The role of Rho GTPases in Complement-mediated Glomerular Epithelial Cell injury

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ABSTRACT

In glomerular epithelial cells (GEC), the actin cytoskeleton is a key determinant of cell morphology and functions, including permselectivity. Complement C5b-9 induces sublytic GEC injury associated with GEC morphological changes and proteinuria. This study addressed the role of Rho GTPases in complement-mediated GEC injury. We demonstrated that the amount of active RhoA increased; while the amount of active Rac1 and Cdc42 were decreased in C5b-9 mediated sublytic GEC injury both in vitro and in glomeruli from rats with PHN in vivo. Complement mediated inactivation of p190RhoGAP may contribute to complement-induced RhoA activation. Overexpression of constitutively active or dominant negative mutants of RhoA, Rac1 and Cdc42 distinctly altered GEC morphology and F-actin pattern. Complement caused changes in GEC actin cytoskeleton, at least in part mediated by a downstream kinase of RhoA--Rho kinase (ROCK). Activation of RhoA exacerbated complement-mediated cytotoxicity in GEC, while inhibition of ROCK attenuated it.

RESUME

Dans les cellules épitheliales glomérulaires (GEC), le cytosquelette d'actine joue un rôle detérminant dans la morphologie, la fonction et la permsélectivité. La fraction C5b-9 du complément induit des effets sublytiques dans les GEC, ces effets sont associés à des changements morphologiques et, dans un modèle in vivo, à la protéinurie. Notre étude examine le rôle des protéines Rho-GTPases dans l'action du complément sur les GEC. Nous avons montré une augmentation de la forme active de RhoA et une diminution de l'activité des protéines Rac1 et Cdc42 in vitro et in vivo, après traitement des GEC avec le complément et dans les lysats de glomérules de rats PHN, respectivement. Cette activation de RhoA est mediée par l'inactivation de p190RhoGAP. La surexpression constitutive de la forme active ou dominante négative des proteines RhoA, Rac1 et Cdc42 conduit à differents changements morphologiques et des patterns de F-actine distincts. Ces effets du complement que nous avons observé sur l'organisation de l'actine et du cytosquelette seraient partiellement mediés par la RhoA kinase (ROCK) en aval de RhoA. En effet la surexpression de la forme active de RhoA exacerbe l'effet cytotoxique du complément sur les cellules GEC alors que l'inhibition de ROCK atténue cet effet. En conclusion, le complément induit le débalancement de RhoA, Rac1 et Cdc42 dans les cellules épitheliales glomérulaires. Ceci peut contribuer aux changements morphologiques dans les GEC et à la protéinurie. L'activation de la voie de signalisation RhoA/Rho-kinase a un effect important sur la fonction des GEC.

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SECTION 1: LITERATURE REVIEW

Introduction

In glomerular epithelial cells (GEC), the actin cytoskeleton is a key determinant of cell morphology and function, including permselectivity. GEC injury is associated with the alteration of the well-organized actin cytoskeleton into a dense network (1). Therefore, understanding how the GEC actin cytoskeleton is regulated is of critical importance. Rho family GTPases are members of the Ras superfamily of monomeric 20-30 kDa GTP-binding proteins. RhoA, Rac1 and Cdc42 are three well-characterized members of Rho-GTPases. They have various functions in cellular events, including regulating the assembly and organization of the actin cytoskeleton (2) . In the rat passive Heymann nephritis (PHN) model of membranous nephropathy, complement C5b-9 induces sublytic GEC injury, associated with morphological changes of GEC and proteinuria. Rearrangement of the actin cytoskeleton can partly explain why C5b-9 leads to a GEC morphological change and proteinuria (3). However, the activity and role of Rho-GTPases in complement-mediated GEC injury remains poorly studied.

Structure and function of the kidney

An important organ, the kidney is responsible for regulating water and inorganic ion balances, removing metabolic waste products and foreign chemicals from the blood and excreting them in urine. Moreover, the kidney can produce hormones and enzymes, such as erythropoietin, renin and 1, 25-dihydroxyvitamin D_3. During prolonged fasting, the kidney can release glucose into the blood from the breakdown of amino acid.

Each human kidney has up to 1.2 millions nephrons, which are connected by connective tissues, blood vessels, nerves and lymphatics. The nephron is the functional subunit of the kidney. Each nephron is composed of a renal corpuscle and a tubule. The glomerulus, the filtration unit of the kidney, protrudes into Bowman's space and forms a renal corpuscle. The glomerulus can filter water and small molecules including body waste products, while retaining cells and large molecules such as proteins. When this barrier function is impaired, proteinuria occurs and often, kidney failure follows. The tubule is the component, which regulates secretion and reabsorption.

In summary, when the blood encounters the filtration barrier, only ultrafiltrates free of cells and proteins pass by the barrier and enter the tubule, where they are modified, concentrated and excreted as urine (4; 5).

The glomerular capillary wall

The glomerular capillary wall is composed of a monolayer of endothelial cells, lining the capillary lumen, which is covered by a glomerular basement membrane (GBM), and finally surrounded by GEC. GEC are also referred to as podocytes. The glomerular capillaries are supported by the mesangium, including mesangial cells and their surrounding matrices. As Fig.1 shows, when the blood passes by the glomerular capillary wall, it first encounters glomerular endothelial cells. With a diameter of approximately 30nm, the fenestrations prevent the passage of blood cells into the subendothelial space. Most likely, the negative surface charge of the endothelial cells partially restricts passage of plasma proteins. The next barrier encountered by the filtrate is the GBM. The net negative charge of the GBM helps to repel large negative proteins. The final and the most important line of prevention from protein leakage is the GEC (6).

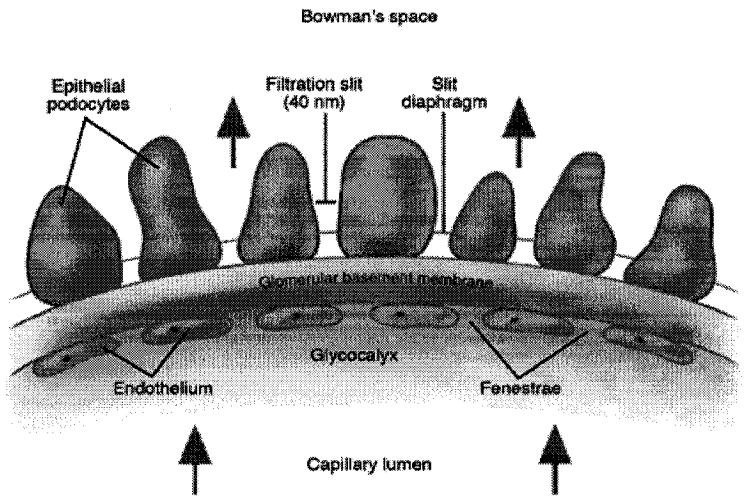


Fig.1*. Composition of glomerular capillary wall. From the direction of capillary lumen to Bowman's space, it includes a glycocalyx (applying negative-charge) coated fenestrated (~30 nm in diameter) endothelium, a basement membrane (200-400 nm in*

thickness), and epithelial foot processes consequently. The neighboring foot processes form the filtration slit (~ 40nm) bridged by the slit diaphragm. The filtrates pass by the slit diaphragm into Bowman's space (6).

Structure and function of GEC or podocytes

GEC are critical for structural integrity as well as barrier function of the glomerulus. GEC are highly specialized cells with a complex cytoarchitecture (Fig. 2). The most outstanding features are the long fingerlike foot processes, interdigitating to cover the basement membrane, forming a ~40 nm-wide filtration slit bridged by a continuous membrane-like structure called the slit diaphragm. Proteins and some macromolecules that pass by the endothelial cells and basement membrane are retarded by the slit diaphragm. It therefore forms the final barrier to protein loss, which explains why GEC injury is typically associated with marked proteinuria, a hallmark of most glomerular diseases. The normal slit diaphragm function is crucial in maintaining the integrity of the foot process (7).

There are three distinct domains in the membrane of the foot processes: the basal membrane domain, the apical membrane domain and the slit diaphragm protein complex (Fig. 3). The basal membrane domain is in contact with the GBM. The apical domain is covered by sialoglycoproteins, which are responsible for providing the negative charge of the GEC. The slit diaphragm protein complex is at the border of the basal and apical membranes. The recent discovery of several novel slit diaphragm proteins, including nephrin, P-cadherin, CD2AP, ZO-1, FAT, podocin, and Neph1 and their mutation analysis have shed light on the pathogenesis of proteinuria and emphasized

the critical role of the slit diaphragm (1). A milestone in podocyte research was the finding that the nephrin is a critical structural component of the slit diaphragm and bridges the distance between interdigitating GEC foot processes. Nephrin is a transmembrane adhesion protein of the Ig superfamily. It is encoded by *NPHS1*, the gene mutated in congenital nephrotic syndrome of the Finnish type (8). Podocin is encoded by the *NPHS2* gene, which is mutated in autosomal recessive, steroid-resistant nephrotic syndrome in childhood (9). CD2AP interacts with nephrin via a C-terminal domain (10) and is also capable of associating with the actin cytoskeleton. CD2AP knockout mice develop proteinuria and kidney failure (11). Although it has been suggested that the slit diaphragm represents the ultimate filtration barrier, additional roles for this structure as a signaling center and in endocytosis have also been identified (12).

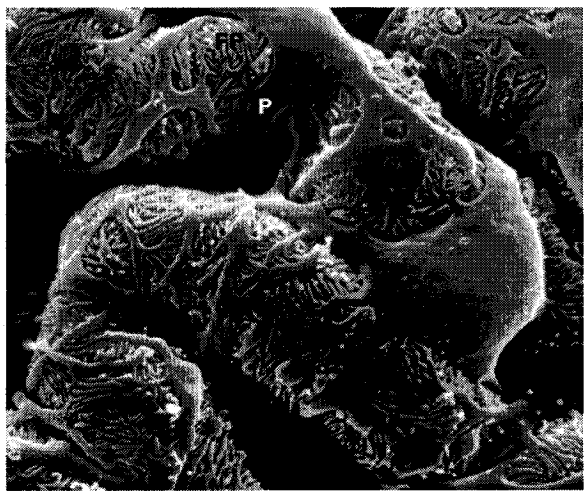


Fig.2. *Scanning electron micrograph of normal rat glomerular capillaries. View from Bowman's space showing highly branched GEC (P) and its foot processes (FP) surrounding the glomerular capillaries (13).*

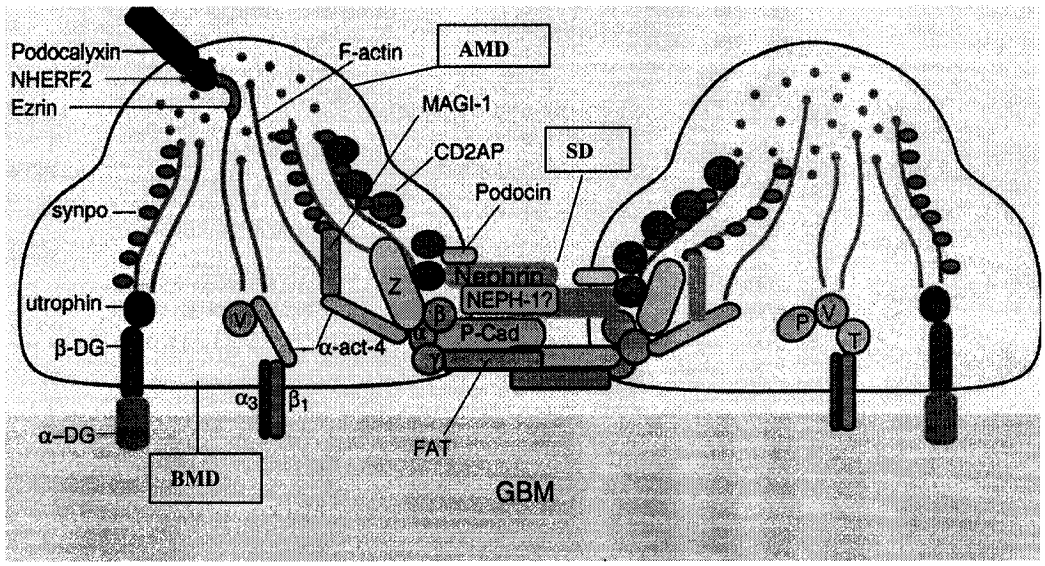


Fig.3. *The molecular anatomy of GEC foot processes. There are three membrane domains of GEC, which are the slit diaphragm protein complex (SD), the basal membrane domain (BMD) and the apical membrane domain (AMD). They all bind to the actin cytoskeleton. Schematic also shows recently discovered members of the glomerular slit diaphragm, nephrin, NEPH-1, P-cad (p-cadherin), FAT, podocin, Z (ZO-1) and CD2AP. Molecular components linking the apical, basal, slit diaphragm domain to the actin cytoskeleton: α-act4 (α-actinin-4), α3β1 (α3β1 intergrin), α-DG (α-dystroglycan), ezrin, T (talin), V (vinculin) (1).*

The GEC actin cytoskeleton

The cytoskeleton is formed of three structural elements: microfilaments (7-9nm in diameter, consisting of actin), intermediate filaments (10nm) and microtubules (24nm). In GEC, the actin cytoskeleton is a key determinant of cell morphology and function. In the cell body of GEC, microtubules and intermediate filaments, *e.g.*, vimentin and desmin,

make the majority of the cytoskeleton. In the foot processes of GEC, microfilaments are the predominant cytoskeletal component (14).

GEC foot processes are not static, but rather contain a contractile system. This contractile apparatus is composed of actin, myosin-II, α-actinin-4, talin, and vinculin (1)(Fig.3). In GEC foot process, membrane domain proteins are linked to the foot process actin cytoskeleton. The slit diaphragm protein nephrin is linked to the actin cytoskeleton (15). On the apical membrane domain, podocalyxin associates with the actin cytoskeleton through interactions with ezrin and Na^+/H^+-exchanger regulatory factor 2 (NHERF2), which is a scaffold protein (16; 17). The actin filaments are connected to the underlying GBM at focal contacts via α3β1 integrin and dystroglycans (18). The foot process actin cytoskeleton is highly dynamic and ultimately determines the structural maintenance of the filtration slits. A functional glomerular filter is dependent on an intact slit diaphragm and GEC-GBM interactions (1).

GEC are injured in many forms of human and experimental glomerular diseases, including minimal change disease, focal segmental glomerulosclerosis, membranous nephropathy, diabetic nephropathy, and lupus nephritis (19; 20). Independent of the underlying disease, four major causes lead to foot process effacement and proteinuria: disturbance of GBM or GBM-GEC interaction; disturbance of the slit diaphragm and lipid raft; disturbance of the actin cytoskeleton and its associated proteins; or disturbance of the apical membrane domain of GEC, such as neutralization of negative cell surface charges (1).

Foot process effacement, also referred to as process simplification, retraction, or fusion, has to be initiated at the cytoskeleton of GEC and results in the alteration of the

cell-cell contacts at the slit diaphragm and in a mobilization of the cell-matrix contacts. Foot process effacement is accompanied by an increase in microfilament density that builds a mat of intercrossing stress fibers at the sole of the foot process (19).

An actin binding protein α-actinin-4 is widely expressed in GEC foot processes, co-localizing with actin stress fibers. Induction of proteinuria with puromycin in rats resulted in foot process retraction and progressive GEC damage and an increased α-actinin-4 expression (21; 22). A mutation in the gene encoding for α-actinin-4, which increases the affinity of α-actinin-4 to F-actin, has been associated with late-onset autosomal-dominant focal segmental glomerulosclerosis (23). This study demonstrated the role of the actin cytoskeleton in regulation of GEC structure. Proteins regulating or stabilizing F-actin are therefore of critical importance for sustained function of glomerular filtration.

Complement-mediated GEC injury

GEC injury can be induced by several toxins, antibodies, complement factors, and mechanical stress (13). Complement activation has been determined as one of the mechanisms in developing GEC injury in in vivo and in vitro studies (13). The complement system plays an important role in mediating inflammation, phagocytosis, and cytolysis. Activation of the complement cascade near a cell surface leads to the assembly of terminal components, exposure of hydrophobic domains, and insertion of the C5b-9 membrane attack complex into the lipid bilayer of the plasma membrane. Nucleated cells require multiple C5b-9 lesions for lysis, but, at lower doses, C5b-9 induces nonlytic injury and various metabolic effects (24; 25). It was reported that C5b-9

activates protein kinases, such as protein kinase C, as well as mitogen-activated protein kinases (MAPKs) including extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK)/stress-activated protein kinase and p38 MAPK. C5b-9 also activates cytosolic phospholipase A_2 ($cPLA_2$), which leads to the release of arachidonic acid (AA). In GEC, AA are metabolized to prostanoids via the constitutively expressed cyclooxygenase-1 and the C5b-9-inducible cyclooxygenase-2 (24; 26-28).

Membranous nephropathy is a frequent cause of nephrotic syndrome in adults, which is characterized by injury of the glomerular capillary wall, glomerular permselectivity impairment and proteinuria. The rat model of passive Heymann nephritis (PHN) has been extensively used as a model to study the pathophysiology of human membranous nephropathy (29; 30).

PHN is induced by a single intravenous injection of heterologous antibody to the rat proximal tubular brush border (anti-Fx1A), whereupon a lesion morphologically resembling human membranous nephropathy develops. PHN is characterized by subepithelial immune deposits with marked proteinuria, and is the consequence of antibody depositing in situ and binding to a resident glomerular antigen that has been shown to be a component of GEC. In PHN, assembly of the complement C5b-9 membrane attack complex in GEC plasma membranes leads to nonlytic GEC injury, which in turns leads to foot process effacement, actin cytoskeleton condensation, slit diaphragm dislocation and filtration barrier dysfunction and proteinuria. Activation of $cPLA_2$ and production of prostanoids exacerbates GEC injury and proteinuria (31).

Previous studies reported the relationship between complement and the actin cytoskeleton in GEC. On one hand, foot process effacement and condensation of the

GEC cytoskeleton are manifestations of PHN (3). Complement-mediated sublethal injury was accompanied by a loss of actin stress fibers and focal contacts in GEC in vitro (3). These changes in the actin cytoskeleton are complement-dependent and are associated with altered GEC slit diaphragm integrity and dissociation of nephrin from the actin cytoskeleton (32). On the other hand, the actin cytoskeleton facilitates complement-mediated activation of $cPLA_2$, which partially regulates the pathophysiology of GEC injury. Stable transfection of constitutively active RhoA, which is known to induce formation of stress fibers and stabilize F-actin, can attenuate the complement-activation of $cPLA_2$ in GEC (33).

Rho family GTPases members

Rho-GTPases are members of the Ras GTPase superfamily of monomeric 20-30 kDa GTP-binding proteins and regulate a wide spectrum of cellular functions. Rho-GTPases are ubiquitously expressed across all species, from yeast to humans (34). The structural feature that distinguishes them from other small GTPases is the Rho insert domain located in the small GTPase domain. Most Rho proteins are small (190-250 residues) and consist only of the GTPase domain and short N-terminal and C-terminal extensions. Within their GTPase domains, they share approximately 30% amino acid identity with the Ras superfamily proteins and 40-95% identity within the Rho family (35). To date, 20 genes encoding proteins with a small GTPase domain of the Rho consensus type have been identified in humans. This is likely to be the final number with the human genome being almost completely characterized (36). According to the structure and function analysis, they are roughly divided into 5 groups, those being the

Rho-like, Rac-like, Cdc42-like, Rnd, and RhoBTB subfamilies (37)(Fig.4). RhoD, Rif, and RhoH/TTF are not included in these groups because of the limited knowledge about them. MIRO-1 and MIRO-2 were described as belonging to the Rho family (38). However, due to the absence of homology to other Rho-GTPases and their lack of the Rho-specific insert loop in their GTPase domains, they are not presented in Fig.4.

The Rho-like subfamily members are all very similar in sequence and when overexpressed as activated proteins in fibroblasts, each of them contributes to contractility and formation of stress fibers and focal adhesion (37). Also, in other cell types, such as in epithelial cells, overexpression of RhoA in Mardin-Darby canine kidney (MDCK) cells lead to rearrangement of stress fibers. F-actin was diffuse at lateral membranes and prominent in stress fibers on the basal membrane (39). A few functional differences are reported. RhoA and RhoC promote growth; RhoB inhibits growth (40; 41) and is downregulated in cancer cells (42) and upregulated during the G1/S transition of the cell cycle and during apoptosis (43; 44); RhoC expression has been shown to promote metastatic behavior and is upregulated in many invasive carcinomas (45; 46).

The Rac-like subfamily all stimulate the formation of lamellipodia and membrane ruffles in fibroblasts (47). Rac1 and RhoG are widely expressed, Rac2 is expressed in hematopoietic cells, while Rac3 is found in neural tissues (37) .

The Cdc42-like subfamily all stimulate the formation of filopodia in fibroblasts. They all interact with downstream effectors WASP or N-WASP, which mainly regulate the formation of filopodia (48; 49). Cdc42 is also known to regulate cell polarization. TC10 and TCL are involved in insulin-mediated metabolic events (50). Wrch1 has been shown to be involved in Wnt signaling (51). The role of chp/wrch2 is still unclear.

Compared to the above three subfamilies, the Rnd and RhoBTB subfamilies are less well defined and will not be discussed here.

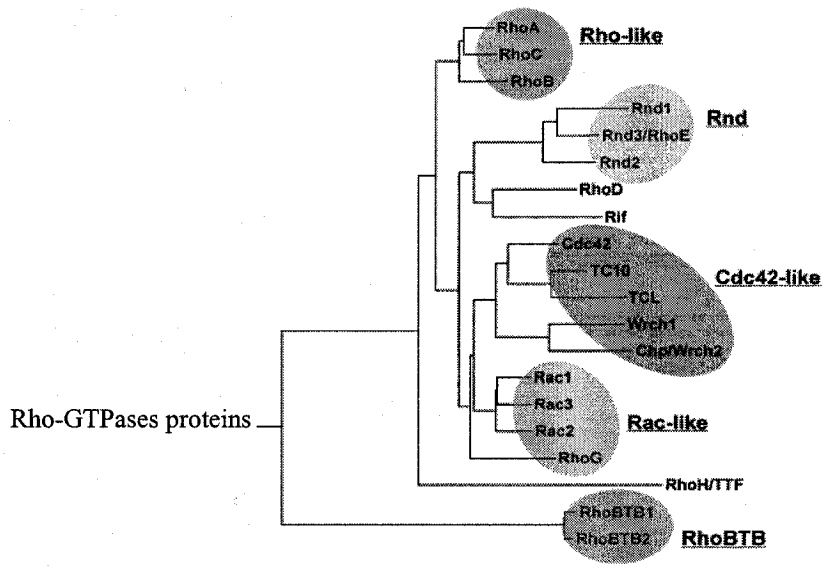


Fig.4. *The Rho-GTPase subfamilies. To date, Rho-GTPase proteins are divided into five subfamilies, Rho-like, Rnd, Cdc42-like, Rac-like, and RhoBTB, which are highlighted by circles (37) .*

Among these Rho GTPase family members, the best-known classical members are RhoA, Rac1 and Cdc42 (52). They are expressed ubiquitously in various cells and tissues. Most of the functional information on Rho-family proteins has come from the study of these three members. Each Rho-family protein interacts with multiple effectors, and some effectors are recognized by multiple family members. Interaction with and activation of the effectors stimulates signaling pathways that mediate the diverse

functions of Rho-family proteins. They play a critical role in a wide range of biological processes, including actin cytoskeleton reorganization, membrane trafficking, transcriptional activation, cell growth control, cell motility, gene expression, and cell cycle progression. Consequently, a major challenge has been to unravel the underlying molecular mechanisms by which the Rho-GTPases mediate these various activities in molecular levels (37) .

Regulation of Rho protein activity

As is the case with Ras and other small GTPases, RhoA, Rac1 and Cdc42 act as binary switches by cycling between an inactive (GDP-bound) and an active (GTP-bound) conformational state. The activity of Rho-GTPases is determined by the ratio of their GTP/GDP-bound forms in the cells. The ratio of the two forms is regulated by three distinct functional classes of regulatory proteins, which mainly control the GDP/GTP cycle (53)(Fig.5). Guanine nucleotide exchange factors (GEFs) stimulate the exchange of GDP for GTP to generate the activated form; activated Rho protein can translocate from the cytosol to the plasma membrane, which is then capable of recognizing downstream targets or effector proteins. GTPase activation proteins (GAPs) accelerate the intrinsic GTPase activity of Rho family members and accelerate the hydrolysis of GTP, thereby deactivating them. Guanine nucleotide dissociation inhibitors (GDIs) interact with the GDP-bound form, recruit Rho-GDP proteins from the plasma membrane and retain them in cytosol (53).

In principle, extracellular signals could act upon any of the three types of regulatory proteins to control the activities and localization of Rho proteins (53). Much

research has been done on GEFs. For example, Vav, a haematopoietic cell-specific exchange factor, is one of the best-characterized GEFs (54). Vav acts predominantly on Rac. Tyrosine phosphorylation of Vav can strongly enhance its exchange activity. Other Rho GEF family proteins, such as p190RhoGEF, a RhoA-specific GEF, are expressed in neuronal cells and in brain tissue extracts (55). p190RhoGEF can be activated by protein-tyrosine kinases (PTKs), such as the focal adhesion kinase (FAK). Overexpression of FAK in Neuro-2α cells increased both endogenous p190RhoGEF tyrosine phosphorylation and RhoA activity. Cdc42-GEF FRG is a Cdc42 specific GEF (56). Cdc42 can be activated by trans-interactions of the cell adhesion molecule nectin through c-Src and Cdc42-GEF FRG.

There is also evidence to suggest that GAPs and GDIs could have a role in the activation arm of the GTPase cycle. It has also been reported that the inhibition of Rho GAP activity, in the absence of GEF activation, is sufficient to induce Rho-mediated actin reorganization (57). More than 80 Rho GAP family members have been identified. Each Rho GAP might play a specialized role in regulating individual Rho-GTPase activity and in influencing their specific functions (58). One of the well-characterized members, p190RhoGAP, binds more specifically to RhoA, when compared with Rac1 and Cdc42. p190RhoGAP is expressed ubiquitously among tissues (58). Several lines of evidence support that p190RhoGAP participated in the rearrangement of the actin cytoskeleton. For example, microinjection of the C-terminal region of p190RhoGAP into Swiss 3T3 cells has been found to block serum-induced stress fiber formation (59). p190RhoGAP, is regulated by Src family tyrosine kinases. Activation of Src in cells leads to phosphorylation of two tyrosine residues of p190, which are located close to the Rho

GAP domain. Upon phosphorylation, p190RhoGAP controls the activity of RhoA (58). Cadherins are transmembrane receptors that mediate cell-cell adhesion in epithelial cells. Cadherins inactivate RhoA through activation of p190RhoGAP (60). The diversity of Rho GEFs and Rho GAPs reflects the fact that Rho-GTPase can be regulated in a variety of ways.

As opposed to Rho GEFs and Rho GAPs, there are only a few known Rho GDIs (34). Three Rho GDIs have been particularly well studied: RhoGDI-1 binds well to RhoA, Rac1 and Cdc42, while RhoGDI-3 binds well to RhoB and RhoG. The targets for RhoGDI-2 remain unclear. The interaction between Rho GDI and Rho can be inhibited by the binding of Rho GDI to members of the ezrin-radixin-moesin (ERM) family that serve as crosslinkers between actin filaments and the plasma membrane (61). Furthermore, overexpression of a fragment of the ERM protein radixin has been shown to induce stress fibers in a Rho-dependent manner, suggesting that the modulation of ERM protein activity might contribute to the activation of GTPases, presumably by sequestering Rho GDI (61).

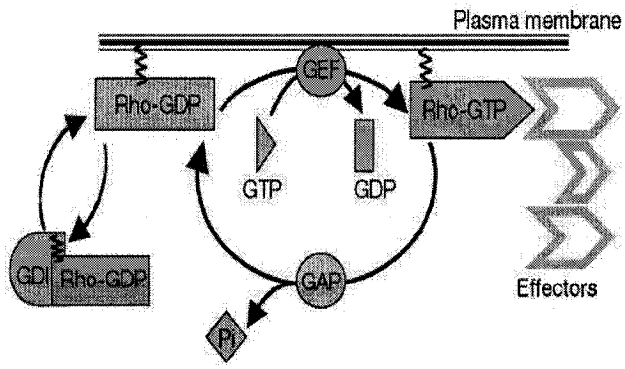


Fig.5. *The Rho-GTPase cycle. Rho-GTPases cycle between an active (GTP-bound) and an inactive (GDP-bound) conformation. In the active state, they interact with downstream effectors. The cycle is highly regulated by three classes of proteins, GEF, GDI, and GAP (53).*

When cells receive extracellular stimuli such as growth factors, cytokines and hormones, which bind to cell surface receptors, adhesive interactions or stimuli from mechanical stresses, these stimuli act upon GEF/GAP/GDI to control the activity of Rho, Rac and Cdc42. Once activated, the GTPases bind to a spectrum of effectors to stimulate downstream signaling pathways (Fig.6).

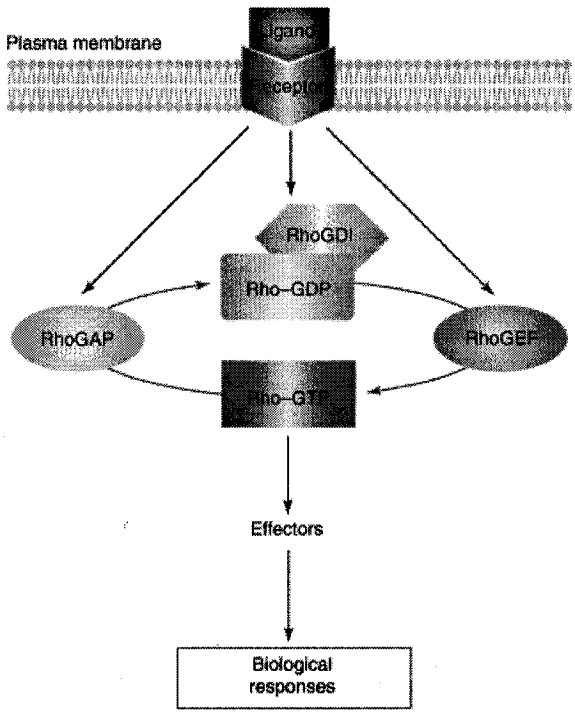


Fig.6. *The upstream regulators of Rho-GTPases. Extracellular signals conveyed through specific cell surface receptors modulate the activities of GEF/GAP/GDI, which in turn regulate the activation state of individual Rho-GTPases (58).*

Rho-GTPases and downstream effectors

One of the key functions of Rho-GTPases is to regulate the actin cytoskeleton (2). In early 1990s, it was first demonstrated that microinjection of recombinant RhoA in Swiss 3T3 fibroblast dramatically induced the formation of stress fibers (62). Later, ROCK (Rho-associated coiled-coil forming kinase, ROK) was identified as the first downstream effector of RhoA to affect actin organization (63-65). It has been shown that ROCK can increase myosin light chain (MLC) phosphorylation either by inhibiting MLC phosphatase or by directly phosphorylating the regulatory MLC, thereby enhancing myosin activation and increasing tension generation. ROCK can also phosphorylate ezrin-radixin-moesin (ERM) family proteins, activating their functions as linkers between the actin and plasma membrane. ROCK also can phosphorylate the Na/H^+ antiporter NHE-1, in order to promote actin-membrane interactions with several intermediate filament proteins (desmin, vimentin and glial fibrillary acidic protein) and to regulate intermediate filament structure (66). The other ROCK substrate is LIM kinase (LIMK), which phosphorylates cofilin, thereby causing the release of actin monomers and the promotion of actin polymerization (66). The other Rho downstream effector mammalian homolog of diaphanous (mDia), induces the assembly of fine arrays of thin stress fibers (67) (Fig 7).

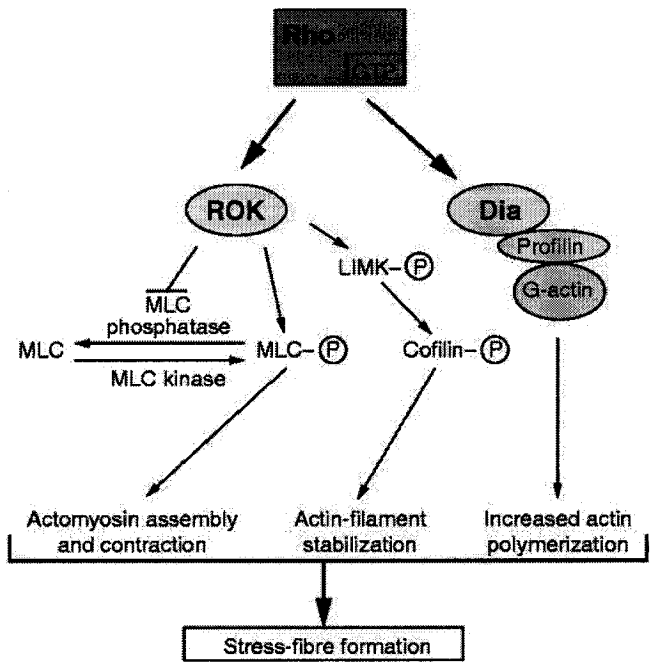


Fig. 7. *Downstream effectors of RhoA involved in stress fiber formation (52).*

Rac and Cdc42 also have numerous effectors that mediate effects on the cytoskeleton (66). Rac binds to the WAVE complex, causing the release of active WAVE, which promotes actin polymerization in lamellipodia through activation of the Arp2/3 complex (68). Both Rac and Cdc42 bind and activate the p21 activated protein kinases (PAKs): PAK1, PAK2 and PAK3 (69). PAKs have multiple substrates, including LIMK, which lead to actin polymerization. PAK activity also regulates myosin phosphorylation and cell contractility through several pathways, including myosin light chain kinase, myosin light chain, myosin heavy chain and caldesmon. WASP and the more widely

expressed N-WASP are important downstream effectors of Rac and Cdc42 to mediate formation of filopodia (37). Rac and Cdc42 also bind to the actin-binding protein IQGAP, which is involved in regulation of cell-cell adhesion (66).

Cdc42 activates the serine/threonine kinase, called myotonic dystrophy kinase-related Cdc42-binding kinase (MRCK), which is related to Rho kinases and promotes myosin phosphorylation (66). Cdc42 also activates the tyrosine kinases ACK1 and ACK2; the latter regulates focal adhesion formation and organization of the actin cytoskeleton (66). Cdc42 is one critical determinant of polarity in developmental systems of the effector PAR6, which regulates positioning of the microtubule organizing center (MTOC) in migrating cells and early embryos, and tight junction formation in epithelial cells (66).

Rho-GTPases and actin cytoskeleton regulation

Through the downstream effectors, Rho-GTPases have the effects on actin cytoskeleton regulation. The actin cytoskeleton mainly determines the ability of a eukaryotic cell to maintain or change its shape and its degree of attachment to the substratum in response to extracellular signals. It plays a crucial role in processes such as cell motility, cytokinesis, and phagocytosis. Monomeric actin (G-actin) has the potential to polymerize into filamentous actin (F-actin) in vitro and in vivo under certain regulation, Regulation of actin polymerization is central to the function of Rho-GTPases in cells(70). The actin cytoskeleton of mammalian cells is composed of actin filaments and many specialized actin-binding proteins (71; 72). Filamentous actin is generally organized into a number of discrete structures (34). Filopodia are found primarily in motile cells and neuronal growth cones. It has finger-like protrusions that contain a tight bundle of long

actin filaments in the direction of the protrusion. Lamellipodia dominate the edges of cultured fibroblasts with thin protrusive actin sheets. Actin stress fibers are bundles of actin filaments that traverse the cell and are linked to the extracellular matrix through focal adhesions (34).

Rho-GTPases regulate many actin-dependent cellular processes, such as platelet aggregation, lymphocyte and fibroblast adhesion, cell motility, contraction, and cytokinesis (73). Although the effects of Rho-GTPases on the organization of the actin cytoskeleton were well characterized first in fibroblasts (62), there is compelling evidence for a similar role for these proteins in other eukaryotic cells. In macrophages, activation of Rho induces a contractile actin-myosin filament network but no focal adhesions; consequently, macrophages round up. In contrast, the activation of Rac and Cdc42 induces the formation of filopodial and lamellipodial protrusions in macrophages (74). In addition, as detailed below, RhoA, Rac1, Cdc42 have different impacts on neural cells, as compared to on fibroblasts. Thus, the effect of Rho-GTPases on cell morphology is cell specific. One reason is that different cell types can be also modified by other parameters, in particular by cell's ability to assemble integrin-based cell-matrix or cadherin-based cell-cell adhesion complexes (2).

Cross talk between Rho, Rac and Cdc42

A balance of signalling by Rho family small GTPases RhoA, Rac1 and Cdc42 coordinates cytoskeletal morphology (75). It has been proposed that the opposing effects of Rac/Cdc42 and Rho might be a general feature of these GTPases (2). Rac is known to inhibit Rho. One proposed mechanism is that Rac-mediated production of oxygen radical

causes an inhibition of the low molecular weight protein tyrosine phosphatase (LMW-PTP), leading to increased phosphorylation and activation of p190RhoGAP. In turn, this results in the inactivation of Rho (76). The signaling events downstream from Rac may also inhibit Rho signaling pathways. For example, it has been reported that constitutively active PAK promotes loss of stress fibers and focal adhesions. This may occur through the inhibition of myosin light chain kinase (MLCK) (77), or by direct phosphorylation of myosin II heavy chain by PAK, both events leading to inhibition of myosin function (78).

Madera and colleagues demonstrated that both activation and inactivation of RhoA, Rac1 and Cdc42 enhance paracellular permeability and contribute to barrier dysfunction in MDCK cells (79). Another study showed both activation of RhoA and inhibition of ROCK reduced complement-mediated extracellular signal-regulated kinase (ERK) activation in GEC (80). Therefore, the balance of active/inactive RhoA, Rac1 and Cdc42 are likely to be critical for maintenance of the structure and function of the actin cytoskeleton.

Function of Rho-GTPases on GEC and neuron

The GEC and the neuron share many cell biological characteristics (81). Both of them exhibit a highly arborized morphology that is supported by the highly organized cytoskeleton. GEC and neurons develop long processes, such as GEC major processes and neuronal axons and dendrites. These processes are supported mechanically by microtubules and intermediate filaments. Both cell types also have thin projections that contain actin filaments as the major cytoskeletal elements, i.e. GEC have foot processes and neurons have dendrite spines (81).

Roles of Rho-GTPases in neuronal cells are well documented. In neuroblastoma cells (N1E-115), Rac and Cdc42 activation promoted the formation of lamellipodia and filopodia, respectively along neurite extensions (2). Activation of Rho in neuronal cells induced neurite retraction and cell rounding, and although this appears different from what is seen in fibroblasts after Rho activation, the underlying cause is same: the Rho-dependent formation of contractile actin-myosin filaments. The difference is that fibroblasts, unlike neuronal cells, can maintain a flattened shape through the formation of strong focal adhesion attachment sites (2). It was reported that Rho-GTPases are responsible not only for axonal growth, but also for dendritic morphogenesis. In mammalian neurons, a constitutively active mutant of Rac1 promoted dendrite branching and spine formation, whereas active RhoA reduced dendritic complexity (81). Multiple roles for Cdc42 in branching and spine formation have been also revealed in dendrites of Drosophila visual system neurons (81).

By analogy, there is also evidence for a role of Rho-GTPases in GEC. In conditionally immortalized mouse podocytes, Y-27632 promoted elongation of major processes. Treatment of the conditionally immortalized mouse GEC with Y-27632 and forskolin in combination further induced foot process projections elongating from major processes. Forskolin is known to inhibit RhoA activity, as well as to increase the activity of Rac1 and Cdc42. These results suggest that Rac1 activation promote process formations in neural cells and podocytes, while RhoA activation has an opposite effect. These results suggest that the well balanced activities of Rho family small GTPases are important for maintenance of the arborized morphology of GEC (82). The role of Rho-GTPases in GEC morphology is also supported by the observation of mice lacking Rho-

GDIα, an intrinsic inhibitory regulator of Rho activity. Rho-GDIα knock out mice developed massive proteinuria mimicking nephrotic syndrome, leading to death due to renal failure within a year (83). In this model, podocytes were severely injured and foot processes were disrupted, suggesting a critical role of Rho-GTPases in GEC morphology and function (83).

SECTION 2: PROJECT OUTLINE AND HYPOTHESIS

In GEC, the actin cytoskeleton is a key determinant of cell morphology and other functions, including permselectivity. Complement C5b-9 causes reorganization of the actin cytoskeleton, leading to GEC morphological changes and proteinuria. Rho-GTPases are known to regulate the actin cytoskeleton through their effectors. However, the expression and function of Rho-GTPases in GEC are poorly understood. We hypothesized that *RhoA, Rac1 and Cdc42 are expressed and activated in GEC and that they are important in regulating the morphology and function of GEC in complement-induced GEC injury in vitro and in vivo.*

We first studied the activities of RhoA, Rac1 and Cdc42 in complement-mediated GEC injury in vitro and in vivo by pull-down assays. We further studied the impact of RhoA, Rac1 and Cdc42 activities on GEC morphology and F-actin organization by overexpressing GFP conjugated constitutively active (CA) or dominant negative (DN) mutants of RhoA, Rac1 and Cdc42 individually into GEC. We observed the morphology and actin cytoskeleton organization by a light microscope and confocal microscope. Furthermore, we studied mechanisms for complement-induced RhoA activation and downstream signaling pathways of RhoA involved in complement-mediated GEC injury. Specifically, we studied whether C5b-9 was required for the RhoA Rac1 and Cdc42 activity changes, whether $cPLA_2$ had an effect on RhoA activation, and also whether p190RhoGAP, an upstream regulator for RhoA, and ROCK, a downstream effector for RhoA, were involved in complement-mediated GEC injury. We also studied the impact of Rho activity on complement-mediated cytotoxicity in GEC.

SECTION 3 : MATERIALS AND METHODS

Materials

Tissue culture media, Ecdysone-inducible Mammalian Expression System, ponasterone A, hygromycin, zeomycin and Lipofectamine 2000 were from Invitrogen-Life technologies (Burlington, ON); NuSerum from Collaborative Research (Bedford, MA); electrophoresis reagents from Bio-Rad Laboratories (Mississauga, ON). Mouse anti-Rho, mouse anti-p190RhoGAP and mouse anti-Rac antibodies from Upstate (Lake Placid, NY). Mouse anti-Phospho-p44/42 MAPK (p-ERK) antibody, mouse anti-phospho-myosin light chain 2 (ser19) and rabbit anti-myosin light chain antibody from Cell Signaling Technology (Beverly, MA). Rabbit anti-Cdc42 antibody and protein G PLUS-agarose were from Santa Cruz Biotechnology, Inc (Santa Cruz, CA). Mouse anti-Phosphotyrosine (PY69) was from BD Biosciences (Missisauga, ON). Enhanced chemiluminescence (ECL) detection reagents and glutathione–sepharose beads were from Amersham Bioscience (Baie d'Urfé, Quebec, Canada). Alexa fluor 488 conjugated deoxyribonuclease (DNase) I was from Molecular Probe (Eugene, OR). Male Sprague-Dawley rats were from Charles River Canada (St. Constant, PQ). The plasmids pRK5-RhoA (L63)-Myc (constitutively active), pRK5-RhoA (N19)-Myc (dominant negative), pRK5-Rac1 (L61)-Myc (constitutively active), pRK5-Rac1 (N17)-Myc (dominant negative), pRK5-Cdc42 (L61)-Myc (constitutively active), pRK5-Cdc42 (N17)-Myc (dominant negative), Rhotekin Rho-binding domain fused to GST protein (GST- RBD) and Cdc42/Rac interactive binding domain fused to GST (GST-CRIB) were kindly provided by Dr. Natalie Lamarche-Vane (McGill University, Montreal, QC). Unless

otherwise specified, other chemicals or biochemical agents were purchased from Sigma-Aldrich Canada (Mississauga, ON).

GEC culture

Rat GEC culture and characterization were described previously (30). Briefly, confluent GEC were suspended by incubating with a 0.05% trypsin-0.02% EDTA mixture in Ca^{2+} and Mg^{2+} free Hank's balanced salt solution. Dissociated cells were resuspended in K1 medium (50% DMEM, 50% Ham F-12, 10% NuSerum, hormone mix) and plated onto tissue culture dishes. All cultures were maintained at 37°C in an atmosphere of 95% air and 5% CO_2. Studies were done with cells between passages 10 and 60. By phase-contrast microscopy, GEC demonstrated polygonal shape at subconfluency. When GEC reached confluence, they had a cobblestone-like appearance. For complement stimulation experiments, a subclone of GEC that stably overexpressed $cPLA_2$ (GEC-$cPLA_2$) or a clone containing only the neomycin-resistance gene (GEC-neo, control) were used. Establishment and characterization of GEC-$cPLA_2$ were described previously (84). For morphological studies, untransfected GEC, which grow on plastic substratum (GEC-pl) were used. Inducible-RhoAL63 clones were generated using Ecdysone-inducible mammalian expression system. First, GEC-pl were stably transfected with the plasmid pVgRXR and selected by zeomycin. Second, cells were transfected with RhoA-L63 subcloned into the pIND/Hygro vector and selected by hygromycin. Expression of RhoA-L63 was induced with an insect hormone, ponasterone A (1μM).

Complement stimulation of GEC

Stimulation of GEC by complement was described previously (30; 84; 85). Briefly, cultured GEC were incubated with rabbit anti-GEC antiserum or sheep anti-Fx1A antiserum (5% vol/vol) in modified Krebs-Henseleit buffer, containing 145 mmol/L NaCl, 5 mmol/L KCl, 0.5 mmol/L $MgSO_4$, 1 mmol/L Na_2HPO_4, 0.5 mmol/L $CaCl_2$, 5 mmol/L glucose, and 20 mmol/L Hepes, pH 7.4 for 40 minutes at 22°C, followed by incubation with normal human serum (NS; 1.5% vol/vol) for 40 minutes (short-term) or 16 hours (long-term) at 37°C in order to assemble C5b-9. In control experiments, heat inactivated serum (HIS; 56°C, 30 min) was used in the place of NS (86; 87). After heating, the complement is not present in the serum. In some experiments, antibody-sensitized GEC were incubated with C8-deficient human serum (C8D) (1.5% vol/vol) with or without reconstitution with purified human C8 (2μg/ml) (86).

Induction of PHN in rats

As stated earlier, PHN was induced in male Sprague-Dawley rats (150-175-g body wt.) by intravenous injection (400 μl/rat) of sheep anti-Fx1A antiserum. Preparation of anti-Fx1A antiserum was described earlier (85; 88). Rats did not show significant proteinuria until 7 days of injection. Significant proteinuria was observed 14 days after injection (>150 mg/day; normal rats excrete less than 10 mg of protein per day). On day 14, rats were sacrificed and glomeruli were isolated by differential sieving (28) . All studies were approved by the McGill University Animal Care Committee.

Immunoblotting

Cells or glomeruli were washed with ice-cold PBS and lysed with IP buffer [1% Triton X-100, 125mM NaCl, 10mM Tris (pH 7.4), 1mM EDTA, 1mM EGTA, 2mM Na_3VO_4, 10mM sodium pyrophosphate, 25mM NaF], and protease inhibitor cocktail (Roche Diagnostics). After insoluble components were removed by centrifugation (14,000 rpm, 5 min, 4°C), protein concentration of supernatants was quantified using a commercial reagent (Bio-Rad). Proteins were separated by SDS-PAGE under reducing conditions and were transferred electrophoretically to nitrocellulose membranes. Membranes were blocked with 5% skim milk and incubated with primary antibodies at 4°C overnight. After three washes, membranes were incubated with secondary antibodies conjugated with horseradish peroxidase. Immunoreactive proteins were identified by the ECL system.

Purification of GST-CRIB and GST-RBD

GST-CRIB (Cdc42/Rac-interactive binding) and GST-RBD (Rhotekin Rho binding domain) were used to isolate activated Cdc42/Rac and RhoA respectively, from the lysate of rat glomeruli and cultured rat GEC (see next section). Only the active (GTP-bound) Rho-GTPases interact with their downstream effectors, the GST-RBD and GST-CRIB can pull-down only GTP-bound RhoA, Rac1 and Cdc42. Escherichia coli transformed with the GST-CRIB or GST-RBD constructs were grown at 37°C to an absorbance level of 0.8. Expression of the fusion proteins was induced with 0.1 mM isopropyl-β-d-thiogalactopyranoside for 3 hours at 37°C for GST-CRIB or 30°C for GST-RBD. Cells were washed once in STE buffer (100 mM Tris-Cl, pH 8.0, 100 mM

NaCl, 1 mM EDTA) before sonication in a sonication buffer (20 mM HEPES, pH 7.5, 120 mM NaCl, 2 mM EDTA, 10% glycerol, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM PMSF). The lysates were cleared by centrifugation, and NP-40 was added in lysate to a final concentration of 0.5%. Glutathione beads (300μl) were added to the lysate for 30 minutes at 4°C with gentle rocking in order to conjugate GST-CRIB or GST-RBD to the beads. Protein yield was verified by gel electrophoresis and Coomassie blue staining with BSA as standard. Per sample, 10-15 μg of GST-RBD (as estimated by Coomassie blue staining) bound to glutathione-Sepharose beads were washed twice with binding buffer (25 mM HEPES, pH 7.5, 30 mM $MgCl_2$, 40 mM NaCl, 0.5% NP-40, 1 mM DTT) before use (89-91).

Active RhoA, Rac1 and Cdc42 assays

Cultured rat GEC, glomeruli from normal rat and rat with PHN were lysed in lysis buffer (25 mM HEPES, pH 7.5, 1% NP-40, 10 mM $MgCl_2$, 100 mM NaCl, 5% glycerol, 5 mM sodium fluoride, 1 mM sodium vanadate, 1 mM PMSF, 10 μg/ml aprotinin, 10 μg/ml leupeptin). Equal protein amounts (250-1000 μg) were incubated for 1 hour at 4°C with purified GST-CRIB or GST-RBD (10-15 μg). Beads were washed twice in binding buffer and boiled in Laemmli sample buffer, and the proteins were separated on a 12% SDS-polyacrylamide gel. The quantities of RhoA, Rac1 and Cdc42 bound to the fusion proteins, as well as the levels present in total lysates (40-50 μg), were evaluated by western blotting with anti-Rho (2μg/ml), anti-Rac (1:2000) and anti-Cdc42 (1:1000) antibodies.

Measurement of active p190RhoGAP

Cell lysates were immunoprecipitated with anti-p190RhoGAP antibody (4μg/ml) for 2 hours, followed by 50μl of protein G PLUS-Agarose for 2 hours. The beads were subsequently collected by centrifugation and washed three times in IP buffer The beads were then resuspended in 25μl of Laemmli buffer and boiled at 100°C for 5 min. Samples were separated by 7.5% SDS-polyacrylamide gel electrophoresis. Immunoblotting was performed using an anti-p190 RhoGAP (1μg/ml) or anti PY-69 (1μg/ml).

Transient transfection of GEC with Rho-GTPase mutants

For morphological studies, GEC were grown on coverslips. CA and DN mutants of RhoA, Rac1 and CDC 42 were subcloned into pEGFP-C1. GEC-pl (40-50 % confluency) were transiently transfected with lipofectamine 2000 transfection reagent, using 3μl of reagent per μg of plasmid. After 24h, cells were fixed with 3% formaldehyde and permeabilized with 0.5% Triton X-100. After blocking with 3% BSA, GEC were stained with Rhodamin-phalloidin (0.05μg/ml) for F-actin and examined under a confocal microscope (Zeiss LSM 510 META).

F/G actin ratio

Cells grown on coverslips were fixed with 3% formaldehyde in PBS for 15 minutes at room temperature and permeabilized with 0.5% Triton X-100 in PBS for 5 minutes. After blocking in PBS containing 3% BSA for 20 minutes, F-actin and G-actin were labeled with rhodamine-labeled phalloidin (0.05μg/ml) and Alexa Fluor 488

conjugated DNaseI (9μg/ml) in 0.2% PBS/BSA for 20min at room temperature, respectively. Cells were examined under a fluorescence microscope. F-actin and G-actin were quantified (NIH Image J software), and F/G actin ratio was calculated.

Measurement of complement-dependant cytotoxicity

When cells are injured, an intracellular enzyme lactate dehydrogenase (LDH) is released into supernatants; thus, it could be used as a marker of cell injury. Complement-mediated cytolysis was determined by measuring the release of LDH as described previously (92). The specific release of LDH into supernatants was calculated for the purpose of quantifying cytotoxicity by the formula, (NS-HIS)/(100-HIS) x100, where NS represents percent LDH release induced by NS, while HIS represents percent LDH release with HIS.

Statistics

Data are presented as mean ± SEM. The *t* statistic was used to determine significant differences between two groups. One-way analysis of variance (ANOVA) was used to determine significant differences among groups. Where significant differences were found, individual comparisons were made between groups using the *t* statistic.

RESULTS

Activities of RhoA, Rac1 and Cdc42 are altered by complement in GEC in vivo

Previous studies in cultured GEC showed that complement-mediated sublethal injury was accompanied by reorganization of the cytoskeleton, including loss of actin stress fibers and focal contacts (3). RhoA, Rac1 and Cdc42 are known regulators of the arrangement and assembly of the actin cytoskeleton (53). For RhoA, Rac1 and Cdc42 to have an impact on the actin cytoskeleton, they must be active (GTP-bound). Thus, we first determined the amount of active RhoA, Rac1 and Cdc42 in glomeruli from rats with PHN using pull-down assay (Methods). Fourteen days after induction of PHN, rats showed significant proteinuria (325±125 mg per day), as compared with normal rats (15±3 mg per day, N=6, $p<0.01$ vs PHN). In glomeruli of rats with PHN, the amount of active RhoA increased by 2.3 fold, as compared with normal rat glomeruli (control 100±17, PHN 228±50 arbitrary units; N=6, $p<0.05$)(Fig. 1A). In contrast, the amount of active Rac1 and Cdc42 were decreased by 38% and 55%, respectively, in glomeruli from rats with PHN (Rac1: control 100±1, PHN 62±12, arbitrary units; N=6, $p<0.05$; Cdc42: control 100±2, PHN 45±19, arbitrary units; N=6, $p<0.05$)(Fig.1B, C). There were no significant differences in the total amount of Rho-GTPases (Fig. 1A-C). Since the GEC is the main cellular target in PHN, it is reasonable to assume that these changes in Rho-GTPase activities occurred in GEC (29). These results indicate that complement-induced injury leads to activation of RhoA and inactivation of Rac1/Cdc42 in GEC in vivo.

Activities of RhoA, Rac1 and Cdc42 are altered by complement in GEC in vitro

In lysates of cultured rat GEC, RhoA, Rac1, and CDC42 were detected as bands of 22kDa, 21kDa, and 25kDa, respectively by immunoblotting, confirming the endogenous expression of these proteins (Fig. 2A). In order to further address the role of Rho-GTPase in GEC, we studied the effect of complement on RhoA, Rac1 and Cdc42 activities in cultured rat GEC. We first determined the optimal concentration of normal serum (NS) to induce GEC sublytic injury. Complement is present in NS. We reported previously that sublytic complement stimulation leads to phosphorylation of extracellular signal-regulated kinase (ERK) (84). When cells were stimulated with various concentrations of complement (NS), 1.5% NS (per dish medium volume) induced most prominent and consistent phosphorylation of ERK (Fig. 2B); thus this concentration was chosen for further experiments.

In the first series of experiments, GEC- $cPLA_2$ was stimulated with complement for 40 min (acute injury). In the previous study, it was shown that $cPLA_2$ augments complement-mediated GEC injury and signaling events(87). Therefore, we used GEC-$cPLA_2$ for this series of experiments. Complement stimulation increased the amount of active RhoA and Rac1 by 2.6 fold and 1.7 fold, respectively (RhoA: HIS 13±5, NS 37±9, arbitrary units, N=4, $p<0.01$; Rac1: HIS 24±8, NS 43±7, arbitrary units, N=4, $p<0.05$), as compared with control (Fig. 2C, D). The amount of active Cdc42 also tended to increase, however the difference was not statistically significant (not shown). Thus, in acute complement incubations, some changes in Rho-GTPase activities did not necessarily parallel the changes in vivo. In the next series of experiments, we studied the impact of

long-term (overnight) complement stimulation on Rho-GTPase activities. The amount of active RhoA was increased by 1.2 fold by complement stimulation, while the amounts of active Rac1 and Cdc42 were decreased by 26% and 46%, respectively (RhoA: HIS 31±10, NS 38±9, N=4, $p<0.05$; Rac1: 55±10, NS 41±8, N=4, $p<0.05$; Cdc42: HIS 35±8, NS 19±5, N=4, $p<0.05$, active RhoA, Rac1 and Cdc42 were presented in arbitrary units) (Fig. 3A-C). These results suggest that RhoA activation observed in vivo is probably occured and is persistent, while Rac1 and Cdc42 activities may be increased initially, but are suppressed during chronic exposure to complement.

To verify if complement-mediated changes of Rho-GTPases were due to C5b-9 assembly, we used C8-deficient human serum (C8D), which assembles only C5b-7, and studied its impact on active RhoA. When antibody-sensitized GEC were incubated with C8D alone, the amount of active RhoA was not different from control (HIS). However, when C8D was reconstituted with purified C8, active RhoA was increased, similar to cells stimulated with NS (Fig. 3D). These results indicate that activation of RhoA is dependent on C5b-9 assembly.

We previously reported that C5b-9 activates $cPLA_2$ in GEC, leading to exacerbation of complement dependent GEC injury and amplification of signaling events, such as an increase in arachidonic acid release, production of prostanoids, and activation of various protein kinases. Thus, several signaling pathways activated by C5b-9 are, at least in part, dependent on $cPLA_2$ (87). Next, we studied if $cPLA_2$ contributes to complement-mediated RhoA activation. When a subclone of GEC that stably overexpress $cPLA_2$ (GEC-$cPLA_2$) was stimulated with complement, active RhoA was increased by 1.4 fold (HIS 25±9, NS 36±9, arbitrary units, N=3, $p<0.01$). This increase was not,

however, significantly different from a 1.6 fold increase observed in control cells (GEC-neo: HIS 23±3, NS 38±6, arbitrary units, N=3, $p<0.05$)(Fig. 3E). These results suggest that $cPLA_2$ does not augment complement-induced RhoA activation.

Activity of p190RhoGAP is decreased by complement in GEC

To begin to address the mechanisms of complement-mediated RhoA activation in GEC, we next studied the effect of complement on the negative regulator of RhoA, p190RhoGAP (58). Upon undergoing tyrosine phosphorylation, p190RhoGAP becomes active and inactivates RhoA (58). Thus, we studied the level of tyrosine phosphorylation of p190RhoGAP as a surrogate marker of its activity. p190RhoGAP was clearly tyrosine phosphorylated in control GEC. When GEC were stimulated with complement, tyrosine phosphorylation of p190RhoGAP decreased by 41% (Fig. 4, HIS 21±3, NS 12±1, arbitrary units, N=4, $p<0.05$). These results suggest that the complement-induced decrease of p190RhoGAP activity may, at least in part contribute to complement-mediated RhoA activation.

Impact of Rho-GTPase activities on GEC morphology in vitro

As the first step to understand the functional consequences of complement-induced changes in Rho-GTPase activities, we studied the impact of constitutively active (CA) or dominant negative (DN) mutants of RhoA, Rac1, and CDC42 (Methods) on the morphology of cultured rat GEC. All mutants were conjugated with GFP in order to identify successfully transfected cells. GEC transfected with GFP alone did not demonstrate any morphological changes, as compared with untransfected cells (Fig. 5A).

GEC transfected with CA-RhoA and CA-Cdc42 showed a smaller and round contour, and the staining with rhodamine-phalloidin exhibited a strong cortical F-actin pattern (Fig. 5B&F). In contrast, GEC transfected with CA-Rac1 demonstrated morphological changes that resembled process formation (Fig. 5D). DN-mutants had less impact on GEC morphology, although each appeared to have the opposite impact from its CA-counterpart (Fig.5C, E, G). These results suggest that each Rho family member has a distinct effect on the F-actin pattern and morphology in GEC.

Impact of complement stimulation on the actin cytoskeleton in GEC

In these studies, we addressed the impact of complement on the actin cytoskeleton in GEC. Phalloidin staining of control GEC showed a distinct cortical F-actin pattern and some stress fibers (Fig. 6A). After cells were stimulated with complement, the intensity of cortical F-actin and stress fibers decreased, while strong perinuclear F-actin staining appeared (Fig. 6C). We also quantified F/G actin ratio. After cells were treated with complement, F/G actin ratio decreased significantly, as compared with control (HIS 0.87±0.009, NS 0.79±0.004, arbitrary units, N=3, $P<0.01$). Thus, complement has a distinct impact on the actin cytoskeleton in GEC both in quantity and quality.

The studies described above have established that RhoA is activated by complement C5b-9 in GEC. ROCK is a major downstream effector of RhoA, which mediates Rho-induced assembly of focal adhesions and stress fibers (52). We addressed whether ROCK contributes to the changes in the actin cytoskeleton induced by complement. In control GEC, F-actin (visualized by rhodamine-phalloidin staining) was

distributed in a cortical pattern, and there was stress fiber apparent in cytosol (Fig. 6A). When GEC was treated with a specific inhibitor of ROCK, Y27632, stress fibers decreased, while the cortical F-actin was relatively maintained (Fig. 6B). Without complement stimulation, Y27632 decreased F/G actin ratio slightly, as compared with control; however, this change was not statistically significant (Fig. 6E). When GEC were stimulated with complement, F/G actin ratio decreased (Fig. 6E, HIS 0.87±0.011, NS 0.78±0.005, arbitrary units, N=3, $P<0.01$). When GEC were stimulated with complement in the presence of Y27632 (Fig. 6D), the perinuclear F-actin staining induced by complement was partly reversed, and the cortical F-actin was well preserved in the presence of Y27632, as compared with in its absence (Fig. 6C). Y27632 also revered the change in F/G actin ratio induced by complement (Fig. 6E, HIS+Y27632 0.84±0.02, NS+Y27632 0.84±0.005, arbitrary units, N=3, no significant difference). The effect of complement on GEC F/G actin, was attenuated with the presence of Y27632. These results suggest that ROCK contributes to changes in the actin cytoskeleton in GEC induced by complement.

It is known that ROCK induces phosphorylation of myosin light chain (MLC) either by inhibiting the MLC phosphatase or by taking the place of MLC kinase (MLCK) and directly phosphorylating MLC, thereby enhancing myosin activation, and increasing tension generation (63-65). Thus, we examined if complement stimulation would lead to phosphorylation of MLC. Immunoblotting with a phospho-Ser 19 specific antibody was used to monitor MLC phosphorylation (93). Weak MLC phosphorylation was present in control cells (data not shown), and acute or chronic complement simulation did not cause consistent changes in this phosphorylation. These results suggest that although ROCK

appears to contribute to complement-mediated cytoskeletal changes in GEC, MLC is unlikely to participate in this process.

Impact of RhoA activity on complement-mediated cytotoxicity in GEC

RhoA activation has a profound effect on GEC morphology (Fig. 5). In order to study the impact of RhoA activation on complement-mediated GEC cytotoxicity, we generated a subclone of GEC that inducibly expresses CA-RhoA (RhoAL63). CA-RhoA was induced by stimulation with the insect hormone, ponasterone A (1μM) for 24 h, and morphological changes were apparent (Fig. 7A), cells are becoming smaller and rounder as compared with control cells (Fig.7B, treated with ethanol in the place of ponasterone A). In control cells stimulated with serially increasing concentrations of complement, concentration dependent cytotoxicity was observed (Fig. 7C). As a cytolysis marker, LDH is used to measure the cytotoxicity in complement-mediated GEC injury. The more cell is injured, the more LDH is released into the medium. Cytotoxicity was augmented significantly by induction of CA-RhoA. (Fig.7C, NS1.5%: control 22±3%, ponasterone A 25± 4%, N=7, $P<0.05$, NS2.5%: control 38±4%, ponasterone A 48±3%; N=7, $P<0.01$). The high LDH release (22%) seen with 1.5% of NS, the nonlytic concentration in the earlier studies (Fig.2 and 3), is likely caused by the different batch of NS used in this series of experiments. We also studied the effect of Y27632 on complement-mediated cytotoxicity (Fig 7D). When GEC were stimulated with complement in the presence of Y27632, cytotoxicity was inhibited significantly, as compared with in the absence of Y27632 (NS 1.5%: control 8±1%, Y27632 7±1%, NS2.5%: control 33±4%, Y27632 28±3%, $p<0.05$; NS5%: control 64±4%, Y27632 56±3%, $P<0.05$, N=8 for all groups).

Together, these results indicate that over activation of RhoA exacerbates complement-mediated cytotoxicity, likely via activation of ROCK.

DISCUSSION

The objective of this study was to characterize the function of Rho-GTPases in complement mediated GEC injury. Using pull-down assay, we demonstrated that the amount of active RhoA increased, while the amount of active Rac1 and Cdc42 decreased in chronic C5b-9 mediated sublytic GEC injury in vitro and in glomeruli from rats with PHN in vivo (Fig. 1-3). The results from in vitro and in vivo study also suggest that RhoA activation in vivo is probably occurred shortly after complement stimulation and is persistent, while Rac1 and Cdc42 activities may be increased initially, but are suppressed during chronic exposure to complement. Complement-mediated inactivation of p190RhoGAP may contribute to complement-induced RhoA activation (Fig. 4). Overexpression of CA or DN-mutants of RhoA, Rac1 and Cdc42 distinctly altered GEC morphology and F-actin distribution (Fig. 5). Complement caused marked changes in the actin cytoskeleton in GEC, which appeared to be, at least in part mediated by a downstream kinase of RhoA, ROCK (Fig. 6). Finally, activation of RhoA exacerbated complement-mediated cytotoxicity in GEC, while inhibition of ROCK attenuated it (Fig. 7). To the best of our knowledge, this is the first study to provide a detailed profile of RhoA, Rac1 and Cdc42 activities in complement-mediated GEC injury. Moreover, we have also identified some of the Rho-mediated signaling pathways, which contribute to complement-mediated GEC injury.

The sophisticated cell shape of GEC/podocyte is supported by the intracellular filamentous network of cytoskeletons, including microtubules, intermediate filaments and actin filaments. In GEC, cytoskeletal system is highly organized to establish and maintain

their major and foot processes, where cytoskeletal proteins are clearly segregated: thick major processes contain bundles of microtubules and intermediate filaments, whereas thin foot processes contain bundles of actin filaments (82). The foot process actin cytoskeleton is highly dynamic and ultimately determines the structural maintenance of the filtration slits (1). Rho-GTPases are known to regulate the actin cytoskeleton assembly. To date, there is limited information on the role of Rho-GTPases in GEC/podocytes. Rho GDIα$^{-/-}$mice developed massive proteinuria (83). In this model, lack of Rho GDIα influenced the structural and functional organization of kidney epithelial cells, and gradually led to renal failure and death. In this model, podocytes were severely injured and foot processes were disrupted, suggesting a critical role of Rho-GTPases in podocyte morphology and function. However, since Rho GDIα non-selectively regulates various Rho-GTPases, including RhoA, Rac1 and Cdc42 (34), specific roles of individual Rho-GTPases could not be identified in this study. In immortalized mouse GEC, Y27632 inhibited actin cytoskeleton redistribution caused by mechanical stretch (94). Y27632 also inhibited redistribution of F-actin fibers and up-regulation of prepro-endothelin (ET)-1 induced by protein overload (95) . These results are suggestive of a pathological role of the RhoA/ROCK pathway in podocytes. The current results demonstrated that RhoA activity is increased by complement stimulation in GEC (Fig. 1-3) and that inhibition of ROCK reversed some of the complement-induced changes in F-actin (Fig. 6). Overexpression of active RhoA exacerbated complement-mediated GEC cytotoxicity, while a ROCK inhibitor attenuated cytotoxicity (Fig. 7). These results support RhoA/ROCK pathway is involved in complement-mediated GEC injury.

Rho-GTPase activities are controlled by three families of proteins, i.e. GAPs, GEFs and GDIs. Among ~80 GAPs known to date, p190RhoGAP is one of the best-studied molecules (58). It is known that the activity of p190RhoGAP is increased by tyrosine phosphorylation mediated by Src-family kinases (58). Miller et al. demonstrated that Receptor for activated C kinase 1(RACK1), upon activation of protein kinase C (PKC), binds to Src and inhibits its function, leading to decreased phosphorylation of p190RhoGAP (96). In this report, a PKC agonist reduced tyrosine phosphorylation of p190RhoGAP by ~40%, similar to what we observed in GEC stimulated with complement (Fig. 4). This decrease of p190RhoGAP phosphorylation was accompanied by a clear increase in active RhoA (96), indicating that a 40% change in p190RhoAGAP phosphorylation is physiologically significant. We have shown previously that complement C5b-9 activates PKC (87), thus one potential mechanism for the complement-mediated decrease of p190RhoGAP phosphorylation is via activation of PKC. It was also reported that low molecular weight-protein tyrosine phosphatase (LMW-PTP), a key mediator of platelet-derived growth factor receptor signaling, counteracts the effects of Src-family kinases and functions as a negative regulator of p190RhoGAP (97). Whether C5b-9 activates LMW-PTP is not known.

GEC and neurons share many cell biological characteristics, including a highly arborized morphology. Concerning the formation of podocyte foot processes and dendritic branches, actin filaments are thought to play a central role in maintaining structure and function in both cells (81). Rho-GTPases are responsible for the establishment of dendritic branching morphology. Activation of RhoA in neuronal cells induced neurite retraction and cell rounding (98), while activation of Rac1 promoted the

formation of lamellipodia, along the extension of neurite-like structures (99). Cells expressing active Rac reduced Rho-mediated cell rounding, suggesting that the activities of Rac and Rho oppose each other in neuronal cells (99; 100). By analogy, Kobayashi and colleagues recently reported that in immortalized mouse podocytes, Y-27632 promoted elongation of thick processes, and Y-27632 and forskolin in combination further induced projections elongating from major processes (82). Forskolin is known to inhibit RhoA activity, as well as to increase Rac1 and Cdc42 activities. These results suggest that Rac1 activation promotes process formation in neural cells and podocytes, while RhoA activation has an opposite effect. In the current study, GEC transfected with CA-RhoA had a smaller and round contour, while cells transfected with CA-Rac1 demonstrated morphological changes that resembled process formation (Fig. 5). Also, in complement-stimulated GEC, RhoA activity was increased while Rac1 activity was decreased (Fig. 1-3). Considering the impact of RhoA and Rac1 activation on cultured podocytes and neural cells, it is tempting to speculate that a certain ratio of active RhoA/Rac1 is critical for the formation and maintenance of foot processes and that increased RhoA activity and/or decreased Rac1 activity would disturb morphological character of podocytes, exacerbate GEC injury induced by complement, leading to proteinuria.

In many physiological and pathophysiological settings, changes in Cdc42 activity often parallels that of Rac1 and cellular effects of Cdc42 are more similar to Rac1 than to RhoA (99; 100). In the current study, both Rac1 and Cdc42 activities were decreased in GEC by complement (Fig. 1-3), similar to observations in other systems. However, CA-Cdc42 had an impact on GEC morphology similar to CA-RhoA, but not to

CA-Rac1 (Fig. 5). This unusual response of GEC to Cdc42 could be cell specific and may be determined by cell's ability to assemble integrin based cell-matrix or cadherin based cell-cell adhesion complexes (2; 101).

In the current study, complement led to a decrease in cortical F-actin and overall F/G actin ratio (Fig. 6). These results were somewhat unexpected because RhoA was consistently activated by complement in vitro and in vivo (Fig. 1-3), which is expected to increase the cortical F-actin and actin polymerization (37; 102). Nonetheless, a decrease in actin stress fibers by complement was also reported by others (3). It was shown that complement-mediated injury cause cellular ATP depletion, which could lead to disassembly of actin fibers (3). Perinuclear F-actin condensation is another manifestation of ATP depletion (103) and its appearance in complement-stimulated GEC (Fig. 6) is in support of ATP depletion in these cells. Alternatively, increased membrane permeability caused by complement may lead to the loss of vital components of cytoskeleton assembly, which also lead to disassembly of actin fibers. Y27632 partially reversed the appearance of perinuclear F-actin and abolished the decrease of cortical F-actin and F/G-actin ratio (Fig. 6). These results suggest that ROCK is involved in complement-mediated GEC cytoskeletal reorganization and that inhibition of the RhoA/ROCK pathway could attenuate the collapse of cytoskeleton in complement-mediated GEC injury.

In an attempt to identify downstream effectors of ROCK, we studied phosphorylation of MLC, however, complement had no significant impact on MLC phosphorylation (not shown). ROCK activated by RhoA leads to phosphorylation of MLC, while PAK activated by Rac1 also increases MLC phosphorylation, and consequently reassembles actin filements (93). Since complement stimulation decreases

Rac1/Cdc42 activity in GEC, subsequent inactivation of PAK may neutralize the effect of RhoA on MLC phosphorylation. Thus, the Rho/ROCK pathway might achieve actin filement rearrangement through other potential downstream effectors of ROCK, which include actin-depolymerizing factor (ADF)/cofilins (104; 105) or ezrin-radixin-moesin (ERM) family proteins (66). Of interest, Pritchard and colleagues demonstrated that in fibroblasts, The gene (BRAF) encoding B-Raf is mutated at a high frequency in human malignancies. B-Raf acts via the ROCK/LIMK/cofilin pathway, instead of via MLC phosphorylation, to regulate actin stress fibers (106). Potential roles of these ROCK effectors in complement-mediated GEC injury will require further investigation.

Epithelial intercellular junctions regulate cell-cell contact and barrier function. Both tight junction and adherens junctions are regulated in part by their affiliation with the F-actin cytoskeleton. Actin cytoskeleton in turn is influenced by Rho-GTPases. Madera and colleagues demonstrated that both activation and inactivation of RhoA, Rac1 and Cdc42 enhance paracellular permeability and contribute to barrier dysfunction in MDCK cells (79). We also reported that both activation of RhoA and inhibition of ROCK reduce complement-mediated ERK activation in GEC (80). Using a subclone of GEC, which expresses CA-RhoA in an inducible manner, we demonstrated that complement stimulated GEC cytotoxicity was exacerbated by activation of RhoA (Fig. 7A). In contrast, the ROCK inhibitor attenuated complement-induced cytotoxicity (Fig. 7B). In complement-mediated chronic GEC injury, activity of RhoA increased, while activities of Rac1 and Cdc42 decreased (Fig1-3). Overexpression of CA-RhoA/Rac1/Cdc42 or DN-RhoA/Rac1/Cdc42 exhibited distinct effect on GEC morphology (Fig 5). These results

further support the view that a critical balance of active RhoA, Rac1, and Cdc42 is essential for maintenance of normal GEC function.

In summary, the present study supports an important role for Rho-GTPases in the morphology and function of GEC. An imbalance of RhoA, Rac1 and Cdc42 activities in GEC might cause derangement of the actin cytoskeleton and contribute to morphological changes of GEC and proteinuria in complement-mediated GEC injury.

FIGURE 1

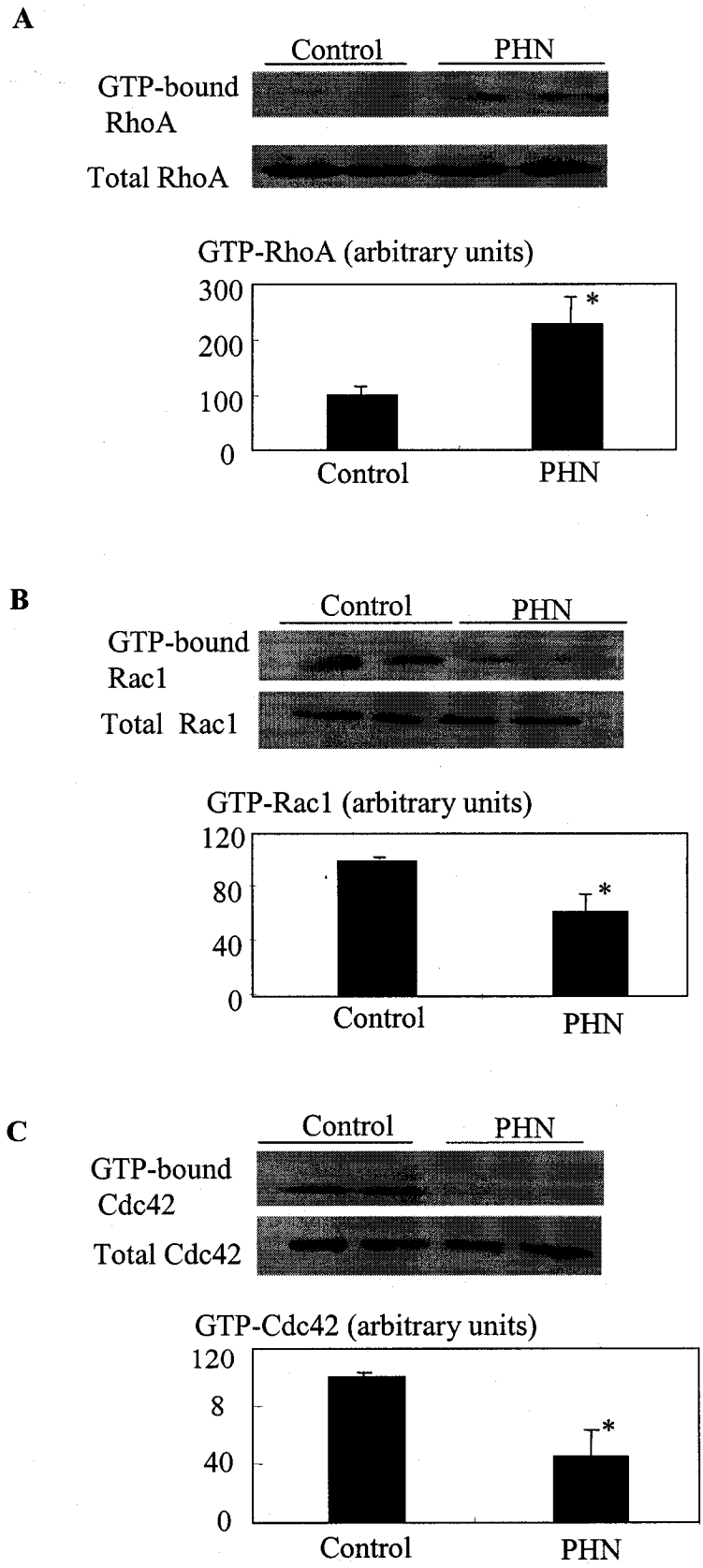

A
Control
PHN
GTP-bound RhoA
Total RhoA
GTP-RhoA (arbitrary units)
300
200
100
0
*
Control
PHN
B
Control
PHN
GTP-bound Rac1
Total Rac1
GTP-Rac1 (arbitrary units)
120
80
40
0
*
Control
PHN
C
Control
PHN
GTP-bound Cdc42
Total Cdc42
GTP-Cdc42 (arbitrary units)
120
8
40
0
*
Control
PHN

Fig. 1. Activities of RhoA, Rac1 and Cdc42 are altered by complement in GEC in vivo

Glomeruli were isolated from normal rats (control) and rats with PHN (day 14). Glomerular lysates were subjected to pull-down assay with GST-Rhotekin and GST-CRIB to assay for active (GTP-bound) RhoA and Rac1/Cdc42, respectively (Methods). Pull-down samples and total lysates were analyzed by immunoblotting using antibodies for RhoA (A), Rac1 (B), and Cdc42 (C). The expression of RhoA, Rac1, and Cdc42 in control and rats with PHN are equal, the GTP-bound Rho-GTPases was qualified without normalization by total Rho-GTPases. For each panel, the top is a representative immunoblot and the bottom is densitometric analysis. *$p<0.05$ vs control, N=6 rats in each group.

FIGURE 2

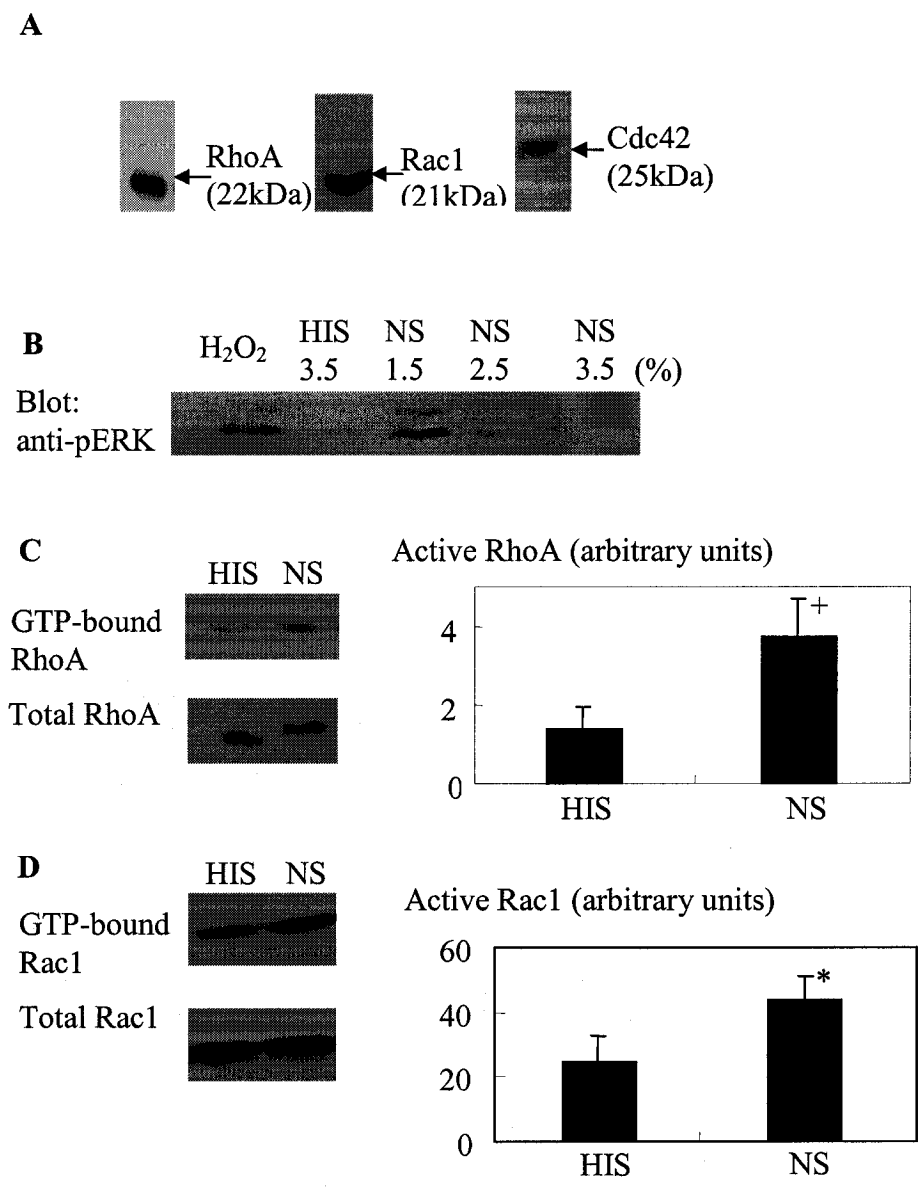
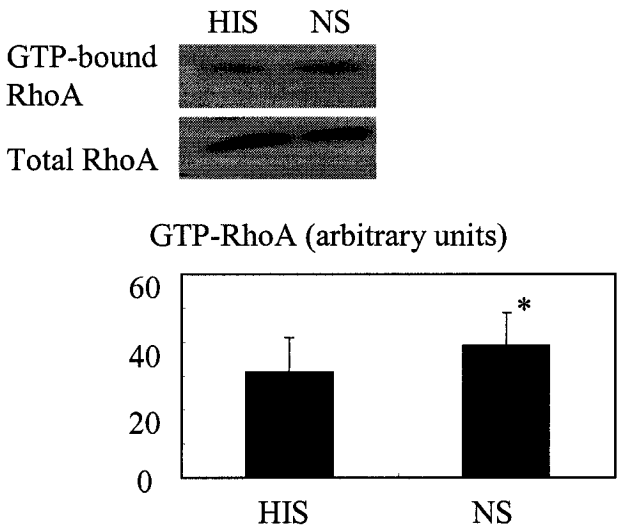

A
RhoA
(22kDa)
Rac1
(21kDa)
Cdc42
(25kDa)
B
H_2O_2
HIS 3.5
NS 1.5
NS 2.5
NS 3.5 (%)
Blot:
anti-pERK
C
HIS NS
GTP-bound RhoA
Total RhoA
Active RhoA (arbitrary units)
4
2
0
+
HIS
NS
D
HIS NS
GTP-bound Rac1
Total Rac1
Active Rac1 (arbitrary units)
60
40
20
0
*
HIS
NS

Fig. 2. Activities of RhoA, Rac1 and Cdc42 are altered by complement in GEC in vitro (short term incubation)

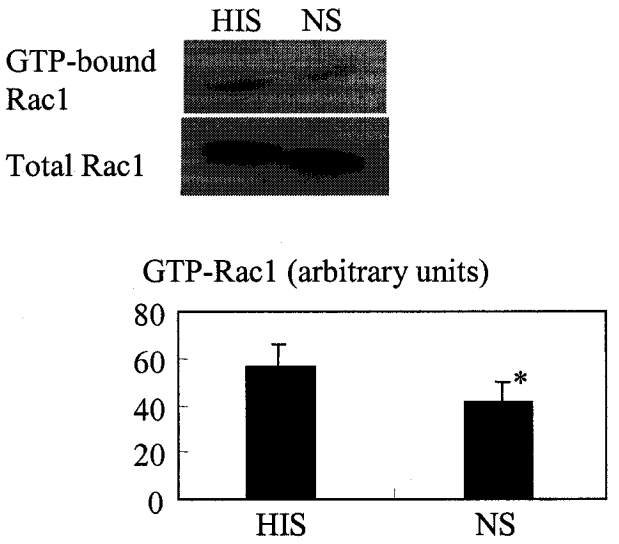
A. Expression of RhoA, Rac1 and Cdc42 in cultured rat GEC was determined by immunoblotting. B. GEC-cPLA$_2$ (Methods) were sensitized with anti-GEC antiserum and incubated for 40minutes at 37°C with serially increasing concentrations of normal human serum (NS) [1.5%, 2.5%, and 3.5% (v/v)] or with 3.5% heat-inactivated human serum (HIS) in control. H_2O_2 (1 mM, 20 min, 37°C) was used as positive control. Cell lysates were immunoblotted for anti-pERK and ERK. C, D) GEC-cPLA$_2$ were stimulated with complement using 1.5% (v/v) of NS for 40 minutes at 37°C and GTP-bound (active) RhoA (C) and Rac1 (D) were quantified as in Fig. 1 (RhoA: *$p<0.01$ vs HIS, N=4; Rac1: +$p<0.05$, N=4).

FIGURE 3

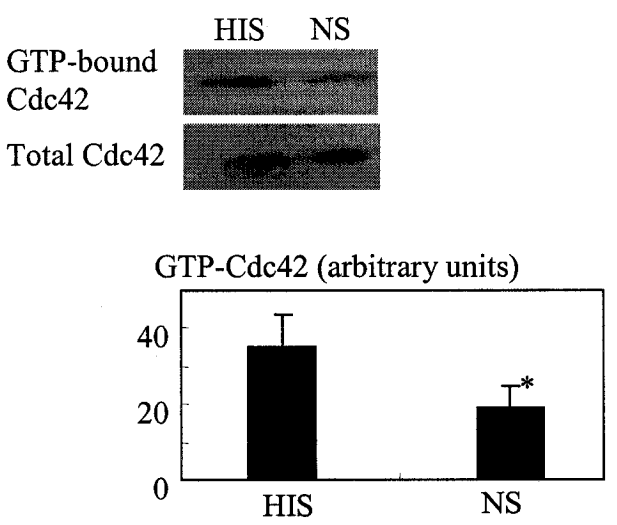
A


HIS
NS
GTP-bound RhoA
Total RhoA
GTP-RhoA (arbitrary units)
60
40
20
0
*
HIS
NS

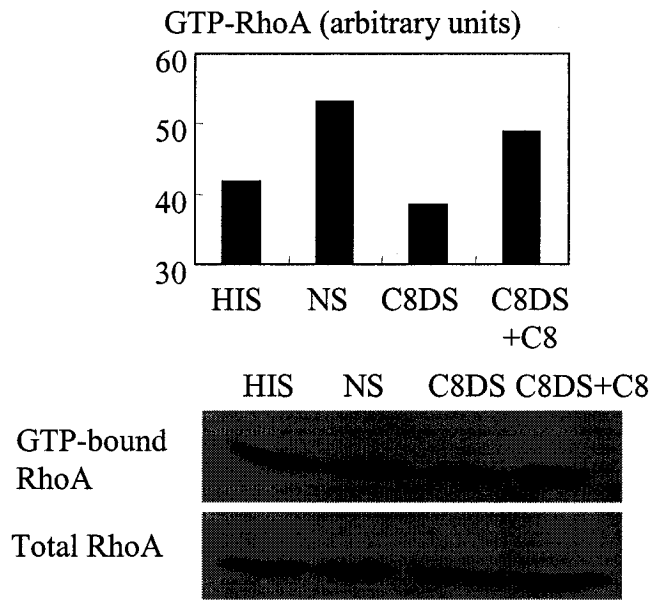
B


HIS
NS
GTP-bound Rac1
Total Rac1
GTP-Rac1 (arbitrary units)
80
60
40
20
0
*
HIS
NS

C


HIS
NS
GTP-bound Cdc42
Total Cdc42
GTP-Cdc42 (arbitrary units)
40
20
0
*
HIS
NS

D


GTP-RhoA (arbitrary units)
60
50
40
30
HIS
NS
C8DS
C8DS +C8
HIS NS C8DS C8DS+C8
GTP-bound RhoA
Total RhoA

E

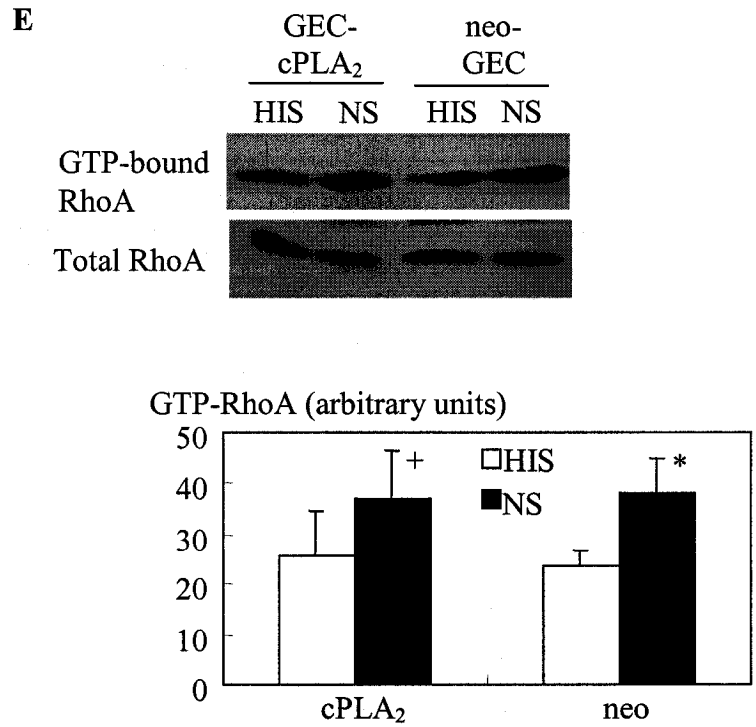

GEC-cPLA2
neo-GEC
HIS NS HIS NS
GTP-bound RhoA
Total RhoA
GTP-RhoA (arbitrary units)
50
40
30
20
10
0
+
*
HIS
NS
cPLA2
neo

Fig. 3. Activities of RhoA, Rac1 and Cdc42 are altered by complement in GEC in vitro (long term incubation)

Antibody-sensitized GEC-cPLA$_2$ were incubated with 1.5% (v/v) NS or HIS (control) for 16 hours at 37°C. GTP-bound (active) RhoA (A), Rac1 (B), and CDC42 (C) were quantified as in Fig. 1. *$p<0.05$ vs HIS, N=4 (RhoA) or 5 (Rac1 and CDC42) D. GEC-cPLA$_2$ was incubated with HIS, NS, C8-deficient serum alone (C8DS; to form C5b-7), C8-deficient serum reconstituted with purified C8 (C8DS+C8; to form sublytic C5b-9) for 16 hours at 37°C and GTP-bound RhoA was quantified by pull-down assay. E. Antibody sensitized GEC-cPLA$_2$ or GEC-neo were incubated with HIS or NS (1.5% v/v) at 37°C for 16 h and GTP-bound RhoA was quantified. *$P<0.05$ vs HIS, N=3, $^{+}P<0.01$ vs HIS, N=3.

FIGURE 4

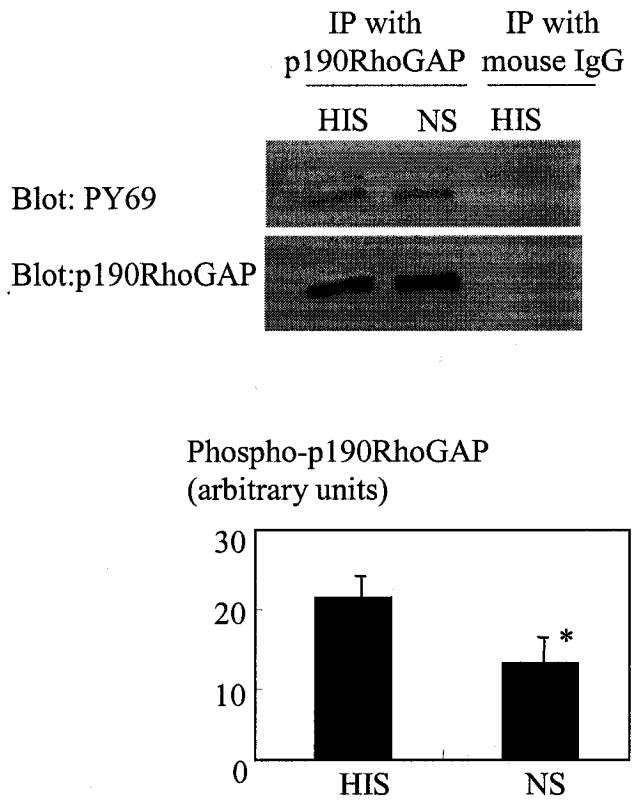
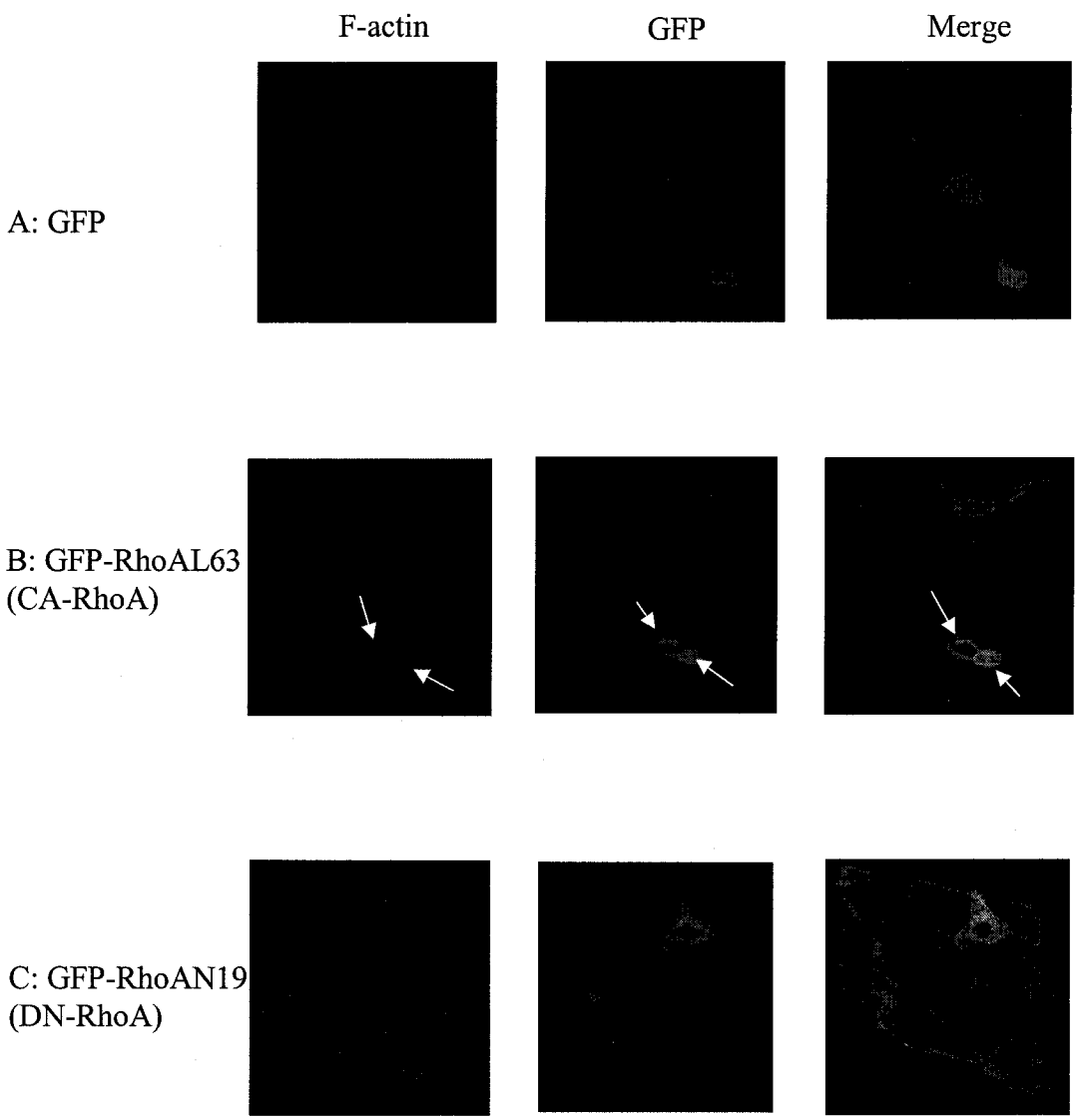

IP with p190RhoGAP
IP with mouse IgG
HIS
NS
HIS
Blot: PY69
Blot:p190RhoGAP
Phospho-p190RhoGAP (arbitrary units)
30
20
10
0
*
HIS
NS

Fig. 4. Activity of p190RhoGAP is decreased by complement in GEC

GEC-cPLA$_2$ were incubated with complement as in Fig. 2. Cell lysates were immunoprecipitated with anti-p190RhoGAP antibody or mouse IgG (control). Immunoprecipitates were analyzed by immunoblotting with anti-p190RhoGAP or anti-PY69 antibodies. Top: representative blot, Bottom: densitometric analysis. *$p<0.05$ vs HIS, N=4.

FIGURE 5


F-actin
GFP
Merge
A: GFP
B: GFP-RhoAL63
(CA-RhoA)
C: GFP-RhoAN19
(DN-RhoA)

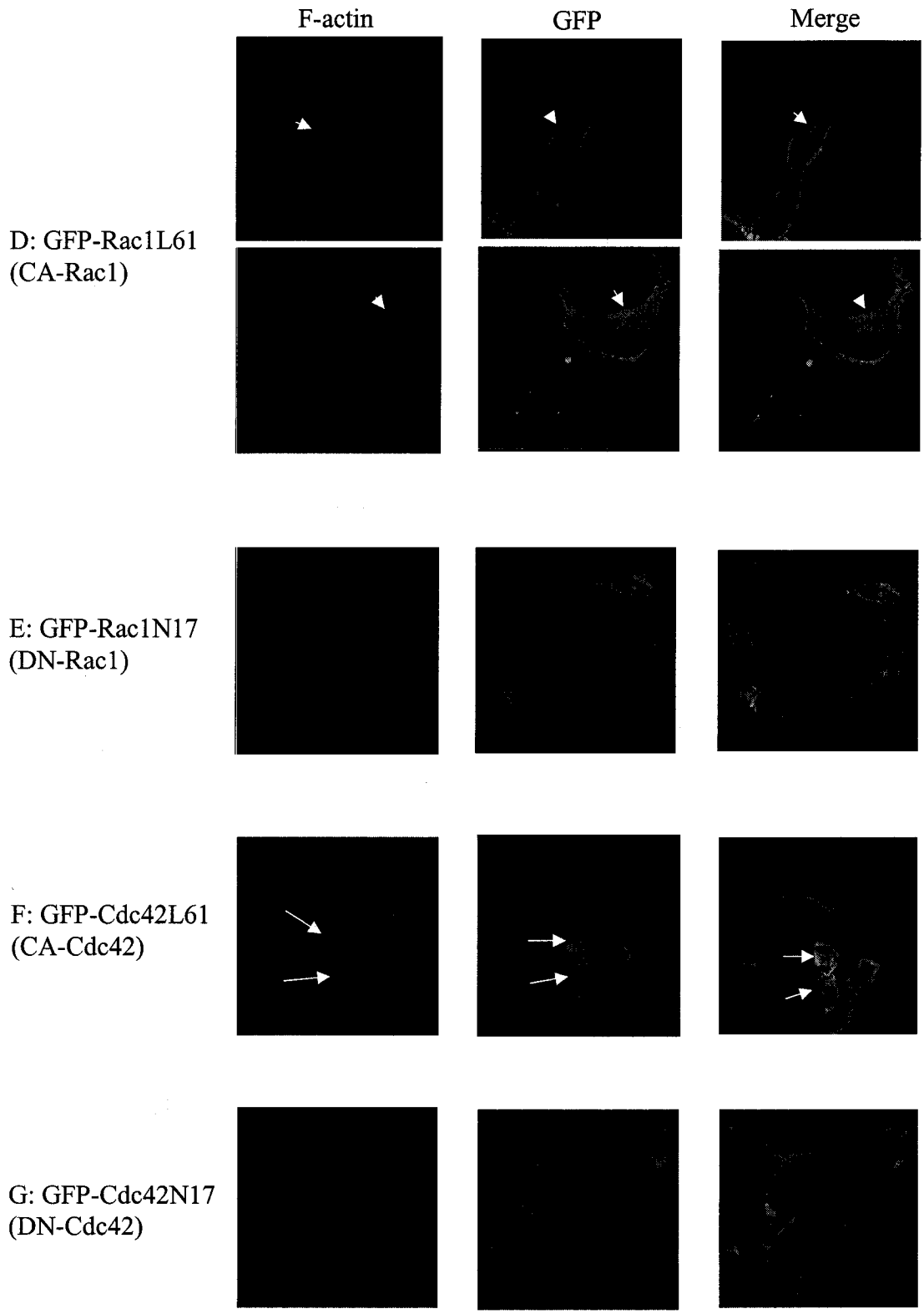
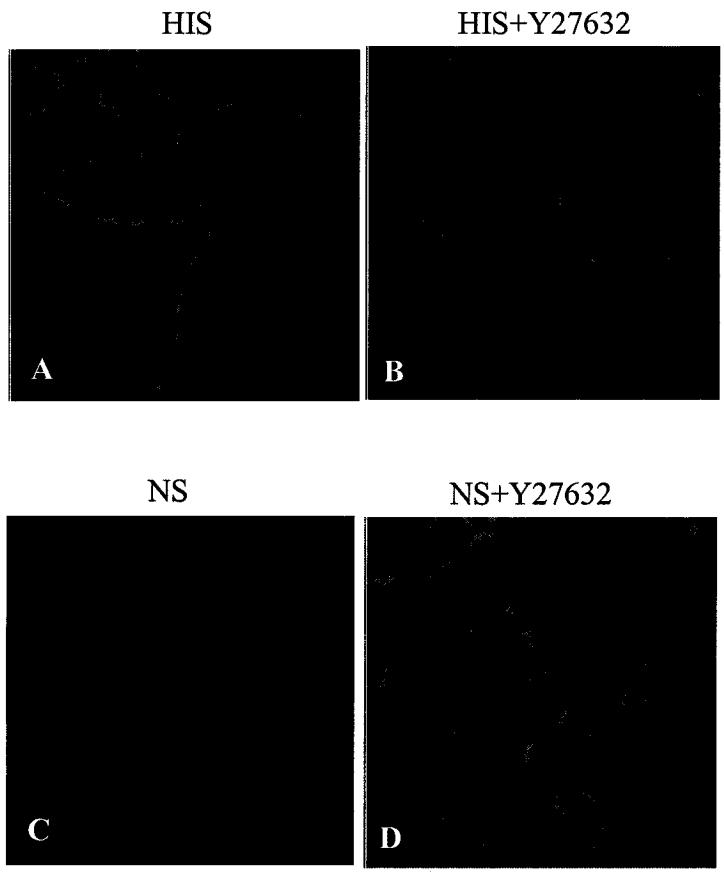

F-actin
GFP
Merge
D: GFP-Rac1L61
(CA-Rac1)
E: GFP-Rac1N17
(DN-Rac1)
F: GFP-Cdc42L61
(CA-Cdc42)
G: GFP-Cdc42N17
(DN-Cdc42)

Fig. 5. Impact of Rho-GTPase activities on GEC morphology in vitro

GFP-conjugated Rho-GTPase mutants (CA and DN) were transiently transfected into cultured rat GEC grown on glass coverslips. After fixation and permeabilization, cells were stained with rhodamine-phalloidin. Cells were studied by confocal microscopy and successfully transfected cells were identified by green fluorescence. B, F. CA-RhoA and CA-Cdc42 resulted in smaller, rounder cells, and the staining with rhodamine-phalloidin demonstrated a strong cortical F-actin pattern (arrow). D-1 and D-2. In contrast, CA-Rac1 demonstrated morphological changes that resembled formation of process (arrow heads). CA-RhoA, CA-Rac1, and CA-Cdc42 are co-localized with F-actin.

FIGURE 6


HIS
HIS+Y27632
A
B
NS
NS+Y27632
C
D

E

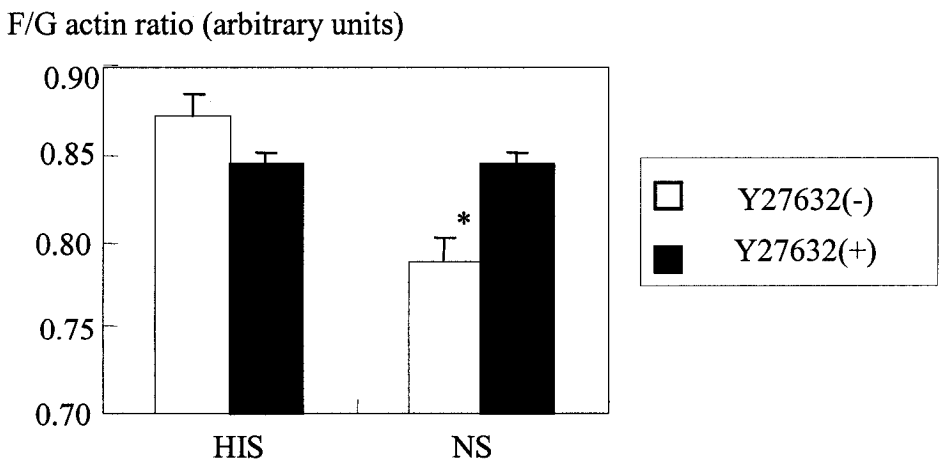
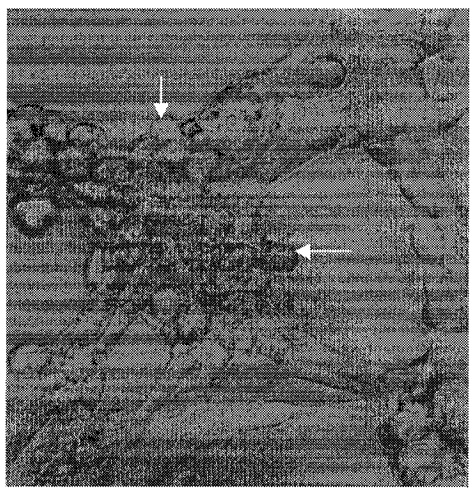

F/G actin ratio (arbitrary units)
0.90
0.85
0.80
0.75
0.70
*
HIS
NS
Y27632(-)
Y27632(+)

Fig. 6. Impact of complement stimulation on the actin cytoskeleton in GEC

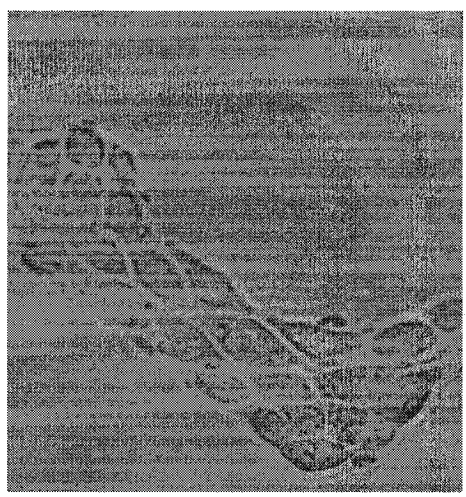
A-D. GEC-cPLA$_2$ grown on glass coverslips were stimulated with complement as in Fig.2, pretreated with or without the ROCK inhibitor, Y27632 (10μM). Cells were stained with rhodamine-phalloidin and examined by confocal microscopy (A-D). E. GEC-cPLA$_2$ were treated as in A-D and F/G actin ratio was quantified as in Methods. *P<0.01 NS vs HIS, N=3.

FIGURE 7

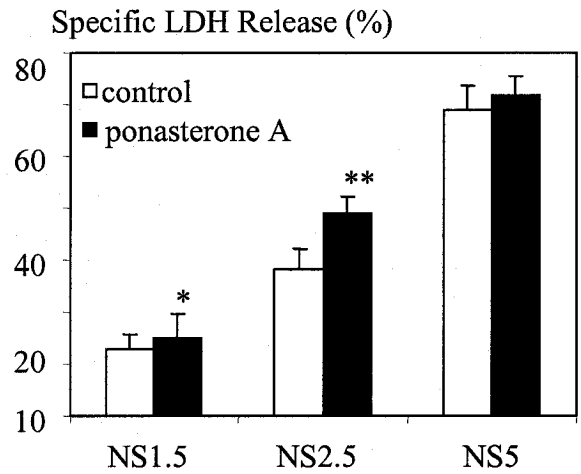
A



B



C


Specific LDH Release (%)
80
60
40
20
10
control
ponasterone A
*
**
NS1.5
NS2.5
NS5

D

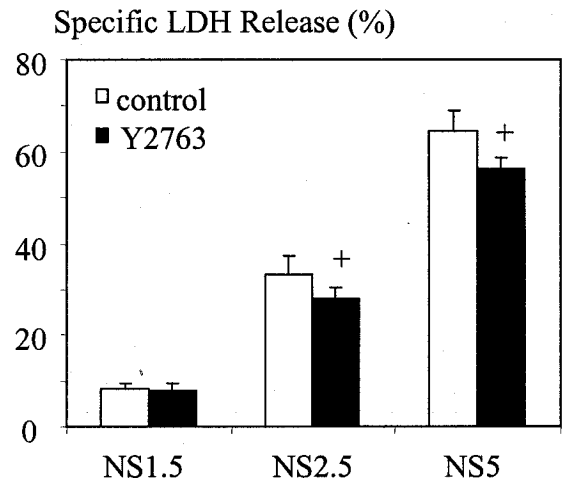

Specific LDH Release (%)
80
60
40
20
0
control
Y2763
+
+
NS1.5
NS2.5
NS5

Fig.7. The Rho/ROCK pathway contributes to complement-mediated GEC injury.

A subclone of GEC that overexpressed CA-RhoA (RhoAL63) in an inducible manner was established (Methods). Cells were incubated with vehicle (control) or ponasterone A (1 μM) for 24 hours. A, B. Morphological changes were studied by light microscopy. After RhoAL63 was induced by ponasterone A (1 μM) for 24 hours, GEC became smaller and rounder. A: (arrow), as compared with control cells (B). C. After inducing the expression of CA-RhoA by ponasterone A (1 μM) or control cells (treated with ethanol in the place of ponasterone A) for 24 hours, cells were then stimulated with anti-GEC antiserum and NS (or HIS) as in Fig. 2. Complement-mediated cytotoxicity was quantified by monitoring release of LDH. *P<0.05 vs Control, **P<0.01 vs Control, N=7. D. GEC-cPLA2 were stimulated with anti-GEC antiserum and NS (or HIS) as in Fig. 2, pretreated with or without the ROCK inhibitor, Y27632 (10μM). Complement-mediated cytotoxicity was quantified as in D. *P<0.05 vs Control, N=4.

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SECTION 6: APPENDIX