Expression of TM4SF10, a Claudin/EMP/PMP22 Family Cell Junction Protein, During Mouse Kidney Development and Podocyte Differentiation

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Cell junctions in the nephron are highly specialized to perform specific and distinct filtration and reabsorption functions. The mature kidney forms complex cell junctions including slit diaphragms that prevent the passage of serum proteins into the filtrate, and tubule cell junctions that regulate specific paracellular ion reuptake. We have investigated the expression of TM4SF10 (Trans-Membrane tetra(4)-Span Family 10) in mouse kidneys. TM4SF10 is the vertebrate orthologue of Caenorhabditis elegans VAB-9, a tetraspan adherens junction protein in the PMP22/EMP/Claudin family of proteins. We found that TM4SF10 localizes at the basal-most region of podocyte precursors before the capillary loop stage, at some tubule precursors, and at the ureteric bud junction with S-shaped bodies. Overall expression of TM4SF10 peaked at postnatal day 4 and was virtually absent in adult kidneys. The very limited expression of TM4SF10 protein that persisted into adulthood was restricted to a few tubule segments but remained localized to the basal region of lateral membranes. In undifferentiated cultured podocytes, TM4SF10 localized to the perinuclear region and translocated to the cell membrane after Cadherin appearance at cell–cell contacts. TM4SF10 colocalized with ZO1 and p120ctn in undifferentiated confluent podocytes and also colocalized with the tips of actin filaments at cell contacts. Upon differentiation of cultured podocytes, TM4SF10 protein disappeared from cell contacts and expression ceased. These results suggest that TM4SF10 functions during differentiation of podocytes and may participate in the maturation of cell junctions from simple adherens junctions to elaborate slit diaphragms. TM4SF10 may define a new class of Claudin-like proteins that function during junctional development. Developmental Dynamics 236:596–605, 2007. © 2006 Wiley-Liss, Inc.

Key words: podocyte; kidney; cell junction; slit diaphragm

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INTRODUCTION

Podocytes are visceral epithelial cells that regulate the glomerular filtration barrier. The filtration barrier is generated in part by a specialized cell junction, the slit diaphragm, which forms between podocyte foot processes. The slit diaphragm is a modified adherens junction that contains proteins of the adherens and tight junction, such as P-Cadherin and ZO-1, as well as specialized junctional proteins such as the Nephrin class of immunoglobulin superfAMILY proteins Nephrin, NEPH1, and the protocad...
herin FAT1 (Kestila et al., 1998; Reiser et al., 2000; Donoviel et al., 2001; Ciani et al., 2003; Kreidberg, 2003). Nephrin, NEPH1, and FAT1 are thought to be central cell adhesion molecules required for the formation of the ladder-like slit diaphragm structure (Kestila et al., 1998; Reiser et al., 2000; Ciani et al., 2003). Nephrin is associated with podocin, an integral membrane Stomatin family protein, and CD2AP, a CD2 associated adapter protein in membrane lipid rafts (Shih et al., 1999; Schwarz et al., 2001). Similar to other junctional complexes, the Nephrin complex is intimately associated with the underlying actin cytoskeleton, and the actin cytoskeleton is essential for cell morphology and slit diaphragm development. The central proteins of the Nephrin complex associate with F-actin at the cell membrane, and depolymerization of actin disrupts complex localization (Saleem et al., 2002; Yuan et al., 2002). CD2AP interacts directly with F-actin and the actin associated molecule α-actinin-4, which binds to MAGI-1, a MAGUK scaffolding protein localized to tight junctions in MDCK cells (Lehtonen et al., 2002; Patrie et al., 2002). Nephrin-family proteins may be linked to the cytoskeleton through C-terminal binding to ZO-1, an actin-associated protein (Itoh et al., 1997; Huber et al., 2003).

Loss of Nephrin, NEPH1, or CD2AP results in nephrotic syndrome (Kestila et al., 1998; Shih et al., 1999; Roselli et al., 2002), and α-actinin-4 mutations result in focal and segmental glomerulosclerosis (FGS), demonstrating that junctional complexes and junctional–cytoskeletal linkages are critical for normal kidney physiology (Kaplan et al., 2000).

The slit diaphragm of the podocyte, like the cell junctions of other types of epithelia, may have several distinct developmental stages, characterized by the sequential assembly of discrete cell junction complexes (Reeves et al., 1978). Cell junctions of undifferentiated confluent cultured podocyte are maintained by classic Cadherins that are linked to the actin cytoskeleton by means of Catenins (Reiser et al., 2000). Nephrin and Nephrin-like NEPH1 are likely required for a late step in the formation of the slit diaphragm, because these proteins are expressed upon podocyte differentiation and are required for the development of the mature filtration barrier, but not for the localization of early cell junction proteins ZO-1 and P-Cadherin (Ruotsalainen et al., 2000). Furthermore, podocyte differentiation is accompanied by reduced Catenin expression, induction of Synaptotagmin expression, and redistribution of P-Cadherin, suggesting that the composition of the “classic” adherens junction may be modified in mature slit diaphragms and that a specialized membrane cytoskeletal linkage is generated (Reiser et al., 2000; Ruotsalainen et al., 2000; Yaoita et al., 2002; Usui et al., 2003).

Despite sharing some similarities in molecular constituency, the slit diaphragm is distinct from both adherens and tight junctions (Reiser et al., 2000; Hunt et al., 2005). In mature podocytes, P-Cadherin and ZO-1 proteins both co-localize at the slit diaphragm (Kurihara et al., 1992; Tassin et al., 1994; Pavenstadt et al., 2003). ZO-1 is normally a tight junction protein, but localizes with Cadherin at adherens junctions either before tight junction formation, or in tissues such as fibroblasts where a typical tight junction is not present (Itoh et al., 1993, 1997; Yonemura et al., 1995). The slit diaphragm is located at a basolateral position of the podocyte, separating the apical domain of the podocyte from the basement membrane that opposes the renal capillary wall. At this location, the slit diaphragm intercellular space is approximately 35–70 nm, which is far wider than the intercellular space of adherens or tight junctions (Karnovsky and Ryan, 1975; Reeves et al., 1978; Wartiovaara et al., 2004). Tight junctions are so named because the intercellular space is essentially absent. This close apposition is maintained by tight junction strands, which are composed primarily of the Claudin family of four-pass integral membrane proteins. Despite the observation that individual Claudin proteins have discrete expression patterns along the proximal distal axis of the nephron, no Claudin or Claudin-like protein to date is known to be expressed in the podocyte under normal circumstances, and freeze fracture preparations of podocytes do not reveal tight junction strands typical of Claudin-based junctions (Humbert et al., 1976; Reiser et al., 2000; Kiuchi-Saishin et al., 2002). Because Claudins generate a tight seal in the intercellular space and regulate ion-specific charge selectivity across the paracellular space of transporting epithelia, a similar role for Claudins at the slit diaphragm is inconsistent with the function and structure of the slit diaphragm (Colecio et al., 2002). Thus, the slit diaphragm is a unique cell junction that may lack the properties of Claudin-based tight junctions.

Here, we present an analysis of the expression of TM4SF10 (Trans-Membrane tetra(4)-Span Family 10) in the developing mouse kidney and in differentiating cultured podocytes. TM4SF10 is a four-pass integral membrane protein belonging to the PMF22/EMP/ Claudin family of proteins. In this family, TM4SF10 has limited homology with the traditional Claudins, with the most significant conservation in the overall protein topology and several well-conserved cysteines (Van Itallie and Anderson, 2006). This distinct structure logically would suggest that TM4SF10 may have novel functions not typical of the traditional Claudins. TM4SF10 is the vertebrate orthologue of VAB-9, a component of the adherens junction in Caenorhabditis elegans (Christophe-Hobertus et al., 2001; Simsk et al., 2003). VAB-9 functions downstream of the C. elegans Cadherin orthologue HMR-1 during epidermal morphogenesis, is required for the organization of contractile actin filaments, and has a redundant role in cell adhesion. We found that TM4SF10 expression and localization at cell junctions was regulated during kidney development and during in vitro differentiation of podocytes. TM4SF10 localized at nascent cell contacts after Cadherin in undifferentiated podocytes, but unlike P-Cadherin, TM4SF10 expression did not persist in the mature slit diaphragm. These studies suggest that TM4SF10 may have a role in podocyte development.

RESULTS

TM4SF10 Is Expressed in Podocytes and Tubule Segments in Developing Kidneys

TM4SF10 (previously known as BCMP1) was originally described as a
strongly expressed gene in the adult canine brain and was also shown to be expressed in the kidney (Christophe-Hobertus et al., 2001). To characterize TM4SF10 expression in the mouse kidney, we first determined the temporal expression pattern by Northern analysis of total cellular RNA from kidneys postnatal days 1 through 30. TM4SF10 expression peaked at day 4, but declined to undetectable levels by day 15 (Fig. 1A,B). This overall expression pattern likely reflected the expression of TM4SF10 in tubular epithelia (see below) due to the large contribution of tubular cells to the total mRNA pool. TM4SF10 also was expressed in (mouse) podocyte cell lines, as determined by reverse transcriptase-polymerase chain reaction (RT-PCR). Amplified RT-PCR products were cloned and sequenced to verify TM4SF10 identity (data not shown).

To determine the cellular localization of TM4SF10 within the kidney, we immunostained mouse kidneys at various stages of development from embryonic day (E) 12.5 to 3 months, using VAB-9 antibodies. We previously found that antibodies raised against the C-terminal cytoplasmic domain of C. elegans VAB-9 could recognize an epitope at cell junctions in Drosophila and MDCK cells (A. Ralston, S. Blair, and J. Simske, unpublished observations), suggesting that the antibody recognized a conserved epitope (Fig. 1C). To determine whether VAB-9 antibodies recognize TM4SF10 protein, we fractionated confluent cultured podocytes and found that both anti-VAB-9 antibodies and anti-TM4SF10 antibodies recognized a band of approximately 20 kDa in membrane fractions, indicating that anti–VAB-9 antibodies recognize TM4SF10 and that it resided in the plasma membrane (Fig. 1D). In addition, transfection of MDCK cells with a TM4SF10 expression plasmid similarly produced bands of the correct molecular weight with both the anti-TM4SF10 and VAB-9 antibodies. Because anti–VAB-9 antibodies performed better than anti-TM4SF10 antibodies with in situ procedures, anti–VAB-9 antibodies were used for the remaining studies. TM4SF10 was detected as early as E12.5 in the stalk and initial branches of the ureteric bud (data not shown). Expression was detected in glomerular precursors in the area of comma- and S-shaped bodies (Fig. 2A,B). There was evidence of TM4SF10 present in both basal membrane cell—cell junctions and the cytoplasm of these precursors. The cytoplasmic distribution likely reflected new protein synthesis and sequestration in the Golgi, which was also observed in undifferentiated cultured podocytes and described in more detail below. In S-shaped bodies (Fig. 2A,C), TM4SF10 expression was present in glomerular precursors and was strong and localized at basal cell—cell contacts in presumptive distal nephron progenitors, which arise from the metanephric mesenchyme. During the period of TM4SF10 expression in glomerular precursors, TM4SF10 colocalized with Cadherin at points of cell—cell contact (Fig. 2D,E). There was also TM4SF10 detected within the cytoplasm, which may reflect newly synthesized protein within the Golgi, as was observed in differentiating cultured podocytes (see below, description of Fig. 3). TM4SF10 was expressed also at the junction between the distal part of the S-shaped body and the branching ureteric bud (Fig. 2A, arrow). In the capillary loop stage of glomerular development (Fig. 2G,H), typically when Synaptopodin and Nephrin expression are first detected (Mundel et al., 1991; Ruotsalainen et al., 2000; Ichimura et al., 2003), TM4SF10 expression in putative podocyte precursors was absent, but strong basolateral expression of TM4SF10 persisted in some tubule segments (Fig. 2H,I). In the adult kidney, although very limited, TM4SF10 was detected in some tubule segments (Fig. 2J), which were AQP1-
Fig. 2. TM4SF10 (Trans-Membrane tetra(4)-Span Family 10) protein distribution in mouse kidneys. 
A: Confocal image of the nephrogenic distal margin of a newborn mouse kidney fluorescently immuno- 
nostained with Cadherin (red) and TM4SF10 (green); arrow indicates location of the putative junction 
between the branching ureteric bud (UB) and developing nephron. B,C: Higher magnification of comma 
stage (B) and S-shaped body stage (C) shown in A; arrowheads indicate basolateral location of 
TM4SF10 expression in a location consistent with glomerular precursors. D–F: Higher magnification of 
inset box in C to show colocalization of TM4SF10 and Cadherin. One point of colocalization at a 
cell–cell contact is indicated with an arrowhead. G: In day 4 kidneys, a differential interference contrast 
(DIC) image of capillary loop stage glomeruli showed no expression of TM4SF10 in glomerular precursors. 
The fluorescent signal detected in these glomeruli were red cells in the vascular space, which were 
autofluorescent. H: Nonfluorescent immunostaining showing no TM4SF10 expression in capillary loop 
stage glomeruli; however, expression remained in some tubule segments. I: Section through the basal 
surface of tubules demonstrating that TM4SF10 localized around the basolateral circumference of 
these cells. J: Adult TM4SF10 expression persisted only in some tubule segments and localization 
remained basal. K,L: In tubules, TM4SF10 (green, K) localized along the basolateral membrane, but 
ZO-1 (red, L) localized at basal and apical (luminal) positions. M: Merged image of K and L. N: Merged 
image superimposed on the DIC image. No TM4SF10 was detected on the apical (luminal) surface. 
Apical ZO-1 is indicated with an arrow, TM4SF10 is indicated with an arrowhead. Lumen is indicated 
with an asterisk. Scale bar = 20 μm.

Fig. 3. TM4SF10 (Trans-Membrane tetra(4)-Span Family 10) expression in differentiating cul- 
tured podocytes. A,B: Localization of TM4SF10 in undifferentiated cells (A) and undifferentiated 
cells with older cell–cell contacts (B). Perinu- 
clear localization (A, arrow) diminishes in favor 
of localization at cell–cell contacts (B, arrow- 
head). C,D: TM4SF10–green fluorescent pro- 
tein (GFP) expression in undifferentiated podo- 
cytes. C: In undifferentiated noncontacting podocytes, TM4SF10-GFP had a perinuclear 
localization (arrow). D: In contacting podocytes, 
TM4SF10-GFP also localized to cell contacts 
(arrowhead). E,F: Localization of TM4SF10 in 
contacting cells during differentiation at 37°C. 
Podocytes were induced to differentiate for 4 
days and stained for the differentiation marker 
Synaptopodin and for TM4SF10 (E), with the 
merged image shown in F (with 4,6-diamidine- 
2-phenylidole-dihydrochloride [DAPI]). The 
differentiation state of podocytes was determined 
by abundant and well-organized Synaptopodin 
expression. Nuclei are numbered to indicate 
differentiated cells (#5 and #6) and undifferen- 
tiated cells (#1–#4). The arrows indicate a typical 
cell contact between differentiated cells (#5 and 
#6), and a differentiated cell (#5) and a undiffer- 
entiated cell (#4), and the arrowheads indicate a 
typical cell contact between undifferentiated 
cells.
AQP2-negative (data not shown), suggesting they were neither proximal tubule nor collecting tubule (the distal nephron segment derived from the ureteric bud). Due to their location in maturing kidneys, and the prior detection of TM4SF10 staining in the region of distal nephron precursors in the S-shaped body stage, these may represent distal nephron segments derived from the metanephric mesenchyme: the distal convoluted tubule or thick ascending limb. In all stages examined, TM4SF10 was found to localize along the basolateral surface of the tubular epithelial precursors, with basolateral TM4SF10 localizing around the entire periphery of these epithelial cells (Fig. 2H–N). In contrast to TM4SF10, the tight junction protein ZO-1 localized at the most apical (luminal) region of the lateral cell membrane in tubular epithelia (Fig. 2K–N).

**TM4SF10 Localization and Expression Is Dynamic in Differentiating Cultured Podocytes**

Our observed transient expression of TM4SF10 in mouse podocytes suggests that TM4SF10 may function during podocyte development. To test this hypothesis, we examined TM4SF10 expression during differentiation of conditionally immortalized cultured podocytes (Mundel et al., 1997). The conditionally immortalized podocytes are a useful system in which to analyze the development of podocyte cell junctions, because these cells can be maintained in a mitotic, undifferentiated state at 33°C and can be induced to differentiate by culturing for approximately 7–10 days at 37°C. In addition, many of the cell junction proteins are expressed and localize normally at cell–cell contacts (Reiser et al., 2000; Saleem et al., 2002). At 33°C, cultured podocytes proliferate to confluence and establish cellular junctions. After switching to 37°C, proliferation rates decrease significantly, and the podocytes differentiate to a more mature phenotype as determined by the appearance of a well-organized cytoskeleton containing Synaptopodin and extension of numerous lamellipodia (Mundel et al., 1997). It is important to note that the differentiation process takes several days, and that not all podocytes progress through the differentiation process at the same rate. Therefore, in partially differentiated cultures (i.e., growth at nonpermissive conditions for 3–4 days) some cells may differentiate more quickly than others, and the extent of podocyte differentiation is determined by the expression level and organization of Synaptopodin.

We found that, in undifferentiated, noncontacting podocytes at 33°C, TM4SF10 localizes to perinuclear regions that most likely correspond to the endoplasmic reticulum or Golgi (Fig. 3A). As undifferentiated podocytes form cell–cell contacts, perinuclear TM4SF10 localization diminished and localization was observed at points of cell–cell contact (Fig. 3B). Next, we introduced green fluorescent protein (GFP)-tagged TM4SF10 into podocytes and found that TM4SF10-GFP displayed similar localization patterns as the native protein (Fig. 3C,D). Upon initiation of differentiation of the culture (4 days nonpermissive conditions), podocyte TM4SF10 expression was reduced or absent in those cells that expressed high levels of Synaptopodin (Fig. 3E,F). Specifically, TM4SF10 expression was absent at cell contacts between cells strongly expressing Synaptopodin (Fig. 3E,F, cells #5 and #6). In contrast, TM4SF10 expression remained strong in undifferentiated cells expressing little or no Synaptopodin (Fig. 3E,F, cells #1–4, arrowheads). In Figure 3E,F, cell #5 (a differentiated podocyte with strong Synaptopodin expression) was in contact with both a differentiated cell (cell #6) and an undifferentiated cell (cell #4). Of interest, the presence of TM4SF10 in the distinct cell–cell contacts with this individual cell was different. The cell contacts with the undifferentiated cell contained TM4SF10, but not in the cell contacts with the differentiated cell. The presence of TM4SF10 in contact with the undifferentiated cell may reflect TM4SF10 expression only in the undifferentiated cell. These observations are consistent with the notion that TM4SF10 is a transient participant in cell junction formation during podocyte differentiation, which is no longer required to preserve a cell contact once a Synaptopodin-rich cytoskeleton has been established.

The dynamic expression pattern of TM4SF10 in podocytes suggested that TM4SF10 may have a role in the maturation of podocyte cell junctions. To test this hypothesis, we compared the localization of TM4SF10 with other podocyte cell junction proteins during in vitro podocyte differentiation. First, we examined expression in newly plated, undifferentiated podocytes. At this stage, TM4SF10 localized to a perinuclear cytoplasmic compartment (Fig. 4A), while Cadherin was just beginning to localize at cell junctions (Fig. 4B,C). As undifferentiated podocytes became confluent, TM4SF10 and Cadherin colocalized at cell junctions (Fig. 4D–F). Four days after the switch to differentiation conditions, TM4SF10, Cadherin, p120ctn, and ZO-1 all localized at cell junctions (Fig. 4G–N). Since C. elegans VAB-9 is known to affect actin organization, we also examined the relationship between F-actin and TM4SF10. In Figure 4O–Q, TM4SF10 localized along actin filaments only at cell–cell junctions. Drawing parallels from our more extensive studies on VAB9 function in C. elegans (Simmske et al., 2003), this association of TM4SF10 with actin filaments may suggest that TM4SF10 similarly may be involved in actin organization in podocytes.

**DISCUSSION**

We have provided an initial description of TM4SF10 expression in the mouse kidney. TM4SF10 is a four-pass integral membrane protein that localized to podocyte and tubule cell–cell contacts during kidney development and at nascent podocyte cell contacts in culture. TM4SF10 expression was lost upon podocyte differentiation. Despite the similarity of TM4SF10 to the Claudin family proteins, our results suggest that TM4SF10 does not function like a prototypical Claudin. TM4SF10 was almost exclusively expressed during development and was localized along the basolateral membrane at cell–cell contacts. In contrast, most Claudins and other tight junction proteins localize at the most apical or luminal region of the lateral membrane, where the tight junction and paracellular barrier is formed. Claudins have been identified in every tissue type in the developing kidney and different Claudins have discrete ex-
expression domains in kidney epithelia that correspond to distinct functions in filtration and paracellular reabsorption (Kiuchi-Saishin et al., 2002). Claudins form the protein strands of the tight junction, and homotypic or heterotypic interactions between Claudin molecules on opposing cells essentially eliminates the intercellular space. Thus, Claudins would not be expected to localize at the mature slit diaphragm, because this cell junction structure spans a region of 70 nm (Reeves et al., 1978). Correspondingly, the intramembrane fibril strands typical of Claudin-based tight junctions are not found in freeze-fracture preparations of normal podocytes (Pricam et al., 1975; Kerjaschki, 1978). Furthermore, expression of Claudins in podocytes may only occur during tight junction formation after injury (Kurihara et al., 1992; Reiser et al., 2000). We cannot rule out the possibility that TM4SF10 forms tight junction-like strands at cell junctions in undifferentiated podocytes; however, unlike Claudins, we have observed that TM4SF10 expressed in mouse L-fibroblasts did not form strands (J.S. Simske, unpublished observations). In C. elegans, other Claudin-like proteins exist and are more likely to function in a manner similar to vertebrate Claudins than the TM4SF10 orthologue VAB-9, suggesting that both VAB-9 and TM4SF10 are cell junction proteins with novel functions (Asano et al., 2003; Simske et al., 2003).

Assembly of the slit diaphragm is likely to proceed from the formation of a rudimentary Cadherin-based adherens junction providing the scaffold for the formation of the slit diaphragm, to the development of mature, highly specialized slit diaphragms demarcated by Nephrin family protein localization. Adherens junction components are expressed in comma- and S-shaped body stage podocyte precursors, when these cells are columnar epithelia, but appear to be absent in the mature slit diaphragm. For example, p120 and β-Catenin have been reported to be absent in the mature slit diaphragm (Yaöita et al., 2002; Usui et al., 2003), and most Cadherins are not detected in the slit diaphragm as well. There is some controversy regarding this conclusion because there are counter-examples of Catenins and P-Cadherin expression in podocytes and localization at the slit diaphragm (e.g., Piepenhagen and Nelson, 1998; Reiser et al., 2000). In either case, our results indicated that TM4SF10 colocalized with all tested cell junction proteins (p120ctn, Cadherin, ZO-1) in cultured, undifferentiated podocytes, supporting the assertion that TM4SF10 is a component of cell junctions in developing podocytes in vivo. Given the transient expression of TM4SF10 in podocyte precursors in

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**Fig. 4.** TM4SF10 (Trans-Membrane tetra(4)-Span Family 10) localization at cell junctions relative to Cadherin and ZO-1. Differentiation time course of TM4SF10 localization at junctions with respect to other known junctional components. A,B: Newly plated, undifferentiated podocytes have TM4SF10 (A, green) localized at a perinuclear region and not in the plasma membrane when Cadherin (B, red) localized at nascent cell contacts. C: In the merged image, nuclei are identified with 4',6-diamidino-2-phenylindole-dihydrochloride (DAPI) staining (blue). D,E: As undifferentiated cells become confluent and cell contacts mature, TM4SF10 (D, green) appeared at the plasma membrane along with Cadherin (E, red). F: The merged image of D and E. G–I: TM4SF10 (G, green) colocalized with Cadherin (H, red), and ZO-1 (I, blue) colocalized at cell junctions after TM4SF10 translocation. J: The merged image with colocalization of all three epitopes is indicated in white, and ZO-1 and Cadherin colocalization only indicated in purple. Note that circumferential ZO-1 and Cadherin cell junction localization was more extensive than TM4SF10 at this stage. K–M: Similarly, adherens junction protein p120ctn (M, blue) colocalized with TM4SF10 (K, green) and Cadherin (L, red) at cell contacts. N: Merged image of K–M. O–Q: TM4SF10 (O, green) localized along actin filaments (P, red) only at cell contacts, seen as yellow in the merged image (Q). Scale bar = 20 μm.
vivo and the lack of exclusive markers for these progenitor cells, it was difficult to unequivocally demonstrate TM4SF10 localization at podocyte precursor cell junctions. During podocyte differentiation in vivo, these "primitive" occluding cell junction structures are highly transient as they move from an apical to basal localization along the lateral cell membrane (Reeves et al., 1978). Immunostaining indicated that TM4SF10 localized to cytoplasmic as well as membrane locations in the podocyte precursors of S-shaped bodies, mirroring the changing subcellular distribution observed in cultured podocytes. Immunolocalization of TM4SF10 in comma- and S-shaped kidney sections using transmission electron microscopy will ultimately determine precise localization of TM4SF10 with respect to the occluding junctions of immature podocytes.

Because Cadherin localization preceded TM4SF10 at cell junctions, this finding suggests that TM4SF10 is recruited to the cell junction either directly by one of the cadherins expressed at this stage (N-, P-, R-Cadherin, or Cadherin-6), or is targeted to the plasma membrane after Cadherin engagement (Dahl et al., 2002). Consistent with this model is the observation that C. elegans Cadherin HMR-1 is required for VAB-9 localization (Simsko et al., 2003). The pseudocadherin FAT1 is another candidate Cadherin that may serve to localize TM4SF10, although it is not known whether FAT1 is expressed early enough to mediate this function. Furthermore, we detected Cadherin using a pan-Cadherin antibody that does not recognize FAT1, indicating that at least one classic Cadherin localizes at cell junctions before TM4SF10. In C. elegans, VAB-9 is required for actin organization downstream of HMR-1 (Simsko et al., 2003). Consistent with a role for TM4SF10 in F-actin organization in podocytes, we also observed that TM4SF10 at cell contacts colocalizes with F-actin oriented orthogonal to the plane of cell contact. This organization of F-actin is similar to that observed in filopodia that initiate adhesion zippers in nascent (immature) cell contacts (Vasiukhin et al., 2000), suggesting that TM4SF10 may participate in organizing the actin cytoskeleton in undifferentiated podocytes.

We also observed that TM4SF10 expression disappeared temporally as slit diaphragms develop and replace occluding junctions. Slit diaphragm proteins are likely not required for early podocyte development because slit diaphragm proteins such as Nephren and NEPH1 are not expressed in podocyte precursors, are not required for the localization of adherens junction proteins, and are not required for podocyte differentiation and process extension. (Kestila et al., 1998; Ponnasi et al., 1999; Cox et al., 2000; Reiser et al., 2000; Ruotsalainen et al., 2000; Ciani et al., 2003). Thus, these mature slit diaphragm proteins assemble at podocyte cell junctions downstream of TM4SF10 recruitment.

The dramatic change in TM4SF10 expression and localization during the differentiation of cultured podocytes suggests that regulation of TM4SF10 expression may be essential for podocyte development and differentiation. Based on work here and our previous studies on VAB-9 function in C. elegans, we speculate that TM4SF10 may function to promote differentiation by driving cell junction assembly and establish polarity. Further experiments will be required to differentiate between these and other working models for TM4SF10 function in cell junction development. One general hypothesis is that VAB-9 and TM4SF10 function during junctional assembly and participate in the transition from nascent junctions in which junctional components are closely associated to mature junctions in which junctional components are functionally separated. Such a role may be particularly relevant during development, when components of different junctions are closely associated spatially and temporally, such as in the thin epithelial cells in C. elegans, in newly contacting epithelial cells, and during slit diaphragm morphogenesis (Suzuki et al., 2002).

In contrast to podocytes, expression of TM4SF10 in developing renal tubule epithelia was only monitored in situ. TM4SF10 appeared to be expressed in a restricted set of tubule segments and localized specifically along the basal-most region of the lateral membrane. What is the role of basal TM4SF10? One possibility is that, in these tubules, TM4SF10 is required for unique adhesive or permeability functions, either through cell–cell or cell substrate interactions. There is precedence for Claudin family proteins localizing along the basolateral membrane, in particular Claudin-7 along descending limbs of the distal nephron (Wing et al., 2004).

Further experimentation will be required to determine whether TM4SF10 has a similar function in podocyte and in tubular epithelia.

In summary, our results suggest that TM4SF10 is a novel integral membrane cell junction protein that may function specifically during the development of podocyte and tubule cell junctions. These functions may be unique and atypical of the role of traditional Claudins in cell junction assembly and homeostasis. Determining TM4SF10's function and testing of these models awaits the characterization of TM4SF10 mouse knockout phenotypes. In addition, a role for TM4SF10 during repair or regeneration after renal injury or disease would be a prediction of our current working model and is currently under investigation.

**EXPERIMENTAL PROCEDURES**

**Antibodies, Western Blotting, and Cell Lines**

We have adopted the in vitro system of podocyte differentiation originally described by Mundel (Mundel et al., 1997). The murine podocyte cell line used for these studies has been published (Schwarz et al., 2001) and was propagated under both permissive and nonpermissive conditions as previously described (Mundel et al., 1997). The in vitro system used here to model stages of podocyte differentiation consisted of comparisons of (1) undifferentiated podocytes that were newly plated and not confluent (growth at permissive conditions); (2) undifferentiated podocytes that were confluent and had established cell contacts (growth at permissive conditions); (3) podocytes undergoing differentiation (growth at nonpermissive conditions for 3–4 days); and (4) fully differentiated podocytes (growth at
nonpermissive conditions for 10–14 days). The degree of differentiation was determined by the expression level and organization of Synaptodom by immunostaining, which is low or absent in undifferentiated podocytes but very intense and well organized in fully differentiated cells.

Immunoreagents, antibodies, and antibody dilutions used for immunohistochemistry include the following: VAB-9, 1:400 (Simske et al., 2003); TM4SF10 C-terminal antibodies (Biosource, Invitrogen); 1:1,000, pan-Cadherin (1:100 Sigma); ZO1 (1:100 Chemicon); p120ctn (Upstate); Synaptodomin, undiluted (Missouri Biotechnologies, Inc., Portland, MA). Secondary antibodies (Jackson ImmunoResearch) include goat anti-rabbit Cy3, goat anti-mouse fluorescein isothiocyanate, and goat anti-rat Cy5 (1:200). Actin localization was with rhodamine-conjugated Phallolidin (Molecular Probes).

Coverslips and sections were mounted with 4',6-diamidino-2-phenylindole-dihydrochloride (DAPI) -containing mounting medium to visualize nuclei.

Western blots were performed as previously described (Simske et al., 2003), with primary antibody for 12 hr at 4°C and alkaline phosphatase–conjugated secondary antibodies (Jackson Immunoresearch, 1:10,000), and development with ECL plus (Amersham), according to manufacturer's instructions. Podocytes were plated at confluence and allowed to form cell contacts for 4 days at 33°C. Standard cell fractionation methods were used to isolate nuclei, membrane, and cytoplasmic fractions.

RNA Analysis

Mouse kidneys were harvested from fetuses of timed pregnant females, neonatal, and adult mice. All animal studies were conducted in accordance with animal care and use requirements of Case Western Reserve University. For Northern analysis, kidneys were homogenized in TRIzol (Invitrogen, Carlsbad, CA) and RNA extracted according to the manufacturer's instructions. For blotting, 10 μg of total cellular RNA was resolved on 1% agarose formaldehyde gels and transferred to nylon membranes. Membranes were hybridized with a radioactively labeled full-length cDNA of TM4SF10 (MNCb-0941, gift from K. Hashimoto, Japanese Collection of Research Bioresources; Suzuki et al., 2000) and washed under maximum stringency at 50°C in 0.2× standard saline citrate/0.1% sodium dodecyl sulfate. For RT-PCR, RNA from podocyte cell lines was extracted using TRIzol, and 1 μg of total cellular RNA was used for synthesis of cDNA. PCR primers for TM4SF10 were selected from the 3′-untranslated region of the murine gene (GenBank accession no. 7670345; forward: 5′-CAGTGTGGAGAACCTCAAG, and reverse: 5′-TTTACATGTTGCTTATGC) generating a 280-bp fragment. These RT-PCR products were cloned into the vector pGEM-T Easy (Promega, Madison, WI) and were sequenced to confirm TM4SF10 amplification.

Immunohistochemistry

For immunohistochemistry, mouse kidneys were fixed in 4% paraformaldehyde (PFA) in phosphate buffered saline for 1 (fetal) to 4 (adult) hr, dehydrated, and blocked in paraffin using standard protocols. Sections (5 μm) were cut onto poly-L-lysine slides, deparaffinized with xylene, and rehydrated using standard methods. Detection of TM4SF10 was performed using one of two techniques, either an avidin–biotin based system using horseradish peroxidase–driven chromagen production (Vectastain ABC Elite Kit, Vector Laboratories) or a fluorescent-conjugated secondary antibody followed by epifluorescence or confocal microscopy. For immunocytochemistry, podocytes were grown on collagen-coated glass coverslips and fixed in 2% PFA, 4% sucrose in phosphate buffered saline for 7 min, permeabilized with 0.1% Triton X-100 for 3 min, blocked with 1% bovine serum albumin, 1% fetal bovine serum, 0.1% fish gelatin for 30 min, and incubated 1 hr at room temperature with primary antibodies. After washing, a fluorescent-conjugated secondary antibody was applied for 1 hr, followed by epifluorescence or confocal microscopy to visualize antibody localization.

Construction of GFP-Tagged TM4SF10-GFP and In Vitro Podocyte Infections

TM4SF10 was cloned into the pEGFP-N2 vector (Invitrogen) placing a GFP tag on the C-terminus of TM4SF10. The resulting TM4SF10-GFP fragment was subcloned into pH1-CMV-MCS-ires2-Δ3-B, the viral backbone for a packaging system to generate recombinant vesicular stomatitis virus (VSV) -psuedotyped virus (Naldini et al., 1996). In this construction, CMV drives the expression of TM4SF10-GFP, and because this is a lentivirus-based system, infection with virus results in stable transformants. Virus was generated as described (Naldini et al., 1996) by liposome transfection (lipofectamine 2000, Invitrogen) of the pRH-CMV-MCS-ires2-Δ3-B plasmid, with two additional plasmids containing Gag, Pol, and the VSV G protein into HEK293T. Conditioned medium from this transfection contains high titer virus that was subsequently used to infect podocytes growing at 33°C. One week after infection, cells were fixed and analyzed for GFP expression by epifluorescence.

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REFERENCES


