Role of Guanine Nucleotide Exchange Factor-H1 in Complement-mediated RhoA Activation in Glomerular Epithelial Cells

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ABSTRACT

Visceral glomerular epithelial cells (GEC), also known as podocytes, are essential components of the kidney glomerulus, and play a pivotal role in maintaining normal glomerular permselectivity. The regulation of the actin cytoskeleton is central to podocyte morphology and function. In the rat model of membranous nephropathy, passive Heymann nephritis (PHN), heterologous antibody binds to GEC antigens. Following immune complex formation the complement system is activated with the assembly of the C5b-9 membrane attack complex. In GEC, C5b-9 induces sublytic injury associated with morphological changes and proteinuria. We have previously reported that complement activates RhoA, member of the Rho family of small GTPases, \textit{in vitro} and \textit{in vivo}. The current study addresses the role of GEF-H1, a Rho GEF protein, in complement-mediated RhoA activation in GEC injury. In glomeruli from rats with PHN and cultured GEC, complement stimulation increased GEF-H1 activity, in a parallel fashion with RhoA activation. Activation of GEF-H1 by complement was at least partially dependent on the extracellular signal-regulated kinases (ERK) pathway, but not on the epidermal growth factor (EGF) receptor, or Src-family kinases, or microtubules. To address the functional effects of GEF-H1, GEC were transduced with lentivirus-mediated shRNA of GEF-H1, to knockdown the expression. GEC transduced with shRNA of GEF-H1 significantly blocked RhoA activation and augmented cytotoxicity in response to complement stimulation, implicating a cytoprotective effect of GEF-H1 on GEC in complement-mediated injury.
RÉSUMÉ

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Chapter 1

Literature review and Objectives
1.1 THE PODOCYTE

1.1.1 Introduction to the podocyte cytoskeleton

The nephron, the basic structural and functional unit of the kidney, consists of a glomerulus and renal tubule. The glomerulus is a network of capillary loops surrounded by the Bowman’s capsule and performs the first step of blood filtering. It consists of 3 resident cell types: the mesangial cells, glomerular endothelial cells, and visceral epithelial cells (also known as podocytes). The podocyte, which sits on the outside of the glomerular capillary loop, consists of a large cell body, located in the urinary space, major processes, and foot processes linking the podocyte to the underlying glomerular basement membrane (GBM).[1] Podocyte foot processes interdigitate with foot processes of the neighboring cell forming in between the filtration slits bridged by the glomerular slit diaphragm that is highly permeable to water and small molecules, but not to larger proteins, such as albumin[1, 2]. Injury to mesangial or endothelial cells often leads to inflammatory glomerular diseases (glomerulonephritis), because they are in direct contact with the blood containing leukocytes, complement, and other inflammatory proteins[3]. On the other hand, injury to podocytes disrupts the filtration barrier, leading to proteinuria, a hallmark of many primary glomerular diseases such as minimal change disease, idiopathic focal segmental glomerulosclerosis (FSGS), and membranous nephropathy, or secondary glomerular diseases such as diabetic nephropathy (DN). The best characterized podocyte injury involves the reorganization of foot process actin cytoskeleton leading to alterations in podocyte charge or shape (effacement) and to disruption of the slit diaphragm [3-5]. Thus, the spatiotemporal profile of cytoskeleton arrangement has to be highly controlled in normal podocyte functions, and a profound understanding of the dynamics and regulation of the cytoskeleton of podocytes is essential to enable the study of glomerular diseases and eventually develop targeted therapies.
1.1.2 Overview on structure and functions of podocytes

In order to understand recent advances in glomerular pathogenesis, it is necessary to understand normal structure and functions of podocytes. Glomerular development is divided into 4 stages: renal vesicle stage, S-shaped body stage, capillary loop stage, and mature glomeruli stage[6]. The progression from the S-shaped body stage to the capillary loop stage is critical for the differentiation of podocytes[1, 2]. During this transition, undifferentiated epithelial cells (immature podocytes) lose their mitotic activity, and begin to establish their unique and complex cell architecture marked with the appearance of foot process and the establishment of cell-cell junctions forming the slit diaphragm[2]. The mature podocyte presents both epithelial features (established cell polarity with an apical and basal side), and mesenchymal features (de novo expression of vimentin, an intermediate filament protein, loss of epithelial markers such as desmosomal proteins and E-cadherin, and partial motility)[7]. In addition, podocytes have long been compared in analogy to smooth muscle cells, such as capillary pericytes due to similar functional analogies including spontaneous and angiotensin-induced contraction, as well as glucose uptake[8].

Fully differentiated podocyte consists of a cell body (located in the urinary space), major processes, rich in microtubules, and foot processes branching from major processes and binding to the GBM by means of integrins, α and β dystroglycans[1, 3]. Podocyte foot processes are arranged in a characteristic interdigitating pattern with foot processes of neighboring podocytes, thereby forming the filtration slits that are bridged by the slit diaphragm, a modified adherens junction[1, 2, 9]. Foot process cytoskeleton consists of highly organized parallel and contractile actin filament bundles. The dynamic regulation of the latter organization is crucial for the proper function of podocytes. Three distinct membrane domains functionally define foot process: the apical membrane domain, the slit diaphragm, and the basal membrane domain, all of which contain specialized proteins responsible for the dynamic regulation of podocyte[2, 4]. These three domains are linked to each other and to the contractile apparatus by means of the actin skeleton[2]. Any disruption in the expression or function of
these domains and their specialized proteins leads to actin rearrangement and loss of foot process architecture, a process known as foot process effacement marked with proteinuria. The main structural elements of podocyte and the slit diaphragm are illustrated in Figure 1.1. (Please also refer to Table 1 for more details)[2, 5].

Under normal physiological conditions, podocyte functions can be classified as follow:

1) **Podocyte: a mechanosensing contractile cell in the glomerulus:** As described earlier, podocytes show a unique microtubule and actin rich architecture with an intermediate phenotype between epithelial and mesenchymal cells showing analogy with smooth muscle cells, particularly capillary pericytes[8]. These features enable podocytes to contract in response to upstream mechanical signals potentially controlling blood flow in addition to the historically known mesangial cells[8, 10]. Moreover, when mouse podocytes were subject to fluid shear stress, to mimic the flow of the glomerular ultrafiltration, tyrosine kinases and Rho kinases were activated leading to actin reorganization in order to withstand the fluid shear stress[11]. The findings clearly indicate that podocytes serve as mechanosensing cells to sustain the normal capillary structure and barrier function[10-12].

2) **Regulation of glomerular fluid flux across the glomerular barrier:** It has been long recognized that the slit diaphragm, which connects adjacent foot processes, is essential in blocking the leakage of large molecules (e.g. protein) into the urine. However, it was not known how this physical barrier could escape from “clogging” with large molecules. The identification of a subpodocyte space using serial transmission microscopy in 2007 shed some light on this issue[13]. Subpodocyte space consists of the restricted area bound by podocyte cell body and its major processes covering 60% of the entire GBM[13]. Using isolated, perfused glomerulus and an in vivo multiphoton imaging technique, Salmon and colleagues showed that in these areas of subpodocyte space, proteins were washed out[14]. One hypothesis of this reverse filtration (flushing back) is the constant movement of the podocyte along the GBM[7]. Although some studies suggest that
detectable movement of podocytes *in vitro* and *in vivo* occurs[15], direct and solid evidence still needs to be provided. Endocytosis is another mechanism by which podocytes prevent filter clog[16]. Disappearance of aggregates residing on the GBM due to sequential intravenous injections of protamine and heparin into rats was shown to be due to podocyte phagocytosis[16]. More recently, albumin endocytosis was described in conditionally immortalized mouse and human podocytes in a statin-sensitive manner[17]. New evidence suggests the existence of an active transport mechanism (FcRn, an IgG and albumin receptor) in the podocyte to remove immunoglobins accumulated at the filtration barrier[18].

3) **Regulation of the GBM itself:** GBM is mainly composed of polymers of laminin and type IV collagen[19]. This composition changes temporally during glomerular development[20]. Initially, both endothelial cells and podocytes produce laminin α1β1 and later during development, laminin replacement occurs resulting in the production of laminin α5β2 by the same cells[21]. Collagen α1α2α1 (IV) network is first produced by the endothelial cells during fetal development[20]. This network is then replaced by the α3α4α5 network produced solely by the podocyte[19]. Absence of this network results in several kidney disorders such as Alport disease[19]. The mechanism and reason of the GBM composition isoform switch during development are still unknown, but this may be important for the maintenance and achievement of the differentiated states of podocytes.

4) **Importance of cross-talk between podocytes and adjacent cells:** A detailed description of podocytes in isolation can only provide information of their individual biology. In order to evaluate the contribution of the podocyte to the physiology of the entire glomerulus, we should recognize the interactions of the podocyte with its adjacent cells[22]. Podocyte-specific knockout systems have identified the role of podocytes in maintaining healthy fenestrated endothelium by the production of vascular endothelial growth factor (VEGF) acting on VEGF receptors on the endothelial cells[23]. Interaction between podocyte and mesangial cell is also important especially when we consider glomerular injury. Chemokines produced by either podocyte or mesangial cell can interact with their
respective receptor on these same cells and influence their local migration and adherence to the GBM[24, 25].

5) **Immunological functions of podocytes:** The emerging immunological role of podocyte is recently getting more attention. Cultured podocytes express Toll-like receptor 4 (TLR4) on their surface, which upon stimulation, resulted in a marked induction of chemokines[26]. In addition, it was shown that in response to the binding of LPS to TLR4, podocytes start expressing on their surface B7-1 (T cell co-stimulatory molecule involved in antigen processing), which led to actin cytoskeleton rearrangement and slit diaphragm reorganization resulting in proteinuria[27]. Thus, podocytes may play a role in the innate immunity, in the surveillance for pathogens and initiation of danger signaling in the Bowman’s space.

**1.2 MEMBRANOUS NEPHROPATHY**

**1.2.1 Introduction to membranous nephropathy (MN)**

Membranous nephropathy (MN) is one of the most common causes of nephrotic syndrome in adults, constituting up to one-third of biopsy diagnoses in some countries[28-30]. MN is divided into 2 main groups; primary (‘idiopathic’), with no identified cause (consisting 70-80% of cases) and secondary forms. Secondary MN is associated with hepatitis B infection, systemic lupus erythematosus, thyroiditis, malignancies, and the use of drugs such as penicillamine, and nonsteroidal anti-inflammatory drugs[29, 31, 32]. MN is characterized by the development of immune complexes (containing mainly immunoglobulins and complement members such as C3 and C5b-9) in the subepithelial space leading to podocyte damage, causing increased production of extracellular matrix proteins rendering the GBM thick[33]. Neutral endopeptidase is a protein expressed on the human podocyte and considered as a podocyte antigen involved in the formation of immune deposits in animal models[34]. Neutral endopeptidase expressed in podocytes in babies born to mothers deficient of this enzyme was shown to be responsible in several cases of neonatal MN [35]. Debiec et al reported a case in which the mother, deficient in
neutral endopeptidase, became immunized against the antigen, and the antibodies produced were transferred to her fetus, which developed a severe form of neonatal MN [35]. More recently, Salant and colleagues reported that antibody against the M-type phospholipase A₂ receptor (PLA₂R) was found in 80% of patients with idiopathic MN[36]. This breakthrough discovery established that the PLA₂R expressed on the cell surface of podocytes as a common antigen causing MN.

Treatment of MN has proven to be challenging, mainly because of the uncertainty regarding the beneficial and adverse effects of immunosuppressive treatments, and the lack of reliable biomarkers[37-39]. Thus, a profound understanding of the pathogenic mechanisms of MN is required to better monitor disease progression and develop effective therapeutic treatments. Over the last decade, immense advances have been made to unravel some of the fundamental molecular pathomechanisms in human MN. Most of the findings are derived from experimental models of idiopathic MN, mainly Heymann nephritis model [40-42], involving in situ formation of immune complexes, and cationic bovine serum albumin (cBSA) animal model[43], illustrating the case of "planted" or "exogenous antigen bound to the basement membrane of the podocyte.

1.2.2 Heymann nephritis model

In 1959, Heymann et al developed an experimental rat model mimicking human membranous nephropathy[40]. Early studies used an active immune model utilizing the production of autologous antibodies to renal antigen(s) by the animal’s own immune system[40]. However, this active model is rarely used because of the long disease induction latency, and the high frequency immunization administered into footpads of rats, which can be uncomfortable for the animal[44]. Subsequently, passive model of immunization, now known as passive Heymann nephritis (PHN), was introduced utilizing antiserum to the renal antigen(s) generated in another animal (such as rabbits, goats, or sheep)[44]. These antigens are then injected into the rat to induce immune complex formation. In both active and passive Heymann nephritis, the antibodies are targeted against
a large podocyte transmembrane protein known as megalin. Megalin, ~600 kDa molecular weight molecule, also referred as LDL receptor-related protein 2, is a glycoprotein expressed at the brush border of proximal tubular cells and at the sole of podocyte foot processes in the form of clathrin-coated molecule [45]. In both models of immunization, immune deposits are accumulated in the subepithelial space until they block the slit diaphragm [46]. As a consequence, sublethal complement activation occurs resulting in intracellular signaling activation influencing podocyte metabolic pathway, structure and function please refer to Figure 1.2 for mechanism of complement –mediated podocyte injury[46].

It has been reported that the onset of proteinuria occurs four to seven days after injection depending on the initiating antibody [44]. Histological changes include thickening of the GBM and foot process effacement, leading to glomerular and tubulointerstitial sclerosis [47]. Although studies of Heymann nephritis have greatly contributed to our understanding of the pathological mechanisms of membranous nephropathy, there is no evidence that megalin is expressed in human podocytes, and therefore cannot be considered as the pathogenic human antigen.

1.2.3 Other animal models of MN

Another rat model of MN reported in the literature is the anti-dipeptidyl peptidase IV (DPPIV) rat model [48]. DPPIV is another important antigen found on the same tubular brush border fraction used in Heymann nephritis model (Fx1A)[48]. Passive immunization is based on the generation of anti-DPPIV in rabbit, and injection of these antibodies in the rat[48]. Proteinuria is observed on day 1, reaches a peak on day 2 and then gradually decline[48]. Unlike, Heymann nephritis model, the immune deposits are transient (5 days) and occur in the absence of C3[48]. Few published reports of mouse model of MN include cationic BSA (cBSA) mouse model, implicating the concept of ‘planted’ antigen in the subepithelial space of the GBM [49], anti-renal tubular antigen model [50], and aminopeptidase A (APA) model describing a transient complement independent albuminuria [51]. In addition, non-rodent systems of MN have been developed but
are rarely used. These models include (cBSA) rabbit model [43], and anti-
angiotensin converting enzyme (ACE) pig model [52].

Despite the development of several systems, PHN rat model remains the
only system of study because of its nearly identical pathology to human MN. The
model demonstrates the distinct concept of *in situ* immune complex formation due
to antibodies binding to a podocyte antigen analogous to human MN.

1.3 Rho-GTPASES: FUNCTION AND REGULATION

1.3.1 Introduction to Rho-GTPases

Rho-family GTPases control many aspects of cell behavior. The three
best-characterized members are RhoA, Rac1 and Cdc42 discovered in the early
1990s for their role in the formation of stress fibers, lamellipodia, and filopodia,
respectively [53]. In eukaryotic cells, it has been shown that Rho-GTPases
regulate actin-dependent processes such as those involved in migration, adhesion,
morphogenesis, axon guidance, and phagocytosis [54-56]. In addition to the
regulation of actin cytoskeleton, Rho-GTPase play an important role in the
regulation of microtubules, cell polarity, gene expression, cell cycle progression
and cell survival [57, 58]. Because they regulate such a wide range of biological
behaviors, it is not surprising that the Rho-GTPases are themselves highly
regulated.

Like Ras, Rho proteins act as a bi-molecular switches cycling between two
states: inactive (GDP-bound) form, and the active (GTP-bound) form. Primarily
three regulatory molecules regulate these conformational states: GAPs (GTPase
activating proteins) enhancing the intrinsic GTPase activity of Rho proteins
promoting the inactive form, GEFs (guanine exchange factors) catalyzing the
exchange of GDP for GTP and thus promoting the active form of the GTPase[59],
and GDI (GDP dissociation inhibitor) which inhibits Rho protein function by
sequestering them in the cytoplasm preventing nucleotide dissociation [60].
Please refer to Figure 1.3 for more details about Rho regulation and signaling to
the actin cytoskeleton.

### 1.3.2 Rho-GTPases in podocyte

The majority of the data available to date concerning Rho-GTPases in podocytes are on the prototypical Rho-GTPase, RhoA. RhoA is expressed in immortalized mouse podocytes and is upregulated upon differentiation, which is accompanied by the increased activity [61]. RhoA activity was shown to be important for cell migration in cultured mouse podocytes and calcineurin overexpression, which causes RhoA degradation, in podocytes induced proteinuria in mice, suggesting an important role of RhoA in the maintenance of podocyte function in vivo[61]. On the other hand, in cultured mouse podocytes, filamentous actin reorganization by mechanical stretch was dependent on Rho-kinase (ROCK, downstream kinase of Rho) [12]. Inhibition of ROCK promoted elongation of processes in cultured mouse podocytes, implying that the activation of the Rho pathway may be inhibitory to podocyte process formation [62, 63]. Sublytic concentrations of complement membrane attack complex C5b-9 increases RhoA activity in cultured rat GEC [64]. Transfecting mouse podocytes or rat GEC with constitutively active RhoA caused the loss of cellular processes and cell contraction [64, 65]. These data from cultured podocytes may suggest that activation of RhoA has a negative impact on podocyte morphology and/or function. To further support this notion, there have been numerous reports using Rho-kinase inhibitors (Y27632 and fasudil) in the setting of renal injury and proteinuria. All these studies reported amelioration of proteinuria and/or kidney function [66-81]. While most of the studies utilized hypertension-related models, many reported beneficial effects on the kidney without a significant change in systemic blood pressure [67, 70-72, 75, 76, 78, 79]. It is of interest that other models such as diabetic nephropathy [68, 69, 73, 77], crescentic glomerulonephritis [66], lupus nephritis [81], and puromycin aminonucleoside nephrosis[74] also responded to the treatment with fasudil. In addition, doxycycline-inducible and podocyte-specific expression of constitutively active
RhoA in mice showed that expression of constitutively active RhoA induces foot process effacement and proteinuria [65, 82]. These effects were reversible upon withdrawal of doxycycline, at least when the changes were mild, suggesting that RhoA induced reversible changes in the actin cytoskeleton [65, 82]. All these studies support the notion that activation of RhoA in adult podocytes could be detrimental to their function. On the other hand, several reports suggest that the activation of the Rho pathway may be beneficial for podocytes by means of protection or even recovery of podocytes under certain conditions [83, 84]. These apparently conflicting views may come from several factors; although cultured podocyte system is an invaluable tool to study podocyte biology, its correlation with in vivo structures is not convincing. Most importantly, cultured podocytes do not form intricate interdigitation of foot processes observed in vivo. Similarly, the cell-cell contacts seen in cultured podocytes are similar but not identical to the slit diaphragm, which is a key structure and signaling center of podocytes[9, 85, 86]. Moreover, no study has addressed the spatial temporal profile of RhoA activation, which will be critical for actin dynamics. It is also possible that RhoA activation has dual effects on podocytes. For example, expression of constitutively active RhoA induced cellular process retraction but also made the cells more resistant to complement-mediated cell death [64]. It is possible that RhoA protects the cells from cell death at the expense of the intricate foot process structure.

Information on Rac1 in podocytes in the literature is more limited. In the kidney of GDIα-knockout mice (Arhgdia−/−), Rac1, but not RhoA or Cdc42, was activated and the Rac1 inhibitor ameliorated proteinuria [87]. The same group also demonstrated that the Rac1 inhibitor ameliorates glomerular damage in salt sensitive Dahl rats loaded with salt in a mineralcorticoid-dependent manner [88]. Thus, Rac1 activation likely activates the mineralcorticoid receptor in the kidney thereby contributing to kidney damage. It should be noted, however, that the above studies did not study the cellular localization of Rac1 activation within the kidney. In immortalized mouse podocytes, Rac1 activity was increased at 1 week of differentiation as compared with undifferentiated cells, before declining to the
basal level at 2 weeks [89]. Undifferentiated mouse podocytes transfected with constitutively active Rac1 showed increased cell size and increased number of lamellipodia, the features seen in differentiated cells [89]. *In vivo*, glomerular Rac1 activity was decreased in PHN [64] and was increased in PAN [89] but it is unclear how these changes relate with the development of proteinuria. These results suggest that Rac1 may contribute to podocyte differentiation.

The potential role of Cdc42 in podocytes has been typically discussed in conjunction with Rac1 and on its own, Cdc42 has attracted limited attention thus far. A recent study clearly demonstrated that the gene deletion of Cdc42 in podocytes, but not of RhoA or Rac1, causes congenital nephrotic syndrome and glomerulosclerosis[90]. Interestingly, actin polymerization induced by nephrin clustering was impaired in the absence of Cdc42 but was not affected by RhoA or Rac1 deficiency [90]. More comprehensive approaches would be necessary to address the role of Cdc42 in podocytes.

1.3.3 Regulatory proteins of Rho-GTPases: RhoGAPs

More than 70 members of the RhoGAP family have been identified, all of which contain at least one GAP domain that enhances the intrinsic GTPase activity, leading to the inactivation of the Rho proteins [91]. Although GAPs were initially considered as simple signal terminators, accumulating evidence suggest that GAPs are also tightly regulated by external factors and play an important role in mediating different signaling pathways and maintaining an equilibrium between different Rho proteins’ activity [91]. Substrate specificity and function of many of the RhoGAPs still remain unknown [92]. Several human diseases have been identified that are caused by RhoGAP mutations such as X-linked mental retardation, limb anomalies, and cancers[91, 93]. Akilesh *et al* reported that mutations of *ARHGAP24*, which encodes one of the RhoGAPs, are genetically linked to familial FSGS [94]. Focusing on the mutation, Q158R, located in the GAP domain of ARHGAP24, it was demonstrated that the mutant has less GAP activity, resulting in the increased Rac1 activity and the authors concluded that
Rac1 activation in podocytes is responsible for the pathogenesis of FSGS [94]. It should be noted, however, that ARHGAP24 knockdown in mouse podocytes resulted in the activation of both Rac1 and Cdc42 and the impact of the ARHGAP24 mutation on other glomerular cells was not studied [94]. Therefore, contribution of Cdc42 and potential impact on other glomerular cells need to be investigated before drawing a firm conclusion. Nevertheless, this was the first direct evidence that dysregulation of Rho-GTPases can cause podocyte dysfunction in humans.

1.3.4 Regulatory proteins of Rho-GTPases: RhoGDIs

In contrast to the many GEFs and GAPs only 3 members of the GDI family of proteins have been identified in humans, GDI 1, 2 and 3 among which RhoGD11, also known as RhoGDIa is the most abundant and is ubiquitously expressed [60][95]. RhoGDIs are small proteins of ~25 kDa comprising two main domains: the ‘regulatory arm” at the N terminus and the C-terminus domain. The N terminus initiates contact with Rho-GTPases while the C-terminus domain is required to maintain binding to Rho-GTPases. GDIs sequester Rho-GTPases in their inactive form, away from the membrane and regulate their intracellular localizations. The main function of GDIs is to maintain an intracellular pool of soluble inactive Rho-GTPases, allowing a rapid response and translocation to the membrane after an external stimulation. Thus GDIs can be viewed as chaperones to stabilize and maintain mature inactive Rho-GTPase proteins[60]. Implication of RhoGDIs in cancer has been thoroughly studied. For example, RhoGDI2 was shown to be upregulated and overexpressed in invasive human ovarian cancer [96], RhoGDI3 was also shown to be upregulated in malignant tissues such as colon polyps and ovarian cancer [97], and RhoGDI2 was demonstrated to be overexpressed in pancreatic cancer [98]. Systemic knockout of RhoGDIa in mice resulted in a severe renal phenotype including heavy proteinuria and glomerulosclerosis, leading to progressive renal failure and death within a year.
Currently, there is no data regarding the role of the other two isoforms of RhoGDIs in the kidney.

1.3.5 Rho-GTPases regulatory proteins: RhoGEFs

1.3.5.1 Structural features of GEFs

Dbh proteins (also known as GEFs) get their name from the first GEF discovered, which was a transforming gene found in diffuse B-cell lymphoma [100]. DH (Dbh Homolgy) domain is found to be conserved in all the Dbh family proteins and is necessary for GEF activity as it interacts with and modifies the binding pocket of the GTPase facilitating the exchange reaction [59]. And with the completion of many genome-sequencing studies, ~80 human GEF’s were identified which several appear to be specific to a single GTPase, such as p115 RhoGEF which act only on RhoA, whereas other may activate many, like Vav which can act on RhoA, Rac1, Cdc42 and RhoG[101, 102].

Another conserved domain between GEF’s is the plekstrin homology domain (PH) located C-terminally from the DH domain [59]. PH domains can participate in the GTPase binding interface facilitating nucleotide exchange. The PH domain of LARG (Leukemia associated RhoGEF) interacts directly with RhoA and is necessary for proper activation of the GTPase[103]. Although limited data exist to generalize this role, other functions of PH domain include targeting GEF’s to specific intracellular locations and serving as a docking site to many proteins associated with signaling cascades regulated by Rho-GTPases. These findings are important in the research field as it may help linking the activation of Rho-GTPases to distinct cellular sites and signaling cascades [59, 104].

In most cases DH-PH module is the smallest functional unit of the Dbh family proteins that can promote nucleotide exchange in vivo. But there exists additional functional domains such as SH2, SH3, Tyr kinase, Ras-GEF, PDZ and many others underlying the unique cellular functions of different family members [104].
Until recently, only the Dbl family proteins of GEFs were known, but a second family of proteins have been discovered, known as CZH proteins. These proteins contain a DOCKER or CZH2 (CDM-zizimin homology2) domain that act on Rac1 and Cdc42 [105-107].

1.3.5.2 Regulation of GEFs

The number of mechanisms by which Rho GEF are activated has made it difficult to study the activation profile of GEF’s in general. However, some general mechanisms have emerged and include: 1) release of intramolecular inhibitory interactions by phosphorylation or by binding to another protein, 2) stimulation by protein-protein interactions, 3) recruitment to specific intracellular location and regulating the spatio-temporal profile of Rho-GTPases activation, and 4) turning off GEF’s when stimulation is terminated [104].

1.3.5.3 Mechanism of guanine exchange reaction

Activation of the small Rho-GTPases by GEF, which stimulates GDP/GTP exchange involves many steps [108]. The first step of engagement consist of forming a low affinity complex with the GDP-bound GTPase, dissociating the GDP and binding with high affinity to the nucleotide free state of GTPase[109, 110]. This intermediate does not accumulate in the cell because the high GTP:GDP concentration ratio in the cell drives the reaction towards the GTP-bound GTPase[108]. This reaction implies that Rho GEF (DH domain in particular) causes the dissociation of GDP and transiently stabilizing a nucleotide-free transition state of the protein.

Despite the fact that most of the Rho-GTPase protein regions are conserved between the GDP and GTP-bound conformational state, some regions are altered in response to the nature of the nucleotide and are known as the switch I and switch II regions [111]. These regions bind only to the phosphate groups of GTP. This face of interaction is called the P-loop[108, 111]. Along with the P-loop, the switch regions reside at the heart of interaction of the Rho-GTPase with their GEF’s, forming the nucleotide-binding pocket.
In general, for the nucleotide exchange reaction to occur, Rho GEF’s probably first interact with the P-loop of the protein dissociating its GDP. As a consequence, the switch regions are severely altered: the switch II region binds to GEF, inducing or promoting the disrupted conformation of the P-loop, and stabilizing the nucleotide free state of the protein by forming large regions of interface [108]. The rate-limiting step of the reaction is the binding of the GTP, which binds to the phosphate groups and causes the dissociation of the GEF.

1.3.5.4 GEFs in diseases

Many human diseases involve impairment in many signaling pathways controlled by small GTP-binding proteins. For instance, Rho-GTPase proteins have been implicated in cancer playing a pivotal role in the progression of the disease mainly in the morphology and motility of the tumor cells [112, 113]. Mutations can target the GTPase expression itself, their protein regulators (GEF, GAP, GDI) or even their downstream targets [114].

With respect to GEF’s, many were found mutated in many human cancers [104]. The best-studied examples are the products of the chimeric Bcr-Abl protein. Because Bcr contain both a DH-PH GEF domain and a GAP domain, its fusion with the tyrosine kinase Abl can result in either a 185-KDa protein which lacks the GEF and GAP domains of Bcr and is associated to acute lymphatic leukemia (ALL), or a 210-KDa protein which lacks only the GAP domain of Bcr and is associated with chronic myeloid leukemia (CML)[115]. In both cases, the chimeric protein is constitutively active due to the kinase activity of Abl, and thought to be responsible for the oncogenic property of both diseases [115]. Although this concept has been well established, the potential role and contribution of the DH domain of Bcr has been poorly studied. Another example of the involvement of GEFs in cancer includes the chimeric protein found in acute myeloid leukemia (AML), which is the fusion of LARG (leukemia-associated-Rho guanine-nucleotide exchange factor) and the mixed lineage leukemia (MLL) gene [116]. Although the expression of this fusion protein is under the control of
the MLL promoter, it is not surprising to observe an alteration in the levels of LARG expression and thus Rho activation.

Most recently, the identification of RacGEF P-Rex1 as an essential mediator of Rac1 activation and tumorigenesis in response to ErbB receptors in breast cancer cells reveal a pivotal mechanism crucial for breast cancer progression and therapeutic implications [117].

In addition, Rho GEF alterations have also been identified in developmental and neurodegenerative disorders. Mutations in the DH domain of Cdc42-GEF Fgd1 result in the development of faciogenital dysplasia, a developmental disorder characterized by short stature, facial, skeletal and genital abnormalities[118, 119]. Although the detailed mechanism of function is not well understood, a recent study suggests that activation of MLK3 (mixed-lineage kinase 3) specifically by Fgd1/Cdc-42 is important for skeletal mineralization in cultured osteoblasts[120]. Many regulators of Rho-GTPase have been involved in X-linked mental retardation (MXR) notably ARHGEF 6-also called a-Pix, a Rac-specific GEF [121]. Although it has been suggested that the underlying cause of MRX is due to a defective Rac signaling, a recent group have identified a missense mutation in the Cdc42-GEF-9 (ARHGEF9) suggesting that it is likely to be responsible for syndromic X-linked mental retardation associated with epilepsy [122].

Another implication of GEF’s is detected in viral and bacterial pathogenesis. Some viruses and bacteria take control of some cellular pathways by inserting their own GEF into the host genome cells such as the Legionella ArfGEFRalF[123], and the Salmonella RacGEF SopE[124]. Other pathogens can block the function of GEF’s resulting in the loss of RhoA activation such as viral peptide 3A [125].

In the kidney and in the rat passive Heymann nephritis (PHN) model of membranous nephropathy, complement C5b-9 induces nonlytic Glomerular Epithelial Cells (GEC) injury associated with morphological changes of GEC and proteinuria [64]. Because actin cytoskeleton is crucial for the maintenance of GEC morphology and function, implication of Rho-GTPases is of no surprise
Our current study has identified GEF-H1 as a RhoA GEF to be activated in podocytes in response to complement and responsible for RhoA activation contributing to proteinuria in vivo and morphological changes in vitro (thesis project- unpublished data).

All these examples illustrate the pivotal role of GEF’s in the progression of a vast majority of diseases. Because GEF’s are important in the activation of the small GTPases and in the specificity of downstream signaling, their inhibition by small molecules could potentially inhibit the uncontrolled activation of the small GTPases, especially in the context of disease.

1.3.5.5 Rho Guanine Nucleotide Exchange Factor-H1 (GEF-H1)

The Rho guanine nucleotide exchange factor GEF-H1 was first identified by screening a human HeLa cell cDNA library in 1998 [126]. GEF-H1 shows nucleotide exchange activity towards both RhoA and Rac1 [126], and localize to microtubules in many non-polarized cell lines[101, 127-129]. In a first study trying to unravel the mechanism by which GEF-H1 mediates RhoA activation by means of microtubule depolymerization in HeLa cells, it was shown that microtubule-bound GEF-H1 is inactive, and disassembly of microtubule networks, in response to an upstream stimulus, releases GEF-H1 and results in GEF-H1-mediated RhoA activation[101]. Please refer to Figure 1.4. In addition, overexpression of a dominant negative mutant of GEF-H1 or GEF-H1 knockdown using siRNAs, inhibited RhoA activation in response to microtubule disrupting agent, nocodazole[101, 130]. Thus, GEF-H1 is the first known mediator linking microtubule dynamics to RhoA activation and actin polymerization [101, 131].

GEF-H1 activity has been implicated in many cellular processes involving regulation of actin cytoskeleton. In the setting of epithelial barrier permeability, GEF-H1-mediated ROCKII signaling cascade was shown to be activated in calcium-depleted human epithelial cells leading to the physical disruption of apical junction complexes, which regulate epithelial barrier function [132]. The authors suggested that microtubule depolymerization in epithelial cells causes GEF-H1 activation, which thereby stimulates RhoA and leads to apical junction
complex disassembly [132]. Another mechanism of GEF-H1 activation was demonstrated in an attempt to understand renal tubular permeability [133]. Tumor necrosis factor alpha (TNF-alpha) is known to increase paracellular (between cell) permeability in tubular epithelial as a result of increased RhoA activation[134]. In that paper, the authors identified the ERK/GEF-H1/Rho/Rho kinase/phosphor-MLC pathway as the mechanism mediating TNF-alpha- induced tubular epithelial permeability[133]. The physiological importance of GEF-H1 in regulating RhoA-dependent signaling pathways has also been demonstrated in regulation of vascular endothelial permeability[128], dendritic spine morphology, mitosis, apoptosis, and cell motility [131].

Structural features of GEF-H1 include: 1) C1-domain, region containing zing-finger motif, 2) DH-domain, the catalytic domain of the protein responsible for the prompting loading of GTP on RhoA and Rac1, 3) PH-domain, located C-terminally to the DH-domain, and involved in microtubule binding and targeting the protein to specific subcellular compartments, 4) Coiled-coil domains, promoting recruitment of GEF-H1 to actin-based structures, and inducing oligomerization and protein-protein interactions, 5) Proline-rich regions and 14-3-3-binding sites, containing many phosphorylation sites by several kinases such as Aurora A, Pak1, ERK1 and/or ERK2. The function of the proline-rich regions in GEF-H1 is not fully understood. Please refer to Figure 1.5, for more details.

It is clear that the mechanisms of GEF-H1 are more complex than just microtubule binding status; they involve phosphorylation-dependent regulation, and protein-protein interactions. These mechanisms of action seem to be cell and/or stimulus context-specific, and thus, further studies are required to enlighten us on the detailed mechanisms of GEF-H1 activation, which will be important for a better understanding of the physiological and pathological roles of GEF-H1, and potentially consider it as a therapeutic target.
FIGURES FOR CHAPTER 1

Figure 1.1 The components of the foot process and the slit diaphragm.

Nephrin interacts with podocin, a transmembrane hairpin-like scaffolding protein and with CD2AP, an adapter protein. The nephrin complex induces actin polymerization. The actin cytoskeleton is stabilized by α-actinin-4, synaptopodin, INF2, and myosin 1E, and linked to the slit diaphragm through CD2AP. PLCε1 catalyzes the hydrolysis of phosphatidylinositol-4,5-bisphosphate (PIP2) and generates the second messengers inositol 1,4,5-triphosphate (IP3) and diacylglycerol (DAG). IP3 releases Ca2+ from the endoplasmic reticulum. The concentration of Ca2+ in the cytoplasm regulates actin polymerization. Transient receptor potential canonical (TRPC) 6 is a Ca2+ ion channel and has a role as a sensor of mechanically induced membrane stretch by translating mechanical tension to ion-channel action. The foot processes are anchored to glomerular basement membrane through integrins (predominantly α3β1 integrin) and α- and β-dystroglycans (DGs). Almost all the mutations of familial focal segmental glomerulosclerosis are slit-diaphragm components and/or the regulators of the actin cytoskeleton[135].

Abbreviation: INF2, inverted formin 2.
Antipodocyte antibodies targeting integral membrane proteins cross the basement membrane induce clustering of antigens, and shedding of immune complexes in the extracellular space. This process results in complement activation and formation of C5b–9 on the membrane of podocytes, which in turn leads to a variety of intracellular events, including production of reactive oxygen species and proteases, cytoskeletal changes and a stress response. In addition to forming immune complexes, antipodocyte antibodies can directly alter podocyte biology by activation of signaling pathways irrespective of complement activation. Under disease conditions, cytoplasmic proteins can be routed to the cell membrane, and a variety of neoantigens or neoepitopes may be induced by injury. Circulating antibodies raised against those antigens could be nephritogenic and implicated in the perpetuation of the disease. The multiple antigen–antibody systems that are generated could be involved at different time points and in different subcategories of patients with membranous nephropathy[46].
Figure 1.3 Signaling from Rho-GTPases to the cytoskeleton.

**Abbreviations:** LIM, acronym of the three gene products Lin-11, isl-1, and Mec-3; LIMK, LIM kinase; P-MLC, phosphorylated myosin II light chain; PAK, p21-activated kinase; MLCK, myosin light chain kinase; Arp2/3, actin-related protein 2/3; WASP, Wiskott–Aldrich syndrome protein; WAVE, WASP-family verprolin-homologous protein.
The diagram illustrates the regulation of Small GTPases, specifically Rho, Rac1, and Cdc42, and their roles in cellular processes.

- **Rho-GTP** is regulated by GEF (Guanine Nucleotide Exchange Factor) and GAP (Guanine Nucleotide Dissociation Inhibitor).
- **RhoA-GTP** activates Rho-kinase and mDia, leading to actin polymerization and stress fibers and focal adhesions.
- **Rac1-GTP** activates WAVE, leading to lamellipodia and filopodia outgrowth.
- **Cdc42-GTP** activates PAK1 and N-WASP, leading to Arp2/3 and filopodia formation.

These processes involve various downstream effectors and are interconnected, highlighting the complexity of cellular signaling pathways.
Figure 1.4 GEF-H1 activation by microtubule depolymerization.

Microtubule dynamic instability is characterized by the rates of growth and shrinkage, and the frequencies of catastrophe and rescue of the ends of individual microtubules. Stimulus-induced disassembly of microtubular networks results in GEF-H1-mediated activation of RhoA-dependent signaling pathways that facilitate paracellular permeability of epithelial barriers, antigen, presentation, dendritic spine morphology, mitosis, and cell motility[131].

Abbreviations: MT, microtubule.
Figure 1.5 Structural features of GEF-H1.

(a) GEF-H1 is the human homolog of murine Lfc. They are 88% identical overall at the amino acid level and share a similar domain structure.

C1 domain: This zinc-finger motif-containing region is similar to the C1 diacylglycerol (DAG) binding domain of the atypical protein kinase C (PKC) family.

Dbl-homology domain: A common feature of all members of the Rho-GEF family is a ~200 amino acid conserved region that confers enzymatic guanine nucleotide exchange activity. In GEF-H1, the DH domain interacts physically with RhoA and Rac1 but selectively promotes loading of GTP on RhoA.

Plextrin homology domain: Plextrin homology (PH) domains are found in many signaling proteins that bind phospholipids, such as phosphatidylinositol- 3,4,5-triphosphate (PtdIns(3,4,5)P3). In all Dbl family members, the PH domain is located C-terminally to the DH domain. In GEF-H1, the PH domain is involved in microtubule binding and targeting of the protein to different subcellular compartments, such as tight junctions.

Coiled-coil domains: a-helical coiled-coil (CC) structures are found in a multitude of proteins. They can provide mechanical stability and also promote oligomerization and protein–protein interactions.

Proline-rich regions and 14–3-3–binding sites: Two short proline-rich (PR) stretches that can be recognized by Src-homology 3 (SH3) domains flank a central 14–3-3 protein binding motif resembling the consensus sequence RSXpS885XP (where pS885 is phosphorylated). Several kinases, including Aurora A and Pak1, have been shown to phosphorylate Ser885 and induce 14–3-3 binding to GEF-H1. Recruitment of 14–3-3 proteins to GEF-H1 correlates with reduced catalytic activity in some cases, although this has not been explicitly demonstrated. Accessibility of the 14–3-3 binding motif might be regulated by other C-terminal phosphorylation sites, (e.g. Ser959 by Cdk1). An enhancement of GEF-H1 exchange activity towards RhoA has been reported as a result of the
phosphorylation of Thr678 by ERK1 and/or ERK2; the mechanism remains unknown. The function of the proline-rich regions in GEF-H1 is not yet clear.

Abbreviations: Akt, kinase product of the Akt oncogene; AurA and AurB, Aurora kinases A and B; Cdk1, cyclin-dependent kinase 1; GSK3, glycogen synthase kinase 3; Pak1 and Pak4, p21-activated kinases 1 and 4; PKA, protein kinase A or cAMP-dependent protein kinase.

(b) GEF-H1 exists in an active form when it is not bound to microtubules. Studies using deletion mutants and isolated domains of GEF-H1 suggested that interactions between the C1 and the PH domain of GEF-H1 are required for microtubule binding and hence inhibition of GEF-H1, most probably by blocking access of the DH domain to its substrate (inhibited state). However, a requirement for the GEF-H1 C terminus for full inhibition of GEF-H1 activity upon microtubule binding was also noted[131].
TABLES FOR CHAPTER 1

Table 1.1. Podocyte proteins: structure, function and associated disease.
<table>
<thead>
<tr>
<th>Structure component</th>
<th>Site</th>
<th>Function</th>
<th>Associated disease</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nephrin</td>
<td>Slit diaphragm</td>
<td>Regulation of podocyte actin dynamics</td>
<td>Congenital nephrotic syndrome of the Finnish type (CNF)</td>
<td>[136]</td>
</tr>
<tr>
<td>Podocin</td>
<td>Slit diaphragm</td>
<td>Adaptor protein mediating the connection of slit diaphragm to the actin cytoskeleton</td>
<td>Corticosteroid-resistant nephrotic syndrome</td>
<td>[137]</td>
</tr>
<tr>
<td>PLCε1</td>
<td>Cytoplasm: podocyte cell bodies, major and intermediate processes</td>
<td>Unknown</td>
<td>Inherited Nephrotic syndrome</td>
<td>[138]</td>
</tr>
<tr>
<td>α-actinin4</td>
<td>Cytoskeleton</td>
<td>Actin binding protein</td>
<td>FSGS</td>
<td>[139]</td>
</tr>
<tr>
<td>TRPC6</td>
<td>Slit diaphragm</td>
<td>Regulation of calcium homeostasis</td>
<td>FSGS</td>
<td>[140] [141]</td>
</tr>
<tr>
<td>Wilms Tumor-1 (WT-1)</td>
<td>Nuclear</td>
<td>Important during development</td>
<td>Deny's Drash syndrome (DDS) Frasier's syndrome (FS)</td>
<td>[142, 143]</td>
</tr>
<tr>
<td>CD2AP</td>
<td>Slit diaphragm</td>
<td>Adaptor protein mediating the connection of slit diaphragm to the actin cytoskeleton</td>
<td>Sporadic FSGS Nephritic syndrome in K.O mice</td>
<td>[144, 145]</td>
</tr>
<tr>
<td>Synaptopodin</td>
<td>Cytoskeleton</td>
<td>Regulator of RhoA signaling and cell migration</td>
<td>Sporadic FSGS</td>
<td>[146]</td>
</tr>
<tr>
<td>Myosin 1E</td>
<td>Basolateral membrane and slit diaphragm</td>
<td>Important for podocyte motility and may also stabilize the podocyte cytoskeleton</td>
<td>Childhood FSGS</td>
<td>[147]</td>
</tr>
<tr>
<td>MYH9</td>
<td>Cytoskeleton</td>
<td>Interacts statically with F-actin to maintain membrane tension</td>
<td>Epstein syndrome Fechtner syndrome</td>
<td>[148, 149]</td>
</tr>
<tr>
<td><strong>Gene Name</strong></td>
<td><strong>Location</strong></td>
<td><strong>Function</strong></td>
<td><strong>Disease</strong></td>
<td><strong>Reference</strong></td>
</tr>
<tr>
<td>--------------</td>
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<td>-------------</td>
<td>---------------</td>
</tr>
<tr>
<td>APOL1</td>
<td>Unknown</td>
<td>Unknown</td>
<td>End stage renal disease (non-diabetic)</td>
<td>[150, 151]</td>
</tr>
<tr>
<td>LIM-homeodomain protein (LIMX1B)</td>
<td>Nuclear</td>
<td>Transcriptional regulation of collagen expression by podocytes</td>
<td>Nail-patella syndrome</td>
<td>[152]</td>
</tr>
<tr>
<td>Zonula Occludens-1 (ZO-1)</td>
<td>Basolateral and slit diaphragm</td>
<td>Linker protein for the attachment of slit diaphragm to actin cytoskeleton. Membrane associated guanylate kinase protein</td>
<td>Unknown</td>
<td>[153]</td>
</tr>
<tr>
<td>Glomerular Epithelial protein-1 (GLEPP-1)</td>
<td>Apical cell surface</td>
<td>Important for charge and size of filtration characteristics of podocytes</td>
<td>GLEPP-1−/− mice had reduced glomerular filtration function and a tendency to hypertension. IgA Nephropathy</td>
<td>[154, 155]</td>
</tr>
<tr>
<td>Inverted Formin-2 (INF-2)</td>
<td>Cytoskeleton</td>
<td>Important for the regulation of actin de/polymerization</td>
<td>FSGS</td>
<td>[156]</td>
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</tbody>
</table>
RATIONALE AND OBJECTIVES

Rationale

There is no doubt that the actin cytoskeleton is central to podocyte function and that Rho-GTPases play an important role in cytoskeletal regulation. Over the last several years, the role of the Rho-GTPases in podocyte morphology and function has been unfolding and there is now compelling evidence that the Rho-GTPase, RhoA, is an important signaling element in the podocyte. In addition, it has been also shown that RhoA plays a role downstream of many actin derangement stimuli. We have previously shown that RhoA is activated by complement in GEC, and that RhoA activation in podocytes in mice results in proteinuria. However, the intracellular molecular mechanisms by which RhoA is activated in pathological conditions are still unclear. RhoA activation is generally regulated by the group of enzymes called GEFs. Among them, GEF-H1 appears to have an important pathological role in the disruption of the junctional complexes and epithelial barrier in tubular epithelial cells. The main goal of the work presented in this thesis was to investigate the role of GEF-H1 in complement-induced RhoA activation in GEC.

Objectives

1. Investigate the activation of GEF-H1 in vivo.
2. Investigate the activation of GEF-H1 in cultured podocytes.
3. Study the impact of GEF-H1 gene knockdown on cell function and/or behavior.
4. Study the possible contribution of extracellular signal-regulated kinase (ERK) to GEF-H1 activation by complement.
5. Study the intracellular localization of GEF-H1 in GEC
Chapter 2

Role of Guanine Nucleotide Exchange Factor-H1 in Complement-mediated RhoA Activation in Glomerular Epithelial Cells

Flaviana Mouawad, Lamine Aoudjit, Ruihua Jiang, Andrey V. Cybulsky, Katalin Szaszi, Tomoko Takano

Will be submitted to the American Journal of Physiology-Renal Physiology
PREFACE TO CHAPTER 2

We have previously shown that complement induces increased RhoA activity in cultured GEC and the rat model of podocyte injury and proteinuria, passive Heymann nephritis (PHN) presented with a significantly increased glomerular RhoA activity concomitant with proteinuria. Furthermore, podocyte specific expression of CA (constitutively active)-RhoA in cultured rat GEC and mouse podocytes (MP) induced cell contraction and rounding in adult mice. The mechanisms by which RhoA is activated in complement-mediated GEC and glomeruli are still unclear. We propose that GEF-H1, a Rho GEF, activates RhoA in response to complement stimulation. In this study we used rat glomeruli and cultured GEC to study the implication of GEF-H1 in complement-mediated RhoA activation in addition to the mechanism by which GEF-H1 is activated.
CONTRIBUTION OF AUTHORS

This thesis is a manuscript-based structure. According to the faculty regulations, manuscripts co-authored by authors must be accompanied with an explicit statement as to who contributed to such work and to what extent.

The candidate performed all the experiments described in chapter two entitled “Role of guanine nucleotide exchange factor-H1 in complement-mediated RhoA activation in glomerular epithelial cells” except for the induction of rats with PHN, which was done by Lamine Aoudjit. All FRET experiments have been taught and supervised by Ruihua Jiang. The candidate wrote the manuscript with support from Dr. Tomoko Takano. The manuscript will be submitted to the American Journal of Physiology-Renal Physiology.
Role of Guanine Nucleotide Exchange Factor-H1 in Complement-mediated RhoA Activation in Glomerular Epithelial Cells.

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Will be submitted to the American Journal of Physiology- Renal Physiology

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INTRODUCTION

Glomerulus is the filtration unit of the kidney where the first step of urine formation occurs. It filters water and small solutes such as waste products while retaining large proteins essential for body function such as albumin. When this barrier function is impaired, protein leakage into the urine (proteinuria) occurs. Proteinuria is not only a marker of glomerular injury but also a prognostic predictor for progression of renal failure. Visceral glomerular epithelial cells (GEC), commonly known as podocytes, are highly specialized epithelial cells residing on the outside surface of the glomerular capillary loop and have an important role in maintaining the barrier function of the glomerulus [1]. They consist of three morphologically and functionally distinct parts: large cell body, major processes rich in microtubules, and foot processes consisting of an actin-based cytoskeleton [2]. Foot processes from adjacent podocytes form extensive interdigitations and are linked by a structure called the “slit diaphragm”. These intricate structures are essential for maintaining proper glomerular permselectivity and loss of this interdigitating pattern (“foot process effacement”) is the hallmark of many glomerular diseases accompanied by various degree of proteinuria [2]. In the last decade, many proteins expressed in podocytes have been identified that regulate the actin cytoskeleton either directly or indirectly [157]. Mutations of these proteins change the actin cytoskeleton from parallel, contractile bundles into a dense meshwork of short disorganized actin filaments [157]. Therefore, the regulation of actin cytoskeleton and dynamics are crucial for normal physiology of podocytes.

Nephrotic syndrome is a disease featured by massive proteinuria, hypoalbuminemia, and edema and is primarily caused by GEC injury. Membranous nephropathy is the most frequent cause of idiopathic nephrotic syndrome in adults and is caused by complement-mediated GEC injury. The rat model of passive Heymann nephritis (PHN) has been extensively used to mimic and study the pathophysiology of membranous nephropathy [158]. In PHN
heterologous antibody binds to GEC antigens forming immune complexes [44]. Following subepithelial immune complex formation, the complement system is activated with the assembly of C5b-9 membrane attack complex [44]. Nucleated cells require multiple C5b-9 lesions for lysis, but at lower doses, C5b-9 induces sublethal (sublytic) injury and various metabolic effects [31]. Previous study reported complement-dependent disruption of actin microfilaments in cultured GEC [159], which may explain the altered cell-matrix and impaired permselectivity that occurs in podocyte effacement in membranous nephropathy [159].

Rho family of small GTPases consists of more than 22 members out of which RhoA, Rac1, and Cdc42 are the most extensively studied prototypes [160, 161]. They are important mediators of actin cytoskeleton dynamics, as they regulate changes in cell morphology, motility, adhesion, and malignant transformation [160, 161]. As demonstrated first in fibroblasts, the RhoA pathway mediates formation of stress fibers, whereas Rac1 and Cdc42 mediate the formation of lamellipodia (membrane ruffles, mainly seen at the leading edge of a motile cell) and filopodia (short cell protrusions), respectively. In addition, they play key roles in many other biological processes including apoptosis, gene transcription, cell cycle progression, and endocytosis [160, 161]. Rho proteins act as bi-molecular switches cycling between two states: active (GTP-bound) form, which can bind to effectors and activate downstream pathways, and the inactive (GDP-bound) form. These conformational states are tightly regulated by three families of proteins, GEF (guanine nucleotide exchange factors), GAP (GTPase-activating proteins), and GDI (guanine nucleotide dissociation inhibitors).

The GEF family of proteins promotes exchange of GDP for GTP in response to upstream signals [162] and ensures the proper spatio-temporal activation of Rho-GTPases[59]. To date more than 80 members of the GEF family have been identified in humans, outnumbering those of the Rho-GTPase family [59]. This unequal partition is likely to reflect the complex and context-specific mechanisms of action of GEFs but the substrate specificity and the activation mechanisms of GEF proteins are largely unknown [59]. GEF-H1 (ArhGEF2, also
referred to as Lfc in mice) is one of the few GEF proteins that have been relatively well characterized [131]. It was initially described as an exchange factor for RhoA and Rac1, although its role as a Rho exchange factor is much better characterized [126] and reviewed in [131]. Previous studies have shown an important role of GEF-H1 in the regulation of paracellular permeability in epithelial and endothelial cells [128, 163]. GEF-H1 was also shown to disassemble the apical junctional complexes and the epithelial barrier in renal tubular epithelial cells through activation of RhoA and its subsequent effector, Rho-kinase II (ROCKII) [132]. GEF-H1 is also a target of TNF-alpha signaling in renal tubular epithelial cells, resulting in RhoA activation and increased paracellular permeability [133]. GEF-H1 may thus play a pathological role in the disruption of junctional complexes and epithelial cell barrier.

We have previously reported that complement activates RhoA in GEC in vitro and in vivo [64, 65], but the mechanisms involved remained unknown. The aim of the current study is to identify the upstream signaling mechanism involved in complement-induced RhoA activation in GEC, and to explore a potential role of GEF-H1.
MATERIALS AND METHODS

Materials

Tissue culture media and Lipofectamine 2000 were from Invitrogen-Life Technologies (Burlington, ON). Electrophoresis reagents were from Bio-Rad Laboratories (Mississauga, ON). Enhanced Chemiluminescence (ECL) detection reagent and glutathione-Sepharose beads were from Amersham Bioscience (Baied’Urfé, QC). Protease Inhibitor Cocktail was from Roche Diagnostics (Montreal, QC). RhoA G-LISA™ Activation assay luminescence format was from Cytoskeleton (Denver, CO). The plasmids pLKO.1-TRC cloning vector, pMD2.G, psPAX2, and lentiviral shRNA GEF-H1 were from Addgene (Cambridge, MA) [164]. Male Sprague-Dawley rats were from Charles River Canada (Saint-Constant, QC). Antibodies for GEF-H1, phospho-myosin light chain 2 (Ser19), phospho- and total p44/42 MAPK (ERK1/2) were from Cell Signaling (Beverly, MA). Anti α-tubulin was from Abcam (Cambridge, MA). Anti-RhoA was from EMD Millipore (Billerica, MA). Taxol was from Bioshop (Canada, CA). Human C8-deficient serum, purified human C8, and other chemicals were from Sigma-Aldrich (Mississauga, ON). U0126, AG1478 and PP2 were from EMD Biosciences (Mississauga, ON). GST-RhoAG17A was from Dr. K. Burridge (University of North Carolina, Chapel Hill) [165, 166]. FRET-pRaichu1298x probe was from Dr. M. Matsuda, Osaka University [167]. The GFP-tagged wild type (WT) GEF-H1 and the point mutant T678AGEF-H1T678A were gifts from Dr. M. Kohno [168].

Podocyte culture and transfection

Rat GEC culture and characterization were described previously [169, 170]. Briefly, GEC were cultured in K1 medium (50% DMEM, 50% Ham-F12, 10% Nu Serum, 1% hormone mix) and experiments were carried out between passages 10 and 70. A subclone of GEC that grows on plastic substratum (GEC-
pl) was used for all experiments. GEC-pl were transiently transfected with 0.5 μg per 35-mm plate of DNA [GFP-GEF-H1-WT or GFP-GEF-H1-T678A and a 1:3 ratio of DNA and Lipofectamine 2000 transfection reagent).

Immortalized mouse podocyte (MP) were also used. MP cell culture and morphology has been previously described[171]. Briefly, MPs were cultured in medium containing RPMI 1640, 10% Fetal Bovine Serum (FBS), and 1% Penicillin/Streptomycin (P/S).

**RT-PCR**

Total RNA was extracted from GEC-pl or rat glomeruli using the RNeasy Mini Kit (QIAGEN, Valencia, CA). Synthesis of cDNA was performed with 1 μg of total RNA using the Reverse Transcription Reagents Kit (QIAGEN, Valencia, CA). Primers used for PCR are listed in Table 1. PCR was carried out with DNA Polymerase (ZmTech Scientifique Montreal, QC) by 40 cycles of the following conditions (5 seconds at 98°C, 30 seconds at 65°C, 30 seconds at 72°C). GAPDH was used as a house keeping control.

Primers used are as indicated in Table 2.1.

**Incubation of GEC with complement**

Complement stimulation of GEC was previously described [169, 170]. Briefly, GEC-pl were incubated with anti-GEC antiserum or sheep anti-Fx1A antiserum (5% vol/vol) for 40 min at room temperature, followed by incubation at 37°C with normal human serum (NS; 1, 1.5 or 2.5% vol/vol) to assemble C5b-9 or, in control, with decomplemented, heat-inactivated serum (HIS; 56°C, 1 h) for the indicated times. In some experiments, antibody sensitized GEC were incubated with C8-deficient serum (C8D; 1.5% vol/vol) with or without reconstitution with purified C8 (2 μg/ml in undiluted serum).
Induction of PHN

PHN was induced in male Sprague-Dawley rats by a single intravenous injection of sheep anti-Fx1A antiserum (400 μl/rat) as described previously [172]. 14 days post-injection, rats were sacrificed and glomeruli were collected by differential sieving [173]. All studies were approved by the McGill University Animal Care Committee.

Immunoblotting

Cultured GEC-pl or glomeruli were washed once and lysed with ice-cold lysis buffer [20 mM HEPES (pH 7.5), 5 mM MgCl₂, 150 mMNaCl, 1% Triton-100, 1 mM PMSF, 1mM DTT containing Protease Inhibitor Cocktail]. After insoluble components were cleared by centrifugation (14 000 rpm, 10 min 4°C), supernatant concentration was determined using commercial reagent (Bio-Rad). Equal amounts of proteins were separated by SDS-PAGE under reducing conditions and electrophoretically transferred to nitrocellulose membranes. Membranes were block with either 5% BSA or skim milk and incubated with primary antibody overnight at 4°C. After three washes, membranes were incubated with secondary antibodies conjugated with horseradish peroxidase for 1 h at room temperature. Immunoreactive proteins were then detected by ECL. Densitometric analysis was carried using the Image J software. The following antibody dilutions were used: rabbit anti-GEF-H1 1:1000, mouse anti-tubulin 1/100,000, mouse anti-RhoA 1:1000, mouse anti-P-ERK 1:2000, mouse anti-ERK 1:2000, mouse anti-P-MLC 1:1000, anti-mouse and rabbit secondary antibodies: 1:2000.

Affinity precipitation assay for active GEF-H1

GST-G17A-RhoA, a mutant with high affinity for activated GEFs was described previously [165]. The assay was performed as in[165] with minor modifications. Briefly, treated cells or glomeruli were lysed in lysis buffer (20 mM HEPES, pH 7.5; 150 mM NaCl; 5mM MgCl₂; 1% TX-100; 1mM DTT; 1mM
PMSF, protease inhibitors). Equal amounts of protein (200-900 μg) were incubated with purified GST-G17A-RhoA (15-20 μg) bound to glutathione-Sepharose beads for 1 h at 4°C. After incubation, beads and protein were washed three times with lysis buffer and were subjected to SDS-PAGE (7.5%) and immunoblotting. X-ray films were scanned and band intensities were quantified by ImageJ. Active GEF-H1 was normalized by total GEF-H1 and the values of HIS were further normalized to 1.

Live cell imaging and fluorescence resonance energy transfer (FRET) microscopy

GEC-pl were plated on 35 mm glass bottom dishes (MatTek Corp, Ashland, MA) and transfected with 0.5 μg of FRET Raichu1298x probe (pCFP-RBD-pYFP). FRET measurement was carried out 48 hours after transfection following overnight serum starvation using an inverted microscope (IX81; Olympus). After incubation with anti-GEC antiserum for 40 min at room temperature, cells were placed in the microscope chamber and pre-warmed medium alone was added. After a baseline image was obtained, pre-warmed medium containing NS or HIS (for control) was added to the cells to reach a final concentration of 2.5% vol/vol. Corrected FRET (cFRET) intensity was calculated at each pixel using Metaphorph Software (Molecular Devices, Sunnyvale, CA) using the following formula: (rawFRET-background)/(CFP-background). cFRET was converted to pseudocolour pixel-to-pixel.

Immunofluorescence

Two days after transfection, cells were fixed in 4% paraformaldehyde for 15 min at room temperature, permeabilized with 0.5% Triton X-100 in PBS, and blocked in 3% BSA in PBS for 20 min. α-tubulin was stained with mouse anti-α-tubulin antibody (1:4000) and rhodamine-conjugated goat anti-mouse antibody (1:40, Jackson Immunoresearch, Inc). Nuclei were stained with DAPI (Invitrogen). Images were captured using an AxioObserver-100 microscope.
(Zeiss). Quantification of GEF-H1 localization was done by attributing a “yes” or “no” to transfected cells for showing perinuclear distribution. One hundred cells were counted for each condition and the experiments were repeated three times.

**RhoA activity Assay**

RhoA activity was determined using RhoA G-LISA Activation Assay in the luminescence format (Cytoskeleton, Denver, CO) according to the manufacturer’s protocol. Briefly, treated cells were lysed on ice, cleared by centrifugation, and snap-frozen in liquid nitrogen. Prior to the assay, lysates were thawed and protein concentration was adjusted to 2 μg/μL. Equal amounts of lysates were added to the Rho-GTP affinity plate and incubated for 30 min at 4°C. After washes, each well was incubated with anti-RhoA antibody for 45 min at room temperature and with a secondary antibody containing a detection reagent for another 45 min at room temperature. Absorbance at 490 nm was quantified using a microplate spectrophotometer. The same lysates were analyzed by immunoblotting to ensure that the same amounts of total RhoA were contained in the lysates.

**Transduction of GEC with lentiviral particles**

Lentiviral particles were produced according to the manufacturer’s protocol. Briefly, low-passage HEK293T cells were transfected with pLKO.1-TRC Cloning vector plasmid (control) or GEF-H1 shRNA plasmid, the packaging plasmid psPAX2, and the envelope plasmid pMDG.2 at a ratio of 4:3:1 with Lipofectamine 2000 transfection reagent. The medium was replaced with fresh media 18 h after transfection, and viral particles contained in the media were collected twice at 24 h intervals. Infection of the target cells with lentiviral particles was used at a multiplicity of infection of 5. GEC-pl were exposed to lentivirus encoding GEF-H1 shRNA for 24 h in cell culture medium. Parallel infection with pLKO.1-TRC cloning vector was used as control. All experiments were performed at 48h after the virus induction was started.
Measurement of complement–dependent cytotoxicity

Complement-induced cytotoxicity was assessed by measuring the amount of lactate dehydrogenase (LDH) release in cultured GEC-pl as described previously [174]. Specific release of LDH was calculated using the formula (NS-HIS)/(100-HIS) x100, where NS and HIS represent the percentage of LDH release with NS and HIS, respectively.

Statistics

Data are presented as mean ± SEM. The t-test statistic was used to determine significant difference between two groups. Two-way ANOVA was used to determine significant difference in multiple measurements among groups.
RESULTS

Complement C5b-9 activates GEF-H1 in GEC in vivo

We reported previously that RhoA is activated in the glomerulus in rats with PHN, a model of complement C5b-9-mediated GEC injury and proteinuria [64]. To address the mechanisms involved in RhoA activation, we first wished to investigate whether the Rho exchange factor GEF-H1 is activated in our model of PHN. Active GEF-H1 in glomeruli was detected using affinity precipitation with GST-G17A-RhoA. The G17A mutant of RhoA represents a nucleotide-free form of RhoA that has a high affinity to active GEFs and therefore the amount of a particular GEF binding to GST-G17A-RhoA reflects the amount of activated form of the GEF[165]. Fourteen days after the induction of PHN, rats showed significant proteinuria (384 ± 25 mg/day, n = 5) compared with normal rats (5 ± 2 mg/day, n = 4, P< 0.01 vs. PHN; Figure 2.1A). Control rat glomeruli showed a well-detectable small basal GEF-H1 activity, which was increased significantly by 2.3 fold in PHN (Control 1 ± 0.5, n = 4, PHN 2.3 ± 0.5, n = 5, arbitrary unit, P<0.01; Figure 2.1A). There was no significant difference in the total amount of GEF-H1 (Figure 2.1A). Phosphorylation of MLC, a downstream effector of RhoA and Rho-kinase, was also increased significantly in glomeruli from PHN rats as compared with control (Control 1 ± 0.5, n = 4, PHN 2.5 ± 0.6, n = 6, arbitrary unit, P<0.01; Figure 2.1B) consistent with our previous report that glomerular RhoA activity is increased in PHN [64]. These results are consistent with a notion that complement C5b-9 activates GEF-H1 in GEC in vivo, leading to the activation of RhoA and its downstream effectors.

Complement C5b-9 activates GEF-H1 in GEC in vitro

To further study the mechanisms of GEF-H1 by complement, we next explored complement-induced GEF-H1 activation in two podocyte cell lines. Both rat GEC and immortalized mouse podocytes expressed GEF-H1. The RhoG17A pulldown assay demonstrated that GEF-H1is somewhat active under basal, unstimulated conditions (Figure 2.2A, heat-inactivated serum (HIS)).
Complement activation was induced by serial incubation with antibody and serum (1-2%) that assemble a sublytic concentration of complement C5b-9. Complement activation increased GEF-H1 activity significantly, compared with control (Figure 2.2A, normal serum (NS)), without significantly affecting the total expression of GEF-H1 (Figure 2.2A). GEF-H1 activation started at 15 min after the addition of the serum and continued up to 30 min (Figure 2.2B).

In order to verify that the complement-induced GEF-H1 activation was dependent on assembly of the full C5b-9 complex, we compared the effects of C8-deficient human serum (C8D), which assembles C5b-7 only, with C8 reconstituted serum, into which purified C8 was readded to allow the assembly of C5b-9. When antibody-sensitized GEC were incubated with C8D alone, the amount of active GEF-H1 was not different from control (HIS, not shown) (Figure 2.2C). However, when cells were exposed to the C8D that was reconstituted with purified C8, active GEF-H1 increased significantly (~1.7 fold) (Figure 2.2C), similar to cells stimulated with NS. These results substantiate that complement-induced GEF-H1 activation is dependent on C5b-9 assembly.

It was previously reported that sublytic complement stimulation leads to phosphorylation of ERK [175]. To verify the efficiency of complement stimulation in this experiment, GEC lysates were immunoblotted for P-ERK. All different time points of serum incubation induced prominent and consistent phosphorylation of ERK, indicating a proper stimulation of complement (Figure 2.2D). Total amounts of GEF-H1 and ERK displayed no significant changes. Therefore, complement-induced GEF-H1 activation occurs at 15 min of treatment, and continues to be increased by complement.

**Complement activates RhoA in GEC in a similar time course to GEF-H1 activation**

We reported previously that RhoA activity was increased significantly in rat GEC stimulated with complement for 40 min[64]. In order to establish the spatial-temporal profile of complement-induced RhoA activation, we utilized a
probe that detects RhoA activating through fluorescence resonance energy transfer (FRET). The FRET probe used (Raichu1298x) contains a yellow fluorescent protein (YFP), a Rho binding domain (RBD), a wild-type RhoA, and a cyan fluorescent protein (CFP) in tandem. Upon activation (GTP binding) RhoA within the probe binds to the RBD. This change brings CFP and YFP to close proximity, allowing energy to be transferred from CFP to YFP, resulting in FRET. Thus, the FRET signal intensity correlates with the RhoA activity in a specific site within a cell (Figure 2.3A) [176]. GEC were transfected with Raichu1298x and changes in fluorescent monitored. When antibody-sensitized cells were exposed to serum, RhoA activity started to increase at around 15 min and continued to increase up to 30 min (Figure 2.3B, NS). The change was especially prominent in the perinuclear area of the cells. In contrast, control cells exposed to decomplemented serum did not show RhoA activation (Figure 2.3B, HIS). Thus, complement induced localized activation of RhoA in a time course that parallels changes in GEF-H1 activity.

**GEF-H1 mediates complement-induced RhoA activation in GEC and protects against cytotoxicity**

Having established that GEF-H1 is expressed and activated by complement in rat GEC *in vivo* and *in vitro*, we next studied the functional significance of GEF-H1 activation. Using lentivirus-mediated transduction of an shRNA, we achieved a significant reduction in the expression of GEF-H1 in rat GEC (~55% knockdown, Figure 2.4A). Basal as well as complement-stimulated RhoA activities were significantly lower following GEF-H1 silencing as compared with control cells (Figure 2.4B). GEF-H1 silencing did not affect RhoA expression (not shown). These data support that GEF-H1 plays an important role in complement-induced RhoA activation in GEC.

We next studied the functional consequence of GEF-H1 knockdown. To test complement-induced cytolysis, GEC with or without GEF-H1 knockdown were treated with lytic concentrations of complement (NS 5% and 10%).
Cytolysis was quantified by measuring the released lactate dehydrogenase (LDH) in the culture medium. Complement-induced cytolysis was augmented significantly in GEF-H1 silencing, as compared with control cells (Figure 2.4C). Similar results were obtained when cell viability was quantified by Cell Titer Blue kit (data not shown). These results suggest that GEF-H1 and subsequent RhoA activation is protective against complement-induced cell death (see below for discussion).

**Complement-mediated GEF-H1 activation is mediated by ERK but not by the microtubules**

It was shown previously that GEF-H1 is phosphorylated by extracellular signal-regulated kinase (ERK) at T678, and this phosphorylation is necessary for tumor necrosis factor alpha (TNF-alpha)-induced GEF-H1 activation in tubular cells [133, 168]. As shown before by us, complement C5b-9 activates ERK in rat GEC (Figure 2.2D)[175]. Moreover, the kinetics of complement-induced ERK and GEF-H1 activation are similar. To test whether ERK contributes to GEF-H1 activation by complement, we used the pharmacological inhibitor of the ERK pathway (U0126, inhibitor of the ERK activator, MEK1/2, 50 μM)[177]. U0126 significantly reduced the activation of GEF-H1 by complement, although the inhibition was not complete (Figure 2.5A). Similar results were obtained with another MEK1/2 inhibitor, PD98059 (data not shown). These results indicate that complement-induced GEF-H1 activation is mediated by ERK activation.

In tubular cells the receptor tyrosine kinase, epidermal growth factor receptor (EGFR) was required for TNFα-induced activation of the ERK/GEF-H1/RhoA pathway [178]. In addition, the Src-family of tyrosine kinases was also required for the activation of this pathway [178]. Thus we tested if the EGFR and the Src family kinases also mediated complement-induced GEF-H1 activation. Our data show, that neither the EGFR inhibitor, AG1478, nor the Src family inhibitor, PP2, affected the basal or complement-stimulated GEF-H1 activities in GEC (Figure 2.5B). Thus, complement activates GEF-H1 through signaling
pathways that include ERK activation, but independent of the EGFR and Src.

GEF-H1 is also known to be a microtubule-associated nucleotide exchange factor of the Dbl family of proteins [126]. In 2002, Krendel et al suggested a mechanism of GEF-H1 regulation by microtubule dynamics [101]. They proposed that microtubule depolymerization could liberate the bound inactive GEF-H1, allowing activation of RhoA in many cellular processes, such as endothelial barrier permeability, migration, and dendritic spine morphology [101, 131]. Thus we next tested the role of the microtubules in complement-induced GEF-H1 activation. Neither the microtubule-disrupting agent, nocodazole, nor the microtubule-stabilizing agent, taxol, exerted an effect on basal or complement-stimulated GEF-H1 activities Figure 2.5C). These results suggest that complement activates GEF-H1 independently of microtubule dynamics (see below for discussion).

GEF-H1 translocates to the perinuclear regions upon stimulation with complement in GEC

In order to study the intracellular localization of GEF-H1 in GEC, we transfected GEF-H1 tagged with GFP (GFP-GEF-H1-WT) into cultured rat GEC. In unstimulated cells, GEF-H1 was distributed diffusely in the cytosol, and showed some colocalization with the microtubule filaments (Figure 2.6A, HIS). When transfected cells were stimulated with complement (NS), many cells demonstrated a distinct perinuclear distribution of GEF-H1 (Figure 2.6A). Morphological semi-quantification (see Methods) showed that 43 % complement-stimulated cells showed a distinct perinuclear distribution of GEF-H1, as compared to 13% in control cells (Figure 2.6A). It was noted that complement also altered the structure of the microtubules; in unstimulated cells, microtubules were hair-like filaments projecting from the nucleus to the cell periphery, whereas in complement-stimulated cells, a prominent condensation of the tubulin staining was observed in the perinuclear region (Figure 2.6A, HIS vs. NS). As a consequence, there was a clear overlap of GEF-H1 and tubulin in the perinuclear
region (Figure 2.6A, HIS). To determine if the perinuclear translocation of GEH-H1 is dependent on the microtubules, cells were treated with nocodazole or taxol prior to the stimulation with complement. Nocodazole caused an unwinding of the microtubule filaments, whereas taxol stabilized and strengthened them, demonstrating the efficacy of the two drugs. Nonetheless, these two drugs did not affect the perinuclear translocation of GEF-H1 induced by complement (Figure 2.6A). These results indicate that complement-induced perinuclear translocation of GEF-H1 is independent of the microtubules.

In the previous experiments, we showed that complement-induced GEF-H1 activation was, at least in part, dependent on ERK (Figure 2.5A). We next studied whether ERK also has a role in the subcellular localization of GEF-H1. The MEK1/2 inhibitor, U0126, decreased complement-induced perinuclear translocation of GEF-H1 by ~20%, while it had no effect on the microtubule structure in unstimulated and complement-stimulated GEC (Figure 2.6B). In order to test if the effect of the ERK pathway inhibition was directly on GEF-H1, we next utilized the T678A mutant of GEF-H1 [168]. T678 of GEF-H1 is the known phosphorylation site by ERK and the T678A mutant of GEF-H1 was shown to lose the ability to be activated by TNF-alpha, [133]. GFP-GEF-H1-T678A expressed in GEC showed diffuse cytosolic distribution and colocalization with microtubules, similar to the wild-type GEF-H1 (Figure 2.6B). However, when cells were stimulated with complement, perinuclear translocation was significantly blunted (by ~25%), similar to the effect of the U0126 (Figure 2.6B). These results suggest that the change in GEF-H1 intracellular distribution in response to complement is, at least in part, dependent on ERK. Moreover, ERK is likely exerting its effects by phosphorylating the T678 residue of GEF-H1.

**Supplemental Information**
Expression of GEF genes in rat GEC

To evaluate the expression of the most extensively studied GEFs in rat GEC, we isolated RNA from rat glomerular epithelial cells (GEC) and rat glomeruli and performed reverse transcription polymerase chain reaction (RT-PCR). Transcripts of p190 RhoGEF, GEF-H1, Net1, LARG but not p115RhoGEF genes were detected in both rat GEC and rat glomeruli (Supplemental Figure 1).
DISCUSSION

In the present study, we demonstrated a role of GEF-H1 in complement-mediated GEC injury. We showed that in the glomerulus from rats with PHN (Figure 2.1), and in complement-stimulated cultured GEC (Figure 2.2), GEF-H1 activity was increased coinciding with RhoA activation (Figure 2.3). Activation of GEF-H1 by complement was at least partially dependent on the ERK pathway (Figure 2.5A) but not on Src-family kinases (Figure 2.5B), or the microtubules (Figure 2.5C), or the EGF receptor. GEF-H1 knockdown effectively blocked RhoA activation and augmented cytotoxicity induced by complement in GEC (Figure 2.4).

More than 80 human GEFs have been identified, outnumbering the Rho-GTPases, suggesting that GEFs have important regulatory roles on Rho-GTPase activities in a cell and stimulus-dependent manner. However, the information on the role of GEFs in podocytes is limited. Leukemia-associated RhoGEF (LARG or ArhGEF12) was recently reported to be enriched in a glomerular expression library prepared from human kidney [179]. A recent study showed that the scaffold protein, WT1-interacting protein (Wtip), activates RhoA, in cultured mouse podocytes leading to stress fiber formation, and that ArhGEF12 was implicated in Wtip-induced RhoA activation [180]. Podocyte-specific deletion of αPKCλ/ι (an isoform of atypical protein kinase C) in mice caused proteinuria and severe glomerulosclerosis[181], which was attributed to the upregulation of Def-6, a RacGEF[182-185]. It is likely that the roles of GEFs are complex and dependent on the target Rho-GTPases and context. In the current study, we wished to study GEFs which mainly target/activate RhoA because we have previously shown that RhoA is activated by complement in GEC[64] and that RhoA activation in podocytes in mice results in proteinuria [65]. We focused our initial efforts on GEF-H1 because of its known implication in regulation of cell-cell junctions in renal tubular cells. Nonetheless, we have screened for other RhoGEFs by RT-PCR and found that rat GEC and glomerulus both express transcripts of p190 RhoGEF, Net1, LARG but not p115RhoGEF (Supplemental
Figure 1). Potential roles of other GEFs in podocyte function and pathology will require further studies.

Our results indicate that GEF-H1 mediates complement-induced RhoA activation in GEC. The impact of RhoA activation in GEC/podocytes appears to be complex. In cultured mouse podocytes, filamentous actin reorganization by mechanical stretch was dependent on Rho-kinase, a major downstream target of RhoA[12]. Pharmacological inhibitors of Rho-kinase have been shown to ameliorate proteinuria and/or kidney functions in a variety of animal models including puromycin aminonucleoside nephrosis (PAN) [74, 186] and hypertensive glomerulosclerosis[67, 71, 72, 78, 88]. These effects were independent of systemic blood pressure suggesting that the inhibitors act directly in the kidney, in particular on podocytes[67, 71, 72, 78, 88]. These findings support the notion that RhoA activation has a negative impact on podocyte morphology and/or function. On the other hand, RhoA activation was shown to be important for podocyte migration and development [61]. Thus, RhoA activation could be beneficial or detrimental in podocyte function depending on the context.

In the current study, we showed that GEF-H1 knockdown decreased the basal, as well as complement-stimulated RhoA activity in GEC (Figure 2.4). Interestingly, GEF-H1 knockdown cells showed significantly augmented cytolysis by complement. These results are consistent with our previous observation that GEC that express active RhoA are more resistant to complement-induced cytolysis [64]. One possible interpretation is that activation of RhoA protects GEC from cell death in expense of intricate foot process structures. GEC or mouse podocytes transfected with active RhoA lose cellular processes and become contracted with prominent cortical F-actin [64, 65]. Similar phenotype was observed in mitotic cells and it is speculated that rigid cortex of a rounded cell protects cells against external insults[187].

Previous studies have demonstrated that GEF-H1 activity is regulated by its association with microtubules in that disruption of the microtubules releases GEF-H1 rendering it active [101]. This microtubule-mediated activation of GEF-H1 has been shown in endothelial cells and dendritic cells [131]. Thus, it was
surprising to find that complement-induced GEF-H1 activation was independent of the microtubules; nocodazole, a potent disruptor of the microtubules did not affect the basal or complement-induced GEF-H1 activity (Figure 2.5C). One possible explanation would be that regulation of GEF-H1 activity by the microtubule occurs only in specific cells and does not occur in podocytes. Alternatively, subcellular localization of GEF-H1 in podocytes may influence its mode of activation. In podocytes in vivo, the microtubules are the main cytoskeleton in the primary processes while actins are the dominant cytoskeleton in the foot processes. If GEF-H1 localization is limited in foot processes, it is less likely to be regulated by the microtubules. Further studies are needed to address this hypothesis.

GEF-H1 activation was partially dependent on ERK, a mechanism described in tumor necrosis factor alpha-mediated GEF-H1 activation in renal tubular epithelial cells (LLC-PK1 and MDCK cells) [133, 168]. However, unlike in these cells, complement-induced GEF-H1 activation was independent of the EGF receptor activation [178], suggesting a slightly different mechanism. Other potential candidates that could contribute to complement-induced GEF-H1 activation include increased cytosolic Ca^{2+} concentration, phospholipases C, protein kinase C (PKC), and cytosolic phospholipase A2-a (cPLA2) [169, 175, 188, 189]. Several studies showed that PKC signaling is involved in RhoA activation possibly via phosphorylating and activating p115RhoGEF in thrombin-induced endothelial cells leading to impaired endothelial barrier [190-192]. It was also shown that PKC alpha activation leads to disruption of renal tubular epithelial (MDCK) apical junctions via a Rho-kinase II-dependent pathway [193]. Thus, PKC alpha could be an attractive candidate to contribute to complement-induced GEF-H1 activation.

Activities of some GEFs such as Net1, Ect2, and Tiam1 depend on their translocation within the cell to the site of Rho-GTPase activation [59]. Thus, we postulated that the intracellular movement of GEF-H1 could be another potential mechanism of activation. We found that in unstimulated GEC, GEF-H1 is diffusely distributed in the cytosol with some colocalization with the microtubule
filaments (Figure 2.6A). Upon stimulation with complement, GEF-H1 distribution changed in many cells (~40%) dramatically to the perinuclear pattern. Curiously, complement also changed the pattern of the microtubules from hair-like filaments projecting from the nucleus to the cell periphery in unstimulated cells to loss of hair-like filaments and perinuclear condensation (Figure 2.6A). Whether the change in the microtubules and GEF-H1 distribution are causally related is not know. In the early studies, a mutant of GEF-H1 that lacks the carboxyl-terminal region was found to display dense irregular perinuclear aggregates in COS-7 cells [126]. It is tempting to speculate that complement induces conformational changes of GEF-H1 mimicking the carboxyl-terminal deletion mutant thereby causing the translocation. Of interest, perinuclear translocation of GEF-H1 appears to correlate with its level of activity, since both were inhibited by the inhibitor of the ERK pathway to a similar degree (Figure 2.5 and Figure 2.6A). Furthermore, the T678A mutant of GEF-H1 also demonstrated reduced translocation in response to complement (Figure 2.6B). These results are compatible with a hypothesis that complement-induced ERK activation leads to phosphorylation of GEF-H1 at T678, which induces activation and translocation of GEF-H1. Indeed, the perinuclear localization of GEF-H1 post complement stimulation overlaps with the site of RhoA activation observed in live cells (Figure 2.3). Further studies are required to address this hypothesis.

In summary, we have identified GEF-H1 as an important regulator that links complement stimulation and RhoA activation in podocytes. These data support a regulatory model in which the ERK pathway regulates complement-induced RhoA activation by means of GEF-H1 stimulation, and establishing the ERK/GEF-H1/RhoA signaling pathway in GEC.
FIGURES FOR CHAPTER 2

Figure 2.1 GEF-H1 is activated in the glomerulus of rats with passive Heymann nephritis (PHN).

Passive Heymann nephritis (PHN) was induced by a single injection of anti-Fx1A antiserum. On day 14, urine was collected and glomeruli were isolated. (A) Glomerular lysates were subject to affinity precipitation with GST-RhoG17A to study the amount of active GEF-H1. Precipitates and total lysates were blotted for GEF-H1 (A) or phospho-MLC (B). Top: representative blots, Bottom: densitometric analysis. Each value was normalized to the average of the control values of the same experiment. **P< 0.01 vs. control. (A) n = 4  for control, n = 5 for PHN. (B) n = 4 for control, n = 6 for PHN.
Figure 2.2 Complement activates GEF-H1 in cultured rat GEC.

(A) Cultured rat GEC and mouse podocytes (MP) were incubated with rabbit anti-GEC antiserum and normal human serum (NS, to assemble C5b-9). Decomplemented heat-inactivated serum (HIS) was used as control. Active GEF-H1 was quantified as in Figure 2.1. *P<0.05, **P<0.01 vs. HIS, n = 4 for rat GEC and n = 6 for MP. (B) Antibody-sensitized GEC were incubated with C8-deficient serum (C8D) with or without reconstitution with purified human C8 and active GEF-H1 was quantified as in Figure 2.2. *P<0.05 vs. C8D, n = 4. (C) Antibody-sensitized GEC were incubated with complement (NS) for 10, 15, and 30 min and cell lysates were subject to affinity precipitation for active GEF-H1. *P<0.05 vs. HIS, n = 5.
Figure 2.3 RhoA is activated by complement in GEC.

(A) Schematic representation of the Raichu1298x RhoA FRET probe. YFP (Yellow Fluorescent Probe); RBD (Rho-binding domain of Rhotekin); CFP (Cyan Fluorescent Probe). (B) GEC were plated onto glass-bottom dishes, transfected with the FRET Raichu RhoA probe. Cells were incubated with complement and FRET images were captured as described in Methods. The corrected FRET values of each pixel were converted to pseudo-colors. Representative results of three independent experiments are shown. Complement (NS) induced RhoA activation starting at \(~15\) min, which also led to morphological changes. Control cells (HIS) did not show changes in RhoA activity or morphology.
Figure 2.4 GEF-H1 knockdown inhibits complement-mediated RhoA activation and augments cell injury in GEC.

(A) GEC were transduced with lentivirus encoding GEF-H1 shRNA, or empty vector pLKO.1 (control). Seventy-two hours post-transduction, cell lysates were immunoblotted for GEF-H1. **P<0.01 vs. pLKO.1, n = 3. The pictures show a merged DIC and green fluorescence picture. (B) RhoA activity in GEF-H1 knockdown (shGEF-H1) and control (pLKO.1) cells was assessed by RhoA G-LISA. *P<0.05 vs. pLKO.1, n = 4. Each value was normalized to the average of the treated samples (NS). (C) Cytotoxicity was quantified by LDH release (see Methods). Control and knockdown cells were stimulated with increasing concentrations of complement (NS 1, 2.5, 5, and 10%) **P<0.01 vs. control, n = 4.
Figure 2.5 Complement-induced GEF-H1 activation is dependent on the ERK pathway.

GEC were stimulated with complement in the presence or absence of various inhibitors. The inhibitors were added 30 min prior to the incubation with anti-GEC antiserum and throughout the complement stimulation. After 30 min incubation with NS (or HIS), cell lysates were subject to affinity precipitation for active GEF-H1. (A) U0126 (MEK1/2 inhibitor, 50 μM) inhibited complement-induced GEF-H1 activation. *$P<0.05$ vs. NS control, $n = 6$. (B) AG1478 (EGFR inhibitor) and PP2 (Src inhibitor) (both at 10 μM) did not affect complement-induced GEF-H1 activation. *$P<0.05$, **$P<0.01$ vs. HIS, $n = 4$. (C) Nocodazole (microtubule disrupting agent) or taxol (microtubule stabilizer) (both at 10 μM) did not affect complement-induced GEF-H1 activation. *$P<0.05$ vs. HIS, $n = 5$. (##$P<0.01$ vs. HIS; $#P<0.05$ vs. NS)
Figure 2.6 Complement induces perinuclear translocation of GEF-H1 and alters the microtubule structure in GEC.

(A) GEC transfected with wild-type GEF-H1 (GFP-GEF-H1) pretreated or not with nocodazole, taxol, and U0126 were stimulated with complement for 30 min. Cells were fixed, permeabilised and stained for α-tubulin. The graph shows the percentage of the cells that showed perinuclear pattern of distribution of GEF-H1 (***P<0.01, *P<0.05, vs. HIS, n = 3). (B) GEC transfected with a mutant GEF-H1 (GFP-GEF-H1-T678A), which cannot be phosphorylated by ERK, were treated with complement. The graph shows the percentage of the cells that showed perinuclear pattern of distribution of GEF-H1 (*P<0.05, vs HIS n = 3)(#P<0.05, vs NS).
Supplemental Figure 1. Expression of GEFs transcripts in cultured podocytes and in isolated adult rat glomeruli.

To evaluate the expression of the most extensively studied GEFs in rat GEC, we isolated RNA from rat glomerular epithelial cells (GEC) and rat glomeruli and performed reverse transcription polymerase chain reaction (RT-PCR). Transcripts of p190 RhoGEF, GEF-H1, Net1, LARG but not p115RhoGEF genes were detected in both rat GEC and rat glomeruli.
### Rat GEC

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<th>Net1 (272bp)</th>
<th>LARG (210bp)</th>
<th>P115 RhoGEF (200bp)</th>
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### Rat glomeruli

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# TABLES FOR CHAPTER 2

Table 2.1 PCR primers

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Chapter 4

Summary and final conclusions
3.1 SUMMARY OF ORIGINAL FINDINGS

Before this study, little was known about the signaling mechanisms downstream of complement stimulation in GEC. Although the role of the small Rho-GTPases has been previously described in GEC in response to complement, the implication of Rho GEFs in this system was still unknown before this study.

We have investigated the role of GEF-H1, known for its pathological role in the disruption of epithelial cell barrier, in GEC. We have demonstrated that GEF-H1 is an important element in the signaling induced by complement stimulation. We found that GEF-H1 was activated in vitro and in vivo in response to complement, leading to the activation of the RhoA signaling cascade. Activation of GEF-H1 was surprisingly independent from microtubule polymerization state, a relationship that has always been thought to be the model of regulation. However, GEF-H1 activation was shown to be dependent on ERK pathway. GEF-H1 knockdown blunted RhoA activation and caused increased cytotoxicity induced by complement in GEC, suggesting a cytoprotective role of GEF-H1.
3.2 GENERAL CONCLUSION

The molecular mechanisms of complement-induced GEC injury are not fully understood yet but it is clear that GEF-H1 plays a preponderant role in complement-induced GEC and in PHN rats by activating the RhoA signaling cascade.

Although the PHN model mimics human MN, there are some clear limitations. As previously mentioned, megalin -Heymann nephritis antigen, is not expressed in human podocytes, and therefore cannot be considered as the pathogenic human antigen. In addition, there is some evidence that the central role of C5b-9 may have been over-emphasized [194]. However, the identification of phospholipase A2 receptor (PLA2R) as the target antigen in most cases of idiopathic MN has opened new perspectives for investigation. We can expect development of new animal models of MN consisting of targeting PLA2R antigen. In addition, transgenic models of MN are now under development, which will greatly improve our understanding of the pathogenesis of the disease in vivo. The development of new models of MN may succeed the PHN model, but PHN is still going strong and used as an ultimate model to study the pathomechanisms of MN.

In this thesis, we have focused on the role of GEF-H1 as a RhoA activator in the experimental model of MN. More than 80 GEFs have been identified, and few were reported to be expressed in podocytes[179, 185]. Thus, further studies are required to identify the potential role of other GEFs in podocytes.

We have shown that GEF-H1 activation was dependent on ERK activation, but not on other kinases, such as Src-family kinases, and EGFR, nor on microtubules. Furthermore, the T678A mutant of GEF-H1 reduced translocation to the perinuclear region, a phenomenon observed in wild type GEF-H1, in response to complement. Although it is tempting to speculate that Thr678 of GEF-H1 is the phosphorylation site by ERK, and it is responsible for GEF-H1 activation, further investigation is required to test this hypothesis. One potential experiment could consist of transfecting GEC with GFP-GEF-H1 WT or T678A mutant, stimulate or not with complement, and precipitate GEF-H1 from cell
lysates using an anti-GFP antibody. Precipitates will be analyzed by immunoblotting with anti-phosphothreonine. If the hypothesis holds, then GEF-H1 WT, but not T678A mutant will be phosphorylated at Thr.

When studying the intracellular movement of GEF-H1 in GEC in response to complement, we observed a striking perinuclear distribution pattern. Quantification of GEF-H1 localization was done by attributing a “yes” or “no” to transfected cells showing perinuclear distribution. An additional way of quantification could be useful to make a more objective statement. We could analyze the intensity of GFP in each transfected cell and use the ratio between perinuclear and cytoplasmic intensities. This method will highlight a gradient change rather than a dramatic change of distribution between unstimulated and stimulated cells when treated with different drugs.

Finally, determining the specific GEFs that regulate Rho protein activation downstream of different extracellular signals will be major advance in the field of Rho-GTPases biology. In addition, these discoveries will highlight the importance of targeting these regulatory proteins for therapeutic purposes in diseases demonstrating deregulated Rho signaling, such as glomerular diseases.
REFERENCES


119. Orrico, A., et al., *Phenotypic and molecular characterisation of the Aarskog-Scott syndrome: a survey of the clinical variability in light of


