Podocyte endocytosis in the regulation of the glomerular filtration barrier

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Submitted 31 March 2015; accepted in final form 15 June 2015

Inoue K, Ishibe S. Podocyte endocytosis in the regulation of the glomerular filtration barrier. Am J Physiol Renal Physiol 309: F398–F405, 2015. First published June 17, 2015; doi:10.1152/ajprenal.00136.2015.—Severe defects in the glomerular filtration barrier result in nephrotic syndrome, which is characterized by massive proteinuria. The podocyte, a specialized epithelial cell with interdigitating foot processes separated by a slit diaphragm, plays a vital role in regulating the passage of proteins from the capillary lumen to Bowman’s space. Recent findings suggest a critical role for endocytosis in podocyte biology as highlighted by genetic mouse models of disease and human genetic mutations that result in the loss of the integrity of the glomerular filtration barrier. In vitro podocyte studies have also unraveled a plethora of constituents that are differentially internalized to maintain homeostasis. These observations provide a framework and impetus for understanding the precise regulation of podocyte endocytic machinery in both health and disease.

glomerular disease; podocyte; endocytosis; phosphoinositides

THE KIDNEY IS COMPOSED OF many different segments that work in unison to filter plasma, generating urine essentially devoid of large-molecular weight proteins and regulating electrolyte balance through tubular secretion and reabsorption. This filtration process begins at the glomerulus, which is composed of podocytes, the glomerular basement membrane, and fenestrated endothelial cells, which collectively compose the plasma-nephron interface. Amino acids, glucose, and electrolytes, which filter through the glomerulus, can be reabsorbed in the proximal tubule by sodium dependent cotransporters. Proximal tubular endocytic machinery located at the brush-border membrane and mediated by receptors gp330/megalin and cubilin can also retrieve albumin and low-molecular weight proteins (10, 18, 19, 111, 116). However, breaches to any part of the filtration barrier can result in nephrotic syndrome as massive proteinuria overwhelms the capacity of the proximal tubule to reabsorb filtered protein. Loss of either megalin or cubulin in a mouse disease model results in increased excretion of albumin and low-molecular weight proteins, suggesting that an intact proximal tubular endocytic process is required to maintain homeostasis (2, 58). These two receptors are also believed to be important in human diseases, as patients with megalin (LRP2; Donnai-Barrow and facio-oculo-acoustico-renal syndromes) and cubilin (CUBN) gene mutations have increased low-molecular weight proteinuria (26, 49, 76).

The hypothesis that podocytes internalize and remove proteins was proposed over 50 years ago. Multiple electron microscopy studies have shown vesicles in podocytes which become more pronounced under pathological conditions (29). This fundamental process appears to be conserved in podocytes. However, unlike the proximal tubules, the physiological relevance of the endocytic uptake mechanisms in podocytes has until recently remained elusive. In this review, we will focus on the current understandings of endocytosis in podocyte biology and explain their significance.

Endocytosis

Endocytosis serves as a portal for internalizing and retrieving plasma membrane components and transmembrane receptors by forming vesicles (93, 98). Endocytosis can be divided into clathrin-dependent and -independent pathways. Interestingly, proteins can often exploit either pathway to allow for internalization. Clathrin-mediated endocytosis is characterized by a membrane invagination, which is lined with a triskelion lattice coat. Clathrin-mediated endocytosis occurs in phosphatidylinositol-4,5-bisphosphate [PI(4,5)P2]-enriched regions of the plasma membrane, and these regions recruit clathrin adaptor proteins such as adaptor protein 2 (AP2). The pit further develops through the recruitment of Bin–Amphiphysin–Rvs (BAR)-containing proteins such as endophilin (34, 61). BAR domain-containing proteins dimerize, allowing for their positively charged amino acids to interact with the negatively charged lipid membrane. These dimers impose their innate crescent-shaped curvature on the membrane, thus functioning as membrane-curving or -sensing proteins (33, 34, 64, 70, 79).

Endocytic Machinery in Podocytes

Endophilin also contains a SH3 domain that binds dynamin, which subsequently oligimerizes to constrict the neck, resulting in fission through GTP hydrolysis (60). Following dynamin-induced fission, clathrin uncoating and shedding is initiated by inositol polyphosphate-5-phosphatases such as synaptojanin 1 (22) and then finalized by the arrival of auxillin/GAK and hsc70 (39, 65). Internalization signals appear to exist for proteins that undergo clathrin-mediated endocytosis. Although there appear to be various endocytic signals, two well-described processes are proteins that contain either a dileucine- or
tyrosine-based linear motif that are recognized by AP2, thus coupling the cargo to clathrin (110).

Distinct clathrin-independent endocytosis also plays a critical role in cell trafficking, which can be divided by ultrastructural size by electron microscopy. Large-sized (micrometer) pathways include macropinocytosis and phagocytosis. For example, macropinocytosis is a method of internalization where substances in the extracellular fluid are engulfed and then compartmentalized in vesicles. Small-scale processes (<200 nm) include caveolae, Arf6, flotillin-1, and clathrin-independent carrier (CLIC)/GPI-AP-enriched early endosomal compartment (GEEC)-mediated pathways. Moreover, it has been shown that proteins such as dynamin and endophilin, which are critical for clathrin-mediated endocytosis, also are utilized in clathrin-independent pathways (11, 86). One of the most well-studied clathrin-independent pathway is the caveolar system. Caveolae are cholesterol-abundant invaginations that participate in the uptake of sphingolipids or proteins found in lipid rafts. The major constituent of caveolae is caveolin 1 (Cav1), which oligomerizes to form caveolae (71) before dynamin-dependent fission, which completes its internalization (62). In the GEEC pathway, GPI-anchored protein is internalized in tubular elements. This pathway does not rely on dynamin activity and appears to be responsible for fluid phase uptake (90). Another dynamin-independent pathway is flotillin-1, an integral membrane protein that couples with lipid rafts to select lipid cargo (37). Last, Arf6 is localized at the interface of the plasma membrane and endosomal structure. The cargo proteins of the Arf6-dependent pathway, such as the IL-2 receptor α-subunit, E-cadherin, and β1-integrins, have been identified (14, 77, 85).

Phosphoinositides in Podocytes

Membrane and protein trafficking are both highly coordinated biological processes required for cellular homeostasis. Utilization of endocytic pathways results in the internalization of molecules from the plasma membrane that can be recycled back to the cell surface or degraded in lysosomal compartments. Inositol phospholipids, concentrated on the cytosolic leaflet of membranes, play a central role in orchestrating the fidelity of these systems (24). Reversible phosphorylation of these phospholipids in the cell. PIs mediate the recruitment of dynamin (96). Dynamin has also been shown to interact the interface of various actin-regulatory proteins (105). Recent evidence suggests that endocytic protein networks sit at the interface of various actin-regulatory proteins (105). For example, dynamin, a GTPase that mediates the fission reaction during clathrin-mediated endocytosis, has been shown to interact with actin-nucleating protein Arp2/3 (57) and cortactin (66, 69). In vitro, it has been demonstrated that actin nucleation is either stimulated or inhibited depending on the concentration of dynamin (96). Dynamin has also been shown to interact directly with F-actin and has been implicated in the organization of the podocyte actin cytoskeleton (40). It has been postulated that actin may play a critical role in inducing local tension in the membrane, which is necessary for fission to occur. In vivo, mice injected with either a dominant negative dynamin 1 K44A plasmid or dynamin undergoing proteolytic cleavage by cathepsins demonstrate proteinuria (99). Furthermore, podocyte-specific Dnnl and Dnn2 conditional knockout (KO; Pod-Dnnl-DKO) mice exhibit severe proteinuria and foot process effacement (102). Live cell imaging of wild-type podocytes display actin transiently visiting the neck of clathrin-coated pits before the fission reaction (Fig. 1, A and D). The wild-type podocytes demonstrate an Ω-shaped clathrin-coated pit (Fig. 1B). However, podocytes lacking dynamin manifest with extreme accumulation of Arp2/3 and F-actin at clathrin-coated pits (Fig. 1E) (102), resulting in a tubulated morphology similar to that observed in Dnnl DKO fibroblasts (31) (Fig. 1C). These observations suggest that dynamin in wild-type podocytes serves as a brake, which prevents continued actin

which recruits synaptojanin 1, displays a similar phenotype (102). Despite the presence of several other PI(4,5)P2 phosphates in the mammalian genome, it is unclear why defects in Synj1 have such a profound effect on the glomerular filtration barrier. For example, mutations in the inositol 5-phosphate OCR1 cause Lowe syndrome, an X-linked disorder characterized by mental retardation, congenital cataracts, and proximal tubulopathy (5, 101), while mutations of inositol 5-phosphate INPP5E results in Joubert syndrome, where patients develop cystic kidneys (9). However, both diseases fail to affect the glomerulus. Further evidence suggests the importance of PI metabolism in podocyte function. Loss of podocyte class III phosphatidylinositol (PI) 3-kinase [mammalian homolog of yeast vacuolar protein sorting defective 34 (mVps34)], which phosphorylates phosphatidylinositol to generate PI(3)P, results in severe glomerulosclerosis (16). Loss of mVps34 also demonstrates trafficking defects for intracellular vesicles. In particular, a block between early endosomal Rab5 and late endosomal Rab7 compartments is observed after podocyte mVps34 deletion (7). Furthermore, genetic ablation of class II PI 3-kinase C2 α (Pi3kc2α), which contains a clathrin binding domain and generates PI(3)P as well as PI(3,4)P2, results in severe proteinuria and kidney failure (41). These findings suggest that regulation of phosphoinositides is pivotal in maintaining a healthy glomerular filtration barrier.

Relationship of Podocyte Clathrin-Mediated Endocytosis With the Actin Cytoskeleton

Human genetics research informs our understanding of the actin cytoskeleton’s fundamental role in podocyte biology. Mutations causal for focal segmental glomerulosclerosis have been identified in actin-interacting proteins such as α-actinin-4 (ACTN4), CD2AP, INF2, and MYO1E (13, 50, 52, 68, 95). Recent evidence suggests that endocytic protein networks sit at the interface of various actin-regulatory proteins (105). For example, dynamin, a GTPase that mediates the fission reaction during clathrin-mediated endocytosis, has been shown to interact with actin-nucleating protein Arp2/3 (57) and cortactin (66, 69). In vitro, it has been demonstrated that actin nucleation is either stimulated or inhibited depending on the concentration of dynamin (96). Dynamin has also been shown to interact directly with F-actin and has been implicated in the organization of the podocyte actin cytoskeleton (40). It has been postulated that actin may play a critical role in inducing local tension in the membrane, which is necessary for fission to occur. In vivo, mice injected with either a dominant negative dynamin 1 K44A plasmid or dynamin undergoing proteolytic cleavage by cathepsins demonstrate proteinuria (99). Furthermore, podocyte-specific Dnnl and Dnn2 conditional knockout (KO; Pod-Dnnl-DKO) mice exhibit severe proteinuria and foot process effacement (102). Live cell imaging of wild-type podocytes display actin transiently visiting the neck of clathrin-coated pits before the fission reaction (Fig. 1, A and D). The wild-type podocytes demonstrate an Ω-shaped clathrin-coated pit (Fig. 1B). However, podocytes lacking dynamin manifest with extreme accumulation of Arp2/3 and F-actin at clathrin-coated pits (Fig. 1E) (102), resulting in a tubulated morphology similar to that observed in Dnnl DKO fibroblasts (31) (Fig. 1C). These observations suggest that dynamin in wild-type podocytes serves as a brake, which prevents continued actin
nucleation at the clathrin-coated pit. PI(4,5)P₂ has been shown to facilitate actin nucleation. 5′-Phosphatases, such as synaptojanin 1, can reduce actin assembly due to hydrolysis of PI(4,5)P₂ bound to actin-regulatory proteins (94). Examination of Synj1 KO podocytes revealed increased ectopic Arp2/3 accumulation, suggesting aberrant actin nucleation. It would be of great interest to determine whether Synj1 KO podocytes have actin comets within the cytoplasm due to inability of clathrin uncoating, similar to what has been observed in fibroblasts isolated from Lowe syndrome patients that lack functional OCRL (65).

Proteins previously proven central to the integrity of foot processes, such as Myo1e, CD2AP, and Nck, are also dynamin and synaptojanin 1 interactors (12, 30, 56). Myo1e, an actin-based motor protein, participates in endocytosis through recruitment to the late stages of clathrin-coated pits along with dynamin. Myo1e appears to move in a vectoral manner, driving the clathrin-coated pit inward (17). Loss of Myo1e in cells also reduces clathrin-mediated transferrin endocytosis, reflecting its integral role in the endocytic machinery. Myo1e also colocalizes with F-actin at endocytic pits, suggesting its importance in coalescing actin assembly at sites of endocytosis (102, 105). CD2AP is a close homolog of CIN85, a class of proteins thought to have overlapping cellular functions but partially different tissue distribution. This explains the occurrence of nephrotic syndrome in CD2AP mutant mice and humans, but not in CIN85 mutant mice (100). Some functions of CD2AP/CIN85 are mediated, at least in part, by its direct and indirect interactions with dynamin, synaptojanin 1, and endophilin (12, 80, 103). CD2AP has been shown to visit clathrin-coated pits and late endosomes (114) while also colocalizing with cortactin (117). Nck adaptor protein interacts with the proline rich domain of dynamin and synaptojanin 1 through its SH3 domain (114, 115). Nck adaptor protein interacts with the proline rich domain of dynamin and synaptojanin 1 through its SH3 domain (114, 115). Nck also binds to actin-polymerizing protein N-WASP to induce actin tails (43). Given that the loss of key clathrin-mediated regulatory proteins or their interactors results in aberrant actin dynamics, a coordinated role between regulation of actin and endocytosis likely exists in podocytes.

Fig. 1. Link between actin and endocytosis in podocytes. A: spinning disc confocal images of selected frames 4 s apart from time series of a clathrin-coated pit (GFP-CLC) with merged images depicting late actin (mCherry-Utrophin) arrival before membrane fission and the disappearance of the clathrin-coated pit. Cartoon shows the temporal and spatial relationship of actin (red) and the clathrin-coated pit (green; data from experiments conducted in Ref. 102). B: electron microscope image of wild-type podocyte reveals an Π-shaped clathrin-coated pit (arrowheads). C: electron microscope image of Pod-Dnm-DKO podocyte (data from experiments conducted in Ref. 102) reveals a tubulated clathrin-coated pit (arrowheads). D: wild-type podocytes demonstrate actin (red) and clathrin (green) merging (arrowheads). E: actin (red arrowheads) accumulates near sites of clathrin (green arrowheads) in Pod-Dnm-DKO podocytes.
Furthermore, targeting the endocytic–actin interface with small molecule Bis-T 23 to promote dynamin oligimerization and actin stabilization may have potential therapeutic implications (97).

**Endocytic Process in Slit Diaphragm Regulation**

The slit diaphragm is a modified tight junction that links adjacent podocyte foot processes and serves as a terminal barrier for the retention of circulating macromolecules as blood is filtered in the glomerulus (38, 74). These structures have been identified through the use of electron microscopy, and their biological importance was established through the discovery of the NPHS1 gene. The NPHS1 gene encodes the protein nephrin, a transmembrane protein that belongs to the immunoglobulin superfamily of cell adhesion molecules. Using positional cloning, it was discovered that mutations in NPHS1 result in congenital nephrotic syndrome of the Finnish type, wherein newborns present with massive proteinuria (51, 89). This finding spurred the investigation of slit diaphragm biology. Recent immunogold-tracing electron microscopy and time-lapse fluorescent microscopy experiments suggest that endocytic mechanisms regulate nephrin (Fig. 2, A–D). Following tyrosine phosphorylation by Src family kinases Fyn and Yes, and the binding of Nck protein and the p85 subunit of phosphoinositide 3-OH kinase, nephrin can serve as a signaling platform (42, 47, 73). Nephrin phosphorylation at tyrosine residue 1193 augments podocin interaction, while dephosphorylation at this site induces β-arrestin interaction followed by nephrin internalization (83). Another regulatory mechanism for nephrin endocytosis involves PKC-α (84). Increased PKC-α activity has been demonstrated in mouse models of diabetic nephropathy, where phosphorylation of threonine residues 1120 and 1125 augments β-arrestin interaction and internalization (109). Either treatment with a PKC-α inhibitor or genetic ablation of Prkca results in nephrin retention at the membrane (84). Moreover, induction of the planar cell polarity pathway (PCP) also induces nephrin endocytosis in a β-arrestin-dependent manner (6). Podocyte-specific deletion of Vangl2, a PCP protein, results in reduction of nephrin endocytosis and abnormal glomerulogenesis. These mutant mice also have increased susceptibility to anti-glomerular basement membrane-induced injury (88). Nephrin turnover also involves CIN85/RunkL-mediated ubiquitination in podocytes lacking CD2AP, and it has been postulated that CD2AP can down-regulate CIN85 expression due to SUMOylation at lysine 598 (108). Currently, it is unclear whether nephrin uptake occurs via clathrin-mediated or non-clathrin-mediated pathways. One body of evidence suggests that both are involved, as phosphorylated nephrin appears to undergo raft-mediated endocytosis while the dephosphorylated nephrin results in clathrin-mediated endocytosis (82). However, nephrin internalization involving the dynamin-dependent Notch pathway appears to require clathrin, as cholesterol depletion does not induce nephrin internalization (112). Although nephrin retention at the surface appears critical, evidence suggests that reduction of nephrin internalization is also detrimental to the podocyte (102), as slit diaphragm proteins likely require recycling or turnover to contend with the daily rigors of plasma filtration.

Another slit diaphragm component that appears to be regulated by endocytic proteins is zonula occcludens (ZO)-1. The critical importance of ZO-1 in podocyte biology was recently discovered, as mice with Tjp-1 ablated specifically in podocytes developed severe proteinuria and foot process effacement (44). Furthermore, it has been reported that ZO-1 is mislocalized from tight junctions following pururomycin-induced podocyte injury (87). Actin-based myosin motor Myo1e not only visits clathrin-coated pits but also interacts with ZO-1 (8). Further validating this point, non-muscle myosin 1c also interacts with PI(4,5)P2 and with slit diaphragm protein Nep1 and nephrin, presumably to maintain their proper localization at the membrane (4). Loss of myo1c in zebrafish results in an abnormal glomerular filtration barrier (3). Thus it is likely that the uptake of endocytic proteins plays a fundamental role in quality control of the slit diaphragm. It is unclear where slit diaphragm proteins are destined following endocytosis, as the cargo can be transferred from early to late endosomes followed by lysosomal degradation, the trans-Golgi network, and to recycling endosomes. This complex process is orchestrated and marked by numerous Rab-GTPases. For example, Rab1 and Rab2 are localized at endoplasmic reticulum (ER) exit sites and the pre-Golgi intermediate compartment, affecting ER-Golgi trafficking. In addition, Rab6, Rab33, and Rab40 are localized in the Golgi to mediate intra-Golgi trafficking, while Rab22 functions to traffic cargo between the trans-Golgi network and early endosomes. Conversely, Rab5 is localized at early endosomes, phagosomes, caveosomes, and the plasma membrane mediating early endosome fusion of clathrin-coated vesicles, allowing for Rab11-dependent recycling and/or Rab7-dependent late endosomal trafficking (104). We have recently found that nephrin visits Rab family proteins Rab11 (recycling endosomes) and Rab7 (late endosomes), indicating that pools of nephrin are either recycled or degraded (Inoue K and Ishibe S, unpublished observations).

**Lipoproteins and Albumin Endocytosis**

Plasma membrane and soluble receptors bind to signaling molecules outside the cell and subsequently activate signal transduction pathways. When a ligand binds to a receptor, this can often induce endocytosis of the receptor-ligand complex. Recent evidence suggests that sFLT-1, a soluble VEGF receptor-1 localized at lipid rafts, is internalized and then found in compact punctae that colocalize with endosomal proteins,
fotillin, and Rab5 (46). This process appears to be dynamin dependent, as pharmacological inhibition with dynasore abrogates this process. It was discovered that sFLT-1 associates with sphingolipids at the lipid raft domains, inducing endocytosis. Podocyte-specific deletion of sFLT-1 results in severe proteinuria and foot process effacement, suggesting sFLT-1’s role in the maintenance of the glomerular filtration barrier. The recent discovery of clathrin-dependent internalization of apolipoprotein L1 also underscores the importance of this fundamental process (59). Patients of African ancestry who have mutations in the gene APOL1 have higher risks of various forms of glomerular diseases such as focal segmental glomerulosclerosis and human immunodeficiency virus-associated nephropathy (32, 35, 36). However, it is unclear whether the uptake of apolipoprotein L1 is differentially regulated to induce podocyte injury in affected patients who carry the APOL1 risk alleles.

It is proposed that podocytes are able to internalize and eliminate proteins filtered throughout the glomerular filtration barrier. Immunogold ultrastructural studies have shown that albumin is taken up by rat podocytes following puromycin aminonucleoside administration (53). In addition, albumin endocytosis through caveolae has been observed in cultured podocytes, which are degraded by lysosomes (15, 25). Furthermore, new evidence also suggests that free fatty acid bound to albumin undergoes endocytosis in podocytes (78) and directly elicits injury (1, 75). During nephrotic syndrome in vivo, the fatty acid-bound albumin may augment macropinocytosis due to the free fatty acid, G protein-coupled receptor’s Gβ/Gγ subunit (20), which may lead to podocyte dysfunction directly or indirectly through activation of angiopoietin-like 4, a protein secreted by podocytes that induces proteinuria (21).

**Integrins and Transmembrane Receptor Endocytosis**

Integrins are transmembrane receptors that organize interaction between the cell matrix and extracellular matrix (ECM). There are 24 distinct integrins, each consisting of 1 of 18 α-subunits and 1 of 8 β-subunits (72). The principal integrin that mediates a podocyte’s adhesion to the glomerular base-membrane is the laminin binding integrin αβ (55, 92). Podocyte-specific deletion of Itgb1 or Itga3 in mice induces severe proteinuria and foot process effacement (81, 91). Furthermore, deletion of integrin interactors’ tetraspanin CD151 (Cd151), talin1 (Thn1), integrin-linked kinase (Ilk), and α3β1 ligand β-laminin (Lamb2) results in severe proteinuria (23, 27, 45, 48, 91, 107). Current evidence suggests that clathrin-mediated endocytosis of activated β1-integrins and dynamin involvement with focal adhesion kinase may facilitate focal adhesion disassembly (28, 106). This process may induce cell movement, akin to foot processes undergoing effacement. Activation of the urokinase plasminogen activator receptor (uPAR) has been linked to the pathogenesis of focal segmental glomerulosclerosis (113). A recent study found that podocyte injury occurs through plasminogen activator inhibitor type 1 (PAI-1) mediated endocytosis of both β1-integrin and the uPAR (54). These findings indicate the potential importance of understanding the connection between integrin proteins, focal adhesion turnover, and endocytosis.

**Concluding Remarks**

The endocytic process in podocytes plays a fundamental role in the development and maintenance of the glomerular filtration barrier. The loss of key molecular machinery results in defective actin regulation and faulty slit diaphragm maintenance, while increases in the uptake of lipoproteins or integrins may have deleterious effects on podocyte health (Fig. 3). These current findings provide important insight into podocyte biology and motivate further investigation to garner evidence to understand the complexities displayed by endocytic regulation of diverse cellular processes.

**ACKNOWLEDGMENTS**

We thank Arnaud Marlier for assistance with graphic art.

**GRANTS**

This study was supported by National Institute of Diabetes and Digestive and Kidney Diseases Grants DK083294 and DK093629.
DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

Author contributions: K.I. and S.I. prepared figures; K.I. and S.I. drafted manuscript; K.I. and S.I. edited and revised manuscript; K.I. and S.I. approved final version of manuscript.

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