TM4SF10 and ADAP interaction in podocytes: role in Fyn activity and nephrin phosphorylation

Timur A. Azhibekov,1,2 Zhenzhen Wu,1,3 Aparna Padiyar,1,3 Leslie A. Bruggeman,1,3 and Jeffrey S. Simske1

1Rammelkamp Center for Education and Research, 2Division of Neonatology, Department of Pediatrics, 3Division of Nephrology, Department of Medicine, MetroHealth Medical Center, Case Western Reserve University School of Medicine, Cleveland, Ohio

Submitted 23 May 2011; accepted in final form 29 August 2011

Azhibekov TA, Wu Z, Padiyar A, Bruggeman LA, Simske JS. TM4SF10 and ADAP interaction in podocytes: role in Fyn activity and nephrin phosphorylation. Am J Physiol Cell Physiol 301: C1351–C1359, 2011.—TM4SF10 [transmembrane tetra(4)-span family 10] is a claudin-like cell junction protein that is transiently expressed during podocyte development where its expression is downregulated in differentiating podocytes coincident with the appearance of nephrin at the slit diaphragm. In a yeast two-hybrid screen, we identified adhesion and degranulation-promoting adaptor protein (ADAP), a well-known Fyn substrate and Fyn binding partner, as a TM4SF10 interacting protein in mouse kidney. Using coimmunoprecipitation and immunohistochemistry experiments in cultured human podocytes, we show that TM4SF10 colocalizes with Fyn and ADAP but does not form a stable complex with Fyn. Cytoskeletal changes and phosphorylation events mediated by Fyn activity were reversed by TM4SF10 overexpression, including a decrease in the activating tyrosine phosphorylation of Fyn (Y421), suggesting TM4SF10 may have a regulatory role in suppressing Fyn activity. In addition, TM4SF10 was reexpressed following podocyte injury by puromycin aminonucleoside treatment, and its expression enhanced the abundance of high-molecular-weight forms of nephrin indicating it may participate in a mechanism controlling nephrin’s appearance at the plasma membrane. Therefore, these studies have identified ADAP as another Fyn adapter protein expressed in podocytes, and that TM4SF10, possibly through ADAP, may regulate Fyn activity. Since TM4SF10 expression is temporally regulated during kidney development, these studies may help define a mechanism by which the slit diaphragm matures as a highly specialized cell junction during podocyte differentiation.

kidney; cell junctions; cytoskeleton; posttranslational modifications

The slit diaphragm is a unique cell junction between foot processes of podocytes responsible for the structure and function of the glomerular filtration barrier (31). It is a major component of the protein barrier that separates the circulation from the urinary space, and disruption of this structure due to various inherited or acquired diseases is associated with proteinuria as part of nephrotic syndrome and progressive renal failure. This highly specialized cell junction contains proteins of both adherens and tight junctions such as P-cadherin, Zona occludens-1 (ZO-1), and junctional adhesion molecule A (JAM-A) (7, 39, 42) as well as specialized junctional proteins such as nephrin, Nep1, Podocin, CD2AP, and the protocadherin FAT1 (reviewed in Ref. 8). Developmentally, podocyte cell-cell junctions begin as typical epithelial lateral junctions at the comma and s-shaped body stage and then transit to a more basolateral position with the incorporation of the slit diaphragm-specific proteins at the capillary loop stage when foot processes begin to appear (38). The developmental process of assembling the slit diaphragm and its restructuring in diseases characterized by foot process effacement are not fully understood.

In animal models and in human diseases, the absence of nephrin leads to effacement of foot processes, indicating the inability to form a slit diaphragm that results in severe nephrotic syndrome with massive proteinuria (16, 35, 37). Nephrin mRNA is initially detected in the kidney at the late s-shaped body stage with the protein being detectable in the basolateral plasma membrane between the columnar epithelial cells (putative podocyte precursors) adjacent to the vascular cleft (12, 41). Nephrin is initially synthesized as a 155-kDa protein that undergoes posttranslational glycosylation events increasing its molecular mass to 175- or 185-kDa forms, which are required for its appearance in the plasma membrane (6, 27, 52). Structurally, nephrin is a transmembrane protein of the immunoglobulin superfamily with multiple tyrosine phosphorylation sites in its intracellular domain (29). Experimental deletion of the intracellular domain containing these phosphorylation sites results in changes in nephrin’s subcellular location, concomitant with foot process effacement, and proteinuria. Therefore, it has been suggested that besides a structural role in the slit diaphragm, nephrin likely participates in signal transduction events that may mediate podocyte interactions with environmental events or injury responses to adverse stimuli (13).

The phosphorylation of nephrin is primarily through the Src family kinase Fyn, which binds nephrin directly via an SH2 domain and phosphorylates nephrin at multiple tyrosine residues (18, 21, 51). Fyn is a myristoylated protein that localizes to lipid rafts and is involved in a variety of signal transduction pathways through its association with intracellular signaling molecules (14, 44, 50). In Fyn knockout mice, foot process effacement with marked attenuation of nephrin phosphorylation was observed in detergent-resistant membrane fractions (51). Fyn activity also appears to mediate interaction of nephrin with the cytoskeleton, another factor that determines the structural integrity and proper function of the slit diaphragm (2, 49, 50, 53, 55). In a recent study, the phosphorylation of nephrin was shown to direct nephrin into raft-mediated endocytic recycling vesicles, thereby coupling nephrin phosphorylation with its removal from the plasma membrane (36).

TM4SF10 (also known as brain cell membrane protein-1, human gene name: TMEM47) is a 20-kDa transmembrane junctional protein in the PMP22/EMP/Claudin family of pro-
teins. This cell junction protein is the vertebrate orthologue of VAB-9 in Caenorhabditis elegans, a cell membrane protein we have previously shown to be required for organization of contractile actin filaments with redundant functions in cell adhesion (45). Recently, we have established that TM4SF10 is expressed in podocytes but is temporally restricted to a window of expression in the early stages of glomerular development before the capillary loop stage (3). In subconfluent cultured podocytes TM4SF10 localizes to the perinuclear region but translocates to the cell membrane following cadherin appearance at nascent cell-cell contacts. As podocytes mature by extension of synaptoptin-rich lamellapodia, TM4SF10 expression is downregulated and is no longer present in cell contacts, suggesting a transient role for TM4SF10 in the formation of cell junctions.

To determine a mechanistic role for TM4SF10 in podocyte differentiation and slit diaphragm development, we screened for TM4SF10-binding proteins using a yeast two-hybrid system and identified the Fyn binding protein adhesion and degranulation-promoting adaptor protein (ADAP) also known as Fyb (Fyn binding protein) or SLAP-130, which we shall refer to as ADAP (11). Since Fyn has been shown to be the primary kinase that phosphorylates nephrin, we explored possible connections between TM4SF10 with ADAP and Fyn in association with nephrin. In cultured podocytes, TM4SF10 expression appeared to change Fyn and nephrin phosphorylation, suggesting that TM4SF10 may have a regulatory function on Fyn and nephrin activity in podocytes.

MATERIALS AND METHODS

Cell lines, plasmids, and cell transduction. Human and mouse podocyte cell lines have been previously reported (17, 26) and were cultivated as described in RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 100 mg/ml streptomycin, 0.25 U/ml amphothericin B, and 2 mM L-glutamine (all from Invitrogen, Carlsbad, CA) at 37°C. Madin Darby canine kidney (MDCK), U937, and RAW264.7 cells were obtained from American Type Culture Collection and propagated with recommended conditions at 37°C. Human podocytes (AJP-Cell Physiol • VOL 301 • DECEMBER 2011 • www.ajpcell.org)

plasmids were sequenced to verify absence of PCR and cloning artifacts. MDCK cell were transiently transfected with Lipofectamine 2000 by standard methods and podocytes were transfected with FuGene6 (Roche Applied Science, Indianapolis, IN) as previously described (24). Cells were harvested 48 h after transfection for immunoprecipitation or Western blotting.

Yeast two-hybrid screen. The 22 amino acid cytoplasmic NH2-terminal domain of mouse TM4SF10 was cloned into the DNA binding domain yeast two hybrid clone (Matchmaker, Clontech). The DNA binding domain-TM4SF10 fusion protein was coexpressed in yeast with activation domain clones from 8- to 12-day-old mouse kidney cDNA library (Clontech). One million clones were screened and 20 candidates were identified that grew on selective media (-ADE, -HIS, -TRP, -LEU, +3AT) and were positive for β-galactosidase activity. These putative binding partners were identified by nucleotide sequencing and were validated for interaction in mammalian cells by coimmunoprecipitation.

Cell morphology. MDCK cells were transfected with GFP, Fyn-GFP, and TM4SF10-GFP, and stable lines were established following dilution plating and clonal selection using rings. Coexpression with GFP was achieved by infection with a TM4SF10-GFP expressing lentivirus as previously described (3). These stably expressing MDCK cell lines were plated at low density (105 cells in 6-well dishes), and after 24 h six random fields for each well were photographed using phase-contrast microscopy to visualize individual cells and were photographed again after 72 h to visualize colony formation. To determine total cell numbers and cells with extensions, the images from the 24-h time point were scored by manual counting, in which cells with extension were scored positive if at least one membrane protrusion exceeded the width of the cell body. Data are presented as an average percentage, and statistical significance was determined by t-test.

Immunoprecipitation and Western blotting. Immunoprecipitation was performed as previously reported (47) on cells lysed in a buffer containing PBS with 0.1% SDS, 1% Nonidet P-40, 0.5% deoxycholate with protease (Complete, Roche Applied Science), and phosphatase inhibitors (Phosphatase Inhibitor Cocktail Set II, EMD Biosciences, Gibbstown, NJ). Proteins were resolved on 4–20% Tris-glycine gels under denaturing conditions, except 6% denaturing gels were used to resolve the high-molecular-weight nephrin proteins. Antibodies and dilutions used are as follows: Fyn polyclonal (H-80, sc-28791, Santa Cruz Biotechnology, Santa Cruz, CA), 1:500 dilution; activated Fyn (anti-Src pY418), Invitrogen, inactive Fyn (anti-Src pY527, Cell Signaling Technology, Danvers, MA), both 1:1,000 dilution; nephrin (N2028–50, US Biological, Marblehead, MA), 1:500 dilution; phospho-nephrin pY1217 (Epitomics, Burlingame, CA), 1:1,000 dilution; ADAP (Epitomics), 1:500; GFP (JL-8, Clontech), 1:1,000 dilution; and secondary antibodies were goat anti-mouse or anti-rabbit fluorescent protein (GFP, and TM4SF10-GFP, and stable lines were established following dilution plating and clonal selection using rings. Coexpression with GFP was achieved by infection with a TM4SF10-GFP expressing lentivirus as previously described (3). These stably expressing MDCK cell lines were plated at low density (105 cells in 6-well dishes), and after 24 h six random fields for each well were photographed using phase-contrast microscopy to visualize individual cells and were photographed again after 72 h to visualize colony formation. To determine total cell numbers and cells with extensions, the images from the 24-h time point were scored by manual counting, in which cells with extension were scored positive if at least one membrane protrusion exceeded the width of the cell body. Data are presented as an average percentage, and statistical significance was determined by t-test.

Immunoprecipitation and Western blotting. Immunoprecipitation was performed as previously reported (47) on cells lysed in a buffer containing PBS with 0.1% SDS, 1% Nonidet P-40, 0.5% deoxycholate with protease (Complete, Roche Applied Science), and phosphatase inhibitors (Phosphatase Inhibitor Cocktail Set II, EMD Biosciences, Gibbstown, NJ). Proteins were resolved on 4–20% Tris-glycine gels under denaturing conditions, except 6% denaturing gels were used to resolve the high-molecular-weight nephrin proteins. Antibodies and dilutions used are as follows: Fyn polyclonal (H-80, sc-28791, Santa Cruz Biotechnology, Santa Cruz, CA), 1:500 dilution; activated Fyn (anti-Src pY418), Invitrogen, inactive Fyn (anti-Src pY527, Cell Signaling Technology, Danvers, MA), both 1:1,000 dilution; nephrin (N2028–50, US Biological, Marblehead, MA), 1:500 dilution; phospho-nephrin pY1217 (Epitomics, Burlingame, CA), 1:1,000 dilution; ADAP (Epitomics), 1:500; GFP (JL-8, Clontech), 1:1,000 dilution; and secondary antibodies were goat anti-mouse or anti-rabbit fluorescent protein (GFP, and TM4SF10-GFP, and stable lines were established following dilution plating and clonal selection using rings. Coexpression with GFP was achieved by infection with a TM4SF10-GFP expressing lentivirus as previously described (3). These stably expressing MDCK cell lines were plated at low density (105 cells in 6-well dishes), and after 24 h six random fields for each well were photographed using phase-contrast microscopy to visualize individual cells and were photographed again after 72 h to visualize colony formation. To determine total cell numbers and cells with extensions, the images from the 24-h time point were scored by manual counting, in which cells with extension were scored positive if at least one membrane protrusion exceeded the width of the cell body. Data are presented as an average percentage, and statistical significance was determined by t-test.

Immunohistochemistry. Immunocytochemistry was performed as previously described (24) with cells grown on glass coverslips using TRITC-labeled phalloloid (Sigma Aldrich), 1:1,000 dilution or the following primary antibodies: Fyn monoclonal (clone 1S, Upstate Biotechnology Millipore, Billerica, MA), 1:50 dilution; ADAP (Epitomics), 1:250; nephrin (US Biological), 1:100; and TM4SF10 (3) and secondary antibodies were goat anti-mouse or anti-rabbit fluorescent conjugates (Jackson ImmunoResearch) at 1:400. Coverslips were mounted in a glycerol-based mounting media with DAPI or TOPRO3.
to visualize nuclei and were imaged with epifluorescence or confocal microscopy.

PAN nephrosis model. Young adult rats (~200 g) were divided into two groups (n = 4 each group) and treated with either a single intraperitoneal injection of PAN (150 mg/kg) or an equivalent volume of saline as a control using the standard method as previously described (46). Rats were killed either 2 or 10 days posttreatment, and kidney tissue was formalin-fixed and evaluated for the induction of TM4SF10 expression by immunohistochemistry. All animal studies were approved and conducted in accordance with the animal care and use committee of Case Western Reserve University.

RESULTS

Fyn-binding protein ADAP is a TM4SF10 interacting protein. We identified ADAP as a TM4SF10 binding partner in an 8- to 12-day-old mouse kidney cDNA library using a yeast two hybrid approach. The 22 amino acid cytoplasmic NH2-terminal domain of mouse TM4SF10 was used as bait (Fig. 1A) and identified multiple, partial cDNA clones of ADAP. Based on these partial cDNAs, the TM4SF10 interaction domain with ADAP would be restricted to its COOH-terminal half (amino acids 378 to 819, Fig. 1B), which contains two SH3 domains, the Fyn binding, and phosphorylation sites and the binding sites for other interacting proteins such as VASP and SLP-76 (25, 48). As an initial confirmation of the yeast two-hybrid interaction, the ability for TM4SF10 and ADAP to interact in mammalian cells was tested by transiently expressing Myc-tagged ADAP in MDCK cells that were stably expressing either GFP or TM4SF10-GFP (Fig. 1C). Immunoprecipitating for GFP from the triton soluble fraction found that Myc-ADAP coprecipitated with TM4SF10-GFP but not GFP alone indicating that ADAP forms a stable complex with TM4SF10.

ADAP is a well-known Fyn binding protein in lymphocytes (32), but its expression outside the immune system has not been evaluated. Both human and mouse podocytes cell lines expressed ADAP by RT-PCR (verified by nucleotide sequencing of PCR products, not shown), Western blotting (Fig. 1D), and by immunohistochemistry (Fig. 2). With immunohistochemistry, ADAP expression partially colocalized with both GFP-tagged TM4SF10 (Fig. 2, A–C) and endogenous TM4SF10 (Fig. 2, D–F), predominately in cytoplasmic compartments and also the plasma membrane, consistent with previous reports on the subcellular distribution of the individual proteins (3, 4). As we have previously shown in podocytes (3), TM4SF10 concentrated at the plasma membrane in regions of cell-cell contacts (Fig. 2, G–I, arrow). Endogenous Fyn expression had a similar distribution at cell-cell contacts, and the plasma membrane pattern overlapped with TM4SF10 (Fig. 2I).

Since ADAP is a Fyn-binding protein and Fyn substrate, interactions between TM4SF10 and Fyn were investigated further in podocytes. The ability of TM4SF10, ADAP, and Fyn to complex in podocytes was tested in coimmunoprecipitation experiments. Endogenous ADAP and TM4SF10-GFP coimmunoprecipitated, as well as the expected coimmunoprecipitation of ADAP and Fyn (Fig. 3A), however, a stable interaction between Fyn and TM4SF10 was not observed. With the use of podocytes cotransfected with TM4SF10-GFP and Fyn plasmids (or as a control, GFP and Fyn plasmids), Fyn-specific antibodies failed to detect Fyn in the samples of both TM4SF10-GFP and GFP expressing podocytes when immu...
noprecipitated with GFP antibodies (Fig. 3B). Similar findings were observed when Western blotting with GFP-specific antibodies failed to detect TM4SF10-GFP or GFP in the samples immunoprecipitated using Fyn antibodies. These results suggest that TM4SF10 and Fyn, although localized together at the plasma membrane, may not form a stable immunoprecipitable complex.

Functional effects of TM4SF10 on Fyn activity and cell phenotype. MDCK cells expressing Fyn-GFP exhibited a markedly different morphology from their typical cobblestone appearance (Fig. 4A). The majority of cells expressing Fyn-GFP extended protrusions (Fig. 4B), whereas MDCK cells expressing GFP or TM4SF10-GFP had no differences in cell morphology compared with untransfected controls. Coexpression of TM4SF10-GFP with Fyn-GFP, however, suppressed this morphological change, and the cells resumed more typical individual cell morphology and colony formation (Fig. 4, A and B), suggesting TM4SF10 inhibited this effect induced by Fyn. In addition, Fyn overexpression in MDCK cells caused total cellular increases in phosphotyrosine detectable by Western blotting, including the autophosphorylation of Fyn and phosphorylation of ADAP, a known substrate of Fyn. Similar to the cell morphology observations, coexpressing TM4SF10-GFP blocked Fyn activity resulting in an overall reduced level of tyrosine phosphorylation, including the phosphorylation of both ADAP and Fyn (Fig. 4C). Fyn, like the other Src family kinases, has nine potential tyrosine phosphorylation sites with two of them being well characterized regarding their function (Fig. 4D). Tyrosine (Y)532 mediates an intramolecular interaction with the SH2 domain, blocking its ability to bind phosphotyrosine-containing proteins, thus rendering the kinase inactive when phosphorylated. However, phosphorylation of Y421 in the kinase domain leads to stimulation of kinase activity (23). Western blotting with antibodies specific for these individual tyrosine phosphorylation sites found TM4SF10-GFP overexpression reduced the level of the activating Y421 phosphorylation, with no change in the Y532 phosphorylation (Fig. 4E). Together, these studies suggest that TM4SF10 expression levels was inversely related to an acti-
vation event for Fyn (Y421 phosphorylation) and total cellular phosphotyrosine levels, which were concurrent with changes in cell morphology. In podocytes, TM4SF10-GFP overexpression also had an effect on cytoskeletal structure, inducing predominately peripheral and circumferential F-actin bundling as opposed to the predominant stress fiber cross striations in the absence of TM4SF10-GFP (Fig. 5, A–C).

**TM4SF10 and nephrin interactions.** Using immunohistochemistry, nephrin colocalized with TM4SF10-GFP in intracellular compartments and also in distal cell extensions in podocytes (Fig. 5, D–G), suggesting there may be spatiotemporal events where TM4SF10 and nephrin may interact. A functional effect of TM4SF10 on nephrin was examined using TM4SF10-GFP overexpression or inhibition by RNAi-mediated gene silencing. Normal podocytes and podocytes stably expressing TM4SF10 and nephrin may interact. A functional effect of TM4SF10 on nephrin was examined using TM4SF10-GFP overexpression or inhibition by RNAi-mediated gene silencing. Normal podocytes and podocytes stably expressing TM4SF10-GFP with and without coexpression of a TM4SF10-specific shRNA, were transiently transfected with a full-length nephrin plasmid and examined by Western blotting (Fig. 5H). The presence of both the 175- and 185-kDa forms of nephrin were observed in greater abundance in cells expressing TM4SF10-GFP; however, when TM4SF10 expression was reduced by RNAi, the abundance of both 175- and 185-kDa forms was reduced. To examine nephrin phosphorylation, several prior studies have shown the phosphorylated forms of nephrin can only be observed by Western blotting using a pervanadate pretreatment to block the action of phosphatases (18, 21, 50, 51). Podocytes with and without TM4SF10-GFP overexpression were pretreated with pervanadate and blotted for total nephrin and nephrin phosphorylated at tyrosine1217 (Fig. 5I). Similar to Fig. 5H, overexpression of TM4SF10-GFP in the absence of pervanadate was associated with a greater abundance of the high molecular weight forms of nephrin (Fig. 5I, compare lanes 1 and 3). Pervanadate pretreatment in the absence of TM4SF10-GFP (Fig. 5I, lane 2) resulted in almost undetectable total nephrin but a strong signal for phosphorylated nephrin, suggesting the small amount of total nephrin in lane 2 represented only phosphorylated nephrin. Pervanadate pretreatment in the presence of TM4SF10-GFP (Fig. 5I, lane 4) was associated with a greater abundance of total nephrin compared with treated cells in the absence of TM4SF10-GFP (Fig. 5I, lane 2); however, there was no difference in the level of phosphorylated nephrin. This shows that a greater proportion of nephrin was not phosphorylated in the presence of TM4SF10-GFP, suggesting that TM4SF10-GFP overexpression inhibited nephrin phosphorylation. This is consistent with the decreased Fyn-mediated tyrosine phosphorylation by TM4SF10-GFP observed in MDCK cells (Fig. 4C) and also reinforces the importance of phosphorylation event in regulat-
ing the abundance or stability of high-molecular-weight forms of nephrin as previously reported (18, 21, 50, 51).

**TM4SF10 reexpression during podocyte injury.** Our previous observation that TM4SF10 is expressed only during podocyte development (3) would suggest that TM4SF10 might be reexpressed during podocyte injury repair process where reestablishment of cell junctions is required. The expression of TM4SF10 was examined in differentiated mouse podocytes using an in vitro model of podocyte injury using PAN (40), an agent that causes foot process effacement in vivo. Compared with untreated cells, PAN treatment resulted in new expression of TM4SF10 with concentrations at cell-cell contacts that colocalized with cadherin (Fig. 6A). A similar induction of TM4SF10 was confirmed by Western blot with anti-GFP antibodies and Tubulin was used as a loading control. I: Western blot of total nephrin abundance and presence of nephrin tyrosine phosphorylation with and without TM4SF10-GFP overexpression and with and without preincubation with pervanadate to block tyrosine phosphatase activity.

**DISCUSSION**

This is the first report of ADAP expression in podocytes. ADAP is now the second protein, along with CD2AP (43), which were originally described in T cells with functional roles in formation of the T cell receptor immunological synapse. In T cells, ADAP is a critical scaffolding center for many phosphorylated interactions in T cell receptor signaling and is known to interact and cooperate with Nck to regulate actin reorganization (20, 30, 48). It is required for T cell receptor-induced integrin activity and is itself phosphorylated and activated by Fyn upon T-cell activation (10, 11). In ADAP null (fyb−/−) mice, inside-out signaling via integrins is disrupted during interactions with antigen presenting cells, indicating that ADAP is required in the immunological synapse for signaling pathways through integrins (33). Although a renal phenotype was not described in the original publication of fyb−/− mice (33), our preliminary observations found adult fyb−/− mice have abnormal glomerular histology (L. A. Bruggeman and J. S. Simske, unpublished observations). Further evaluation of these null mice will help determine possible functional effects of ADAP in podocyte signaling events.
mediated by Fyn and TM4SF10. In addition, ADAP immunoprecipitated both Fyn and TM4SF10 in podocytes, however, we did not observe TM4SF10 and Fyn were both present in an immunoprecipitable complex. One explanation is that ADAP cannot simultaneously bind Fyn and TM4SF10 so that TM4SF10-ADAP and Fyn-ADAP complexes are mutually exclusive. Alternatively, the complex containing ADAP, Fyn, and TM4SF10 may be transient. Therefore, future studies evaluating ADAP as a potential regulatory hub in podocytes will likely be important in establishing the mechanism of TM4SF10 in modifying Fyn activity.

Several lines of experimentation in other model organisms indicate ADAP is associated with regions of dynamic actin activity (5, 54). Our observations in MDCK cells and podocytes also indicated TM4SF10 expression altered Fyn-dependent F-actin distribution and blocked cell protrusions. This corroborates data from a published morphological screen that identified TM4SF10 as a protein that, when overexpressed, potently blocks neurite outgrowth in nerve growth factor-stimulated PC12 cells (19). Part of the well-known transformation effects of Src-family proteins is the induction of lamellipodia/filopodia through the reorganization of actin filaments (1, 34). Our observations that TM4SF10 overexpression prevented cell extensions induced by Fyn, and the prior studies in PC12 cells mentioned above may suggest a general role for TM4SF10 in modifying Fyn activity.

Several lines of experimentation in other model organisms indicate ADAP is associated with regions of dynamic actin activity (5, 54). Our observations in MDCK cells and podocytes also indicated TM4SF10 expression altered Fyn-dependent F-actin distribution and blocked cell protrusions. This corroborates data from a published morphological screen that identified TM4SF10 as a protein that, when overexpressed, potently blocks neurite outgrowth in nerve growth factor-stimulated PC12 cells (19). Part of the well-known transformation effects of Src-family proteins is the induction of lamellipodia/filopodia through the reorganization of actin filaments (1, 34). Our observations that TM4SF10 overexpression prevented cell extensions induced by Fyn, and the prior studies in PC12 cells mentioned above may suggest a general role for TM4SF10 in modifying Fyn activity.

Maturation of nephrin by various posttranslational modifications appear to be critical for its function including transport of nephrin to the plasma membrane, stable nephrin-NEPH1 interactions, and that the fully glycosylated 185-kDa plasma membrane form may be the only form susceptible to Fyn phosphorylation (9, 21, 44, 52). Upon appearance at the plasma membrane, nephrin ectodomain engagement and subsequent Fyn phosphorylation of its cytoplasmic domain results in recruitment of Nck, phosphoinositide 3-kinase, and their associated proteins and may initiate directed actin polymerization and elongation at this site (15, 22, 49, 50, 55). Although it is not fully understood how the nephrin molecule is regulated by these posttranslational modifications, the status of nephrin phosphorylation appears to effect its subcellular localization, stability, and signaling to actin. Our combined observations that TM4SF10 decreased Fyn activity and preserved the high molecular weight but hypophosphorylated forms of nephrin is similar to the observation by Lahdenpera et al. (18) where nephrin mutants lacking the cytoplasmic domain containing the Fyn phosphorylation sites were associated with higher nephrin protein abundance. It had been speculated that this may reflect a possible instability of the nephrin protein when hyperphosphorylated (21). In support of this concept, recent studies have implicated hyperphosphorylation of nephrin with its removal from the plasma membrane by raft-mediated endocytosis (36).
Although not fully understood, these studies are beginning to reveal the mechanism by which nephrin phosphorylation ultimately controls both the formation and remodeling of the slit diaphragm and foot processes during development and in disease.

In conclusion, the mechanism of slit diaphragm assembly is a highly orchestrated series of developmentally coordinated sequential events, which may be a much more dynamic process in podocyte response to injury and disease than previously thought. In *C. elegans*, the TM4SF10 orthologue VAB-9 regulates actin organization at the adherens junction complex, resulting in changes in cell morphology. We speculate that TM4SF10 may have a similar role during the formation of the precursor cadherin-based cell junction early in podocyte development and also may modulate signaling by nephrin to the actin cytoskeleton or integrins during slit diaphragm and foot process formation. Our basic model is that TM4SF10 expression correlates with a phenotype in which extension of cellular processes is not supported; in podocytes this may reflect a dedifferentiated state during glomerular development or with foot process effacement in disease. In addition, the novel identification of ADAP emphasizes the growing molecular similarity of podocyte signaling mechanisms to synaptic structures such as the immunological synapse or the neurological synapse, and theories modeling the slit diaphragm with properties of both cell junctions and synapses may be a productive direction for future investigations.

ACKNOWLEDGMENTS

We thank Dr. John Sedor for critical review of the manuscript. Present address of A. Padiyar: Dept. of Medicine, University Hospitals Case Medical Center, Cleveland, OH 44106.

GRANTS

This work was supported in part by National Institutes of Health Grant DK061395 and MetroHealth Medical Center institutional funds. A. Padiyar was supported by NIH Training Grant DK007470.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


REFERENCES


