TRPC6 mutations associated with focal segmental glomerulosclerosis cause constitutive activation of NFAT-dependent transcription

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Schlöndorff J, del Camino D, Carrasquillo R, Lacey V, Pollak MR. TRPC6 mutations associated with focal segmental glomerulosclerosis cause constitutive activation of NFAT-dependent transcription. Am J Physiol Cell Physiol 296: C558–C569, 2009. First published January 7, 2008; doi:10.1152/ajpcell.00077.2008.—Mutations in the canonical transient receptor potential channel TRPC6 lead to an autosomal dominant form of human kidney disease characterized histologically by focal and segmental glomerulosclerosis. Several of these mutations enhance the amplitude and duration of the channel current. However, the effect of these mutations on the downstream target of TRPC6, the nuclear factor of activated T cell (NFAT) transcription factors, has not been previously examined. Here we demonstrate that all three TRPC6 mutations previously shown to enhance channel activity lead to increased basal NFAT-mediated transcription in several cell lines, including cultured podocytes. These effects are dependent on channel activity and are dominant when mutants are coexpressed with wild-type TRPC6. While TRPC6 mutants do not demonstrate an increase in basal channel currents, a subset of cells expressing the R895C and E897K mutants have elevated basal calcium levels as measured by Fura-2 imaging. Activation of NFAT by TRPC6 mutants is blocked by inhibitors of calcineurin, calmodulin-dependent kinase II, and phosphatidylinositol 3-kinase. PP2 partially inhibits NFAT activation by mutant TRPC6 independently of Src, Yes, or Fyn. Differences in channel glycosylation and surface expression do not explain the ability of mutants to enhance NFAT activation. Taken together, these results identify the activation of the calcineurin-NFAT pathway as a potential mediator of focal segmental glomerulosclerosis.

calcium; calcineurin; phosphatidylinositol 3-kinase; podocyte; canonical transient receptor potential channel

MUTATIONS IN TRPC6, a member of the transient receptor potential superfamily of nonselective cation channels, have been identified as causing a familial form of progressive kidney disease in humans characterized by focal and segmental glomerulosclerosis (46, 58). Focal segmental glomerulosclerosis (FSGS) is a clinicopathologic pattern of kidney injury characterized by proteinuria, progressive renal failure, and a signature glomerular lesion with areas of sclerosis in the capillary loops of a subset of glomerular tufts. Studies in human and mouse genetics over the past decade have led to significant advances in our understanding of proteinuric kidney diseases (9, 32, 53). Mutations in a number of genes have been identified as causing autosomal recessive [nephrin (26), podocin (6), laminin-β2 (17), phospholipase Cε (18), limp-2 (2), and PDSS2 (11, 35)] and autosomal dominant [α-actinin-4 (24)] forms of proteinuric kidney disease in humans, with potential recessive (36) and dominant (28) forms of disease associated with CD2AP. These studies have helped focus our attention on the podocyte and its basement membrane as critical structures in maintaining glomerular function.

Winn et al. (58) reported a mutation in TRPC6 in a large kindred with an adult onset, autosomal dominant form of FSGS. Several additional mutations in TRPC6 in other families with inherited FSGS have since been identified (46). All show a dominant mode of inheritance with adult onset of disease and variable penetrance. TRPC6 is a member of the large transient receptor potential (TRP) superfamily of nonselective cation channels (reviewed in Refs. 45 and 55). This superfamily consists of a group of six transmembrane domain-containing ion channels and has been subdivided into six subfamilies, including the canonical TRP or TRPC proteins. All six currently identified TRPC6 mutations localize to either the amino- or carboxyl-terminal cytoplasmic domains. The mutations are thought to lead to a gain of function, as several of the mutations have been reported to increase channel associated currents or slow current decay (46, 58). Furthermore, TRPC6 expression is upregulated in several proteinuric diseases in humans, while nonspecific overexpression of TRPC6 in mice leads to transient proteinuria (38). Although TRPC6 has a broad expression pattern, attention has been focused on its potential role in podocytes. Within podocytes, TRPC6 has been reported to localize to the slit diaphragm and interact with two key components of that structure, nephrin and podocin (22, 46). The interaction with podocin has been reported to enhance TRPC6-mediated currents (22).

Several signaling events have been described downstream of TRPC6 activation, including RhoA activation and actin cytoskeleton rearrangement (50), and the activation of two calcium-dependent transcription factors, NFAT (31, 42, 44) and cAMP response element binding protein (CREB; 23). Overexpression of TRPC6 in cardiac myocytes in vitro and in vivo leads to activation of the calcineurin-NFAT pathway and cardiac hypertrophy in response to endothelin-1 or angiotensin II (31, 42, 44). Several other TRPC channels have also been implicated in cardiac hypertrophy through activation of the calcineurin-NFAT pathway (7, 15, 25, 40, 43, 44, 48). The link between TRPC6 and the calcineurin-NFAT pathway in cell types other than cardiac myocytes has not been assessed.

In the present study, we examine the effect of FSGS-associated mutations on the ability of TRPC6 to activate NFAT-dependent transcription. We find that the three muta-
tions previously reported to display enhanced channel activity (P112Q, R895C, and E897K) lead to constitutive transcriptional activation of NFAT-responsive reporters. The ability of these mutations to activate NFAT is dominant over wild-type TRPC6, and it is inhibited by coexpression of a channel-inactive dominant-negative mutant. Pharmacologic inhibitors of calcineurin, phosphatidylinositol 3-kinase (PI3K) and CaMKII block the activation of NFAT by mutant TRPC6. The basal activation of NFAT does not appear to be mediated by increased channel “leak.” Taken together, we have identified excess activation of the calcineurin-NFAT pathway as a potential mechanism of TRPC6-induced renal disease.

MATERIALS AND METHODS

Reagents and plasmids. Full-length human TRPC6 cDNA containing an amino-terminal FLG tag sequence was cloned into pcDNA3.1 and pcDNA4/TO/myc-His B (Invitrogen) using standard PCR-based techniques. Various FSGS-associated mutations (46, 58), as well as the dominant-negative pore mutation changing amino acids LFWM to AAA (21), were introduced into this construct using the QuickChange II XL site-directed mutagenesis kit from Stratagene. All plasmids were sequenced to confirm the presence of mutations and exclude the presence of unwanted mutations. The angiotensin II type I receptor (AT1R) expression plasmid (16) was kindly provided by M. Lopez-Illasaca. Green fluorescent protein-tagged NFATc3 expression plasmid (NFATc3-GFP) was the gift of F. McKeon (56). The dual luciferase assay kit and reporter vectors pGL4.30 and pGL4.74 were obtained from Promega.

Angiotensin II, carbachol, cycloporsine A, FK506, KN-93, and poly-L-lysine were obtained from Sigma-Aldrich. Genistin, LY-294002, PP2, and U-73122 were purchased from Calbiochem; 1-oleoyl-2-acetylsn-glycerol (OAG) was obtained from Avanti Polar Lipids; and EZ-Link Sulfo-NHS-biotin was from Pierce. Affinity-purified rabbit anti-TRPC6 polyclonal antibody was purchased from Chemicon, and FLAG M2 and anti-actin monoclonal antibodies were purchased from Sigma. Fura-2 AM and probenecid were obtained from Invitrogen.

Cell culture and luciferase assays. Human embryonic kidney (HEK)-293T cells were obtained from American Type Culture Collection (ATCC) and maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% heat-inactivated fetal bovine serum and penicillin G, streptomycin, and amphotericin B. T-Rex-293 cells (Invitrogen) stably expressing the M1 acetylcholine receptor (M1R) were maintained in the same media as the 293T cells supplemented with 5 μg/ml blasticidin and 1 mg/ml G418. M1R cells were transfected with pcDNA4/TO/myc-His containing FLAG-tagged wild-type or mutant TRPC6 cDNA using Fugene 6 (Roche) following the manufacturer’s protocol. After 24 h, the cells were passaged and placed in selection media containing 200 μg/ml zeocin (Invitrogen). Individual clones were selected and expanded. FLAG-TRPC6 expression in individual clones was assessed by Western blot after the cells had been exposed to 5 μg/ml tetracycline, or carrier, for 24–48 h. SYF cells (murine embryonic fibroblasts lacking the Src-family kinases Src, Yes, and Fyn) were obtained from ATCC and cultured as directed by the provider (30). Conditionally immortalized human podocytes were the kind gift of M. Saleem and were maintained under permissive conditions as previously detailed (47). For luciferase assays, cells were plated in 24-well plates and transfected with Fugene 6 (Roche) following the manufacturer’s protocol. Cells were transfected with a 50:1 mixture of pGL4.30 (NFAT-response element) and pGL4.74 (Renilla luciferase under a thymidine kinase promoter), and an equal amount of control vector (pcDNA4) or TRPC6 expression vector. AT, R expression plasmid was included when indicated. Twenty-four hours after transfection, cells were serum starved overnight, followed by a further 6-h incubation in the presence or absence of various stimuli and inhibitors. Cells were washed once in PBS and then lysed in 100 μl of passive lysis buffer (Roche). The dual luciferase reporter assay (DLR; Roche) was performed with 20 μl of lysate using the manufacturer’s protocol and a Veritas microplate luminometer (Turner Biosystems). All results were normalized to the thymidine kinase-Renilla luciferase internal control and are presented as a fold increase relative to the control condition.

Western blot analysis. Residual cell lysates from the dual luciferase assay were spun at 15,000 g for 15 min. The resulting supernatant was mixed with sample loading buffer, separated by SDS-PAGE, and transferred to polyvinylidene difluoride membrane (Bio-Rad). The membrane was blocked with 5% nonfat milk in PBS with 0.05% Tween 20 (PBST) for 1 h at room temperature, followed by overnight incubation in 1:1,000 anti-TRPC6 polyclonal antibody in 5% nonfat milk PBST. After three washes in PBST, blots were incubated in 1:3,000 goat anti-rabbit IgG conjugated to horseradish peroxidase (Pierce) in PBST at room temperature, followed by detection with SuperSignal West Pico chemiluminescent substrate (Pierce).

NFATc3-GFP localization. M1R cells were plated on collagen I-coated coverslips and transfected with Fugene 6 to a 1:3 ratio of NFATc3-GFP and TRPC6 expression plasmid (or control vector). After 18–30 h, cells were fixed in 4% paraformaldehyde in PBS for 10 min at room temperature, stained with 1 μg/ml 4′,6-diamidino-2-phenylindole (DAPI; Sigma) for 5 min, washed several times with PBS, and mounted on slides with Fluoromount-G (Southern Biotech). Cells were scored for GFP localization using a Nikon E-1000 microscope. GFP localization was categorized as either cytoplasmic (C), cytoplasmic greater than nuclear (C > N), or exclusively nuclear (N). At least 100 GFP positive cells over several random high-power fields were scored for each transfection.

Cell surface biotinylation. Cells were processed either 36 to 48 h after transient transfection or 24 to 36 h after TRPC6 expression was induced by the addition of tetracycline. Cells were washed and incubated in ice-cold PBS containing 1 mM calcium and magnesium for 10 min, followed by a 30-min incubation in ice-cold PBS supplemented with 0.5 mg/ml Sulfo-NHS-biotin. The biotin solution was removed and the cells were incubated for an additional 10 min in ice-cold Tris-buffered saline supplemented with 10 mM glycine to quench any remaining reactive biotin cross-linker. Cells were then lysed in PBS with 1% (vol/vol) Nonidet P-40 and Complete protease inhibitor (Roche). Lysates were briefly sonicated and cleared by centrifugation. An aliquot of lysate was set aside and mixed with sample loading buffer. The remaining lysate was incubated with 20 μl of a 50% slurry of streptavidin beads (Pierce) in lysis buffer and incubated at 4°C for 2 h. The beads were washed three times with 1 ml of lysis buffer, and bound material was eluted by boiling in sample loading buffer. Total TRPC6 and biotinylated TRPC6 were detected by Western blot analysis as above. The relative intensities of total and biotinylated TRPC6 were determined using ImageJ software (National Institutes of Health, Bethesda, MD).

Deglycosylation. M1R cells transiently transfected with various TRPC6 expression constructs were lysed 36–48 h after transfection. Cleared lysates were either mock treated or treated with peptide N-glycosidase F (PNGase F; New England Biolabs) as per the manufacturer’s recommendations, followed by SDS-PAGE and detection of TRPC6 by Western blot analysis.

Electrophysiology. HEK-293 cells were transiently transfected with 1 μg of the wild-type or mutant TRPC6 cDNAs and 0.1 μg of GFP. The cells were used for electrophysiological recordings 24–72 h after transfection.

Macroscopic currents were recorded in the whole cell configuration of the patch-clamp technique using an EPC-9 amplifier and PatchMaster software (HEKA). Patch pipettes had a resistance of 1.5–3 MΩ and were filled with a solution consisting of (in mM) 140 CsAsp, 10 EGTA, 10 HEPES, 2.27 MgCl2, and 1.91 CaCl2, pH 7.2 with CsOH. The bath solution contained (in mM) 145 NaCl, 5 KCl, 1 MgCl2, 2
increase in basal NFAT-mediated transcription (Fig. 1, shown]. NFAT-mediated transcription was assessed either in these cells leads to TRPC6 activation [(46) and data not overexpresses the M1 acetylcholine receptor (referred to as TRPC6 pore mutant. We used an HEK-293T cell line that cotransfected with either wild-type TRPC6, one of the FSGS-kinase promoter-driven results and using the Bonferroni correction for multiple comparisons. All statistical analysis was performed using GraphPad Prism4 (GraphPad Software). Luciferase data were analyzed by repeated-measures one-way ANOVA using log-transformed results and using the Bonferroni correction for multiple comparisons. Surface expression results were analyzed by one-way ANOVA using Dunnett’s test. Fura-2 ratios were compared using the Kruskal-Wallis and Dunn’s multiple-comparison tests.

RESULTS

A subset of FSGS-mutations in TRPC6 enhance NFAT activation. To examine the effect of disease-associated mutations in TRPC6 on activation of the calcineurin-NFAT pathway, we used a luciferase reporter assay. An NFAT-response element driven firefly luciferase reporter plasmid and a thymidine kinase promoter-driven Renilla luciferase control plasmid were cotransfected with either wild-type TRPC6, one of the FSGS-associated TRPC6 mutants, or a known dominant-negative TRPC6 pore mutant. We used an HEK-293T cell line that overexpresses the M1 acetylcholine receptor (referred to as M1R cells); stimulation of the M1 receptor with carbachol in these cells leads to TRPC6 activation [(46) and data not shown]. NFAT-mediated transcription was assessed either in the absence of exogenous stimuli, or after stimulation with carbachol.

Overexpression of wild-type TRPC6 led to a modest increase in basal NFAT-mediated transcription (Fig. 1, A and B). Carbachol stimulation for 6 h led to a robust increase in NFAT-responsive reporter activity in the absence of overexpressed TRPC6, as has previously been described (4). This response was not significantly enhanced by the coexpression of wild-type TRPC6. Basal and carbachol-stimulated NFAT activation by amino terminal mutants N143S and S270T, as well as the carboxyl-terminal truncation mutant K874*, was not markedly different from wild-type TRPC6. In contrast, TRPC6 P112Q, R895C, and E897K, all of which have previously been shown to display enhanced current amplitudes or delayed current decay (46, 58), were able to markedly stimulate the NFAT-responsive reporter in the absence of stimulation, with a further increase in reporter activity upon carbachol treatment. This enhanced reporter activity was not due to increased channel expression of these three mutants compared with wild-type TRPC6 (Fig. 1C). Overexpression of a known dominant-negative TRPC6 mutant, generated by altering the conserved pore domain LFW sequence to AAA (21), was not able to suppress basal or carbachol-stimulated NFAT signaling compared with control transfected cells (Fig. 1A), indicating that while TRPC6 is capable of enhancing M1 receptor-mediated NFAT activation, it is not necessary.

To confirm the ability of TRPC6 and FSGS-associated mutants to activate the calcineurin-NFAT pathway, we examined the subcellular localization of a GFP-tagged fragment of NFATc3. This fusion protein, similar to wild-type NFAT proteins, is excluded from the nucleus under basal conditions, but it translocates to the nucleus upon calcineurin-mediated dephosphorylation (56). In unstimulated M1R cells not expressing TRPC6, NFATc3-GFP is exclusively present in the cytoplasm in >80% of cells, with no cells showing a predominantly nuclear GFP signal (Fig. 1, D and E). Cotransfection of wild-type TRPC6 led to a predominantly nuclear GFP signal in slightly more than 10% of cells. This increased to 40–50% when P112Q, R895C, or E897K mutant TRPC6 was expressed. Dominant-negative TRPC6 did not substantially affect NFATc3-GFP localization.

The ability of TRPC6 mutants to activate basal NFAT-mediated transcription was not dependent on the overexpression of M1 acetylcholine receptor, because the P112Q, R895C, and E897K mutants were more effective than wild-type TRPC6 in enhancing NFAT-driven luciferase activity in HEK-293T cells lacking the M1 receptor (data not shown) and in SYF murine embryonic fibroblasts and a human podocyte cell line (see below). Furthermore, the ability of the R895C mutant to enhance G protein-coupled receptor-mediated NFAT activation also extended to signaling via the AT1R (data not shown), consistent with the role of TRPC6 in mediating angiotensin II-mediated NFAT activation and cardiac hypertrophy (44).

NFAT activation by TRPC6 in podocytes. Although it has not been formally demonstrated that it is abnormal TRPC6 function in glomerular epithelial cells (also known as podocytes) per se that leads to FSGS, several lines of evidence are suggestive (22, 38, 46). To begin to address whether NFAT may represent a target of TRPC6 signaling in the podocyte, we assayed the ability of wild-type and mutant TRPC6 to activate an NFAT-responsive luciferase reporter in a cultured podocyte cell line. While the effect was more modest than in M1R cells, wild-type TRPC6 enhanced NFAT-responsive reporter activity in these cells, while the P112Q, R895C, and E897K mutant forms of TRPC6 achieved greater levels of NFAT activation.
To assess which of the four calcium-responsive NFAT genes (NFATc1-4) might be involved, their expression in the human podocyte cell line was assessed by RT-PCR. Transcripts for NFATc1 and c3 were detected in both M1R cells and human podocytes (Fig. 2C); the presence of NFATc3 in podocytes is consistent with publicly available immunohistochemical staining patterns in human kidney samples [www.proteinatlas.org (3)].

**Pore mutations impair NFAT activation by FSGS-mutant TRPC6.** Recently, the L-type voltage-dependent calcium channel Cav1.2 has been reported to have effects on transcription independent of its channel activity (13). We therefore examined whether the ability of R895C and E897K mutant TRPC6 to activate NFAT-mediated transcription is dependent on channel activity by assessing the ability of a dominant-negative pore mutation to inhibit the effects of the FSGS mutations. Cotransfection of dominant-negative TRPC6 substantially inhibited NFAT activation by R895C, E897K (Fig. 3A), and P112Q TRPC6 (data not shown). Introducing the dominant-negative LFW to AAA pore mutation directly into the R895C TRPC6 largely abolished the ability of the mutant channel to enhance basal or stimulated NFAT activation (Fig. 3C), although overexpression of the R895C dominant-negative channel did induce a small but statistically significant increase in reporter activity. These results suggest that the effects of these FSGS-associated mutants on NFAT activation largely rely on their channel activity. However, we cannot exclude that the pore mutation, in addition to impairing channel function, has additional steric effects that inhibit the ability of mutant TRPC6 to activate NFAT independently of its channel activity.

**NFAT activation by FSGS-mutant TRPC6 is dominant over wild-type TRPC6.** Mutations in TRPC6 lead to an autosomal dominant inheritance of FSGS (46, 58). We therefore wanted to assess whether the ability of the R895C and E897K mutants to enhance NFAT activation was dominant in the presence of wild-type TRPC6. Cotransfection of equal amounts of wild-type and mutant TRPC6 expression plasmids in the luciferase assays led to only a slight, statistically insignificant, decrease in NFAT activation compared with transfection of the mutant TRPC6 plasmids alone (Fig. 3A; compare, for instance,
Calcineurin has been reported to bind to TRPC6 via calmodulin upon M1 acetylcholine receptor activation (29). In addition, FKBP12 binds directly to TRPC6, and high-dose FK506 has been shown to inhibit TRPC6 activity (51). To ascertain

R895C/− to R895C/WT). NFAT activation upon cotransfection remained substantially higher compared with cells expressing wild-type TRPC6 alone. These results are consistent with the hypothesis that the mutant TRPC6 channel characteristics, specifically in regard to its ability to activate the calcineurin-NFAT pathway, are dominant over wild-type TRPC6.

Cyclosporine A inhibits TRPC6-mediated NFAT activation without affecting channel activity. The protein phosphatase calcineurin is responsible for dephosphorylating, and thereby activating, NFAT in response to calcium signaling. As expected, the calcineurin inhibitor cyclosporine A is able to abrogate both TRPC6-mediated and TRPC6-independent NFAT activation (Fig. 4A). Basal and carbachol-induced NFAT activation via the mutant R895C was also substantially reduced in the presence of cyclosporine A.
whether cyclosporine A may have a direct effect on TRPC6 activity in addition to downstream events in the calcineurin-NFAT pathway, we assessed the effect of cyclosporine A on TRPC6-mediated whole cell currents. Cells were activated by the diacylglycerol analog OAG, because this represents a more direct mechanism for activating TRPC6 than carbachol (20). While high concentrations of FK506 were able to rapidly inhibit OAG-induced TRPC6 currents, similar to previous reports (51), high-dose cyclosporine A did not appreciably affect current amplitudes (Fig. 4, B and C). Similar results were obtained with the R895C and E897K mutants and when cells were stimulated with carbachol instead of OAG (data not shown). Thus, the effect of cyclosporine A on NFAT activation appears to be mediated solely downstream of TRPC6 without an additional effect on the channel itself. In contrast, FK506 can potentially act at two sites in the TRPC6-calcineurin-NFAT pathway, by blocking the binding of FKBP12 to TRPC6 (51) and by inhibiting calcineurin.

Mechanism of basal activation of NFAT by TRPC6 mutants. The constitutive activation of NFAT-mediated transcription by three of the TRPC6 mutants was intriguing. We postulated that this could be due to constitutive low-level leak through the channels. To address this issue further, we performed whole cell voltage-clamping experiments in HEK-293 cells expressing either wild-type or mutant R895C and E897K TRPC6 (Fig. 5A). Basal currents were not significantly different in cells expressing R895C or E897K mutant TRPC6 compared with wild-type TRPC6, suggesting that constitutive low-level channel leak is unlikely to explain the basal activation of NFAT by these mutants. Upon subsequent stimulation of cells with either OAG or carbachol, cells expressing the R895C or E897K mutants showed substantially higher current amplitudes than cells expressing wild-type TRPC6 (data not shown), as had been previously reported (46).

We also examined Fura-2 fluorescence under unstimulated conditions as a surrogate for basal intracellular calcium levels in both transiently transfected cells (Fig. 5B) and cells stably expressing TRPC6 under a tetracycline-inducible promoter (Fig. 5C). Cells overexpressing wild-type TRPC6 had slightly lower median basal 340/380 fluorescence ratios than control-transfected cells. Median F340/F380 ratios of cells expressing the R895C or E897K mutants were not statistically significantly different (Fig. 5B), or only slightly lower (Fig. 5C) than ratios of control cells. However, the distribution of the ratios in these cells does shows a significant tail at higher ratios, suggesting that at any given time, a subpopulation of cells expressing the mutant channels have elevated intracellular calcium levels.

We wished to identify what signaling pathways might be important for the basal activation of the calcineurin-NFAT pathway by mutant TRPC6. Because several signaling pathways have been implicated in the regulation of TRPC6, including phospholipase C (PLC) (20), Src-family tyrosine kinases (1, 19, 25), PI3K (33, 54), and CaMK II (49), we tested the ability of inhibitors of these enzymes to block mutant TRPC6-mediated NFAT-activation. Cells overexpressing TRPC6 R895C were exposed to various inhibitors for 6 h followed by the assessment of NFAT-mediated transcription (Fig. 6A). To attempt to identify pathways required specifically for activation of the calcineurin-NFAT pathway by mutant TRPC6, and not for TRPC6-independent NFAT activation downstream of the M1 acetylcholine receptor, we also examined the effect of the inhibitors on M1R cells not overexpressing TRPC6, but stimulated with carbachol.

Cyclosporine A effectively inhibited both carbachol- and TRPC6 R895C-mediated NFAT activation (Figs. 4A and 6A). In contrast, PP2 (a Src-family kinase inhibitor), LY-294002 (a PI3K inhibitor), and KN-93 (a CaMK II inhibitor) all partially inhibited NFAT activation by TRPC6 R895C, but not by carbachol (Fig. 6A). The tyrosine kinase inhibitor Genistein enhanced NFAT-mediated transcription by both carbachol and mutant TRPC6 (Fig. 6A), as well as basal NFAT-mediated transcription in unstimulated M1R cells (data not shown).
Surprisingly, the PLC inhibitor U-73122 did not significantly inhibit NFAT activation.

There have been conflicting reports regarding the role of Src-family kinases, specifically Fyn, in the activation of TRPC6 (1, 19, 25). In light of the ability of PP2 to partially inhibit NFAT activation by R895C TRPC6, the ability of the R895C mutant to activate NFAT-mediated transcription was also examined in SYF cells, which are deficient in Src, Yes, and Fyn (30). The R895C mutant was able to activate NFAT-mediated transcription in these cells under serum starvation, although to a lesser extent than in M1R cells, while wild-type

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**Fig. 5.** Basal channel currents and Fura-2 fluorescence. A: average normalized basal TRPC6 currents measured at 80 mV (gray bars) and −80 mV (black bars) from cells expressing the WT TRPC6 channel or the mutant channels R895C or E897K (pA/pF ± SE). An unpaired Student’s t-test did not reveal statistically significant differences between WT and the two mutant channels. B: histogram of ratio of Fura-2 fluorescence upon excitation at 340 and 380 nm under unstimulated conditions of M1R cells transfected with control plasmid (−), or the indicated TRPC6 expression plasmid. Bar indicates median ratio. ***P < 0.001 vs. all other groups, by Kruskal-Wallis and Dunn’s multiple-comparison tests. C: Fura-2 fluorescence ratio of unstimulated M1R cells stably expressing either WT or the indicated TRPC6 mutants under a tetracycline-inducible promoter, or the parental M1R cell line. Bar indicates median ratio of population. ***P < 0.001 vs. parental; †††P < 0.001 vs. WT by Dunn’s multiple-comparison test.

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**Fig. 6.** Pharmacologic inhibition of basal NFAT activation by TRPC6 R895C. A: M1R cells were transfected with the NFAT luciferase reporter construct either alone (control) or with TRPC6 R895C expression plasmid (R895C). Cells were serum starved overnight followed by a 6-h incubation in serum-free media with vehicle alone, 10 μM cyclosporine A, 10 μM U-73122, 50 μM Genistein, 4 μM PP2, 50 μM LY-294002, or 2 μM KN-93. Control cells were also stimulated with 100 μM carbachol during the 6-h incubation. NFAT-response element-driven luciferase activity (means ± SE) is shown as a percentage of the activity in the corresponding vehicle-treated cells. *P < 0.05 vs. vehicle-treated cells; **P < 0.05 vs. vehicle-treated cells. B: Western blot of TRPC6 R895C-transfected M1R cells. C: SYF murine embryonic fibroblasts were transfected with NFAT-responsive luciferase plasmid and either control plasmid or the indicated FLAG-TRPC6 expression plasmid. The dual luciferase assay was performed after cells were treated with either vehicle or 4 μM PP2 for 6 h. Results are means ± SE. *P < 0.001 vs. control, †P < 0.05 vs. R895C vehicle treated.

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TRPC6 had no effect (Fig. 6B). P112Q and E897K also activated NFAT mediated transcription (data not shown). The addition of PP2 was able to partially inhibit NFAT activation in R895C-expressing SYF cells, while it had no effect in the absence of TRPC6, suggesting that the ability of PP2 to affect mutant TRPC6 mediated activation of the calcineurin-NFAT pathway is independent of its inhibition of Src, Yes, or Fyn.

Differential glycosylation of TRPC6 mutants. Transiently overexpressed TRPC6 was observed to run at two distinct molecular masses of approximately 100 and 130 kDa (Fig. 1, top, and Fig. 7A, top). To confirm that the higher-molecular form represents the fully glycosylated form, while the lower represents a precursor, as has previously been reported (5, 37), we subjected lysates from cells overexpressing various forms of TRPC6 to treatment with PNGase to remove all forms of N-linked glycosylation. After deglycosylation, only the lower-molecular-weight form of TRPC6 remained (Fig. 7A, bottom). Furthermore, while the higher-molecular-weight form of various mutant forms of TRPC6 had slightly different mobilities on SDS-PAGE, this difference was no longer detected after deglycosylation, indicating that these mutants are differently glycosylated from wild-type TRPC6.

To assess whether the 100-kDa form of TRPC6 represents a precursor form found only intracellularly, or whether this form can reach the cell surface, transiently transfected cells were surface biotinylated, and biotinylated TRPC6 was compared with total TRPC6 (Fig. 7B). Both the 100- and 130-kDa forms were biotinylated, with the relative abundance of the two forms on the cell surface generally mirroring their relative abundance in total lysates.

TRPC6 activity has been reported to be at least partially controlled through regulated transport to the cell surface (8), and a greater percentage of P112Q mutant TRPC6 has been reported to reside on the cell surface compared with wild-type TRPC6 (58). We therefore examined whether the amount of cell surface expression of the TRPC6 mutants correlated with their ability to activate the calcineurin-NFAT pathway by comparing the amount of cell surface expressed TRPC6 to the total amount of TRPC6 by Western blot analysis (Fig. 7, B and C). When the relative intensities of the TRPC6 bands in the cell surface and whole lysate fractions were compared, there was a trend toward a higher proportion of the carboxyl-terminal mutants being present on the cell surface (Fig. 7C). However, there was significant heterogeneity from experiment to experiment, and the differences were not statistically significant.

The relative abundance of the 100- and 130-kDa forms of TRPC6 differed between the wild-type and mutant forms (Fig. 7, A and B). Specifically, the three mutations that constitutively activate NFAT, as well as the K874* truncation mutant, are predominantly present as the lower form, while a larger fraction of wild-type TRPC6 and the other mutations exist as the 130-kDa species. Interestingly, introducing the dominant-negative pore mutation into the R895C mutation shifted the protein predominantly into the more heavily glycosylated form (Fig. 7A).

Differences in N-linked glycosylation between TRPC3 and C6 have been shown to be important for the different basal channel characteristics of these proteins, with glycosylation deficient TRPC6 displaying increased constitutive channel activity (10). Is it possible that the differences in the relative abundances of the 100- and 130-kDa forms of TRPC6 could explain the ability of some TRPC6 mutants to constitutively activate the calcineurin-NFAT pathway? Alternatively, the presence of the unglycosylated form of TRPC6 could represent an overwhelming of the protein folding and trafficking machinery by transient transfection. We therefore generated stable M1R cell lines in which wild-type, R895C, or E897K TRPC6 is stably expressed under a tetracycline-inducible promoter. Induction of TRPC6 expression in these cell lines leads to the predominant expression of the fully glycosylated, mature form of wild-type and mutant protein (Fig. 8A), suggesting that the lower-molecular-weight form of TRPC6 accumulates at least in part due to a limited ability of the cell to properly glycosylate the channel in the setting of overexpression by transient transfection. Furthermore, surface biotinylation demonstrated that in these cell lines, only the mature 130-kDa form was present on the cell surface (Fig. 8B). To confirm that a predominance of the 100-kDa form of the mutant proteins is not required for the constitutive activation of NFAT-mediated

Fig. 7. TRPC6 mutants exhibit different glycosylation patterns and are transported to the cell surface. A: lysates from M1R cells transiently transfected with the indicated FLAG-tagged TRPC6 constructs were treated without (mock) or with peptide N-glycosidase F (PNGase F), followed by detection of TRPC6 by Western blot analysis. B: M1R cells transiently transfected with the indicated FLAG-tagged TRPC6 constructs were subject to surface biotinylation. Western blot analysis compares total TRPC6 (T; 5% of the total lysate) to surface-expressed TRPC6 (S; streptavidin-agarose bound fraction). C: relative surface expression of WT and mutant TRPC6. The relative intensities of total to surface expressed protein were normalized to that of WT TRPC6; results are means ± SE of four experiments. P > 0.05 for all mutants vs. WT.
transcription, NFAT-responsive luciferase reporter assays were repeated in these stable cell lines (Fig. 8C). Interestingly, induction of wild-type TRPC6 expression did not lead to any change in basal transcription from the NFAT-responsive reporter plasmid. Induction of either the R895C or E897K mutants led to significant activation of NFAT-mediated transcription, though the relative induction was less than was seen with transient transfection. Although we cannot completely exclude a contribution of improper glycosylation as a mechanism for NFAT activation by transiently overexpressed TRPC6, our results making use of the inducible stable cell lines demonstrate that abnormal glycosylation is not the only mechanism involved in constitutive activation of NFAT by mutant TRPC6.

DISCUSSION

Mutations in TRPC6 have been identified as an important cause of autosomal dominant adult-onset FSGS. These mutations are thought to be gain-of-function in nature; that is, for several of these mutations, increased current amplitudes and calcium entry have been documented in response to channel activation (46, 58). However, the effect on potential downstream targets, such as the NFAT transcription factors, had not previously been addressed.

We have found that when overexpressed, TRPC6 mutations with enhanced channel currents lead to increased basal and stimulated NFAT-mediated transcription. Introduction of pore mutations that abolish channel activity also abrogate NFAT activation. Furthermore, the ability of the mutant channels to activate NFAT is dominant over wild-type TRPC6, consistent with the known genetics of TRPC6-associated FSGS. Calcineurin is a critical intermediary between TRPC6 and NFAT-mediated transcription, while pharmacologic inhibitors of PI3K and CaMK II block NFAT activation by mutant TRPC6, but not by carbachol. Interestingly, PP2 partially inhibits NFAT activation by mutant TRPC6 independently of the Src, Yes, and Fyn kinases. Together, these data identify excess activation of the calcineurin-NFAT pathway as a potential mechanism for TRPC6-mediated renal disease.

The ability of TRPC6 mutations with enhanced channel currents to significantly activate basal NFAT-mediated transcription compared with wild-type TRPC6 is surprising. The initial studies identifying TRPC6 mutations in familial forms of FSGS noted increased channel currents and calcium influx in response to G protein-coupled receptor activation, but they did not report increased basal currents (46, 58). Similarly, electrophysiological studies presented here also failed to demonstrate any substantial basal current leak through these channels. This is in contrast to mutations in TRPML3 in the varitint-waddler mouse, which were recently reported to lead to a constitutively active channel (14, 27, 39, 59). Introduction of a dominant-negative pore mutation that silences channel activity (21) into the FSGS-mutant TRPC6, or coexpression of the two mutant channels, largely blocks the basal activation of NFAT, suggesting that TRPC6 channel activity is necessary for the effect. However, we cannot exclude that the pore mutation, in addition to inhibiting channel activation, also inhibits a channel independent function of TRPC6 critical for activation of the calcineurin-NFAT pathway.

While basal current leak was not statistically different between wild-type and mutant TRPC6, basal Fura-2 fluorescence ratios, a surrogate for intracellular calcium concentrations, do show significant differences between the wild-type and mutant TRPC6-expressing cells. Specifically, a subset of cells expressing the R895C or E897K mutants have elevated Fura-2 ratios, although the majority of cells have ratios similar to those of control cells. Taken together, these results are consistent with the hypothesis that the presence of mutant channels leads to intermittent elevations in intracellular calcium levels. Whether
these elevations in Fura-2 ratios are a direct or indirect effect of mutant channel activation, and whether activation of the calcineurin-NFAT pathway is occurring only in the subset of cells demonstrating elevated intracellular calcium levels, remains to be determined. Defining the mechanism whereby mutant TRPC6 activates the calcineurin-NFAT pathway and modulates intracellular calcium levels will need to be addressed in future studies.

There are conflicting reports on the relative importance of Src-family kinases in activating TRPC6. Fyn has been reported to bind and phosphorylated TRPC6, and addition of Fyn and ATP to TRPC6 patches enhances channel activity (19), while two Src-family kinase inhibitors, PP2 and SU6656, are able to inhibit TRPC6-mediated calcium influx (1). In contrast, TRPC6 channel activation is not impaired in SYF cells lacking Src, Yes, and Fyn (25). We have found that the R895C mutant TRPC6 maintains its ability to activate NFAT-mediated transcriptions in SYF cells, and that this activity is partially inhibited by PP2, suggesting that the effect of PP2 on TRPC6 activity is independent of the three Src-family kinases Src, Yes, and Fyn. The mechanism of PP2’s effect remains to be elucidated. We have further shown that inhibitors of PI3K, PLC, and CaMK II impair NFAT activation by mutant TRPC6. However, we cannot yet place the role of these enzymes specifically upstream of TRPC6 as we do not at present have an assay to directly monitor the mutant TRPC6 activity that ultimately leads to NFAT activation.

Previous studies have suggested that both glycosylation patterns (10) and surface expression (8) of TRPC6 regulate its channel activity. We provide evidence that in the setting of transient transfection in M1R cells, TRPC6 mutants able to activate the calcineurin-NFAT pathway show a different glycosylation pattern compared with wild-type TRPC6, and that the abnormally glycosylated form is transported to the cell surface. In contrast, in the setting of stable expression in M1R cells, or transient transfection in other cell types, these glycosylation differences are not apparent. It is tempting to speculate that the differences in glycosylation patterns may explain the striking increase in NFAT-mediated transcription (on the order of hundreds of fold) in transiently transfected M1R cells compared the more modest (on the order of 10- to 100-fold) effect in SYF cells, podocytes, and stably transfected M1R cells. However, it is important to stress that the FSGS-mutants of TRPC6 maintain an enhanced ability to activate NFAT relative to wild-type TRPC6 even under conditions where no differences in glycosylation between the proteins is apparent, suggesting that altered glycosylation alone is not the sole mechanism involved. It is possible that the relative expression level of TRPC6 affects the composition of the channels or complexes it forms, and thus affects its channel properties, as has been reported by Yuan et al (60). M1R cells express TRPC1, -3, and -4 by RT-PCR (data not shown), all of which have been reported to be capable of forming complexes with TRPC6 (21, 52, 60). Although we have not been able to detect TRPC1, -3, or -4 in immunoprecipitates of TRPC6 from M1R cells (data not shown), it is possible that FSGS-associated mutations act by altering the makeup of TRPC6-containing channels.

The ability of TRPC6 mutants to significantly activate basal NFAT-mediated transcription compared with wild-type TRPC6 was restricted to the three mutations that have previously been shown to mediate increased channel currents, P112Q, R895C, and E897K. Three primary explanations can account for this finding: 1) other mutant forms of TRPC6 may show abnormal channel activity or regulation only under certain physiological conditions; 2) increased channel currents and the resultant activation of NFAT may not represent the only possible mechanism by which the mutations cause FSGS; and 3) additional TRPC6 mutations identified may, in fact, not be disease causing, despite the genetic evidence. Identification of the correct explanation will necessitate the development of an animal model of TRPC6-mediated renal disease.

The calcineurin-NFAT pathway was recently established as a central mediator of TRPC6-mediated cardiac hypertrophy (31, 44). Work presented here demonstrates that FSGS-associated TRPC6 mutations lead to inappropriate activation of NFAT via calcineurin, suggesting that this signaling pathway may be involved in TRPC6-associated FSGS. Faul et al. (12) recently demonstrated that calcineurin is involved in regulating the actin cytoskeleton of podocytes, at least in part through promoting the degradation of synaptopodin, and that this regulation is necessary for the development of proteinuria in the lipopolysaccharide model of transient proteinuria. Furthermore, overexpression of a constitutively active form of calcineurin A in podocytes was sufficient to induce proteinuria in mice. Taken as a whole, these results suggest the hypothesis that mutant TRPC6 leads to proteinuria and glomerular disease through excess activation of calcineurin in podocytes. This hypothesis, as well as the relative importance of NFAT-dependent and -independent actions of calcineurin, will need to be addressed in mouse models.

In summary, we have identified constitutive activation of the calcineurin-NFAT pathway as a consequence of FSGS-associated mutations in TRPC6. These results open up novel lines of investigation focusing on the calcineurin-NFAT pathway in modulating glomerular function, and suggest that calcineurin inhibitors be further examined in the treatment of TRPC6-associated FSGS.

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REFERENCES

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