Cellular and Molecular Biology
E-ISSN : 1165-158X / P-ISSN : 0145-5680
www.cellmolbiol.org
Short Communication

Upregulation of Tumor Susceptibility Gene 101 (TSG101) by mechanical stress in podocytes

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Received April 30, 2018; **Accepted** January 25, 2019; **Published** January 31, 2019

Doi: http://dx.doi.org/10.14715/cmb/2019.65.1.15

Abstract: Elevated mechanical stress in glomerular hypertension is thought to damage podocytes, the loss of which leads to development of glomerulosclerosis. Applying cDNA array analysis to mechanically stressed podocytes, we have recently identified TSG101 as a stretch-induced candidate gene among others. TSG101, which is part of the ESCRT-I complex, is involved in multivesicular body (MVB) formation. Here we demonstrate that TSG101 mRNA is strongly upregulated in conditionally immortalized mouse podocytes by cyclic mechanical stress. Differentiation of podocytes does not affect TSG101 mRNA levels. TSG101 immunofluorescence is distributed in a vesicular pattern in podocytes, the staining intensity being enhanced by mechanical stress. In DOCA/salt treated rats, a model of glomerular hypertension, glomerular TSG101 mRNA levels are elevated, and an increased number of MVBs is observed by electron microscopy in podocyte processes. Our data demonstrate that mechanical stress upregulates TSG101 in podocytes, suggesting that glomerular hypertension enhances sorting of cell surface proteins and their ligands into the degradative pathway in podocytes.

Key words: TSG101; Mechanical stress; Glomerular hypertension; DOCA/salt hypertension; Multivesicular bodies; Podocytes.

Introduction

Since we have come to know that the major way of podocyte loss consists of detachment from the glomerular basement membrane (GBM) as viable cells, mechanical factors have taken center stage accounting for the loss of podocytes. Glomerular hypertension and hyperfiltration have been suggested to represent the major challenges to podocytes (1). In addition, glomerular hypertrophy leading to a decrease in podocyte density increases the susceptibility of podocytes to withstand increased pressures and flows (2).

In the recent years, only few groups have studied the response of podocytes to enhanced mechanical stress (3-8). It has been shown that podocytes are highly sensitive to mechanical force, to tension as well as to fluid shear stress responding by reorganization of the actin cytoskeleton (4,9). Furthermore, it has been shown that mechanical stress alters hormone production and signal transduction in podocytes (8,10-13). Therefore, we wanted to know more about potentially involved players in the podocyte's response to mechanical stress.

By cDNA array analysis, we identified several candidate genes that are differentially regulated by mechanical stress (5). Among the stretch-induced genes, TSG101 was one of the most strongly upregulated genes, but has neither been verified nor studied in detail so far. TSG101 was originally discovered as a tumor susceptibility gene in a screen to identify cell immortalizing genes (14). However, it has become clear in the recent years that TSG101 is the mammalian homolog of Vps23, which is necessary for vesicular trafficking to the yeast vacuole (15). TSG101 is a component of the ESCRT-I complex (16,17). This complex is responsible for sorting endocytosed membrane proteins, such as the epidermal growth factor receptor, into the degradative pathway (15,18). In the present study we investigated the regulation of TSG101 and its possible role as a vesicle sorting protein in response to enhanced mechanical stress in cultured podocytes and in a rat model of glomerular hypertension.

Materials and Methods

Cell culture

Cultivation of conditionally immortalized mouse podocytes (kindly provided by Dr. Peter Mundel, Massachusetts General Hospital, Charlestown, MA) was done as recently reported (4). In brief, podocytes were maintained in RPMI 1640 medium (Life Technologies, Darmstadt, Germany) supplemented with 10% fetal bovine serum (FBS, Roche, Mannheim, Germany), 100 U/ml penicillin and 0.1 mg/ml streptomycin (Life Technologies). To propagate podocytes, cells were cultivated

at 33°C (permissive conditions) and the culture medium was supplemented with 10 U/ml mouse recombinant γ-interferon (Life Technologies) to enhance expression of the temperature-sensitive large T antigen. To induce differentiation, podocytes were maintained at 38°C without γ-interferon (non-permissive conditions) for at least one week.

Mechanical stress experiments

Mechanical stress experiments were performed as reported previously (2). Differentiated mouse podocytes were seeded in 6-well collagen-coated plates with a flexible bottom (Bioflex, Flexcell International, Hillsborough, NC). Three days after seeding, the 6-well plate was mounted on a manifold connected to the stretch apparatus (CLS Cell Lines Service, Eppelheim, Germany). Cyclic variations in air pressure caused upward and downward motion of the flexible membranes (0.5 Hz and 5% linear strain). One to three wells were not subjected to mechanical stress and served as controls.

RT-PCR

Isolation of RNA and RT-PCR were done as reported previously (5). For detection of TSG101 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), the following primers were used: mouse TSG101 (GenBank accession # U52945), sense 5' CTC CCG GCC TAC TGT TTC TG 3', antisense 5' GGC GGA CGT GTT TCA GGA AC 3', 671 bp product size (the same primer pair was also used in rats with the identical predicted product size); mouse GAPDH (GenBank accession # NM_008084), sense 5' GTG AAG GTC GGT GTG AAC GGA TTT G 3', antisense 5' ACA TTG GGG GTA GGA ACA CGG AAG G 3', 710 bp product size; rat GAPDH (GenBank accession # AF106860), sense 5' CAT GGA GAA GGC TGG GGC TC 3', antisense 5' AAC GGA TAC ATT GGG GGT AG 3', 415 bp product size. Cycle numbers and primer concentrations were worked out to yield linear amplification in a simultaneous RT-PCR for TSG101 and GAPDH. To amplify RNA of cultured mouse podocytes, 300 ng of total RNA, 30 cycles, 61°C annealing temperature, and concentrations of 200 nM TSG101 primer and 67 nM GAPDH primer were used. To amplify RNA of isolated rat glomeruli, 300 ng of total RNA, 28 cycles, 61°C annealing temperature, and concentrations of 200 nM TSG101 primer and 20 nM GAPDH primer were used. Specificity of RT-PCR reactions was confirmed by product size, by omission of RT, and by endonuclease restriction analysis.

To quantify RT-PCR products, ethidium bromide-stained gels were digitized (Intas, Göttingen, Germany) and analyzed with Molecular Analyst software (Biorad, München, Germany). A range of 20-fold concentration variations could be quantified reliably as determined in calibration experiments. RNA isolation, RT-PCR and gel analysis of TSG101 and GAPDH mRNA expression were always performed in a pair wise fashion to compare two experimental conditions (e.g. stretch vs. control). TSG101 and GAPDH product intensities were expressed in arbitrary units (AU); product intensities under control conditions were set to 1 AU. RT-PCR was performed on duplicate tubes. Relative variations in product intensity between tubes were 8% for GAPDH (n=15 pairs in 6 experiments) and 12% for TSG101 (n=15 pairs in 6 experiments). Relative variations in product intensity between experiments were 9% (GAPDH) and 8% (TSG101) as determined by amplification of the same RNA sample in three independent RT-PCR reactions.

Isolation of glomeruli from DOCA/salt rats

Experiments were performed on eight adult male Wistar rats (500 g) in accordance with national and local animal protection guidelines. Four animals were treated by weekly s.c. injection of 30 mg/kg desoxycorticosterone acetate (DOCA; Streuli, Uznach, Switzerland) and by supplementing the drinking water with 1% NaCl. The other four animals remained untreated and served as controls. Kidneys were removed and glomeruli were isolated by the sieving technique as reported previously after 2.5 weeks of treatment (5). The purity of isolated rat glomeruli was >95% as assessed by light microscopy.

Immunofluorescence

Immunofluorescence procedures employed were identical to those reported previously (19). Briefly, cells were fixed (2% paraformaldehyde, 4% sucrose), permeabilized (0.3% Triton X-100) and blocked in blocking solution followed by incubation with a goat anti-TSG101 antibody (M-19, Santa Cruz, Heidelberg, Germany). Antigen-antibody complexes were visualized with a Cy3-conjugated secondary antibody (Dianova, Hamburg, Germany). F-actin was visualized using Alexa 488-conjugated phalloidin (Molecular Probes, Life Technologies). Specimens were viewed with a confocal laser scanning microscope (TCS-SP, Leica Microsystems, Wetzlar, Germany).

Transmission electron microscopy

For transmission electron microscopy, material of an earlier study on DOCA/salt and control rats was used (20). Kidneys of these rats had been perfusion-fixed with 3% glutaraldehyde in 0.1 M cacodylate buffer, post-fixed with OsO_4 and tannic acid as a contrast agent, embedded in Epon 812, cut into 90 nm sections, placed on Formvar-coated copper grids, and stained with uranyl acetate and with lead citrate (20). Sections were observed under a Phillips EM 301 electron microscope. Sections from at least five animals per group were examined. Micrographs were taken only of regions that did not exhibit foot process effacement.

Data analysis

Data are presented as means±SEM. Statistical significance was tested by Student's t-test. Statistical analysis and linear regression were done with SigmaStat (Systat Software, Erkrath, Germany). A P value <0.05 was considered as statistically significant.

Results

Application of cyclic mechanical stress increased the level of TSG101 mRNA 6.3±0.9-fold in podocytes as compared to unstretched controls (P=0.001, n=4; Fig. 1A,B). GAPDH mRNA levels were not altered by mechanical stress (P=0.4). Since mechanical stress was

reported to inhibit proliferation by Petermann et al. (6), we determined TSG101 expression in proliferating vs. differentiated podocytes. Proliferation and differentiation in conditionally immortalized podocytes are governed by the thermosensitive SV40 T large antigen, whose expression and activation was controlled by appropriate culture conditions (cf. Materials and Methods). Neither TSG101 nor GAPDH mRNA levels differed significantly between proliferating and differentiated podocytes (P=0.14 and P=0.8, respectively, n=4; Fig. 1C,D). Thus, upregulation of TSG101 mRNA by mechanical stress in podocytes appears to be independent of the proliferation inhibiting effect of mechanical stress.

Vesicular sorting has emerged as the main function of TSG101, which is the mammalian homolog of yeast Vps23, a class E vacuolar protein sorting (Vps) protein (15). We therefore studied the localization of TSG101 in podocytes by immunofluorescence (Fig. 2). Unstretched podocytes, which possess a well developed actin cytoskeleton (Fig. 2A), displayed a weak staining for TSG101 (Fig. 2C). In stretched podocytes, the actin cytoskeleton was reorganized into radial stress fibers and an actin-rich center as reported previously (4) (Fig. 2B). The staining intensity for TSG101 was clearly enhanced in stretched podocytes (Fig. 2D), indicating increased protein levels of TSG101 in stretched podocytes as compared to unstretched controls. TSG101 showed a vesicular staining pattern at higher magnification (Fig. 2D inset). Thus, TSG101 can be assumed to act as a vesicular sorting protein in podocytes, too.

To extend our in vitro findings to the in vivo situation, we used DOCA/salt treatment of rats as an established model of glomerular hypertension. DOCA/salt treatment of rats is known to induce focal segmental glomerulosclerosis through elevation of glomerular capillary pressure (20,21). The glomerular level of

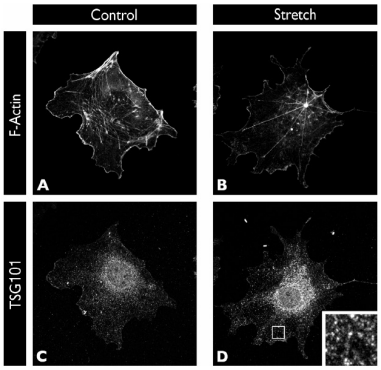

Figure 2. Immunolocalization of TSG101 in unstretched and stretched podocytes. Mechanical stress induces reorganization of the actin cytoskeleton in podocytes as visualized by confocal laser scanning microscopy (panel B vs. A). Stretched podocytes possess radial stress fibers and an actin-rich center. By double immunofluorescence, TSG101 intensity is enhanced in stretched podocytes (panel D) as compared to unstretched controls (panel C). At higher magnification the vesicular pattern of TSG101 immunostaining becomes visible (inset in panel D). Image width is 140 µm (10 µm for the inset).

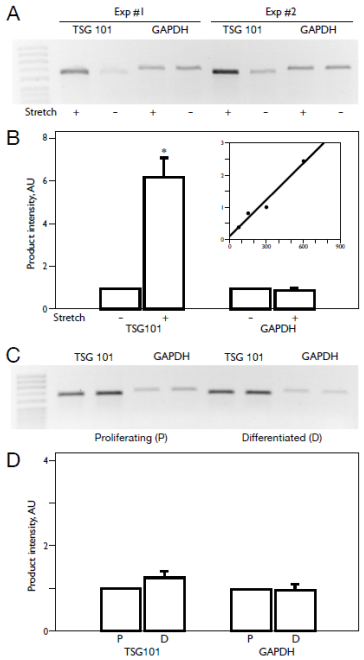

Figure 1. Upregulation of TSG101 mRNA by mechanical stress but not by differentiation in podocytes. The inverted gel image shows increased RT-PCR product intensities for TSG101 in stretched podocytes in two representative experiments (panel A). RT-PCR product quantification is shown in panel B. The inset demonstrates linearity between mouse total mRNA (x-axis in ng) and RT-PCR product intensity for GAPDH (y-axis in AU; r2=0.97 by linear regression analysis). TSG101 mRNA levels were similar in proliferating (P) and differentiated (D) podocytes as illustrated by the inverted gel image of RT-PCR products obtained in duplicate reactions in one representative experiment (panel C). RT-PCR product quantification is presented in panel D. Data are means±SEM of 4 experiments; * P<0.05.

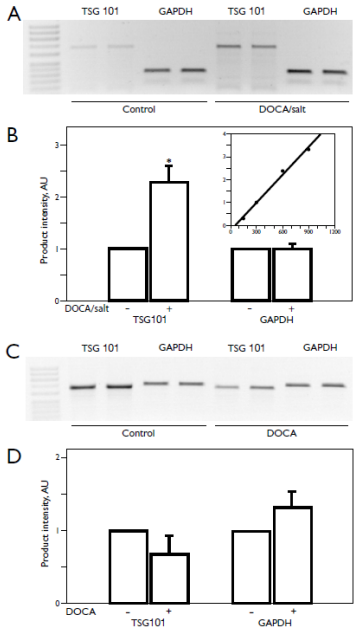

Figure 3. Upregulation of TSG101 mRNA in glomeruli of DOCA/salt rats. TSG101 mRNA levels were increased in isolated glomeruli of DOCA/salt treated rats as illustrated by the inverted gel image of RT-PCR products obtained in duplicate reactions in one representative experiment (panel A). RT-PCR product quantification is shown in panel B. The inset demonstrates linearity between rat total mRNA (x-axis in ng) and RT-PCR product intensity for GAPDH (y-axis in AU; r2=1.00 by linear regression analysis). TSG101 mRNA levels were not significantly affected by treatment of podocytes with 1 µM DOCA for 3 d as shown in the inverted gel image of RT-PCR products obtained in duplicate reactions in one representative experiment (panel C). RT-PCR product quantification is presented in panel D. Data are means±SEM of 4 experiments; * P<0.05.

TSG101 mRNA was increased 2.3±0.5-fold in glomeruli of DOCA/salt treated rats as compared to untreated controls (P<0.05, n=4; Fig. 3A,B), whereas the glomerular level of GAPDH mRNA was not altered by DOCA/salt treatment (P=1). To exclude the possibility that DOCA upregulates TSG101 expression in podocytes, DOCA (1 µM) or solvent were added to cultured podocytes for 3 d. Neither TSG101 nor GAPDH mRNA levels in cultured podocytes were significantly altered by exposure to DOCA (P=0.2, n=4; Fig. 3C,D); TSG101 mRNA levels even tended to decrease. These findings indicate that mechanical stress is the relevant factor for the upregulation of TSG101 in podocytes in glomerular hypertension in vivo.

TSG101 is part of the ESCRT-I complex that is required to sort ubiquitinated membrane proteins of late endosomes into the lumen of MVBs via budding (16,17). Since the staining pattern of TSG101 in cultured podocytes is consistent with its role as a vesicular sorting protein, and since TSG101 is upregulated by DOCA/salt treatment of rats, we asked whether MVBs are more abundant in podocytes of DOCA/salt-treated rats as compared to untreated controls. MVBs were detected by transmission electron microscopy in foot processes and in larger processes of podocytes in untreated rats. MVBs were scarce in podocytes under control conditions (Fig. 4A). The number of MVBs was clearly augmented in podocyte processes in DOCA/salt-treated rats. We regularly observed several MVBs within one microscopic field (Fig. 4B and C). Thus upregulation of TSG101 by mechanical stress might contribute to increased formation of MVBs in podocytes.

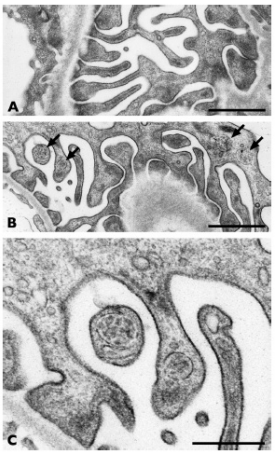

Figure 4. The number of MVBs is increased in podocyte processes in DOCA/salt rats. Transmission electron micrographs of podocyte foot processes and major processes in control rats (panel A) and in DOCA/salt rats (panel B and C). MVBs are scarce in podocyte processes of control animals (no MVB in panel A), while they are frequently found in podocyte processes of DOCA/salt rats (arrows mark four MVBs in panel B). The higher magnification of the left region in panel B shows the characteristic morphology of MVBs (panel C). Scale bars represent 1 µm (panel A and B) and 0.5 µm (panel C), respectively.

Discussion

TSG101 together with VPS28 and VPS37 form the ESCRT-I complex (16,17). Activated receptors are marked by ubiquitination for endocytosis and degradation. The ESCRT-I complex binds ubiquitinated receptors via the ubiquitin binding domain of TSG101. The cargo is then relayed to the ESCRT-II complex, and is finally sorted into a budding intraluminal vesicle that forms under control of the ESCRT-III complex. Thus TSG101 plays an important role in the regulation of membrane receptor degradation, e.g. the epidermal growth factor receptor (15,18). Due to the formation of ligand/receptor complexes, ligands are sorted into MVBs together with their receptors (22,23).

Podocytes in vivo exposed to intolerably high mechanical stress undergo a variety of structural changes (24) including cytoplasm shedding and foot process effacement. These changes have been interpreted as measures counteracting the danger of podocyte detachment from the GBM. Foot process effacement strengthens the attachment of podocytes to the GBM by changing the delicate adhesion pattern by interdigitating foot processes into a continuous simplified attachment area (24). This leads to the loss of the filtration slits, including the slit membrane, functionally to a sealing of the filtration route and, likely, to a stop of any filtrate flow at this site. This rearrangement of the podocyte-GBM-connections is a highly catabolic process including an enormous reduction in total podocyte cell mass. To get rid of the dispensable material, podocytes appear to use two mechanisms: (i) direct shedding of membrane bound large portions of cytoplasm (24) and (ii) increasing degradation of cell components. Beside proteasomal degradation and autophagy (25,26), MVBs represent an additional pathway sorting ubiquitinated membrane proteins into the interior of a vesicle for degradation (23). As shown here, the TSG101 system is prominently upregulated in this process but its particular role is so far unknown. Moreover, the local sealing of the flow route by foot process effacement may increase stranding of plasma proteins just beneath the basal portion of podocytes, inducing an increased uptake by receptor-mediated endocytosis into podocytes. Megalin-mediated uptake of lipoproteins finally localizing to MVBs has been demonstrated by Kerjaschki and colleagues (27).

In conclusion, the strong enhancement of the degradative pathway via MBVs under conditions of the adaptation of podocytes to increased mechanical stress may serve two requirements. First, it acts to decrease total single podocyte mass in correlation with the progressing effacement of foot processes and, second, to remove endocytosed plasma proteins that had become trapped within the filtration barrier under conditions of a hindered outflow from the GBM.

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