

C3G overexpression in glomerular epithelial cells during anti-GBM-induced glomerulonephritis

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The guanine nucleotide exchange factor C3G, along with the CrkII adaptor protein, mediates GTP activation of the small GTPase proteins Rap1 and R-Ras, facilitating their activation of downstream signaling pathways, which had been found to be important in the pathogenesis of glomerulonephritis. We found that expression of C3G protein was upregulated in glomerular epithelial cells in an experimental model of accelerated anti-GBM antibody-induced glomerulonephritis expression. To determine the consequence of its increased expression, we transfected C3G (using adenoviral constructs) into cultured glomerular epithelial cells and measured the activated forms (i.e., GTP-bound) forms of Rap1 and R-Ras. Activation of Rap1 was not affected by C3G; however, the basal level of GTP-bound R-Ras was decreased. Further, C3G over-expression enhanced the activation of R-Ras in response to endothelin. Overexpression of C3G also led to a significant reduction in glomerular epithelial cell spreading and decreased the cells' E-cadherin expression and augmented their migration. We found that C3G was overexpressed in accelerated anti-GBM antibody-induced glomerulonephritis and suggest that this modulates glomerular epithelial cell morphology and behavior.

Kidney International (2009) **75**, 31–40; doi:10.1038/ki.2008.448; published online 10 September 2008

KEYWORDS: C3G; glomerular epithelial cells; small GTPases; glomerulonephritis

In experimental models of proliferative (crescentic) glomerulonephritis (GN), induced by antibody against the glomerular membrane (GBM), and in human crescentic GN, injury to the glomerular visceral epithelial cells (GECs), also known as podocytes, appears to be of pivotal importance.^{1–3} To support the glomerular filtration rate, the renal GEC develop microtubule-based thick processes with sophisticated branching morphology and have thin actin-based projections (podocyte foot processes), which spread and form cell-cell contacts in mature cells. Actin filaments are thought to be important in orchestrating the function of various molecules during the formation of podocyte foot processes. The molecular mechanism of foot process formation seems to include small guanosine triphosphate (GTP)-binding proteins. One common mechanism utilized by small GTPases to regulate cellular function is to cycle between the inactive guanosine diphosphate-bound state and the active GTP-bound state. Guanine nucleotide exchange factors (GEFs) facilitate guanosine diphosphate dissociation and allow the more abundant GTP to rebind, whereas GTPase-activating proteins accelerate GTP hydrolysis to complete the cycle. In a number of pathological processes, small GTPases are upregulated due to overexpression, mutation, intensification of particular signaling pathways, or abnormal GEFs activity that are critical for the onset or progression of the disease.⁴ This suggests that GEFs can be promising targets for the inhibition of small GTPase-dependent pathways that are amplified or diminished in diseases.

The GEF, C3G, predominantly catalyzes the guanine nucleotide exchange reaction for the Ras family of GTPases. Absence of C3G causes embryonic lethality in mice via the primary defect in vascular-supporting cells of mesenchymal origin, and cells derived from such embryos demonstrate impaired cell adhesion, delayed spreading, and enhanced cell migration.⁵ However, the mechanisms by which C3G regulates these actin-dependent cellular functions are poorly understood. C3G-mediated Rap1 activation is required for cell adhesion and cell spreading, but suppresses cell migration.⁶ Recently, a functional link was uncovered between C3G/Rap1 and the FGF2/Akt/Gsk3 β / β -catenin pathway, where C3G is involved as a self-limiting mechanism

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Received 10 October 2007; revised 9 June 2008; accepted 17 June 2008; published online 10 September 2008

for proliferation signals.⁷ Furthermore, York *et al.* proposed that the activation of Rap1 by C3G represents a common mechanism to induce sustained activation of the mitogen-activated protein (MAP) kinase cascade in cells that express B-Raf.⁸ Our previous studies in rat models of proliferative GN have demonstrated that both extracellular signal-regulated kinase (ERK) and stress-activated protein kinase exhibit disease-associated activation, albeit with differing kinetics, and that ERK activation in glomeruli is accompanied by increased expression of its specific upstream activator, MEK1.⁹ Significant evidence has emerged that members of Ras family of small GTPases modulate ERK activation in a cell-context-dependent manner.^{8,10-12} Signaling through the small GTPases Rap1 and R-Ras contributes to the regulation of a wide variety of integrin-mediated processes including cell-cell and cell-matrix adhesion, actin polymerization, cell migration, and spreading.^{13,14} Interestingly, enhanced Rap1 activity was found to be involved in p38 MAP kinase regulation, ERK suppression, increased cell adhesion, and was observed in samples from heart with right ventricular outflow tract obstruction and renal tumors.^{15,16} By contrast, decreased R-Ras activity was linked to such biological effects as augmented cell motility and migration.¹⁴ Taken together, these data allow us to hypothesize that the Ras family of GTPases GEF, C3G, can be important for controlling the signaling cascades in proliferative GN. We have carried out a study of C3G expression in inflammatory kidney disease followed by analysis of C3G biological significance in cultured podocytes.

RESULTS

C3G is upregulated in glomeruli after induction of experimental GN

Western blot analysis of protein lysates obtained from isolated glomeruli revealed a significant increase in levels of GEF C3G 1, 3, and 7 days after induction the accelerated (crescentic) form of anti-GBM GN (Figure 1). Levels of the adaptor protein Grb2 were not different between glomeruli of control and experimental animals (Figure 1, lower panel).

On day 14 following administration of anti-GBM antibody, we observed significantly increased proteinuria (Figure 2), crescent formation (Figure 3a and b), glomerular sclerosis (Figure 3c), and periglomerular inflammatory cell infiltrates (Figure 3, compare a to d). C3G expression (assessed by immunostaining) was prominent in GEC (black arrows in Figure 3c), within cellular crescents (Figure 3b) and, occasionally, in tubular epithelium. Very little or no C3G staining was observed in glomerular cells of control animals. To confirm the localization of C3G staining, we carried out double immunostaining for C3G and nestin as a mature podocytes marker, which is upregulated in response to injury¹⁷ (Figure 4). Glomerulus from control animal was virtually free of brown C3G staining and was characterized by prominent blue signal from anti-nestin antibody (Figure 4a). After GN induction, C3G staining was enhanced and colocalized with nestin forming gray to black color (Figure 4b).

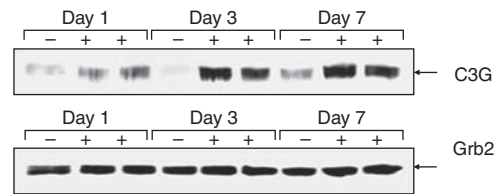


Figure 1 | Expression of C3G protein in anti-GBM antibody induced glomerulonephritis. One control (–) and two experimental (+) animals were studied at 1, 3, and 7 days following injection of anti-GBM serum. Enhanced expression of C3G in experimental animals is identifiable by western blotting as early as 1 day after induction of GN (upper panel). The nitrocellulose membrane was also probed with anti-Grb2 antibodies to confirm equal loading (lower panel). Shown is a representative result. Experiment was repeated twice.

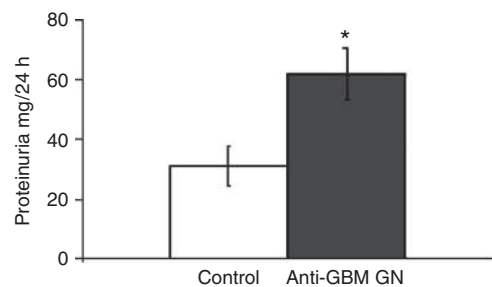


Figure 2 | Proteinuria 14 days after induction of glomerulonephritis. Urinary protein excretion was significantly (marked with star, $P < 0.05$) increased as measured on day 14 following onset of anti-GBM glomerulonephritis (anti-GBM GN) compared with control animals (control).

To investigate the possibility that C3G protein in glomeruli originates from infiltrating macrophages, levels of the macrophage marker ED-1¹⁸ were assessed in glomeruli isolated from control animals and animals with anti-GBM antibody-induced GN. At 12 h following injection of anti-GBM antibody, C3G levels in glomeruli isolated from two experimental animals were indistinguishable from levels in glomeruli from a control animal (Figure 5a, upper panel). In contrast, ED-1 levels were greatly increased only in glomerular lysates of the two experimental animals (Figure 5a, lower panel). At later time points (24 and 48 h and on days 3 and 7 following induction of GN), the differences in glomerular C3G expression between control and experimental animals were clearly manifested (Figures 1 and 5b). In contrast to the striking upregulation of C3G expression, Grb2 did not exhibit a disease-associated increase in expression levels (Figures 1 and 5b). Similarly, the level of expression of glomerular CrkII did not significantly change in animals with GN (Figure 5b). Immunohistochemistry data also did not reveal significant C3G staining in inflammatory infiltrates.

Overexpression of C3G modulates activation of R-Ras in GEC

As detected by Rap1 affinity binding assay, Rap1 activation in response to endothelin-1 (ET-1) was not significantly affected in GEC infected with adenovirus encoding C3G (Figure 6a,

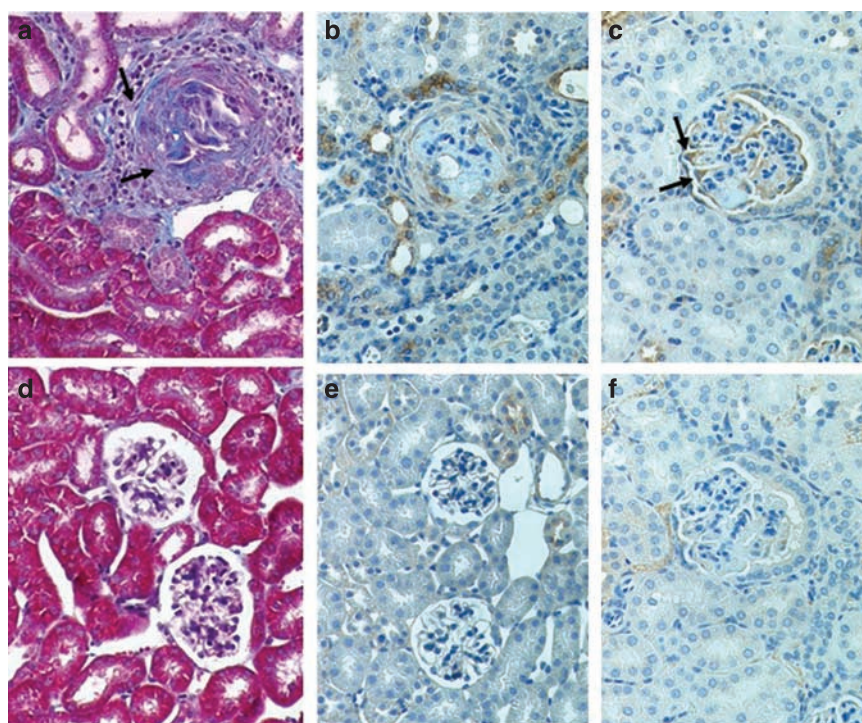


Figure 3 | Kidney morphology and C3G immunohistochemical detection in glomerulus after induction of glomerulonephritis. Representative morphological (Masson's Trichrome, **a**, **d**) and immunohistochemical staining for C3G (**b**, **c**, **e**) in kidney cortical sections obtained on day 14 following onset of anti-GBM glomerulonephritis (**a**–**c**, **f**) and in control animals (**d**, **e**). (**b**) and (**c**) represent two different animals with GN. In (**a**), a crescentic glomerulus with periglomerular infiltrate is shown. In (**c**), arrows indicate immunolocalization of C3G in glomerular epithelial cells. Also shown is a negative control (stained with an isotype-matched IgGs) (**f**), corresponding to the glomerulus shown in (**c**). Cell nuclei in (**b**), (**c**), (**e**), and (**f**) are counterstained with hematoxylin, original magnifications $\times 200$.

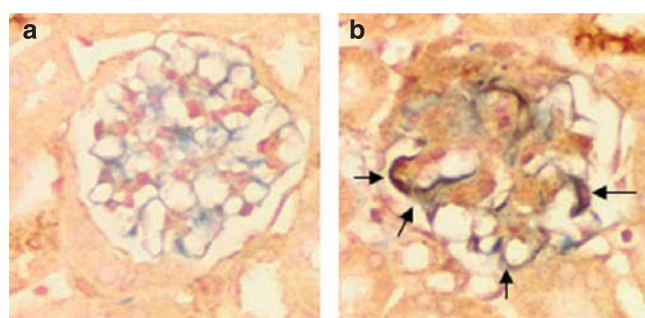


Figure 4 | C3G and nestin immunohistochemical detection in glomerulus after induction of glomerulonephritis. Double immunostaining for C3G (brown cytoplasm) and for nestin (blue cytoplasm) showing changes after glomerulonephritis induction. (**a**) Normal glomerulus from control animal demonstrates very little if any C3G signal and many cells are nestin-positive. (**b**) Crescentic glomerulus characterized by increased C3G staining. Majority of nestin-positive cells express C3G (gray to black color, marked with arrows). Cell nuclei are counterstained with Nuclear Fast Red, original magnifications $\times 400$.

upper panel). The amount of GTP-bound Rap1 from three independent experiments was further quantified using densitometry analysis (Figure 6b).

We observed a marked effect of C3G overexpression on ET-1-induced changes in R-Ras GTP loading (Figure 7a and b). First, the basal level of R-Ras GTP-loading was

decreased in cells overexpressing C3G (Figure 7a, upper panel, lane 6 versus lane 1 or 3). These differences were quantified by densitometry (Figure 7b). Statistically significant alterations between the two groups (AdGFP and AdC3G) were found at basal level (time 0 min, $P < 0.01$) and following a 20 min exposure to ET-1 ($P < 0.05$). Within the AdGFP group, R-Ras GTP loading following 5 min of ET-1 stimulation was significantly lower than that at 0 min ($P < 0.01$). The same effect was observed in uninfected GEC control (Figure 7a, U). Within the AdC3G group, R-Ras GTP loading at 20 min was significantly higher than that at 0 min ($P < 0.001$) or that at 5 min ($P < 0.02$). In summary, GEC overexpressing C3G demonstrated a dramatic decrease in the basal level of GTP-bound R-Ras and a significant increase in the level of GTP-bound R-Ras 20 min following ET-1 stimulation.

Overexpression of C3G affects spreading and migration in GEC

In our experiments, C3G overexpression had no effect on cultured GEC attachment to fibronectin with or without ET-1 stimulation (data not shown). In accordance with R-Ras activation data, C3G upregulation significantly reduced cell size during spreading (Figure 8a). In addition, GEC spreading under ET-1 treatment was significantly diminished in control cells, reflecting normal actin dynamic response to contractile stimulus, but remained unaffected in C3G-

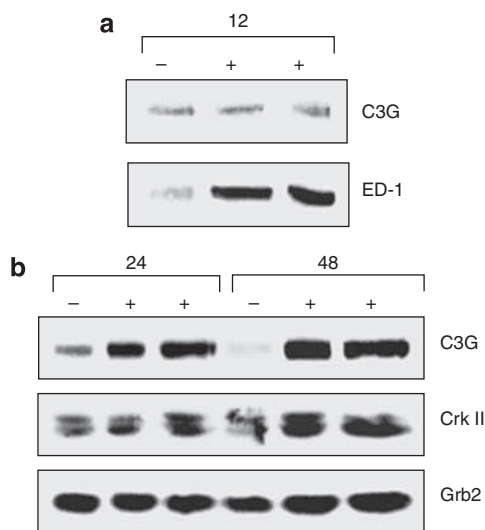


Figure 5 | Glomerular levels of C3G protein and the macrophage marker ED-1 in anti-GBM glomerulonephritis. (a) At 12 h following administration of anti-GBM serum, C3G levels in glomeruli from two experimental rats (Figure 3a, lanes 2 and 3) were indistinguishable from the level in glomeruli of a control animal (lane 1) despite the prominent macrophage migration into glomeruli (Figure 3a, lower panel, lanes 2 and 3) as assessed by western blot analysis of levels of the macrophage marker ED-1. One control (–) and two experimental animals (+) were studied. **(b)** Western blot analysis of C3G (upper panel) and CrkII (middle panel) expression was performed in glomeruli isolated 24 and 48 h following administration of anti-GBM serum. One control (–) and two experimental animals (+) were studied for each time point. The nitrocellulose membrane was also probed with anti-Grb2 antibodies to confirm equal loading (lower panel). Shown is a representative result, the experiment was repeated two times.

overexpressing population (Figure 8a). To further explore changes in intracellular actin balance with or without C3G overexpression and ET-1 stimulation, we labeled filamentous actin in cultured GEC with phalloidine, conjugated with fluorochrom (Figure 8b). After C3G overexpression and ET-1 stimulation, amount of polymerized actin fibers was increased compared with unstimulated GFP-expressing control.

After C3G upregulation, cell migration was augmented compared with GFP control as measured by wound area closing (Figure 9a and b) and by number of migrating cells into the opened area (Figure 9c) in scratch assay. Next, we analyzed proteins, involved into adherens and tight-junction formation and/or into migration signaling. In line with observed changes in cell morphology and behavior, we detected significant decrease in E-cadherin and podocin expression after C3G upregulation (Figure 9d). ET-1 treatment for 24 h led to similar reduction of E-cadherin protein level in GFP-expressing group compared with C3G counterpart (Figure 9d), correlating with spreading data (Figure 8a). We observed no change in vinculin and p130Cas expression. However, substrate-binding domain activation of p130Cas (measured as Tyrosin 165 phosphorylation) was enhanced after C3G overexpression without any significant changes in response to 24 h ET-1 stimulation.

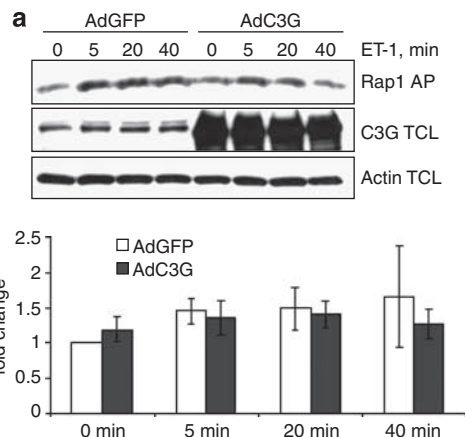


Figure 6 | Adenoviral-mediated transfer of C3G into glomerular epithelial cells does not affect Rap1 activation in response to endothelin-1 (ET-1). **(a)** Lysates from adenovirus encoding green fluorescent protein (AdGFP)- and AdC3G-infected rat glomerular epithelial cells, quiescent or stimulated with ET-1 (100 nM) for indicated periods of time (min), were subjected to a Rap1 affinity binding assay. GTP-bound active Rap1 was isolated by affinity precipitation with a GST-RBD RaIGDS fusion protein followed by immunoblot analysis with an anti-Rap1 antibody (upper panels). Lysates from AdGFP- and AdC3G-infected rat glomerular cells were resolved by SDS-PAGE and immunoblotted with either anti-actin antibodies for equal loading (lower panel) or anti-C3G antibodies (middle panel); AP, affinity precipitation and TCL, total cell lysates. **(b)** The amount of GTP-bound Rap1 was quantified using densitometry. Densitometry data were normalized to the AdGFP '0 min' point and presented as mean \pm s.e.m. In four separate experiments, there was no statistically significant difference between AdGFP- and AdC3G-infected cells.

DISCUSSION

We demonstrated for the first time that GEFs C3G is upregulated in the model of anti-GBM GN. Little is known about regulation of C3G expression. There are few studies reporting downregulation¹⁹ as well as overexpression of C3G isoforms^{20,21} during development of human cancers. However, mechanism of differentiated expression of C3G currently remains unclear.

Intense glomerular macrophage infiltration is known to occur during the early stages of the accelerated variant of anti-GBM antibody-mediated crescentic GN employed in the present studies. Isolated glomeruli western blotting results (Figures 1 and 5) indicate that glomerular C3G overexpression accompanies the course of anti-GBM antibody-induced GN and, although it could be triggered by macrophage infiltration in glomeruli, it did not result from infiltrating macrophages overexpressing C3G. Two weeks after disease induction when macrophage number inside the glomerulus mostly reverts to normal, based on immunolocalization of C3G (Figures 3c and 4b), we propose that GEC are the likely site where C3G overexpression takes place in the anti-GBM model of immune glomerular injury.

We demonstrate that C3G overexpression in cultured GEC results in modulation of ET-1-induced signaling via the small GTPase R-Ras. Our choice of ET-1 stimulation was based on

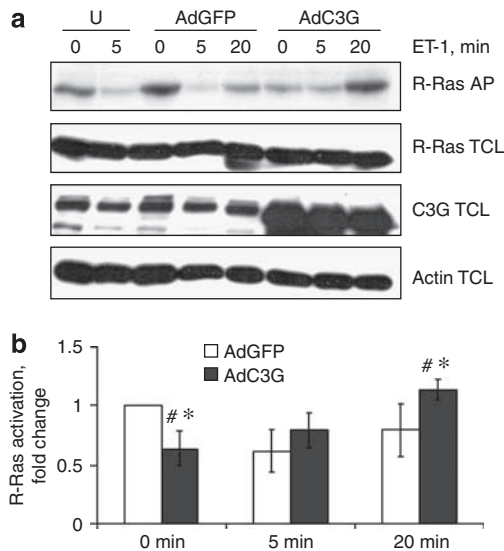


Figure 7 | Adenoviral-mediated transfer of C3G into glomerular epithelial cells modulates R-Ras GTP level. (a) Lysates from uninfected (U), and from AdGFP- or AdC3G-infected epithelial cells, quiescent or stimulated with endothelin-1 (100 nM) for indicated periods of time (min), were subjected to R-Ras affinity binding assay. GTP-bound active R-Ras was isolated by affinity precipitation with a GST-RBD Raf fusion protein followed by immunoblot analysis with an anti-R-Ras antibody (upper panel). Lysates from AdGFP- or AdC3G-infected rat glomerular cells were resolved by SDS-PAGE and immunoblotted with either anti-R-Ras, anti-C3G, or anti-actin antibodies (for verification of equal loading). A representative analysis of five separate experiments is shown; AP, affinity precipitation and TCL, total cell lysates. (b) The amount of GTP-bound R-Ras was quantified using densitometry analysis. Densitometry data are normalized to AdGFP '0 min' point and presented as mean \pm s.e.m. Statistically significant differences between the two groups (AdGFP and AdC3G) were found at time 0 (marked with asterisk, $*P < 0.01$) and following a 20 min exposure to ET-1 ($*P < 0.05$). Within the AdGFP group, R-Ras GTP loading following 5 min of ET-1 stimulation was significantly lower than that at 0 min ($#P < 0.01$). R-Ras GTP loading at 20 min was not different from that at 0 or 5 min. Within the AdC3G group, R-Ras GTP loading at 20 min was significantly higher than that at 0 min ($#P < 0.001$) or that at 5 min ($#P < 0.02$). There was no significant difference in R-Ras GTP loading at 5 min compared with 0 min.

knowledge that ET-1 is important in both the normal and the diseased kidney.^{22,23} ET-1, acting in concert with other constrictor peptides, cytokines, and growth factors, contributes to proliferative renal diseases.²² In immune complex GN there is increased glomerular ET-1 expression²⁴ and G-protein-coupled ET-A and ET-B receptor blockade reduces glomerular cell proliferation and proteinuria.²⁵ We observed diminished GTP loading of R-Ras in cells overexpressing C3G, but increased level of GTP-bound R-Ras at later time-points after ET-1 stimulation (Figure 7). Recently, in wide range of cell lines, it was demonstrated that R-Ras regulates exocytosis.²⁶ Several reports describe that podocytes are capable of secretory exocytosis.^{27,28} Endosomes, before recycling to membrane, contain R-Ras in GTP-bound form. When endosomes are fused with the cell membrane, R-Ras is converted to guanosine diphosphate-bound form by

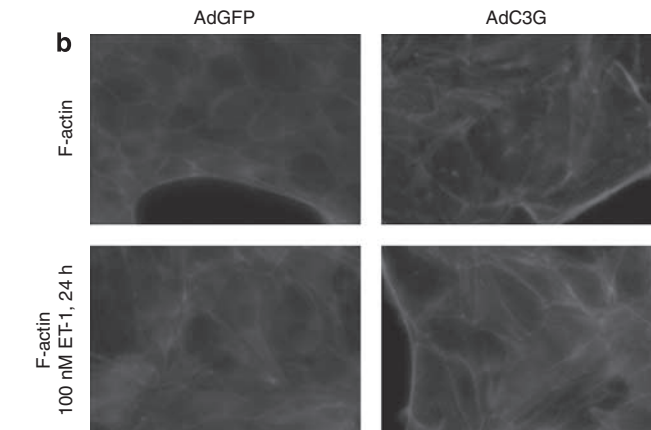
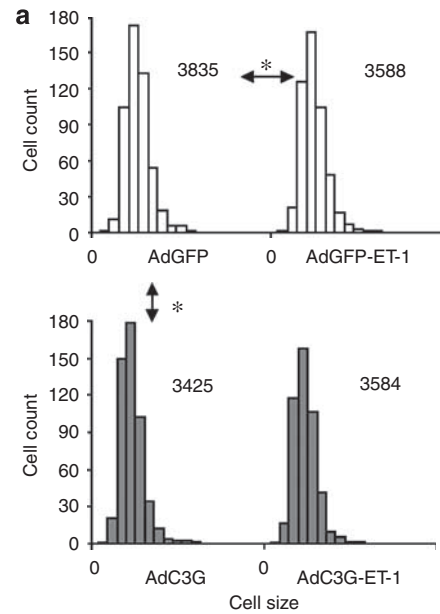


Figure 8 | Adenoviral-mediated transfer of C3G into glomerular epithelial cells reduces cell spreading. AdGFP- and AdC3G-infected rat glomerular epithelial cells were subjected to spreading assay (see section 'Material and Methods', for details). (a) Histograms represent distribution of cell number (y-axis) of a given size (x-axis) counted in the GFP- or C3G-expressing cell populations after spreading (1.5 h). Numbers near the histogram top represent the median values for each particular cell distribution. Arrows with stars indicate significant difference ($P < 0.05$ based on one-way ANOVA) between marked cell populations. A representative example of three separate experiments is shown. (b) In AdGFP- and AdC3G-infected rat glomerular epithelial cells with or without 100 nM ET-1 stimulation F-actin was visualized with AlexaFluor568-phalloidine. Enhanced F-actin staining visible as separated cell long lines was observed in C3G-overexpressing cells and in cells after 24 h ET-1 stimulation.

membrane-located GTPase-activating proteins. ET-1 could stimulate endosome recycling²⁹ to plasma membrane compartments containing GTPase-activating proteins and hence decrease R-Ras GTP binding. We speculate, that C3G overexpression in GEC promotes fusion of endosomes with the cell membrane, an event that may not require involvement of GEF activity of C3G, but could be controlled through C3G-mediated protein-protein interactions.³⁰ This

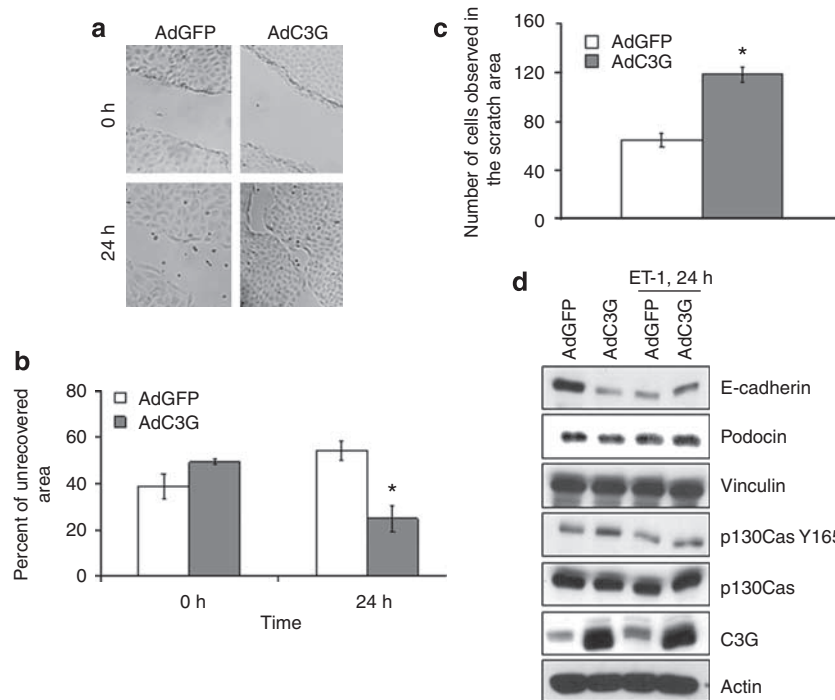


Figure 9 | Adenoviral-mediated transfer of C3G into glomerular epithelial cells promotes cell migration and reduces E-cadherin expression. AdGFP- and AdC3G-infected rat glomerular epithelial cells were subjected to scratch assays (see section ‘Material and Methods’, for details). **(a)** Representative pictures of wound area in different cell populations at 0 and 24 h time points after scratch performed. **(b)** Quantification of percent of unrecovered wound area 24 h after scratching the cell monolayer. **(c)** Number of cells migrated into the wound area presented. Star indicates significant difference ($P < 0.05$) between experimental cell populations. Representative examples of four separate experiments are shown in **(a)–(c)**. **(d)** AdGFP- and AdC3G-infected rat glomerular epithelial cells were subjected to 24 h either starvation or 100 nM ET-1 stimulation. Lysates from AdGFP- or AdC3G-infected rat glomerular cells were resolved by SDS-PAGE and immunoblotted with anti-E-cadherin, anti-podocin, anti-vinculin, anti-p130Cas Y165, anti-p130Cas, anti-C3G, or anti-actin antibodies (for verification of equal loading). A representative analysis of three separate experiments is shown.

could explain why C3G overexpression reduced the basal R-Ras GTP level in unstimulated GEC (Figure 5, lane 6 compared with lane 1). To the extent that ET-1 stimulates GEF activity of C3G in plasma membrane compartments, C3G would facilitate GTP binding of R-Ras in ET-1-stimulated GEC.

Both increased and decreased GTP loading can be important in GTPase signaling.³¹ Reduced R-Ras GTP level usually correlates with reduction in cell–cell and cell–matrix interactions presumably through the inactivation of integrins.³² H-Ras and its downstream effector kinase Raf-1 suppress integrin activation in a manner that is independent of protein synthesis and mRNA transcription and correlates with activation of the ERK/MAP kinase pathway.³³ GTP-bound R-Ras antagonizes the Ras/Raf-initiated integrin suppression pathway in a manner that does not involve inhibition of the Ras/Raf-induced MAP kinase activation.³⁴ Although R-Ras signals through specific integrin- α cytoplasmic domains to promote cell migration,³⁵ the N terminus of R-Ras is involved for the efficient dissemination of R-Ras signals that govern cell shape and migration capacity. The N terminus positively regulates Rac activation and cell spreading but negatively regulates R-Ras-mediated cell migration.³⁶ Both reduced R-Ras GTP levels and its tyrosine 66 phosphorylation contribute to the ephrin inhibitory effects

on COS cell migration and to ephrin-dependent growth cone collapse in primary neurons.³⁷ This suggests that the R-Ras inactivation or a non-GTPase function may be linked to the reduced GEC spreading and increased migration capacity. During GN progression, podocytes reduce foot processes formation and detach from GBM.³⁸ Experiments addressing C3G-mediated spreading of cultured GEC are relevant for understanding intracellular actin dynamics which is important for podocyte foot process formation. Detached podocytes can migrate between parietal epithelial cells forming bridges between them and glomerular tuft. Bridge formation with denuded GBM was proposed to be a precursor to crescent development.³⁹ Together with observation of C3G-positive cells in glomerular crescents (Figures 3b and 4b) formed after induction of anti-GBM GN, our findings allow us to speculate that possible C3G-R-Ras function is to enable cells to migrate in response to kidney injury.

In support of this hypothesis, we found that C3G overexpression in cultured GEC resulted in the reduced spreading associated with enhanced filamentous actin formation and in the increased migration. Since endogenous C3G directly interacts with E-cadherin at initial cell–cell contacts,¹⁰ colocalizes with filamentous actin, and is involved into cell adhesions formation and maturation, C3G overexpression indeed can modulate signaling involved in

attachment to substrate and to neighboring cells. Adhesion of GEC within crescents is a regulated process as several adhesion molecules are expressed within forming crescents and their expression is modified during evolution of crescents.⁴⁰ Of the various adhesion molecules identified, platelet/endothelial cell adhesion molecule-1 was shown to recruit Crk-C3G complexes¹², whereas crosslinking of ICAM-1 induces tyrosine phosphorylation of the cytoskeleton-associated protein p130Cas, which associates with Crk and C3G and is involved into filamentous actin assembling.⁴¹ Formation of cadherin-based cell-cell junctions (adherens junctions) among GEC present within crescents also occurs.^{42,43} Rap1 is important in formation of cadherin-based cell-cell junctions.¹⁰ It is, therefore, conceivable that the C3G overexpression in GEC observed in the present studies could modulate crescent formation, the likely mechanisms being: (1) stabilization of Crk-C3G complexes by the increased C3G levels and modulation of phosphorylation status of scaffolding protein p130Cas and (2) C3G-mediated modulation of the level of GTP-bound R-Ras. The later effect would reduce cell spreading as we observed in cultured GEC overexpressing C3G (Figure 8a). However, we have not observed enhanced and prolonged Rap1 activation after C3G overexpression in response to ET-1 stimulation in GEC when compared with cells, overexpressing reporter protein GFP. This could reflect cell specificity of Rap1 function in epithelial cells, whereby this small G protein and its GEF, C3G, are involved in maturation of cadherin-based cell-cell contacts.⁴⁴ Our observation of unchanged Rap1 activation in cultured GEC after C3G overexpression further suggests that Rap1 is not involved during formation of glomerular epithelial cell adherens-type junctions in crescentic glomeruli. Rather, the C3G-activated small GTPase R-Ras could be important in this process.

Interestingly, in our study, C3G overexpression was associated with decreased E-cadherin and podocin protein levels in cultured GEC. Such reduction can partially explain pro-migratory effect of C3G upregulation in GEC. The mechanism of noticeable decline in adherent protein expression remains obscure. C3G acts as a part of self-limiting mechanism for proliferation signals due to a functional link between C3G/Rap1 and FGF2/Akt/Gsk3 β / β -catenin pathways.⁷ Thus, it is plausible that C3G can initiate negative feedback signaling aimed to reduce cell-cell contacts formation after overexpression in glomeruli. Weak C3G expression in normal glomerulus and dramatic increase of glomerular C3G protein level after GN induction were observed. Therefore, cadherin and podocin downregulation may reflect GEC-specific signaling associated with not only pro-migratory effect of C3G, but also with known de-differentiation of GEC during kidney injury. Indeed, in several models of experimental renal disease, as well as in human biopsies, a decrease of manifestation of podocyte-specific markers (such as podocin, synaptopodin, nephrin) was detected.^{17,45} The loss of these proteins, involved in slit diaphragm formation and function, results in podocyte

de-differentiation contributing to glomerular crescents. Furthermore, observed ET-1-induced decrease in GEC E-cadherin expression finds similarity with known ET-1-induced disruption of intercellular communications and stimulation of cell migration in human cancers.⁴⁶

Here we report that C3G upregulated in nephritic glomerulus and C3G overexpression modulates activity of R-Ras and contributes to migratory phenotype in podocytes. The increase in glomerular C3G expression observed in the present study represents a further example of transcriptional regulation of a molecule which can be involved in MAP kinase signaling via sustained activation of small GTPases. The data presented herein link the enhanced expression of C3G with dramatic change in GEC behavior, suggesting hypothetical mechanism explaining podocyte loss and de-differentiation during renal inflammation.

MATERIALS AND METHODS

Materials

The enhanced chemiluminescence system was obtained from Amersham Corp. (Arlington Heights, IL, USA). Complete Freund's adjuvant and all other reagents, unless specified differently, were from Sigma Chemical Co. (St Louis, MO, USA).

Animals

Male Sprague-Dawley rats weighing 180–200 g (Charles River Laboratories, Wilmington, MA, USA and Harlan, Indianapolis, IN, USA) were used for induction of accelerated proliferative GN, tissue collection, glomerular isolation, and cell culture. Male BALB/c mice were used for induction of accelerated proliferative GN and immunohistochemistry analysis. Animals were cared for according to NIH guidelines. All protocols were reviewed and approved by the Institutional Committee for care and use of animals at the Medical College of Wisconsin. Animals were maintained at the approved animal care facilities and had free access to food and water throughout the study.

Induction of accelerated proliferative GN

Accelerated anti-GBM proliferative GN was induced exactly as described previously.⁹ Briefly, male Sprague-Dawley rats were immunized intraperitoneally with 1 mg rabbit immunoglobulin G emulsified in complete Freund's adjuvant. Five days after this immunization, experimental animals were injected in the tail vein with a subnephritogenic dose of rabbit immune serum raised against rat particulate GBM. This injection was repeated 24 h later. Control rats were preimmunized with rabbit immunoglobulin G in complete Freund's adjuvant and subsequently given two intravenous injections of nonimmune rabbit serum. BALB/c mice (20–25 mg body weight) were subjected to the same protocol of immunization except they received sheep anti-rat GBM serum for induction of GN. Preimmunization was performed with 1 mg of sheep nonimmune serum. At the time of immunization, each mouse in the GN group received 1.5 mg of anti-GBM serum. Control mice were injected with the same amount of nonimmune serum. Studies were performed at 12, 24, and 48 h and on days 3, 7, and 14, following the injection of anti-GBM serum or of nonimmune serum. The day before study animals were placed in metabolic cages for urine collection to access urinary protein excretion. On completion of this collection, animals were killed and nephrectomized. Cortical tissue

was used to generate a preparation of isolated glomeruli using standard sieving methods⁴⁷ before lysis in Triton X-100 containing lysis buffer.

Morphological and immunohistochemical analysis

Paraffin-embedded renal slices were cut at 4 μ m on a standard microtome. Cross-sections included the full thickness of the cortex and medulla and were serially used for histopathological (Masson's Trichrome) and immunohistochemical evaluation. Sections were deparaffinized in xylene and hydrated gradually through graded alcohols. For C3G and nestin detection, sections were incubated in 10 mmol/l citrate buffer pH 6.0 (InnoGenix, San Ramon, CA, USA) at 98 °C for 25 min and then cooled at room temperature for 10 min. Sections were then quenched in 3% H₂O₂ (DakoCytomation, Carpinteria, CA, USA) for 20 min at room temperature, blocked using avidin/biotin blocking kit (Vector Laboratories Inc., Burlingame, CA, USA) and then blocked with Protein-free blocker (DakoCytomation) for 15 min at room temperature. Primary antibody for C3G (1:500; Santa Cruz Biotechnology, Santa Cruz, CA, USA) was applied to the sections and incubated at room temperature for 45 min. Immunodetection for C3G antigen was performed by using the streptavidin-biotin peroxidase system (BiocareMedical, Concord, CA, USA), diaminobenzidine as chromogen and counterstained with hematoxylin (both from DakoCytomation). For double immunostaining of C3G and nestin (1:100 antibody dilution, 1 h incubation; Santa Cruz Biotechnology) second antigen was visualized by using mouse-on-mouse polymer detection system conjugated with alkaline phosphatase (BiocareMedical) and blue substrate with Nuclear Fast Red (Vector Laboratories Inc.) counterstain. Control reactions were performed in the absence of primary antibody and in the presence of isotype-matched immunoglobulins.

Cell culture

Rat glomerular epithelial cells were isolated and characterized based on formation of monolayers with cobblestone appearance with granular inclusions, presence of cilia on their surfaces, presence of receptors for complement (C3), and presence of angiotensin receptors as described.^{48–51}

Tissue and cell lysis, immunoprecipitation, and Western blot analysis

Cell lysis, immunoprecipitation of samples normalized for protein content, sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis and Western blotting were performed as described previously.⁵² Polyclonal anti-C3G, anti-Rap1, anti-R-Ras, anti-Grb2 and anti-CrkII antibodies were from Santa Cruz Biotechnology. Anti-podocin antibody was a gift from Dr C Antignac (Paris, France). Antibody specific for macrophage ED-1 antigenic marker was from Serotec (Raleigh, NC, USA). Secondary goat anti-rabbit or goat anti-mouse antibodies conjugated with HRP were from Bio-Rad (Hercules, CA, USA).

Expression cloning of mouse C3G and generation of adenovirus encoding C3G

Details of screening of a λ EXlox library of 16-day mouse embryo (Novogene) to isolate mouse C3G cDNA and generation of adenovirus-encoding C3G will be reported elsewhere. Infection of glomerular epithelial cells was performed as follows: cells were incubated either with adenovirus-encoding green fluorescent

protein (AdGFP) or C3G at a multiplicity of infection of 100 plaque-forming units per cell for 1 h at 37 °C with periodic shaking, followed by addition of medium supplemented with 0.5% serum. At 48 h after infection, cells were stimulated and lysed for further analysis. Efficiency of gene transfer in glomerular epithelial cells was about 70–90% as determined by fluorescence microscopy visualization of AdGFP control.

Activated Rap1 and R-Ras affinity binding assay

Commercial kits (Upstate, Lake Placid, NY, USA) were used to measure Rap1 and Ras GTP-loading. Aliquots (250–500 μ g) of the supernatants from lysed cultured glomerular epithelial cells were mixed for 35 min at 4 °C with glutathione-agarose containing 30 μ g of Ral GDS-Rap-binding domain (Ral-GDS-RBD), or the Ras-binding domain of Raf (Raf-RBD) and were precipitated by centrifugation. Complexes were boiled in a Laemmli sample buffer and then separated on 4–15% sodium dodecyl sulfate-polyacrylamide gels. The separated proteins were immunoblotted using corresponding antibody against R-Ras or Rap1 small GTPases.

Spreading assay

AdGFP- and AdC3G-infected glomerular epithelial cells were detached by trypsinization, and resuspended in serum-free RPMI-1640 medium. The cell concentration was adjusted to 180,000 cells/ml for replating in 35 mm tissue culture dishes. Serum-free RPMI with or without ET-1 in final concentration 100 nM was poured into each culture dish before addition of the suspended cells. After incubation at 37 °C for 1.5 h, cell spreading was stopped by removing the medium and incubating with 3.7% formaldehyde in phosphate-buffered saline for 20 min. Cells were stained with AlexaFluor568-phalloidin. At least five images from different regions of the dish were captured by video camera interfaced with an inverted microscope. At least 300 individual cells were analyzed.

Scratch assay

AdGFP- and AdC3G-infected glomerular epithelial cells were starved for 24 h in serum-free RPMI to reduce cell proliferation. Scratch was made by yellow automatic pipette tip, and marked on the bottom of 60 mm tissue culture dish (time point 0 h). New serum-free RPMI medium with or without 100 nM of ET-1 was added to each plate for additional incubation at 37 °C for 24 h (time point 24 h). At least 10 images from different regions of the scratch were captured by video camera interfaced with an inverted microscope at 0 h and at 24 h time points. Migrated into the wound area cells were counted and percent of unrecovered area was calculated in each picture.

Statistical analysis

Unless otherwise indicated, results represent one of three identically performed experiments. Western blot data were subjected to densitometry evaluation and then expressed as relative units compared with unstimulated controls (mean \pm s.e.m.). Significance was tested by a two-factor (time and treatment) analysis of variance with corrections for within experiment-repeated measures. For analysis of Rap1 activation, Tukey's honest significance difference was used for multiple comparisons adjustment. For analysis of R-Ras activation, the Waller-Duncan multiple comparison adjustment was used. *P*-values <0.05 were considered statistically significant. The digitized images from spreading and scratch experiments were analyzed in semiautomatic mode by MetaVue 5.0r1 software (Universal Imaging Corp.). Using SigmaStat 3.5 software (Systat

Software Inc.), one-way analysis of variance on ranks was used for further statistical analysis of spreading assay and unrecovered wound area changes in scratch assay. Student's *t*-test was used for analysis of cell migration into the wound at 24 h.

DISCLOSURE

All the authors declared no competing interests.

ACKNOWLEDGMENTS

This work was supported by National Institutes of Health Grants HL22563 and DK41684 to AS and American Heart Association 0525801Z Greater Midwest Affiliate Postdoctoral Fellowship to VAR. We thank Dr Raymond J Hoffman (Division of Biostatistics, Department of Population Health, Medical College of Wisconsin, Milwaukee, WI, USA) for the help with statistical analysis. We are gratefully acknowledging Lynn Gruman (Histology and Imaging Core, Medical College of Wisconsin, Milwaukee, WI, USA) for teaching and consulting of the immunohistochemical part of our presented research. We are grateful to Dr Hasan Mukhtar and Dr Naghma Khan (Department of Dermatology, University of Wisconsin Medical Science Center, Madison, WI, USA) for generously providing us with positive control samples for our immunohistochemical detection of C3G.

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