A Novel Role for c-Myc in G Protein–Coupled Receptor Kinase 4 (GRK4) Transcriptional Regulation in Human Kidney Proximal Tubule Cells

John J. Gildea,* Hanh T. Tran,* Robert E. Van Sciver, Dora Bigler Wang, Julia M. Carlson, Robin A. Felder

Abstract—The G protein–coupled receptor kinase 4 (GRK4) negatively regulates the dopaminergic system by desensitizing the dopamine-1-receptor. The expression control of GRK4 has not been reported, but here we show that the transcription factor c-Myc binds to the promoter of GRK4 and positively regulates GRK4 protein expression in human renal proximal tubule cells (RPTCs). Addition of phorbol esters to RPTCs not only increased c-Myc binding to the GRK4 promoter but also increased both phospho-c-Myc and GRK4 expression. The phorbol ester–mediated increase in GRK4 expression was completely blocked by the c-Myc inhibitor, 10074-G5, indicating that GRK4 is downstream of phospho-c-Myc. The autocrine production of angiotensin II (Ang II) in RPTCs increased the phosphorylation and activation of c-Myc and subsequently GRK4 expression. 3-Amino-4-thio-butyl sulfonate, an inhibitor of aminopeptidase A, increased RPTC secretion of Ang II. 3-Amino-4-thio-butyl sulfonate or Ang II increased the expression of both phospho-c-Myc and GRK4, which was blocked by 10074-G5. Blockade of the Ang II type 1 receptor with losartan decreased phospho-c-Myc and GRK4 expression. Both inhibition of c-Myc activity and blockade of Ang II type 1 receptor restored the coupling of dopamine-1-receptor to adenylyl cyclase stimulation in uncoupled RPTCs, whereas phorbol esters or Ang II caused the uncoupling of normally coupled RPTCs. We suggest that the Ang II type 1 receptor impairs dopamine-1-receptor function via c-Myc activation of GRK4. This novel pathway may be involved in the increase in blood pressure in hypertension that is mediated by increased activity of the renin–angiotensin system and decreased activity of the renal dopaminergic system. (Hypertension. 2013;61:1021-1027.) ● Online Data Supplement

Keywords: angiotensins • cAMP receptor protein • c-Myc genes • dopamine • losartan • promoter regions, genetic • renin angiotensin system

Blood pressure is maintained in the normal range, in part, by renal regulation of sodium balance. Under conditions of moderate sodium intake, >50% of renal excretion is caused by the renal dopaminergic system. Dopamine, secreted from the renal proximal tubule cell (RPTC), acts as an intracrine, autocrine, and paracrine hormone in the renal proximal tubule (RPT) and other nephron segments to stimulate dopamine receptors (D1R, D2R, D3R, D4R, and D5R). Simultaneous stimulation of D1R and D5R with D1-like agonists leads to an increase in cAMP accumulation through adenylyl cyclase, whereas stimulation of D2R, D3R, and D4R decreases adenylyl cyclase activity. Stimulation of the D1R and D5R receptors leads to natriuresis through inhibition of sodium/hydrogen exchanger 3 (NHE3) and the sodium-potassium pump NaKATPase. The G protein–coupled receptor kinase 4 (GRK4) is 1 of the 7-member GRK family, which are serine-threonine kinases that are involved in the desensitization of G protein-coupled receptors (GPCRs). GRK4 phosphorylates the D1R to cause D1R internalization, thus increasing the reabsorption of sodium. The regulation of GRK4 expression is particularly important because increased renal protein expression of GRK4, in rodents, may be implicated in hypertension. High renal GRK4 expression inhibits the ability of the D1R to increase sodium excretion. In spontaneously hypertensive rats, basal levels of GRK4 are 90% higher in the kidney than in their normotensive control, Wistar-Kyoto Rats (WKY). Selective renal cortical inhibition of GRK4 expression with GRK4 antisense oligonucleotides increased sodium excretion and ameliorated the hypertension in spontaneously hypertensive rats. The hypertensive phenotype was further attenuated when renal AT1R expression was also inhibited.

The molecular mechanisms involved in the regulation of GRK4 are not well understood. Promoter analyses of GRK4...
revealed that the 2125 base pairs immediately upstream of the coding sequence are involved in regulating GRK4 expression and are sufficient for high transcriptional activity in transfected human embryonic kidney cells. When successive regions of the 2125 promoter region were deleted, transcriptional activity was decreased or increased in a cell-specific manner. However, little is known of the actual transcription factors that activate or repress the expression of GRK4.

c-Myc, a human homolog of the avian myelocytomatosis viral oncogene v-Myc, is involved in cancer progression in >50% of all cancers. With respect to hypertension, c-Myc has been implicated in hypertrophy and fibrosis of the heart and atherosclerosis but not yet in kidney-mediated hypertension. In Burkitt lymphoma cells overexpressing c-Myc as a result of chromosomal translocation, c-Myc was shown to bind to the promoter region of the GRK4. This finding suggests that c-Myc may be a transcription factor for GRK4. In addition, phorbol esters can transiently increase c-Myc mRNA expression in human myeloid leukemia cells and have been shown to increase promoter activity of GRK4 in transfected human embryonic kidney 293 cells. In cells other than renal proximal tubule, phorbol esters have also been shown to increase phospho-c-Myc, suggesting that phospho-c-Myc may activate GRK4 expression. Therefore, we hypothesized that c-Myc could negatively regulate the dopaminergic system by activating GRK4 expression via its promoter and may be involved in the pathogenesis of hypertension.

Materials and Methods

Cell Culture
We selected several cell lines from our collection of human RPTC isolated from normal tissue of nephrectomies under an institutional review board–approved protocol according to the Declaration of Helsinki, Title 45, Part 46, US Code of Federal Regulations. Primary cell lines were used for most of our experiments except in experiments involving the real-time intracellular cAMP biosensor. Immortalized RPTC lines were used in the cAMP assay because of low transfection efficiency of primary cells (>5 transfected cells per imaging field of view are needed for reproducible results). Previously characterized primary RPTC lines used were 19, a D1R/AC-uncoupled RPTC (uRPTC), and 22, a normally D1R/AC-coupled RPTC (nRPTC). RPTCs for the cAMP assay were immortalized with hTERT, using the methods documented by Kowollik et al and Wieser et al. Culture conditions are detailed in the online-only Data Supplement.

Drug Treatment
The cells were incubated with different combinations of the following drugs: phorbol 12-myristate 13-acetate (PMA; Sigma, 100 nmol/L), 10074-G5 (c-Myc inhibitor; Sigma, 30 μmol/L), losartan (angiotensin [Ang] II type 1 receptor antagonist; Sigma, 10 μmol/L), Ang II peptide (Sigma, 10 nmol/L), 3-amino-4-thio-butyl sulfonate (EC-33; 500 nmol/L), and a fenoldopam mesylate (FEN; Hospira Inc., 1 μmol/L). EC-33 was used to inhibit aminopeptidase A (which converts Ang II to Ang III). The duration of incubation and combinations of drugs are indicated in the figure legends.

Angiotensin II ELISA
The Ang II ELISA was performed using a commercial Ang II EIA Kit (Cayman Chemical). Details are provided in the online-only Data Supplement.

Angiotensin and CD13 Confocal Microscopy
Cells were fixed and permeabilized and stained with B93 anti-angiotensinogen antibody (made in house) and anti-CD13 antibody. See details in the online-only Data Supplement.

Determination of cAMP Accumulation
We quantified the accumulation of cAMP using a FRET biosensor as previously reported. Before stimulating cells with FEN, a specific agonist for D1R and D5R, we incubated the RPTCs for 3 hours with various drugs: Ang II (10 nmol/L), losartan (10 μmol/L), 10074-G5 (30 μmol/L), and PMA (100 nmol/L).

Chromatin Immunoprecipitation
Chromatin immunoprecipitation (ChiP) was performed using Human c-Myc ExactaChiP Chromatin IP Kit (R&D Systems) to isolate c-Myc-bound DNA promoters. DNA isolated from the assay was amplified with qPCR using primers specific to the GRK4 promoter: forward primer (5′–TCC CAA ACA ACG TTA CG–3′) and reverse primer (5′–CCT TCC GCC GTT ACT TTG AG–3′). See the online-only Data Supplement for details.

Immunoblotting
Proteins in cellular lysates were detected using the following primary antibodies: H70 rabbit polyclonal antibody to GRK4 (1:200 dilution, Santa Cruz sc-13079); rabbit mononclonal antibody to phospho-c-Myc (1:1000 dilution, Epitomics1203-1); and monoclonal mouse antibody to β-actin (1:30,000 dilution, Sigma). See the online-only Data Supplement for details.

GRK4 Confocal Microscopy and Plasma Membrane Localization
GRK4 localized on the plasma membrane was measured using RPTCs that were labeled with biotin, fixed and permeabilized, then stained with anti-GRK4 H70 antibody (1:200 dilution). See details in the online-only Data Supplement.

Results
We evaluated the possibility of c-Myc binding to the GRK4 promoter by performing a chromatin immunoprecipitation. Primary cultures of RPTCs were stimulated with either DMSO vehicle (VEH) control or PMA (100 nmol/L) for 3 hours. Because PMA is known to activate phospho-c-Myc through a PKC-mediated pathway, we hypothesized that PMA stimulation should increase the amount of c-Myc bound to the GRK4 promoter. After formaldehyde cross-linking, we purified the DNA bound to c-Myc using a biotinylated anti-c-Myc antibody provided in the kit and detected the presence of GRK4 gene promoter sequences using qPCR. As shown in Figure 1, c-Myc was bound to the GRK4 promoter under basal, prestimulatory conditions (VEH, 897.52 copies±170.29; n=3). After the 3-hour PMA treatment, c-Myc binding increased >3-fold (3036.09 copies±100.25; n=3; P<0.05).

We next determined whether stimulation with PMA could increase total cellular phospho-c-Myc and GRK4 protein expression. Primary RPTCs treated with PMA (100 nmol/L, 24 hours) had a significant increase in phospho-c-Myc protein levels (127.78%±17.74; n=6; P<0.001) compared with the vehicle control (Figure 2A).

Figure 2B shows that GRK4 protein expression also increased after 24-hour incubation with 100 nmol/L PMA (40.51%±5.71; n=12; P<0.001). Co-incubation of the c-Myc inhibitor, 10074-G5 (30 μmol/L, 24 hours), along with
Gildea et al
No vel c-Myc Transcriptional Regulation of GRK4

1023

PMA blocked this increase, restoring GRK4 expression to basal levels. 10074-G5 is a compound that prevents the binding of c-Myc to its partner, Max, so that it cannot initiate transcription of target genes; 10074-G5 by itself had no effect on GRK4 expression. These results suggest that phospho-c-Myc is in the regulatory pathway for increased GRK4 expression.

We then investigated whether or not the AT1R may act through phospho-c-Myc to regulate GRK4. We first wanted to verify whether an autocrine Ang II signal existed in our primary RPTCs by quantifying Ang II levels in RPTC supernatants. Primary cultures of RPTCs were incubated with DMSO, the vehicle which served as a control for EC-33 (aminopeptidase A inhibitor). Supernatants were collected 3 hours after stimulation (Figure 3). We used EC-33 to block the activity of aminopeptidase A and, therefore, the conversion of Ang II to Ang III. Under nonstimulatory conditions, cells treated with the DMSO vehicle control (VEH) showed an increase in the concentration of Ang II in the supernatants after 3 hours compared with serum-free media (SFM) that was not introduced to cells (0.95 pg/mL±0.036; n=6; P<0.001), suggesting Ang II production by human RPTCs in primary culture.

Cells treated with EC-33 for 3 hours also showed a marked increase in the levels of Ang II in the supernatant relative to SFM (1.40 pg/mL±0.050; n=6; P<0.001). In addition, cells treated with EC-33 for 3 hours also showed a further increase in the concentration of Ang II compared with VEH (0.45 pg/mL±0.050; n=6; P<0.001) indicating that EC-33 is inhibiting the conversion of Ang II to Ang III. To further substantiate our findings, we stained our RPTCs for angiotensinogen protein (Figure S2 in the online-only Data Supplement) and found that all of our cells contain angiotensinogen protein in varying amounts, which can then be used for production of autocrine Ang II. Our results are consistent with the findings of Kamiyama et al who found that in mouse and rat renal proximal tubule cells, the S1, S2, and S3 segments contain angiotensinogen protein.

Figure 1. Chromatin immunoprecipitation of c-Myc binding to the G protein–coupled receptor kinase 4 (GRK4) promoter. Cells were treated with phorbol 12-myristate 13-acetate (PMA; 100 nmol/L, 3 hours) or DMSO vehicle control (VEH). Degree of c-Myc binding was measured using qPCR of the GRK4 promoter. Copy number was greater for PMA-treated cells (3036.09 copies±100.25) than for VEH-treated cells (897.52 copies±170.29), indicating a higher degree of c-Myc binding in response to PMA (>3 fold increase over VEH, n=3, P<0.05).

After confirming the presence of Ang II production in primary RPTC culture, the cells were treated with Ang II (10 nmol/L), EC-33 (500 nmol/L), or a combination of both to determine whether the AT1R could be stimulated with exogenously added or endogenously produced Ang II to affect phospho-c-Myc and GRK4 levels. Cells treated with Ang II showed an increase in the protein levels of phospho-c-Myc (86.86%± 15.21; n=6; P<0.001) and GRK4 (40.51%±5.71, n=12, P<0.001) with PMA (100 nmol/L, 24 hours) and was blocked by the c-Myc inhibitor 10074-G5 (30 μmol/L, 24 hours). (+) control lane indicates lysates of human embryonic kidney (HEK) cells that have been transfected with a constitutively expressed GRK4. We used this lysate to confirm that the Western blot bands we analyzed were GRK4.

Figure 2. A, The expression of phospho-c-Myc was measured by Western blot and graphed as the ratio of cellular actin after treatment with phorbol 12-myristate 13-acetate (PMA; 100 nmol/L, 24 hours) or DMSO vehicle (VEH). Phospho-c-Myc expression was increased in response to PMA (127.78%±17.74, n=6, P<0.001). B, Western blot analysis showing G coupled–protein receptor kinase 4 (GRK4) expression was increased (40.51%±5.71, n=12, P<0.001) with PMA (100 nmol/L, 24 hours) and was blocked by the c-Myc inhibitor 10074-G5 (30 μmol/L, 24 hours). (+) control lane indicates lysates of human embryonic kidney (HEK) cells that have been transfected with a constitutively expressed GRK4. We used this lysate to confirm that the Western blot bands we analyzed were GRK4.
GRK4 levels were not altered by co-incubation of both EC-33 and 10074-G5. Treatment of primary RPTCs with EC-33 yielded the same effects as treatment with Ang II, suggesting that the effects were attributable to autocrine Ang II produced by RPTCs. We tested this hypothesis by inhibiting AT1R with losartan (an AT1R antagonist; 10 μmol/L, 24 hours). The expression of phospho-c-Myc (21.70%±4.10; n=6; P<0.05), as well as GRK4 (18.49%±3.83; n=15; P<0.01), was reduced by losartan (Figure 5A and 5B, respectively). These results further substantiated our findings suggesting human RPTCs are capable of producing autocrine Ang II that acts on AT1R to increase phospho-c-Myc and GRK4 expression.

Using confocal microscopy, we determined that changes in GRK4 expression were not associated with changes in GRK4 plasma membrane localization (Figure S1). Treatment of cells with Ang II, losartan, 10074-G5, Ang II plus losartan, and Ang II plus 10074-G5 did not alter the ratio of GRK4 at the plasma membrane to GRK4 in the cytoplasm.

Previously, we have shown that in uRPTC, the D1R is uncoupled to AC and cannot cause a cAMP increase in response to FEN. This uncoupling is, in part, attributable to higher GRK4 activity, which can phosphorylate and desensitize the D1R, preventing it from reacting with FEN. Inhibition of GRK4 by siRNA in uRPTC restored D1R coupling to AC and caused an increase in cAMP in response to FEN. We therefore wanted to determine whether we could recapitulate the D1R to AC in uRPTCs by inhibiting c-Myc to inhibit GRK4. Because we hypothesized that c-Myc positively regulates GRK4, we expected that an inhibition of c-Myc would also inhibit GRK4 expression, leading to a reversal of the uncoupled phenotype in uRPTCs. The cAMP response was measured using a cAMP FRET biosensor, ICUE3. Because we had to transiently transfect our cells with ICUE3 and primary cells do not have a high transfection efficiency or reproducibility, we used immortalized cells for this experiment.

Figure 6 shows lack of a cAMP accumulation on FEN (1 μmol/L, 15 minutes) stimulation in uRPTCs (i19). However, an increase in cAMP accumulation in response to FEN was observed for nRPTCs (i22; 21.06%±1.77; n=14; P=0.001), compared with the FEN-induced response in uRPTCs. uRPTCs were then pretreated with losartan or 10074-G5 before FEN stimulation. Either losartan (10 μmol/L, 3 hours) or 10074-G5 (30 μmol/L, 3 hours) pretreatment completely rescued the uncoupling of AC in uRPTCs, causing a significant increase in intracellular cAMP levels when compared with uRPTCs that were not pretreated. cAMP accumulation increased 21.04%±1.78 (n=28; P<0.001) for losartan-treated uRPTCs and 19.92%±0.97 (n=47; P<0.001) for 10074-G5-treated cells.

FEN-stimulated cAMP accumulation was reduced to 98.7% (losartan) and 97.9% (10074-G5) of nRPTC levels and not significantly different from FEN-stimulated cAMP accumulation in nRPTCs. These data suggest that inhibiting c-Myc may provide an alternative mechanism to inhibit GRK4 and recouple cells to AC. In contrast, pretreatment of nRPTCs with Ang II
or PMA induced the expression of the uncoupled phenotype. FEN did not induce cAMP accumulation with these treatments. Instead, cAMP accumulation decreased by 24.19%±3.75 in Ang II–treated cells (n=16; \(P<0.001\)) and 18.02%±1.98 in PMA-treated cells (n=14, \(P<0.001\)) when compared to nRPTC without pre-treatment. These data suggest that overactive c-Myc may lead to an increased GRK4 protein expression and uncoupling of D1R from AC.

**Discussion**

Stimulation of RPTCs with Ang II has shown c-Myc to be associated with increases in proinflammatory and profibrotic signaling cascades mediating renal injury in many kidney disease states, including hypertension. Loss of dopamine receptor function in the proximal tubule also leads to hypertension, including a mechanism involving an increased sensitivity of the RAS to angiotensin II.24,25 Gene variants in members of the RAS pathway, leading to increased activity, have been demonstrated to be associated with hypertension: AGT rs2004776 (angiotensinogen) and ACE rs4305 (angiotensin converting enzyme).26 Ang II has also been shown to activate c-Myc, which is a transcription factor that activates many proliferative cellular pathways including renal proximal tubule cells.27 Ang II stimulation has also been associated with inactivation of the dopaminergic system in the kidney.24 PMA has been shown to activate c-Myc and has even been shown to increase the transcript of GRK4.9 In humans, single nucleotide polymorphisms in GRK4 are associated with hyperphosphorylation and inactivation of the D1R.30,31 A negative feedback cycle may be initiated where lack of D1R signaling leads to loss of inhibition of AT1R, which in turn leads to an increase in c-Myc expression, GRK4 expression, and further inhibition of D1R. An interruption of this negative feedback mechanism could lead to re-establishing D1R function.

Our previous studies have shown that the coupling of D1R to AC is tied to sodium transport in RPTCs. We showed that addition of FEN generated a cAMP response and led to decreased sodium transport in nRPTCs.5 Because RPTCs mainly reabsorb molecules from the lumen of the nephron, a decrease in sodium transport correlates to decreased sodium reabsorption or increased sodium excretion. We have also previously shown that uRPTCs fail to accumulate cAMP and fail to decrease sodium transport in response to FEN, suggesting greater sodium reabsorption and less sodium excretion.5

Our present study links c-Myc transcriptional regulation of GRK4 to coupling and possibly sodium transport. Importantly, we show that D1R can be uncoupled from AC stimulation with agents that activate c-Myc and increase expression of GRK4. Furthermore, both the c-Myc–induced D1R-AC uncoupling and innate D1R-AC uncoupling in RPTCs can be reversed to normal D1R-AC coupling by c-Myc inhibition. These experiments using nRPTCs and uRPTCs have identified a novel...
and critical signaling molecule that connects aberrant Ang II activation to D1R-AC uncoupling via c-Myc transcriptional control of GRK4. Although it would seem that using c-Myc inhibitors therapeutically may be ill advised for treatment of hypertension, the use of a dominant negative c-Myc construct in mice showed that they could nearly completely inhibit c-Myc activity in all tissues for extended periods of time, with minimal and reversible side effects.32 The use of a c-Myc inhibitor could likely be used at very low dosages, as many drugs tend to accumulate in the proximal tubule of the kidney. Thus, the inhibition of the c-Myc pathway may lead to a novel therapeutic modulation of GRK4 transcription and potentially hypertension.

**Perspectives**

In summary, we describe a mechanism whereby Ang II stimulation leads to the uncoupling of the D1R from AC via the c-Myc transcriptional activation of GRK4 (a kinase that inactivates the D1R) in human RPTCs. We also show that primary human RPTCs in culture synthesize and secrete Ang II and that blocking the conversion of Ang II to Ang III by addition of the aminopeptidase A inhibitor EC-33 leads to an increase in Ang II concentration, c-Myc activation, GRK4 transcriptional activation, and D1R-AC uncoupling. Blockade of AT1R or inhibition of c-Myc prevents the Ang II-mediated uncoupling of D1R from AC. Addition of the diacylglycerol mimic PKC activator, PMA, also leads to stimulation of c-Myc and to D1R-AC uncoupling. This potentially deleterious phenotype is reversed by the addition of a c-Myc inhibitor. Inhibition of the AT1R or c-Myc inhibitor in RPTCs that are D1R-uncoupled leads to reversion of their uncoupled phenotype and enables the uRPTCs to regain the ability to stimulate AC on D1R stimulation.

**Acknowledgments**

We thank Helen E. McGrath for editorial assistance.

**Sources of Funding**

This work was supported by National Institutes of Health grants HL074940 and DK039308.

**Disclosures**

R.A.F. was awarded US Patent (no 6 660 474) on “GRK mutants in essential hypertension,” assigned to Hypogen Inc.

**References**

What Is New?

• A novel role for c-Myc regulating GRK4 expression and thus modulating the renin–angiotensin and dopaminergic systems
• Autocrine Ang II feedback signal exists in cultured renal proximal tubule cells
• Inhibition of c-Myc can restore the coupling defect in D1R AC uncoupled renal proximal tubule cells.

What Is Relevant?

• The blood pressure–lowering effect of AT1R blockers may be mediated by the downregulation of c-Myc and GRK4

Novelty and Significance

• Novel compounds that inhibit c-Myc or GRK4 could be used to treat hypertension without the negative effects of general AT1R blockade

Summary

This article is the first to show transcriptional regulation of GRK4 by c-Myc, thus placing it as a key regulator of the renal renin–angiotensin and dopaminergic systems.
A Novel Role for c-Myc in G Protein–Coupled Receptor Kinase 4 (GRK4) Transcriptional Regulation in Human Kidney Proximal Tubule Cells
John J. Gildea, Hanh T. Tran, Robert E. Van Sciver, Dora Bigler Wang, Julia M. Carlson and Robin A. Felder

Hypertension. 2013;61:1021-1027; originally published online March 18, 2013;
doi: 10.1161/HYPERTENSIONAHA.111.00321

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://hyper.ahajournals.org/content/61/5/1021

Data Supplement (unedited) at:
http://hyper.ahajournals.org/content/suppl/2013/03/18/HYPERTENSIONAHA.111.00321.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Hypertension can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Hypertension is online at:
http://hyper.ahajournals.org//subscriptions/
ONLINE SUPPLEMENT

A Novel Role for c-Myc in G Protein-Coupled Receptor Kinase 4 (GRK4) Transcriptional Regulation in Human Kidney Proximal Tubule Cells

John J. Gildea¹, Hanh T. Tran¹, Robert E. Van Sciver, Dora Bigler Wang, Julia M. Carlson, , and Robin A. Felder

¹Contributed equally to this work

Department of Pathology, The University of Virginia Health System, Charlottesville, VA 22908

*Running title: Novel c-Myc Transcriptional Regulation of GRK4

To whom correspondence should be addressed: Dr. John J. Gildea, The University of Virginia, P.O. Box 801400, Charlottesville, VA, USA, Tel.: (434) 924-9463, Fax: (434) 924-5718, E-mail: jjg5b@virginia.edu
Expanded Materials and Methods

Cell culture
The RPTCs were grown in humidified conditions at 37°C, 95% air, and 5% CO2, and in DMEM-12 medium (Invitrogen) supplemented with 2% fetal calf serum (FCS), 5 µg/mL plasmocin (InvivoGen), 10 ng/mL epidermal growth factor (Sigma), 36 ng/mL dexamethasone (Sigma), 2 ng/mL triiodothyronine (Sigma), 1x insulin transferrin selenium (Invitrogen), 1x penicillin/streptomycin (Invitrogen), and 0.2mg/mL G418 sulfate (EMD Chemicals). G418 sulfate was used as a selection agent only in the culture medium for immortalized cells.

Chromatin Immunoprecipitation
The Human c-Myc ExactaChIP Chromatin IP Kit (R&D Systems) was used for isolation of c-Myc bound promoters. The assay was carried out following the protocol provided in the kit. Equal numbers of cells were plated before formaldehyde crosslinking. DNA promoters isolated from ChIP samples were amplified by qPCR. The PCR reaction consisted of 25 µL of 2x SYBR Green reaction mix (Bio-Rad), 2.5 µL of 10 µmol/L forward primer (5’ – TCC CAA GGA ACA AGG TTA CG – 3’), 2.5 µL of 10 µmol/L reverse primer (5’ – CCT TCC GCG TTT ACT TTG AG – 3’), and 20 µL of ChIP DNA sample. Touchdown PCR was performed with the following cycle conditions: first denaturation at 95°C for 5 min; next ten cycles, denaturation at 94°C for 30 seconds; annealing temperature started at 61°C for 30 seconds and decreased 1°C successively for 10 cycles; extension at 68°C; and the next 55 cycles, denaturation at 95°C, annealing temperature at 51°C, and extension at 68°C. Copy number was determined using a standard curve with 6 log linear controls. The efficiency of the standard curve was 95.8%. The p21 promoter (primers provided in the kit) was used as a positive control for the ChIP assay.

Immunoblotting
Cells were lysed with Mammalian Protein Extraction Reagent (Thermo Scientific), supplemented with Halt Protease and Phosphatase Inhibitor (Thermo Scientific). The total cellular lysate was then centrifuged 15,000 rpm for 10 min. Protein concentrations were determined by the BCA protein assay kit (Pierce Biotechnology), using BSA as a standard. The proteins were separated by SDS-polyacrylamide gel electrophoresis using a precast gel (Mini Gel, Bio-Rad TGX 4-20%), and then transferred onto 0.2 µm nitrocellulose membranes (Bio-Rad). Membranes were blocked with Odyssey blocking buffer (LiCor) before being incubated with the primary antibodies. After 3 washes, the membranes were incubated with secondary antibodies, IRDye 680RD Donkey anti-Mouse IgG (1:15,000 dilution, LiCor 926-68072) and IRDye 800CW Donkey anti-Rabbit (1:15,000 dilution, LiCor926-32213). The immunoblots were washed another 3 times before imaging.

Angiotensin II ELISA
Identical cell numbers of primary human RPTCs were plated and cultured in 12-well plates until 100% confluence. Cells were cultured using media described above. Cells were serum starved for 24 hours before treatment with EC-33 for 3 hours.
Angiotensinogen and CD13 Confocal Microscopy
Cells were serum starved for 24 hours, then fixed and permeabilized with 4% paraformaldehyde and 1% Triton-X for 5 minutes. The reaction was quenched for 10 minutes with 100mM Tris-HCl, and then blocked for 1 hour with LiCor blocking buffer. Angiotensinogen antibody B93 (1:100 dilution). The anti-human B93 angiotensinogen antibody was generated by custom antibody service using full length human angiotensinogen as an antigen. Protein A purified antibody was used without further purification. Specificity of antibody was checked by ELISA and lack of cross-reactivity with Angiotensin 1,2,3 and 4 was noted. CD13 (1µg/ml) (clone 452, Dr. Meenhard Herlyn, Wistar Institute of Anatomy and Biology, Philadelphia, PA) was added for 1 hour. Cells were washed 3x with PBS-T before addition of secondary antibodies, Alexa Fluor 488 Donkey Anti-Rabbit (1:500 dilution) and Alexa Fluor 647 Donkey Anti-Mouse (1:500 dilution).

Images were obtained with Olympus IX81 spinning disk confocal microscope using a 20X water immersion objective. Three images were taken for each capture, one through the FITC filter to look at angiotensinogen, another through the Cy5 filter to look at CD13, and a final DIC image. We observed substantial variation in staining intensity of both CD13 and AGT suggesting that the RPTC cultures may contain S1, S2 and S3 cell types, but all cells had detectable signal well above background for both antigens.

GRK4 Confocal Microscopy and Plasma Membrane Localization
Cells were serum starved for 24 hours before incubation for 3 hours with either DMSO vehicle control, Ang II, 10074-G5, LOS, Ang II + 10074-G5, or Ang II + LOS (concentrations are as indicated previously). Cells were washed 3 times with PBS containing Calcium Chloride and Magnesium Chloride to remove existing FBS. 300µM of biotin was added to cells on ice to label the cell surface. Cells were then fixed and permeabilized with 4% paraformaldehyde and 1% Triton-X for 5 minutes. The reaction was quenched with 100mM Tris-HCl for 10 minutes before blocking with LiCor blocking buffer for 1 hour. H70 primary rabbit polyclonal antibody (1:200 dilution) was added for 1 hour after the blocking step. Cells were washed 3 times before addition of secondary antibodies Alexa Fluor 647 Goat Anti-Rabbit (1:500 dilution, Invitrogen) and Alexa Fluor 750 Streptavidin (1:2000 dilution, Invitrogen). All images were obtained with an Olympus IX81 spinning disk confocal microscope using a 60X water immersion objective. Three images were taken per cell, one through the Cy5 filter to look at GRK4, another through the Cy7 filter to look at the biotin-labeled surface, and a final DIC image to differentiate the nucleus from the cytoplasm. Images were taken with the same exact settings, and quantified using ImageJ version 1.47g. To quantify, both Cy5 and Cy7 images were opened in two separate windows and the macro “Synchronize Windows” was run. This macro allows selections to interchangeably measure intensities between the two windows without having to draw a new selection. A 5µ thick shape was traced along the membrane surface using the Cy7-acquired image, which depicted biotin labeled cell surface. The average intensity of the Cy5-acquired image was then measured for the GRK4 on the surface. The same shape was then moved 10µ toward the cell interior to measure the average intensity of GRK4 in the cytoplasm. The plasma membrane to cytoplasm ratio was the average surface intensity divided by the average
cytoplasmic intensity. Background intensity was measured from a cell free region and subtracted before calculating the intensity ratio. The DIC image was used to visualize where the nucleus was so that the shape did not measure nuclear GRK4 when it was moved toward the interior of the cell.

**Figure S1.** Confocal Microscopy of Surface to Cytoplasmic GRK4 ratio in RPTCs. Incubation with Ang II (10nmol/L), 10074-G5 (30µmol/L), LOS (10µmol/L), Ang II + 10074-G5, and Ang II + LOS for 3 hours did not change the localization of GRK4 to the surface of the plasma membrane.
Figure S2. Confocal microscopy of angiotensinogen protein (A), CD13 (B), DIC (C) and composite (D). All RPTCs were verified by the presence of CD13 stain for angiotensinogen.