A Novel Role for the Adaptor Molecule CD2-associated Protein in Transforming Growth Factor-β-induced Apoptosis*

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CD2-associated protein (CD2AP) is an adaptor molecule involved in T cell receptor signaling and podocyte homeostasis. CD2AP-deficient mice develop nephrotic syndrome and renal failure caused by glomerulosclerosis. Here we report that increased transforming growth factor-β (TGF-β) expression and apoptosis were present in podocytes at the onset of albuminuria and were followed by depletion of podocytes associated with progressive focal-segmental glomerulosclerosis in CD2AP−/− mice. Conditionally immortalized podocytes derived from CD2AP−/− mice were more susceptible to TGF-β-induced apoptosis compared with CD2AP+/+ podocytes. Reconstitution of CD2AP rescued CD2AP−/− podocytes from TGF-β-induced apoptosis. CD2AP was required for early activation of anti-apoptotic phosphatidylinositol 3-kinase (PI3K)/AKT and extracellular signal-regulated kinase 1/2 by TGF-β. In contrast, activation of pro-apoptotic p38 MAPK by TGF-β was accelerated and enhanced in the absence of CD2AP. CD2AP was not required for PI3K/AKT activation by insulin and epidermal growth factor, indicating that CD2AP is a selective mediator of anti-apoptotic TGF-β signaling. In summary, we identified CD2AP as a novel mediator for selective activation of survival pathways and repression of apoptosis signaling by TGF-β in podocytes. Together, our in vitro and in vivo findings suggest that TGF-β-induced podocyte apoptosis is an early pathomechanism in mice developing focal-segmental glomerulosclerosis associated with functional impairment of CD2AP.

The transforming growth factor-β (TGF-β) superfamily consists of secreted peptides, of which the three TGF-β isoforms (TGF-β1–3), activins, and bone morphogenetic proteins are best known in mammalian development, homeostasis, and pathobiology. The TGF-β isoforms are widely expressed and act on virtually every cell type in mammals by engaging a ubiquitous intracellular signaling cascade of Smad family proteins through ligand-induced activation of heteromeric transmembrane TGF-β receptor kinases. Receptor-activated Smad protein complexes accumulate in the nucleus where they participate directly in transcriptional activation of target genes. In addition, TGF-β receptors can activate Smad-independent signaling mechanisms, including mitogen-activated protein kinases (MAPKs), and PI3K (1, 2). However, molecular mechanisms of activation of Smad-independent pathways by TGF-β receptors remain unclear.

The mouse CD2 receptor-associated protein (CD2AP) and its human orthologue Cas ligand with multiple SH3 domains (CMS) belong to a family of ubiquitously expressed adaptor molecules that also includes the human Cbl-interacting protein of 85 kDa (CIN85) and its rat and mouse orthologue regulator of ubiquitous kinase (Ruk) and SH3 domain-containing gene expressed in tumorogenic astrocytes (SETA) (for review, see Ref. 3). These proteins are defined as cytoplasmic adaptor or scaffolding proteins by three N-terminal SH3 domains, a proline-rich region, and a C-terminal coiled-coil domain. CIN85/CMS family proteins have been shown to interact with proline-rich regions present in a variety of signaling proteins, including Cbl oncogene, CD2 receptor, AIP1 apoptosis-inducing protein-1, and the p85 subunit of phosphatidylinositol 3-kinase (PI3K), mediated through the SH3 domains, and with focal adhesion kinase p130Cas, Src family kinases Fyn, Src, and Yes, Grb2, and endophilins, mediated through the proline-rich region (for review, see Ref. 3). Other structural features include FXXDF motifs, mediating interactions with endocytic proteins, and actin binding motifs, mediating interactions with actin cytoskeleton. Based on these observations, CIN85/CMS proteins are thought to exert multiple and diverse signaling functions in the organization of the immunological synapse in T cells, endocytosis and signaling of receptor tyrosine kinases, and neuronal apoptosis (3).

Degeneration of differentiated glomerular, tubular, and vascular cells in the normal nephron is a hallmark of chronic progressive kidney disease along with readily apparent renal scarring (4–6). Sclerosing glomeruli are characterized by progressive depletion of podocytes, resulting in denuded glomerular basement membrane areas and tuft adhesions, which may be considered as initial lesions of irreversible glomerular injury (4, 7). Indeed, recent studies demonstrate that podocyte numbers are reduced early in glomeruli of diabetic patients with nephropathy (8, 9). We have shown that TGF-β induces apoptosis in podocytes in vitro and in vivo via a p38 MAPK and caspase-dependent mechanism. We further demonstrated in a TGF-β1 transgenic mouse model of glomerulosclerosis that podocyte apoptosis is an early glomerular phenotype leading to progressive podocyte depletion. Together, our studies suggest...
that TGF-β may induce podocyte apoptosis and depletion in glomerulosclerosis (10).

An important functional role for CMS/CD2AP in the kidney was suggested initially by phenotype features in CD2AP knockout mice (CD2AP−/−), which manifest high grade albuminuria at a young age followed by progressive glomerulosclerosis and tubulointerstitial fibrosis and death from renal failure (11). We therefore used this novel and essential role for CD2AP in TGF-β signaling in podocytes. We demonstrate that CD2AP is required for rapid activation of PI3K and ERK MAPK pathways by TGF-β. Failure of TGF-β receptors to engage these anti-apoptotic pathways early in the absence of CD2AP is associated with hyperactivation of proapoptotic p38 MAPK and accelerated apoptosis in podocytes in vitro. Finally, rates of apoptosis and expression of TGF-β1 were greatly increased, specifically in podocytes in the kidneys of CD2AP−/− mice, coincident with the onset of albuminuria, and this precedes podocyte depletion and glomerulosclerosis.

EXPERIMENTAL PROCEDURES

Mice—CD2AP knockout mice in mixed C57Bl/129J background were described previously (11). Kidney tissue was harvested and either immersed fixed in formaldehyde for paraffin embedding or embedded in O.C.T. compound (Sakura Finetek, Torrance, CA) for frozen sections. Animal protocols and procedures were reviewed for ethical and humane standards and approved by an institutional Animal Use Committee.

Cell Culture—Conditionally immortalized podocytes were derived from CD2AP−/− mice and CD2AP+/− littermates and cultured as previously reported (12). In brief, podocytes were grown on collagen type I (BD Biosciences) at 33°C (St. Cruz, CA), and anti-active TGF-β1 antibody (LC 1 (14), rabbit polyclonal anti-WT-1 antibody (Santa Cruz Biotechnology), St. Cruz, CA), and wortmannin (100 nM) (Calbiochem), or epidermal growth factor receptor (EGF) (20 ng/mL) and insulin (25 ng/mL) (Sigma).

Immunostaining in S itu and in Vitro—For in situ detection of proteins we used the following antibodies: monoclonal anti-synaptopodin antibody (14), rabbit polyclonal anti-WT-1 antibody (Santa Cruz Biotechnology, St. Cruz, CA), and anti-active TGF-β1 antibody LC1 (13–300) (a gift from Anita Roberts, National Cancer Institute). The samples were evaluated on a Bio-Rad MR600 confocal microscope, and images were captured using a Kanton Electronic Prog/Res/3012 digital video camera and digitally processed using Adobe Photoshop 5.0.2 (Adobe Systems, Inc., San Jose, CA).

Scoring of Apoptotic Nuclei in Situ—To detect DNA fragmentation in situ, apoptotic nuclei were detected by a terminal dUTP nick-end labeling assay of kidney sections using peroxidase and counterstaining with hematoxylin and periodic acid-Schiff stain, as described earlier (10). For reconstitution experiments from 2-, 3-, and 4-week-old mice for TGF-β1 protein in podocytes in CD2AP−/− mice, we associated with TGF-β expression profiles in glomeruli, we examined functional and histopathological manifestations of FSGS in CD2AP−/− and CD2AP+/+ littermates at 1, 2, 3, and 4 weeks of age. Immunoperoxidase labeling of kidney sections from 2-, 3-, and 4-week-old mice for TGF-β1 protein demonstrated that podocytes in 3-week-old (n = 5) (Fig. 1d) and 4-week-old CD2AP−/− mice (n = 5) (Fig. 1f) stained strongly positive for TGF-β1, whereas 3- and 4-week-old CD2AP+/+ controls (Fig. 1, c and e) and 2-week-old mice of either genotype showed no or minimal TGF-β1 staining in podocytes (Fig. 1, a and b). The strong increase in TGF-β1 protein synthesis in podocytes in this model is similar to the increase in Smad7 protein in podocytes that we observed previously (15). Lysates were subjected to 10% SDS-PAGE before transfer to polyvinylidene difluoride membranes (PerkinElmer Life Sciences). Western blotting was performed as previously described, and the following primary antibodies were used for antigen detection: mouse monoclonal anti-total AKT1 (Santa Cruz Biotechnology), rabbit polyclonal anti-SIP1/TOK AKT, rabbit anti-phospho-Smad2, mouse anti-phospho-Erk1/2, rabbit anti-total Erk1/2, rabbit anti-phospho-p38, and mouse anti-total p38 (all from Cell Signaling Inc., Beverly, MA).

Quantitative Real-time PCR—Glomerular fractions were prepared by a sieving method to >95% purity from fresh mouse kidneys. Total RNA was prepared from glomerular fractions using RNeasy columns (Qiagen, Hilden, Germany) and reverse-transcribed with SuperScript II reverse transcriptase (Invitrogen), and the cDNA was amplified using SYBR-Green PCR Master Mix (Applied Biosystems, Foster City, CA), and specific primers for murine TGF-β1 (5’ primer, CCGTATTTG-GAGCCCTGGA; 3’ primer, GTTTGTTTGTAGAGGGCAGAG, TGF-βRI (5’ primer, TCAAAACAGATGGGACCAGG; 3’ primer, TCAATTGGCAT- ACCAGCAT), TGF-βRII (5’ primer, CAGTGTTGTCAGAGGACCG; 3’ primer, ACCACTGCTGCAATGTCCTA), murine WT-1 (5’ primer, ATCTATGCCAGACCCACAC, 3’ primer, AAGCCGGAGACTTTTGTCG), and murine Smad7 (5’ primer, ATCTTATCAAGTCGCGCCAC; 3’ primer, AACCCAGGAAACTTCTGTCG). The anti-histone antibody binds to the histone components of the nucleus, and the anti-DNA peroxidase reacts with the DNA component of the nucleus. After incubation and washing, color was developed using the provided 2,2'-azino-bis-3-ethylbenzthiazoline-6-sulfonic acid (substrate solution to the wells. Absorbance was measured at 405 nm. Western Blotting—To detect levels of total protein and phosphorylated proteins, cells were lysed in radioimmune precipitation assay buffer containing protease inhibitors and phosphatase inhibitors as previously described (15). Lysates were subjected to 10% SDS-PAGE before transfer to polyvinylidene difluoride membranes (PerkinElmer Life Sciences). Western blotting was performed as previously described, and the following primary antibodies were used for antigen detection: mouse monoclonal anti-total AKT1 (Santa Cruz Biotechnology), rabbit polyclonal anti-SIP1/TOK AKT, rabbit anti-phospho-Smad2, mouse anti-phospho-Erk1/2, rabbit anti-total Erk1/2, rabbit anti-phospho-p38, and mouse anti-total p38 (all from Cell Signaling Inc., Beverly, MA).

Increased Expression of TGF-β1 in Podocytes in CD2AP−/− Mice Is Associated with Onset of Albuminuria and FSGS—We previously reported that Smad7 and TGF-β1 induce apoptosis in podocytes in vitro and in vivo (10). To determine whether manifestations of FSGS typically observed in CD2AP−/− mice were associated with TGF-β expression profiles in glomeruli, we examined functional and histopathological manifestations of FSGS in CD2AP−/− and CD2AP+/+ littermates at 1, 2, 3, and 4 weeks of age. Immunoperoxidase labeling of kidney sections from 2-, 3-, and 4-week-old mice for TGF-β1 protein demonstrated that podocytes in 3-week-old (n = 5) (Fig. 1d) and 4-week-old CD2AP−/− mice (n = 5) (Fig. 1f) stained strongly positive for TGF-β1, whereas 3- and 4-week-old CD2AP+/+ controls (Fig. 1, c and e) and 2-week-old mice of either genotype showed no or minimal TGF-β1 staining in podocytes (Fig. 1, a and b). The strong increase in TGF-β1 protein synthesis in podocytes in this model is similar to the increase in Smad7 protein in podocytes that we observed previously in CD2AP−/− mice (15).

During the first 2 weeks, kidney structure and function were not significantly different in CD2AP−/− and control littermates, respectively. However all CD2AP−/− animals had developed high grade albuminuria when examined at 3 weeks of age; significant glomerular histopathology characterized by mesangial proliferation and early FSGS lesions was detectable at 3 weeks and progressed to sclerosis in more than 50% of examined glomeruli in CD2AP−/− mice at 4 weeks (data not shown). Thus, the onset of glomerular disease in this model...
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with high grade proteinuria is rapid and occurs between 2 and 3 weeks of age.

Next, we isolated glomeruli from CD2AP+/+ and CD2AP−/− kidneys at 4 weeks of age to quantitate mRNA expression of key components of TGF-β signaling directly in primary glomerular fractions by quantitative real-time PCR. Transcript levels for TGF-β1, type II TGF-β receptor (TGF-βRII), and Smad7 were all significantly increased in glomeruli obtained from CD2AP−/− compared with glomeruli from CD2AP+/+ mouse kidneys (Fig. 1g). In contrast, type I TGF-β receptor mRNA was not significantly different (Fig. 1g). Together, these in situ protein and mRNA expression data indicate that increased expression of TGF-β1 and Smad7 by differentiated podocytes in CD2AP−/− mice is associated with albuminuria and mesangial activation at early stages of glomerulosclerosis.

CD2AP Deficiency Predisposes Podocytes to Undergo Apoptosis Induced by Autocrine and Paracrine TGF-β in Vitro—After we determined that increased expression of mediators of TGF-β signaling in podocytes of CD2AP−/− mice coincided with onset of albuminuria, we wished to examine whether TGF-β signaling and apoptosis were affected by CD2AP deficiency in vitro. Conditionally immortalized CD2AP−/− and control CD2AP+/+ podocyte cell lines were established from littermate mice as described previously (12). After 14 days of culture in non-permissive conditions, morphology and expression patterns of podocyte markers such as WT-1, synaptopodin, and podocin was indistinguishable in CD2AP−/− and control CD2AP+/+ podocyte cell lines (data not shown). Three independent cell clones for each genotype were examined. Quantitation of apoptotic nuclei showed that base-line (untreated) rates of apoptosis were significantly higher in CD2AP−/− podocytes compared with CD2AP+/+ (2.4 ± 0.9 versus 0.7 ± 0.3%, respectively) (Fig. 2a). TGF-β treatment for 24 h increased apoptosis rates by 2.5 ± 0.5-fold over base line in CD2AP+/+ cells and 4.8 ± 1.7-fold over base line in CD2AP−/− podocytes (Fig. 2a). Thus, the apoptosis-inducing
effect of TGF-β was significantly enhanced in CD2AP−/− compared with CD2AP+/+ podocytes (4.8 ± 1.7 versus 2.5 ± 0.5-fold induction; p < 0.03). These findings suggest that CD2AP exerted an anti-apoptotic function in podocytes both at base line and in particular when exposed to exogenous (paracrine) TGF-β.

Because we have found that podocytes also secrete latent and active autocrine TGF-β, we examined base-line apoptosis rates in the absence or presence of pan-neutralizing anti-TGF-β antibody 2G7. Neutralizing antibody (2G7) significantly reduced base-line apoptosis in CD2AP−/− podocytes by 49% compared with control IgG (1.09 ± 0.39 versus 2.13 ± 0.22%, p < 0.004) (Fig. 2b). Thus, increased base-line apoptosis observed in CD2AP−/− podocytes is largely, but not exclusively, dependent on autocrine TGF-β signaling, indicating that TGF-β receptor activity and survival pathway signaling are coupled in podocytes by the CD2AP adaptor molecule. We conclude that CD2AP deficiency predisposes podocytes to apoptosis induced by both exogenous (paracrine) and autocrine TGF-β.

CD2AP Is Required for Rapid Activation of PI3K and ERK1/2 by TGF-β but Not by EGF and Insulin—We reasoned that the anti-apoptotic effect of CD2AP on TGF-β-induced apoptosis could be due to essential survival signaling and/or inhibition of pro-apoptotic pathways or both. Because PI3K/AKT functions as a primary cell survival pathway in many cell types (16), we examined activation profiles of PI3K/AKT in response to TGF-β in three independent lines of CD2AP−/− and CD2AP+/+ podocytes, respectively. In CD2AP+/+ podocyte lines, TGF-β1 induced a strong and rapid (minutes) phosphorylation of AKT on serine 473 (Fig. 3a). Serine 473 phosphorylation peaked between 1 and 2 h and remained elevated for up to 4 h (Fig. 3b). In contrast, S(P)473-AKT was not detectable until 1 h of TGF-β treatment in CD2AP−/− podocyte lines (Fig. 3a), and peak phosphorylation was delayed at 4 h of TGF-β1 treatment (Fig. 3b). Among MAPKs, the ERK1/2 mediates primarily mitogenic and/or anti-apoptotic signaling (17, 18). Interestingly, the profile of ERK1/2 activation in control CD2AP+/+ podocytes treated with TGF-β1 was very similar to the S(P)473-AKT profile, including a strong and rapid activation detectable at 5 min (Fig. 3a) followed by peak phosphorylation at 2 h (Fig. 3b). Similar to S(P)473-AKT, phosphorylation of ERK1/2 was not detectable during the initial 45 min (Fig. 3a), and peak phosphorylation was delayed between 2 and 4 h in CD2AP−/− podocytes (Fig. 3b). Thus, the anti-apoptotic mediators PI3K/AKT and ERK1/2 are activated by TGF-β in podocytes with similar kinetic profiles, consistent with previous reports that ERK1/2 and PI3K are synergistically activated to mediate anti-apoptotic pathways (19, 20). Our results presented here demonstrate for the first time that early activation of PI3K/AKT and ERK1/2 MAPK by TGF-β in podocytes requires the CD2AP adaptor protein.

To examine whether CD2AP and PI3K function in a linear pathway, we quantitated and directly compared the extent of activation profiles of anti-apoptotic signaling mediators induced by TGF-β in CD2AP−/− and control CD2AP+/+ podocytes. a, Western blot analysis demonstrates levels of phospho-proteins as indicated in total cell protein lysates of conditionally immortalized CD2AP+/+ and CD2AP−/− podocytes after short term treatment with TGF-β1 (5 ng/ml) for 0, 5, 30, and 45 min. b, Western blot analysis shows total cell protein lysates harvested from CD2AP+/+ and CD2AP−/− podocytes after stimulation with TGF-β1 (5 ng/ml) for extended time periods as indicated. Membranes were probed with antibodies detecting S(P)473-AKT (pS473-AKT) and phospho-ERK1/2 (pp44/pp42). Membranes were reprobed with antibodies detecting the respective total protein levels. Data shown are representative results for three independent experiments in three different CD2AP+/+ and CD2AP−/− podocyte clones.

Inactivation of PI3K were associated with enhanced TGF-β-induced apoptosis signaling in a non-additive, quantitatively comparable manner, indicating that CD2AP and PI3K function in a linear pathway downstream of TGF-β receptors.

To confirm this hypothesis at a molecular level by direct reconstitution experiment, we transfected CD2AP−/− podocytes with CD2AP expression plasmid. Expression of epitope-tagged, reconstituted CD2AP in transfected CD2AP−/− cells was validated by Western blot analysis (data not shown). Rates of apoptotic nuclei were significantly reduced in CD2AP−/− cells transfected with CD2AP expression plasmid compared with empty vector (control)-transfected cells (Fig. 4c). Together these findings demonstrate that PI3K/AKT mediates survival signals in podocytes exposed to TGF-β and show that CD2AP is required for activation of this survival pathway by TGF-β.

To examine whether CD2AP is a general or pathway-selective mediator of PI3K/AKT activation in podocytes, we treated CD2AP−/− and control CD2AP+/+ podocytes with insulin and EGF, two well characterized extracellular stimulators of PI3K/AKT-dependent anti-apoptotic signaling (21, 22). In contrast with TGF-β stimulation, insulin and EGF induced early AKT phosphorylation rapidly and to a similar extent in both CD2AP+/+ and CD2AP−/− podocytes, respectively (Fig. 4d), suggesting that CD2AP is required for activation of PI3K/AKT pathway by TGF-β signaling through receptor serine/threonine kinases but not by insulin or EGF signaling through receptor tyrosine kinases. We reasoned next that if CD2AP is not re-
required for PI3K/AKT pathway activation by insulin and EGF, then pretreatment with insulin should reduce the increased apoptotic response induced by TGF-β irrespective of the absence or presence of CD2AP. Indeed, pretreatment with insulin significantly inhibited TGF-β-induced apoptosis in both control CD2AP+/+ and CD2AP−/− cells, providing further functional
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Fig. 5. Activation profiles of pro-apoptotic signaling mediator p38 MAPK induced by TGF-β in CD2AP<sup>+/−</sup> and control CD2AP<sup>+/+</sup> podocytes. a, Western blot analysis demonstrates levels of phosphoproteins as indicated in total cell protein lysates of conditionally immortalized CD2AP<sup>+/+</sup> and CD2AP<sup>−/−</sup> podocytes after short term treatment with TGF-β1 (5 ng/ml) for 0, 5, 30, and 45 min. b, Western blot analysis shows total cell protein lysates harvested from CD2AP<sup>+/+</sup> and CD2AP<sup>−/−</sup> podocytes after stimulation with TGF-β1 (5 ng/ml) for extended time periods as indicated. Membranes were probed with antibodies detecting phospho-p38 and phospho-Smad2. Membranes were reprobed with antibodies detecting the respective total protein levels. Data shown are representative results for three independent experiments in three different CD2AP<sup>+/−</sup> and CD2AP<sup>−/−</sup> podocyte clones.

Fig. 6. Quantitative analysis of apoptosis in glomerular cells in kidneys of CD2AP<sup>+/+</sup> and CD2AP<sup>−/−</sup> mice. Histograms show the average ± S.D. of terminal dUTP nick-end labeling-positive cells per 30 glomerular sections in podocytes (a), parietal cells (b), and endocapillary/mesangial cells (c) in CD2AP<sup>+/+</sup> (open bars) and CD2AP<sup>−/−</sup> mice (black bars) at 2, 3, and 4 weeks of age. 50 glomeruli per cortex section/animal (5 animals in each genotype and age group) were examined.
Bars and CD2AP/H11002/H11002/weeks of age in CD2AP 30 glomeruli per animal at 2, 3, and 4 din/WT1-positive cells determined from CD2AP/H11001/H11001/average number cross-section in CD2AP/H11001/H11001/WT1 double-positive cells per glomerular section, respectively.

red antibody (using a Cy3-conjugated goat anti-rabbit) and WT-1 staining was visualized (green nate-conjugated goat anti-mouse IgG used, and synaptopodin staining was visualized using a fluorescein isothiocyanate-conjugated goat anti-mouse IgG (green) and WT-1 staining was visualized using a Cy3-conjugated goat anti-rabbit antibody (red), g, Histogram shows the average number ± S.D. of synaptopodin/WT1 double-positive cells per glomerular cross-section in CD2AP+/+ (open bars) and CD2AP−/− (black bars) kidneys. Bars represent the number of synaptopodin/WT1-positive cells determined from 30 glomeruli per animal at 2, 3, and 4 weeks of age in CD2AP+/+ (n = 5) and CD2AP−/− (n = 5) animals, respectively.

The average counts of podocytes in 30 glomeruli per animal and 5 animals per experimental group were not significantly different between CD2AP+/+ and CD2AP−/− animals at 2 and 3 weeks of age (Fig. 7g). In contrast, WT1/synaptopodin double-positive cells per glomerular section were significantly (p < 0.01) fewer in the kidneys of 4-week-old CD2AP−/− compared with CD2AP+/+ mice (4.7 ± 1.5 versus 11.3 ± 0.2 podocytes per glomerular section, respectively) (Fig. 7g). These findings demonstrate that with the onset of albuminuria in CD2AP−/− mice an increase in podocyte apoptosis occurs that leads to subsequent loss of podocytes associated with progression of FSGS. Thus, CD2AP deficiency in podocytes caused increased susceptibility to undergo apoptosis associated with TGF-β/α signaling in vitro and in vivo.

DISCUSSION

Our findings presented in this report demonstrate a novel role for the adaptor molecule CD2AP in TGF-β signaling. Specifically, CD2AP is required for rapid and kinetically defined early (up to 1 h) activation of anti-apoptotic PI3K/AKT and ERK1/2 MAPK survival signaling pathways by TGF-β in podocytes. In addition, the activation of pro-apoptotic p38 MAPK by TGF-β is accelerated and enhanced in the absence of CD2AP. Interestingly, TGF-β treatment was associated with kinetically defined late (1–4 h) activation of both pathways in CD2AP−/− podocytes, indicating that secondary, delayed activation mechanisms are CD2AP-independent. Nevertheless, the CD2AP-dependent early activation of anti-apoptotic signaling by TGF-β is important for counterregulation of the pro-apoptotic signaling activity of TGF-β in podocytes previously described by our group (10), because podocytes lacking CD2AP are significantly more susceptible to TGF-β-induced apoptosis, and reconstitution of CD2AP directly reduces TGF-β-induced apoptosis. CD2AP is not required for PI3K/AKT activation by EGF and insulin, suggesting that CD2AP functions selectively in TGF-β receptor-activated pathways but not in receptor-tyrosine kinase pathways. Importantly, we demonstrate in vivo that a 6-fold increase in apoptosis coincides with increased expression of TGF-β1 in podocytes detectable at the onset of albuminuria in kidneys of CD2AP−/− mice. Moreover, sustained apoptosis and TGF-β expression in podocytes is associated with a progressive loss of podocytes per glomerulus and with progressive FSGS in this model. In conclusion, we have identified a previously unrecognized molecular mechanism linking TGF-β expression, TGF-β receptor, and CD2AP signaling as the candidate pathway to specify the irreversible cell fate of podocytes in glomerular injury. The presence of CD2AP restricts podocyte apoptosis by enhancing critical PI3K/AKT and ERK1/2 survival pathways, whereas absent or reduced function of CD2AP may result in accelerated podocyte apoptosis because of insufficient activation of survival pathways and enhanced pro-apoptotic p38 activation in podocytes exposed to increased TGF-β in vitro and in vivo.

Our study is further significant because it provides an alternative model to the prevailing paradigm of the functional role of CD2AP in podocytes. Studies of the functional significance of CD2AP in the kidney were first prompted by the surprising phenotype of CD2AP−/− mice (11), manifesting with high grade albuminuria, FSGS, and renal failure. Subsequent stud-
ies demonstrated that CD2AP can be localized in podocyte foot processes in vitro (23, 24) and is able to interact directly with the slit-diaphragm proteins nephrin and podocin in vitro (25). Because defects in the genes encoding nephrin and podocin, NPHS1 and NPHS2, respectively, cause congenital forms of nephrotic syndrome and FSGS in humans (26) and because this was consistent with the phenotype observed in CD2AP−/− mice, it has been proposed that CD2AP together with nephrin and podocin is important for slit diaphragm and foot process formation (27). However, the functional role of CD2AP in this context remains unclear. A recent report indicates that nephrin and/or CD2AP can interact with the p85 regulatory subunit of PI3K, associated with AKT phosphorylation (28). The authors propose that nephrin and/or CD2AP initiate signaling from the slit diaphragm (28). However, regulation and functional significance of proposed "slit diaphragm signaling" are unknown and remain to be validated in the context of endogenous CD2AP function in podocytes.

In contrast, our results support an alternate model where CD2AP functions as mediator of ligand-inducible, transmembrane TGF-β receptor-regulated anti-apoptotic signaling, which may be independent of slit diaphragm proteins. First, phosphorylation of AKT and ERK1/2 occurs with very rapid kinetics, suggestive of a protein synthesis-independent activation cascade. Because this rapid activation cascade is interrupted in the absence of CD2AP, we propose that CD2AP may provide a molecular linker between TGF-β receptor protein complexes and the p85 subunit of PI3K and/or Ras. Second, CD2AP is not required for the direct phosphorylation of Smad2 by TGF-β receptors, and receptor-regulated Smads are known to bind directly with TGF-β type I receptors (29). Third, TGF-β receptors and CD2AP are both present in lipid rafts, as shown by Schwarz et al. (25) for CD2AP and our group for TGF-β receptor complexes (30). Finally, the adaptor protein Dab2, which contains proline-rich domains similar to CD2AP, has been shown to interact with TGF-β receptor complexes including type I and type II receptors and facilitates activation of Smad2 and Smad3 (31). Thus, these intriguing findings are consistent with a model where CD2AP may interact with TGF-β receptors to enable activation of PI3K/AKT and/or Ras/ERK1/2 signaling. Further studies to test this hypothesis seem warranted and are under way in our laboratory.

Glo merulosclerosis in animal models and humans is characterized by depletion of podocytes, and denuded glomerular basement membrane is thought to form aberrant adhesions and synechiae with Bowman’s capsule (7, 32, 33). If tuft adhesions and synechiae are associated with collapse of appendant glomerular capillaries, irreversible glomerulosclerosis becomes established (34). Because podocyte depletion may be a key initiating lesion in this process, it is considered important to determine the underlying molecular and cellular mechanisms. Several possibilities need to be considered in this context. It is intriguing to note that experimental reduction of podocytes per glomerulus using puromycin amino nucleoside cytotoxicity in rats (34) was correlated directly with the extent and severity of glomerulosclerosis. Second, a recent report proposed mechanistic detachment of podocytes from glomerular basement membranes and loss in urinary space as a candidate mechanism for podocyte depletion, based on the identification of viable cells expressing podocyte proteins in urine collected from humans (35). Interestingly, puromycin injury of podocytes resulted in up-regulation and activation of integrin-linked kinase ILK, which is known to activate AKT, and stable expression of active ILK was associated with decreased adhesiveness of podocytes in vitro (36). Third, it is thought that podocytes are unable to proliferate and to regenerate depleted podocytes in most forms of glomerular injury, leading to a state of "regenerative podocyte insufficiency" (7, 37–39).

In vivo and in vitro studies from our laboratory provide compelling evidence for injury-dependent activation of TGF-β signaling leading to apoptosis of susceptible podocytes (40). For example, the rate of podocyte apoptosis is increased 6-20-fold and peaks already at the onset of albuminuria in three models of progressive glomerulopathy, including CD2AP−/− mice (this report), TGF-β1 transgenic mice (10), and diabetic db/db mice. It is in vitro studies from our laboratory provide compelling evidence for injury-dependent activation of TGF-β signaling leading to apoptosis of susceptible podocytes (40). For example, the rate of podocyte apoptosis is increased 6-20-fold and peaks already at the onset of albuminuria in three models of progressive glomerulopathy, including CD2AP−/− mice (this report), TGF-β1 transgenic mice (10), and diabetic db/db mice. 

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