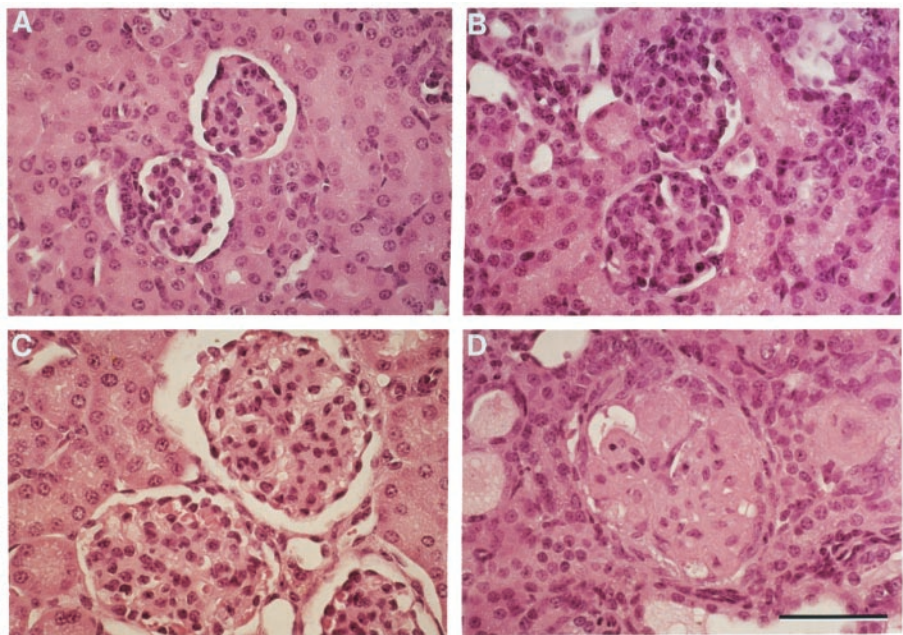


Fig. 2. Severe glomerular disease in CD2AP KO mice. Kidneys from wild-type (7-day-old) and from 7-, 14-, and 28-day-old KO animals were analyzed by hematoxylin and eosin staining. (A) Kidney section of a 7-day-old wild-type mouse. (B) Kidney section of a 7-day-old CD2AP KO mouse demonstrating glomerular hypercellularity. (C) Kidney section from a 14-day-old CD2AP KO mouse demonstrating glomerular hypercellularity and mesangial deposits. (D) Kidney section from a 28-day-old CD2AP KO mouse demonstrating glomerulosclerosis and kidney tubule dilatation. Scale bar, 50 μ m.

REPORTS

Compared with the glomeruli of age-matched wild-type mice (Fig. 2A), some of the glomeruli in 1-week-old KO mice showed

increases in size and cellularity, suggesting a congenital defect (Fig. 2B). Kidney tubules appeared normal. At 2 weeks of age, almost

all glomeruli were affected, and many had evidence of mesangial deposits (Fig. 2C). By 4 weeks, glomeruli were sclerotic, with increased deposits and distended capillary loops (Fig. 2D). There was never any evidence of inflammation.

Electron microscopic (EM) examination of the kidneys indicated that the initial defect involved the podocytes (8). Kidneys from 1-week-old animals showed loss of foot process integrity with obliteration of the spaces between the foot processes (Fig. 3, B and C). These changes were found in all glomeruli and in all capillary loops. However, at this stage, normal foot processes were also present (Fig. 3C). No anomalies of the GBM or endothelial cells were detected. Older animals showed worsening of the foot process damage. In addition, mesangial deposits be-

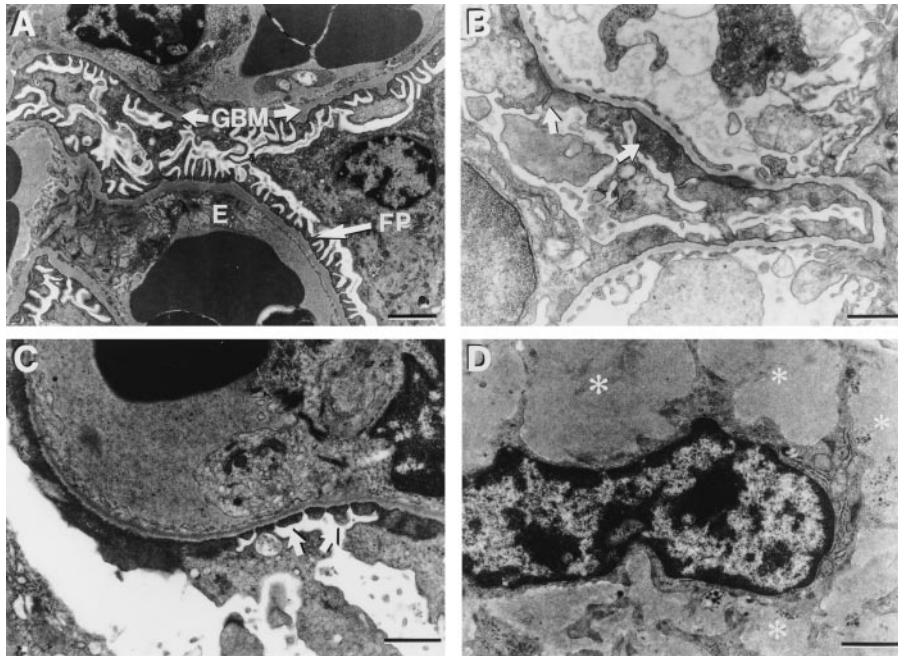


Fig. 3. Electron microscopic analysis of CD2AP KO glomeruli. (A) Ultrastructural analysis of a glomerular capillary wall from a 7-day-old wild-type mouse demonstrates the normal morphology of the glomerular filtration barrier with normal fenestrated endothelial cells (E), glomerular basement membrane (GBM), and normal podocyte foot processes (FP). (B and C) Analysis of glomerular filtration barrier from a 7-day-old KO mouse shows foot process effacement [arrows in (B)], but normal endothelial cells and basement membranes. In (C), normal foot processes (arrows) adjacent to the damaged foot processes are shown. (D) Ultrastructural analysis from a 28-day-old mouse demonstrating massive extracellular matrix deposition (*) surrounding a mesangial cell. Scale bars, 1 μ m.

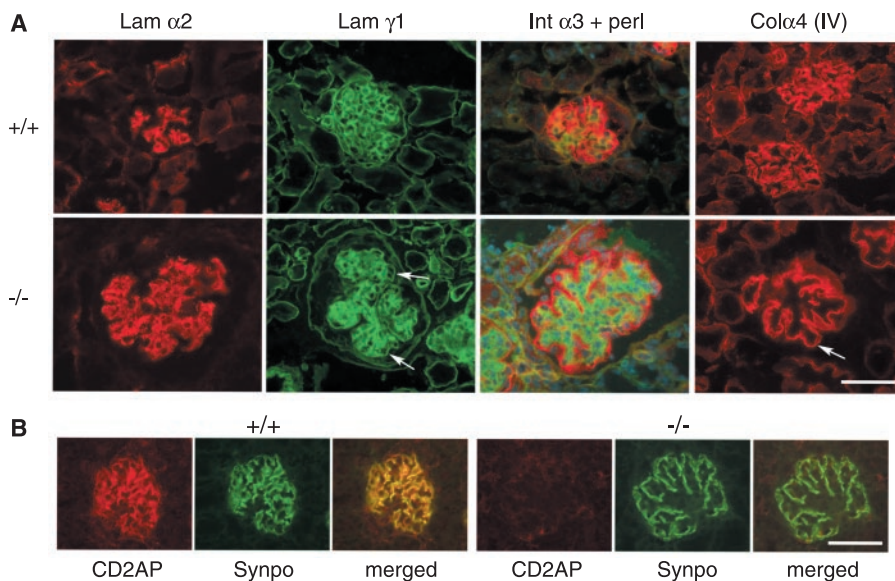


Fig. 4. Immunofluorescence studies of wild-type and CD2AP KO glomeruli. (A) The expanded mesangium of mutant glomeruli contains laminin (Lam) α 2, laminin γ 1, and perlecan (perl; shown in green) as well as other matrix proteins normally secreted by mesangial cells (see text). The composition of the GBM (arrows) is not affected by the mesangial defect; it contains the laminin α 5, β 2, and γ 1 (shown) chains and the collagen (Col) α 3, α 4 (shown), and α 5(IV) chains. Integrin α 3 (Int α 3; shown in red) appears properly localized juxtaposed to the GBM on the basal surface of podocytes in mutant glomeruli. (B) In glomeruli, CD2AP (shown in red) is expressed exclusively by podocytes, as determined by double-labeling with synaptopodin (Synpo, shown in green), a podocyte marker. Scale bar, 50 μ m.

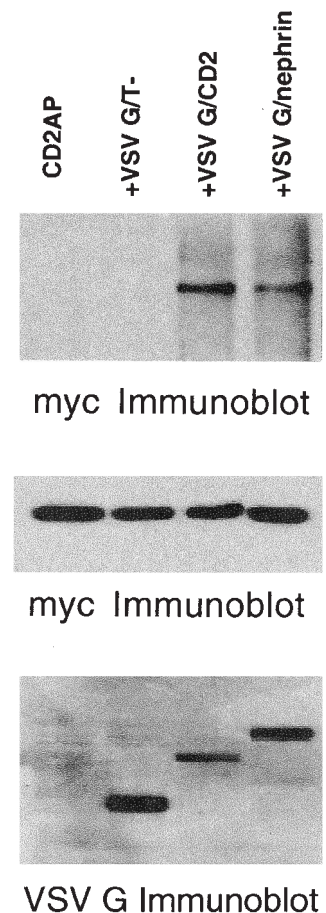


Fig. 5. Association of CD2AP with nephrin. A myc-tagged form of CD2AP was expressed either in HeLa cells alone (lane 1) or with a chimeric protein containing the nephrin cytoplasmic domain (VSV G/nephrin, lane 4), a chimeric protein known to interact with CD2AP (VSV G/CD2, lane 3), or the VSV G protein lacking a cytoplasmic domain (VSV G/T⁻, lane 2). Cell lysates were immunoprecipitated with antibody to VSV G and immunoblotted with antibody to myc (top). Immunoblotting of whole-cell lysates with antibody to myc (middle) or with antibody to VSV G (bottom) was performed to demonstrate similar levels of expression.

gan to accumulate starting around 4 weeks of age (Fig. 3D). In some areas, these deposits were extensive, encroaching upon the lumen of the capillary loops. There were no subendothelial or subepithelial deposits indicative of immune complex pathology.

Deposits were composed of extracellular matrix normally secreted by mesangial cells. Deposits were strongly positive for fibronectin, collagens $\alpha 1$ and $\alpha 2(IV)$, perlecan, and the laminin $\alpha 1$, $\alpha 2$, $\alpha 5$, $\beta 1$, and $\gamma 1$ chains, indicating the presence of laminins-1, -2 and -10 (Fig. 4A) (6, 9). The GBM contained the usual laminin (laminin-11) and collagen IV isoforms ($\alpha 3$, $\alpha 4$, and $\alpha 5$ chains), and these isoforms were not found in the expanded mesangium (Fig. 4A) (6), suggesting that the deposits were of purely mesangial cell origin. In addition, integrin $\alpha 3$ was properly localized adjacent to the GBM on the basal face of podocytes (Fig. 4A), indicating that the podocytes maintained their proper polarity despite the multiple insults to the glomerulus.

Immunofluorescence studies of wild-type kidney demonstrated that CD2AP was expressed primarily in podocytes (9). CD2AP exhibited an overlapping pattern of expression with synaptopodin, a podocyte foot process marker (Fig. 4B) (10). No CD2AP staining was detected in mesangial cells, but a subset of tubules did stain. CD2AP staining was absent in the kidneys of the KO mice (Fig. 4B).

Recently, mutations in the nephrin gene were identified as the cause of congenital nephrotic syndrome of the Finnish type (11). Nephrin, an immunoglobulin superfamily member, is expressed exclusively in podocytes and is thought to be the major component of the slit diaphragm (12). Because the function of nephrin is reminiscent of that of CD2, we tested whether CD2AP associates with nephrin. CD2AP and nephrin, however, could not be solubilized from purified glomeruli with non-ionic detergents, so we could not determine whether the two proteins associate in the podocyte. Therefore, we generated a chimeric protein containing the extracellular and transmembrane domain of vesicular stomatitis virus (VSV) G protein fused to the cytoplasmic domain of nephrin. The fusion protein was expressed with myc-tagged CD2AP, and coimmunoprecipitation was assessed by immunoblotting VSV G immunoprecipitations with antibodies to myc (Fig. 5) (13). The nephrin fusion protein coimmunoprecipitated CD2AP at levels similar to those of the CD2 fusion protein (1). This was specific because a VSV G molecule lacking its cytoplasmic domain (G/T⁻) did not coimmunoprecipitate CD2AP. In addition, nephrin and CD2AP interacted in vitro with the use of purified proteins and by yeast two-hybrid analysis (14).

Given the role of CD2AP in T cells, we also examined T cell function. Stimulation of

KO T cells with antibodies to CD3 or the lectin concanavalin A (Con A) demonstrated impaired T cell function (15). To rule out a relation between the kidney and immune dysfunction, we transplanted bone marrow from KO animals into irradiated wild-type animals (16). Transplanted mice still demonstrated T cell deficits but showed no evidence of kidney dysfunction, demonstrating that the defect is intrinsic to the kidney.

Here, we demonstrated that CD2AP is critical to the integrity of the renal glomerulus. Disease progression appears to begin with epithelial cell injury leading to a mesangial reaction consisting of hyperplasia and massive matrix deposition. This conclusion is supported by the specific expression of CD2AP in podocytes and EM studies demonstrating that damage to podocyte foot processes is the initial lesion. Coimmunoprecipitation studies suggest that CD2AP associates with nephrin, a protein critical for podocyte function. As foot processes can apparently develop in the absence of CD2AP, we suspect that the association of CD2AP with nephrin mainly functions to anchor nephrin to the cytoskeleton.

The role of CD2AP in the renal glomerulus may be similar to its role in the T cell. CD2 and nephrin are both immunoglobulin superfamily proteins involved in forming specialized cell adhesions. In the T cell, this adhesive complex is the immunological synapse (1); in the kidney, it is the slit diaphragm. Supporting a general role for CD2AP in specialized cell contacts, CD2AP was recently shown to associate with the focal adhesion protein, p130^{CAS} (17). Given the phenotype of the mouse, it will be important to determine whether CD2AP plays a role in the pathogenesis of human kidney diseases.

References and Notes

1. M. Dustin *et al.*, *Cell* **94**, 667 (1998).
2. Hybridization with mouse CD2AP cDNA was used to clone a genomic fragment from a 129/SvJ phage library. A 6-kb fragment 5' of the exon encoding the first SH3 domain was generated by polymerase chain reaction (PCR) with the following primers: 5'-GCGG-CCGCTGATATGATGATGACTACACTGTAG-3' and 5'-GCGGCCGCACATTCATACATACTGCATTC3'. After digestion with Not I, it was cloned into the Not I site of the targeting vector pTK.NEO.UMS [L. F. Reis, H. Ruffner, G. Stark, M. Aguet, C. Weissman, *EMBO J.* **13**, 4798 (1994)]. A 1.1-kb fragment 3' of the exon encoding the first SH3 domain was generated with the two primers 5'-TAGAACATCGATGTCAGAAA-TAAATGCATATG-3' and 5'-AATCTAATCGATTCCCA-GCATCCACAGCTC-3' and cloned into the Cla I site of the targeting vector.
3. Transfection of RW-4 embryonic stem (ES) cells was performed by the Washington University ES Cell Core. Homologous recombinants were screened by Southern blotting with a 500-base pair fragment (encompassing the Bam HI-Kpn I fragment) generated with the primers 5'-GGATCCCCCTGGAGCTGTG-GATG-3' and 5'-GGTACCATTTCATTTCTGCTAGG-3', which is external to the 3' arm of the targeting vector. Three positive clones were identified, and two clones were microinjected into C57/BL6 blastocysts with standard methods [S. L. Mansour, K. R. Thomas, M. R. Capecchi, *Nature* **336**, 348 (1988)].
4. For immunoblotting, tissues were homogenized in a

- buffer containing 1% NP-40, 40 mM Hepes (pH 7.6), 100 mM NaCl, 1 mM sodium vanadate, and protease inhibitors. Equal amounts of tissue samples were loaded and resolved by SDS-polyacrylamide gel electrophoresis (PAGE), transferred to nitrocellulose filters, and blotted with a rabbit antiserum to CD2AP as described (7).
5. Tissues from CD2AP KO mice were fixed in 4% buffered formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin by the Research Histology Facility in the Washington University Department of Pathology.
6. N.-Y. Shih *et al.*, data not shown.
7. One-, two-, and three-week-old animals were killed, and urine was collected. Proteinuria was analyzed by SDS-PAGE and Coomassie staining. By 3 weeks of age, proteinuria in KO animals was quite substantial, measuring over 1000 mg/dl.
8. Kidneys from wild-type or KO animals were fixed in glutaraldehyde and embedded in epoxy resin. Thin sections stained with uranyl acetate were examined in the Diagnostic EM Facility of the Washington University Department of Pathology.
9. Freshly dissected kidneys were frozen in OCT compound and sectioned at 7 μ m. After fixation with 2% paraformaldehyde in phosphate-buffered saline (PBS) and clearing in 100 mM glycine in PBS, sections were incubated with primary antibodies for 1 hour, washed in PBS, and incubated with Cy3- or fluorescein isothiocyanate-conjugated second antibodies (or with both). After rinsing in PBS, sections were viewed and digitally imaged under epifluorescent illumination. Primary antibodies were as follows: rabbit antibodies to CD2AP (7), laminin $\alpha 2$ [Y.-S. Cheng *et al.*, *J. Biol. Chem.* **272**, 31525 (1997)], integrin $\alpha 3$ [C. M. DiPersio, S. Shah, R. O. Hynes, *J. Cell Sci.* **108**, 2321 (1995)], and collagen $\alpha 4(IV)$ [J. H. Miner and J. R. Sanes, *J. Cell Biol.* **127**, 879 (1994)]; mouse antibody to synaptopodin [P. Mundel *et al.*, *J. Histochem. Cytochem.* **39**, 1047 (1991)]; and rat antibodies to laminin $\gamma 1$ (MAB1914; Chemicon) and perlecan [M. Kato, Y. Koike, S. Suzuki, K. Kimata, *J. Cell Biol.* **106**, 2203 (1988)].
10. P. Mundel *et al.*, *J. Cell Biol.* **139**, 193 (1997).
11. M. Kestila *et al.*, *Mol. Cell* **1**, 575 (1998).
12. V. Ruotsalainen *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **96**, 7962 (1999).
13. G/Nephrin was generated by replacing the Bam HI-Hind III fragment of pGem G/epsilon [L. T. Gauen, A. N. Kong, L. E. Samelson, A. S. Shaw, *Mol. Cell Biol.* **38**, 5438 (1992)] with a PCR-generated Bgl II-Hind III fragment encoding residues 1086 to 1241 of the nephrin cytoplasmic domain with an expressed sequence tag clone (AA678622) as a template. Myc-CD2AP (7) was coexpressed with VSV G fusion proteins in HeLa cells and analyzed as described previously (7).
14. N. Shih and A. Shaw, unpublished data.
15. Lymph node T cells were stimulated with antibody to CD3 and Con A. Cell proliferation was measured by [³H]thymidine incorporation 48 and 72 hours after stimulation. KO T cells proliferated about 20 to 30% compared with wild-type T cells.
16. T cell-depleted bone marrow cells (1×10^7) from 129SvJ/CD2AP KO mice were injected into irradiated (950 rads) C57/BL6 mice. Graft reconstitution was confirmed with antibody Ly9.1 staining, which recognizes the 129/SvJ T cells but not the C57/BL6 T cells. No kidney abnormality was detected up to 1 year after transplant. T cells from mice reconstituted with KO bone marrow exhibited proliferative responses of about 60 to 70% compared with T cells from animals reconstituted with wild-type bone marrow when stimulated with antibody to CD3.
17. K. H. Kirsch, M. M. Georgescu, S. Ishimaru, H. Hanafusa, *Proc. Natl. Acad. Sci. U.S.A.* **96**, 6211 (1999).
18. N. Shih and J. Li contributed equally to this manuscript. We thank E. Unanue and R. Cotran for invaluable help and suggestions; J. Kissane, J. Saffitz, and M. LaRegina for help with pathological analysis; M. White for ES cell microinjections; A. Chan for help with isolation of the genomic clone; C. Li for antibody staining; and M. A. Arnaout, P. Mundel, P. Yurchenco, M. DiPersio, and M. Yamagata for providing antibodies.

22 June 1999; accepted 30 August 1999