0026-895X/08/7304-1037–1043$20.00
MOLECULAR PHARMACOLOGY
Vol. 73, No. 4
44214/3324960
Mol Pharmacol 73:1037–1043, 2008
Printed in U.S.A.

ACCELERATED COMMUNICATION

The RGS2 Gene Product from a Candidate Hypertension Allele Shows Decreased Plasma Membrane Association and Inhibition of G_q [S]

Steven Gu, Sam Tirgari, and Scott P. Heximer

Department of Physiology, Heart and Stroke/Richard Lewar Centre of Excellence in Cardiovascular Research, University of Toronto, Toronto, Ontario, Canada

Received December 11, 2007; accepted January 23, 2008

ABSTRACT

Hypertension is a leading risk factor for the development of cardiovascular disease. Data from human and animal studies suggest that RGS2, a potent inhibitor of G_q signaling, is important for blood pressure regulation. Several *RGS2* mutations in the Japanese population have been found to be associated with hypertension. The product of one of these alleles, R44H, is mutated within the amino terminal amphipathic α-helix domain, the region responsible for plasma membrane-targeting. The functional consequence of this mutation and its potential link to the development of hypertension, however, are not known. In this study, we showed that R44H was a weaker inhibitor of receptor-mediated G_q signaling than wild-type RGS2. Confocal microscopy revealed that YFP-tagged R44H bound to the plasma membrane less efficiently than wild-type RGS2. R44 is one of the basic residues positioned to stabilize lipid bilayer interaction of the RGS2 amphipathic helix domain. Tryptophan fluorescence and circular dichroism studies of this domain showed that the R44H mutation prevented proper entrenchment of hydrophobic residues into the lipid bilayer without disrupting helix-forming capacity. Together, these data suggest that decreasing the side-chain length and flexibility at R44 prevented proper lipid bilayer association and function of RGS2. Finally, the R44H protein did not behave as a dominant-negative interfering mutant. Thus, our data are consistent with the notion that a R44H missense mutation in human RGS2 produces a hypomorphic allele that may lead to altered receptor-mediated G_q inhibition and contribute to the development of hypertension in affected subjects.

Heterotrimeric G-proteins are important mediators of cardiovascular cell signaling and physiology. G-protein-coupled receptors (GPCRs) mediate the biologic function of neurotransmitters and hormones including catecholamines, angiotensin II (Ang II), and endothelin-1 (ET-1) to control a number of cardiovascular parameters, such as vascular resistance, cardiac output, and plasma volume. It is therefore important to maintain proper regulation and coordination of G-protein signaling to ensure proper blood pressure homeostasis. Regulators of G-protein signaling (RGS) proteins are one family of more than 35 proteins that serve this function through their activity as GTPase activating proteins for G-protein α subunits. Within the RGS superfamily, RGS2 is comparatively well suited to regulate signaling events required for blood pressure homeostasis. RGS2 is a selective and efficient inhibitor of G_q, the primary mediator of vasoconstrictive stimuli including norepinephrine, Ang II, and ET-1. RGS2 also inhibits some types of adenylyl cyclase (Sinnarajah et al., 2001; Salim et al., 2003), a pathway important for dopamine-mediated regulation of blood pressure in the kidneys.

Several studies from our laboratory and others have dem-

This work was supported by grants from the Ontario Heart and Stroke Foundation (NA 5291), Ontario Graduate Scholarships in Science and Technology (S.G.), and the Canada Research Chairs Program of the Canadian Institute for Health Research (S.P.H.).
Article, publication date, and citation information can be found at http://molpharm.aspetjournals.org.
doi:10.1124/mol.107.044214.
[S] The online version of this article (available at http://molpharm.aspetjournals.org) contains supplemental material.

ABBREVIATIONS: GPCR, G-protein-coupled receptor; Ang II, angiotensin II; ET-1, endothelin-1; RGS, Regulators of G-protein signaling; NTD, amino-terminal domain; YFP; yellow fluorescent protein; HEK, human embryonic kidney; PBS, phosphate-buffered saline; TFE, trifluoroethanol; PM, plasma membrane; FR, fluorescent ratio.

onstrated the potential role of RGS2 in blood pressure regulation. The RGS2 knockout animal is hypertensive and shows increased sensitivity and prolonged responsiveness to vasoconstrictor agonists, such as Ang II and ET-1 (Heximer et al., 2003; Gross et al., 2005; Obst et al., 2006; Hercule et al., 2007). Moreover, RGS2 seems to be an important mediator of nitric oxide-dependent vasodilatory signaling, particularly at the level of attenuating GPCR-mediated calcium responses in vascular smooth muscle cells (Tang et al., 2003; Sun et al., 2005; Obst et al., 2006).

Yang et al. (2005) also identified a large number of mutations and single nucleotide polymorphisms within the *RGS2* locus of a Japanese cohort that they implicate as candidate alleles in the development of hypertension. Indeed, Bodenstein et al. (2007) recently showed that one such mutation, Q2L, reduced the function of RGS2 through the ability of a leucine residue at position 2 to destabilize the protein. Among other mutations identified, a heterozygous missense mutation, R44H, with a predicted frequency of 0.133% in the Japanese general population (Yang et al., 2005), was found in seven persons, six of whom were hypertensive. Note that this mutation changes an arginine to a histidine (R44H) within the amino-terminal (NTD) amphipathic helix domain of RGS2. In light of our previous work describing the importance of this domain for RGS2 function and subcellular localization (Heximer et al., 2001; Gu et al., 2007), we sought to determine whether this single amino acid change was sufficient to attenuate RGS2 function in a manner that could provide some mechanistic explanation for its purported association with high blood pressure in affected persons. Specifically, we set out to determine whether the R44H mutation in RGS2 affected its ability to inhibit receptor-mediated G_q signaling.

Materials and Methods

Materials. The pEYFP-C1 plasmid (Clontech, Mountain View, CA) was used for expression of all RGS protein constructs. The polyclonal anti-GFP antibody (Living Colors A.v. Peptide Antibody) was also from Clontech. Fura2-AM and all tissue culture media and transfection reagents were from Invitrogen (Burlington, ON, Canada). HEK293 cells stably expressing the M1 muscarinic receptor (M1-HEK) were a kind gift from P. Burgon (University of Ottawa Heart Institute, Ottawa, ON, Canada) and E. Peralta (Harvard University, Cambridge, MA). Brain lipids were from Avanti Polar Lipids (Alabaster, AL). The peptides used in the spectroscopic studies were acquired from the Advanced Protein Technology Centre (APTC) at The Hospital for Sick Children (Toronto, ON, Canada). Unless otherwise stated, all other reagents and chemicals were from Sigma-Aldrich (Oakville, ON, Canada).

Cell Culture. M1-HEK cells were grown in Dulbecco's modified Eagle's medium/Ham's F12 medium (1:1), supplemented with 10% (v/v) heat-inactivated fetal calf serum, 2 mM glutamine, 10 μg/ml streptomycin, 100 units/ml penicillin, and 0.5 mg/ml G-418 (Geneticin) at 37°C in a humidified atmosphere with 5% CO_2. Cells were transiently transfected using FuGENE 6 (Roche, Mississauga, ON, Canada) according to manufacturer's instructions.

RGS2 Expression Constructs. RGS2-YFP expression plasmids were generated in the Living Colors pEYFP-C1 vector (Clontech) as described previously (Heximer et al., 2001). Robust expression was ensured by inclusion of an optimized translation initiation signal (Kozak, 1994) in the context of the first methionine codon (GCCACCATGGCG). We have previously shown that inclusion of an optimized translation start site or a carboxyl terminal YFP fusion do not significantly alter the localization or function of the RGS2 protein (Gu et al., 2008). The R44H point mutation was introduced by the QuikChange site-directed mutagenesis system (Stratagene, La Jolla, CA). Mutagenesis primers were designed to simultaneously introduce the R44H mutation and an AflII restriction endonuclease site for screening purposes: forward, 5′AAAGATTGGAAGACCCACTTAAGCTACTTCTTACAA 3′; reverse, 5′TTGTAAGAAGTAGCTTAAGTGGGTCTTCCAATCTTT 3′. All plasmid constructs were purified using the Endofree Maxi kit (QIAGEN, Mississauga, Ontario, Canada) and verified by sequencing of the complete protein-coding region.

Intracellular Calcium Imaging. The function of RGS2 as an inhibitor of G_q-coupled signaling was studied by ratiometric calcium imaging in cells selected for similar RGS2-YFP protein expression levels as described previously (Gu et al., 2007). In brief, M1-HEK cells were seeded on poly-L-lysine-coated number 1 glass coverslips and transiently transfected with the indicated constructs using FuGENE 6. Twenty-four hours after transfection, cells were loaded with fura-2AM in Ca1 buffer (11 mM glucose, 130 mM NaCl, 4.8 mM KCl, 1.2 mM $MgCl_2$, 17 mM HEPES, and 1 mM $CaCl_2$, pH 7.3), and coverslips were washed and loaded into a modified Leyden chamber. Cells were perfused at 37°C with Ca1 buffer for 5 min. Baseline fluorescent ratio (FR) values were collected for 5 to 10 s before the perfusate was changed to contain 200 μM carbachol. Peak relative percent FR increase above baseline = [(peak stimulated FR/unstimulated baseline FR) − 1] × 100%. FR values were converted to calcium concentrations using a standard curve generated from standardized calcium solutions (Invitrogen) as described previously (Gu et al., 2007). Note that changes in calcium concentrations within normal physiologic limits (80–500 nM) varied in a nonlinear fashion with changes in FR values, producing large changes in intracellular calcium levels for a comparatively small change in FR.

Confocal Microscopy. Polylysine-coated 25-mm circular number 1 glass coverslips containing RGS-YFP transfected cells were mounted in a modified Leyden chamber containing Ca1 buffer. Confocal microscopy was performed on live cells at 37°C using an Olympus FluoView 1000 laser-scanning confocal microscope. Images represent single planes on the basal side of the cell obtained with a 60× oil objective and processed after capture with Adobe Photoshop 7.0 (Adobe Systems, Mountain View, CA). Shown are pictures representative of at least 50 live cells. Where indicated, densitometric quantitation of protein expression was performed using the gel analysis function of the ImageJ 1.32j software package (http://rsb.info.nih.gov/ij/).

Tryptophan Fluorimetry. Tryptophan fluorescence spectra of helix domain peptides were measured using an AVIV Ratio Spectrofluorometer ATF105 (Lakewood, NJ). RGS2 NTD helix domain peptides were diluted to 0.2 μM in PBS. Extruded unilaminar liposomes (Encapsula NanoSciences, Nashville, TN) were made from bovine brain lipids (Avanti Polar Lipids) and were diluted in PBS. Liposomes were added to peptide solution for 5 min before measurement to allow consistent lipid association. For each lipid concentration, fluorescence emission spectra after 295 nm excitation were recorded at 2 nm steps from 310 to 400 nm. Similarly generated liposome and PBS alone control emission spectra were subtracted from peptide spectra to account for nonpeptide, background fluorescence emission. In experiments involving trifluoroethanol (TFE), solutions with peptides were thoroughly mixed and incubated for 5 min before measurement.

Circular Dichroism. Peptide secondary structure was assessed using an AVIV Circular Dichroism Spectrometer model 202. Wild-type RGS2, L45D, and R44H mutant peptides were analyzed with or without liposomes. Unilaminar liposomes for CD studies were made as described previously (Bernstein et al., 2000). In brief, a 3:2 solution of dipalmitoylphosphatidylcholine/dipalmitoylphosphatidylglycerol (Avanti Polar Lipids) in chloroform was dried under nitrogen and resuspended in PBS. Lipids in solution were sonicated for 5 min with 20-s pulses and chilled on ice. Liposomes were made fresh for

each experiment. Peptides (7–21 μM) were diluted in PBS with and without lipids or TFE, and spectra were measured from 190 to 260 nm in 1-nm increments averaged over 4 s after a 5-min incubation period. The spectra of lipids and PBS alone were subtracted from sample measurements to account for nonpeptide, background fluorescence emission.

Statistical Analysis. Calcium imaging data represent the averages of >30 cells on one experimental day. All data are representative of at least 3 independent experiments carried out on separate days. Pair wise comparisons between groups were made using the unpaired student's *t* test. A p value of <0.05 was deemed significant.

Results

The R44H Mutation in the Amphipathic α-Helix Domain of RGS2 Results in Reduced Function and PM Localization. Members of the R4/B subfamily of RGS proteins contain an NTD amphipathic helix that is required for both binding to anionic phospholipids on the inner leaflet of the PM and formation of a stable interaction via entrenchment of hydrophobic residues into the lipid core of the bilayer. We showed recently that unique features of the RGS2 α-helical domain mediate its constitutive association with the PM and increased relative function as an inhibitor of G_q signaling (Gu et al., 2007). A single nucleotide polymorphism (Fig. 1a) that results in a missense mutation (R44H) was found to be associated with hypertension in the Japanese population (Yang et al., 2005). The position of the mutant histidine residue relative to the hydrophobic core of the amphipathic helix (Fig. 1b, arrows) suggests that the mutation may have an effect on the normal function of the RGS2 amphipathic helix. Accordingly, we hypothesize that replacement of a flexible strongly basic arginine residue with a less flexible weakly basic histidine might change the amphipathic nature of the α-helical NTD to result in less RGS2 function as a G_q inhibitor.

We have reported a fura-2 ratiometric calcium signaling assay for studying relative RGS-YFP protein inhibition of G_q-dependent signaling (Gu et al., 2007). To examine the effects of the R44H mutation on RGS2 function, we used site-directed mutagenesis to generate RGS2(R44H)-YFP. This construct produces a single protein species on anti-GFP immunoblots and shows levels of expression and SDS-polyacrylamide gel electrophoresis migration patterns similar to those of wild-type RGS2-YFP (Fig. 2a). To assess relative G_q inhibitory function, RGS2 and R44H were transfected into HEK293 cells stably expressing the M1 muscarinic receptor (M1-HEK cells). Changes in intracellular calcium concentrations in response to the M1 muscarinic receptor agonist carbachol were measured by ratiometric imaging of fura-2-loaded M1-HEK cells. Figure 2b shows the kinetics of a typical carbachol-induced response of M1-HEK cells transfected with YFP, RGS2, and R44H. Note that cells transfected with RGS2 show a blunted response to the carbachol stimulus compared with cells transfected with YFP or R44H. The average percent increase of the fluorescence ratio from baseline to peak value is plotted in Fig. 2c. RGS2 transfected cells showed an average of 57% inhibition compared with YFP controls whereas the R44H mutant exhibited only 20% inhibition.

Work from our group and others shows that RGS2 is localized constitutively to the PM, nucleoplasm, and nucleoli with relatively little compartmentalization in the cytosol (Heximer et al., 2001). Because potent G_q-inhibitory activity of RGS2 is dependent on its ability to associate with the PM (Gu et al., 2007), and the R44H mutation

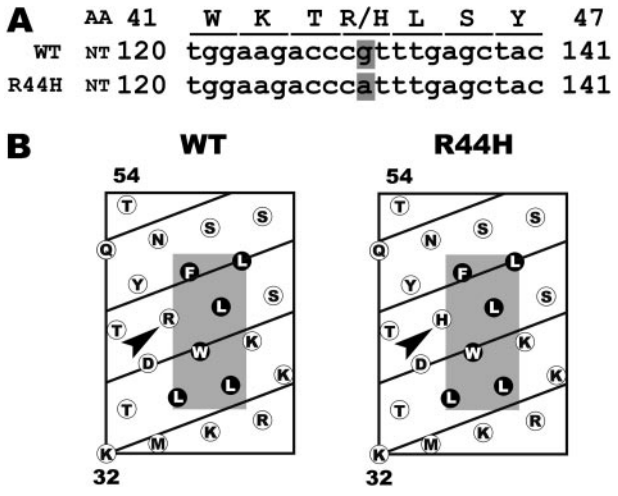

Fig. 1. Single nucleotide polymorphism in *RGS2* results in missense mutation (R44H). A, alignment of WT and R44H mutant DNA sequence shows a single nucleotide change (guanine-adenine) resulting in an arginine-histidine mutation. B, helical net representation of the RGS2 NTD shows the orientation of the mutated residue in R44H. Hydrophobic residues are black and the hydrophobic face of the helix is highlighted in gray. Arrows denote the amino acids affected by the R44H mutation

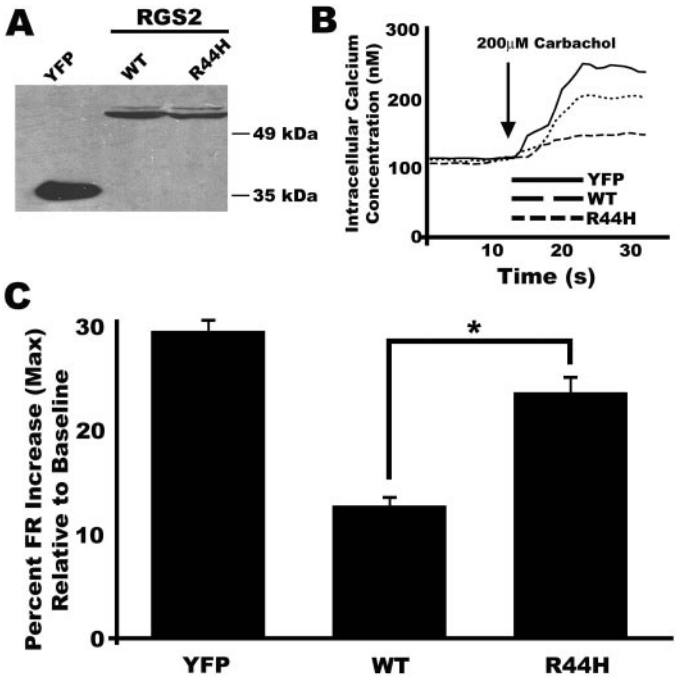

Fig. 2. R44H mutation shows decreased G_q inhibitory function. A, YFP, RGS2, and R44H were transiently transfected into M1-HEK cells and harvested after 24 h. RGS protein expression was evaluated by SDS polyacrylamide gel electrophoresis and immunoblotting using a polyclonal anti-GFP antibody. B, M1-HEK cells were transfected with YFP-tagged constructs as indicated. Cells were loaded with fura-2AM for intracellular calcium measurement as described under *Materials and Methods*. Cells with similar levels of YFP fluorescence (3500 < relative YFP fluorescence units <12,000) were selected for analysis. After recording baseline levels for 10 s, 200 μM carbachol was added to the perfusate (arrow). Shown are mean kinetic traces of intracellular calcium concentration for cells ($n > 30$). C, YFP-transfected M1-HEK cells were processed and selected for calcium imaging as described in B above. All experiments show mean percentage fluorescent ratio (FR) increase above baseline ± S.E.M. for $n > 50$ cells. All data are representative of at least three independent experiments. *, $p < 0.001$.

occurs within the membrane targeting domain, we next tested whether this mutation affected subcellular localization. Indeed, PM association of R44H is greatly reduced compared with that of wild-type RGS2 (Fig. 3a), whereas nuclear and nucleolar localization seem unaffected. Densitometric analysis of the confocal images confirms a markedly higher ratio of PM versus cytosol localization for the wild-type protein (Fig. 3b). However, R44H may retain some weak membrane-binding capacity, because it is sometimes detectable in small amounts within the membrane interface of cell-cell junctions (Fig. 3b, arrows).

The Amphipathic Helix Domain of the R44H Mutant Does Not Stably Associate with Purified Liposomes. The NTD amphipathic helix is required for interaction of RGS2 with negatively charged phospholipids on the inner leaflet of the plasma membrane. Mutations that disrupt amphipathicity are predicted to disrupt its localization and function. Because the R44H mutation specifically changes an arginine residue adjacent to the hydrophobic face of the RGS2 helix to histidine, we hypothesized that this mutation would result in a reduction in RGS2-lipid bilayer affinity. Tryptophan spectroscopy has been used to study the interaction of tryptophan-containing amphipathic α-helical peptides with lipid bilayers (Burstein et al., 1973). This assay measures changes in the fluorescence emission spectrum of a tryptophan residue on the hydrophobic face of an amphipathic helix. As the local environment of the tryptophan changes from polar (solution) to hydrophobic (lipids), there is a blue shift in the spectrum maxima (Burstein et al., 1973). Spectra were corrected for background as described previously. Noncorrected spectra are provided in Supplemental Fig. 1. As seen in Fig. 4, corrected spectra from wild-type RGS2 shows the maxima shift with increasing concentrations of lipids, whereas the R44H spectra is unaffected by the presence of lipids. Likewise, L45D, a mutant peptide previously shown not to interact with the PM or form an α-helix in the presence of lipids (Heximer et al., 2001) shows no change in its tryptophan properties in this assay. One possible explanation for the differences in tryptophan spectroscopy of the R44H mutant is that the long hydrophobic side chain of arginine 44 could contribute to an increased local hydrophobic environment of tryptophan when the peptide is in a helical formation. To address this possibility, we tested whether the tryptophan spectrum was altered by TFE, a helix-promoting solvent, and in the absence of liposomes. As shown in Supplemental Fig. 2, the addition of TFE results in a marked increase in helix formation of the wild-type RGS2 peptide without inducing a blue shift in the tryptophan spectra. Although we cannot exclude the possibility that the Arg44 residue contributes to the hydrophobic environment of the nearby Trp41, such an interaction cannot explain the profound blue-shift observed in the presence of liposomes. Accordingly, these data suggest that the spectral

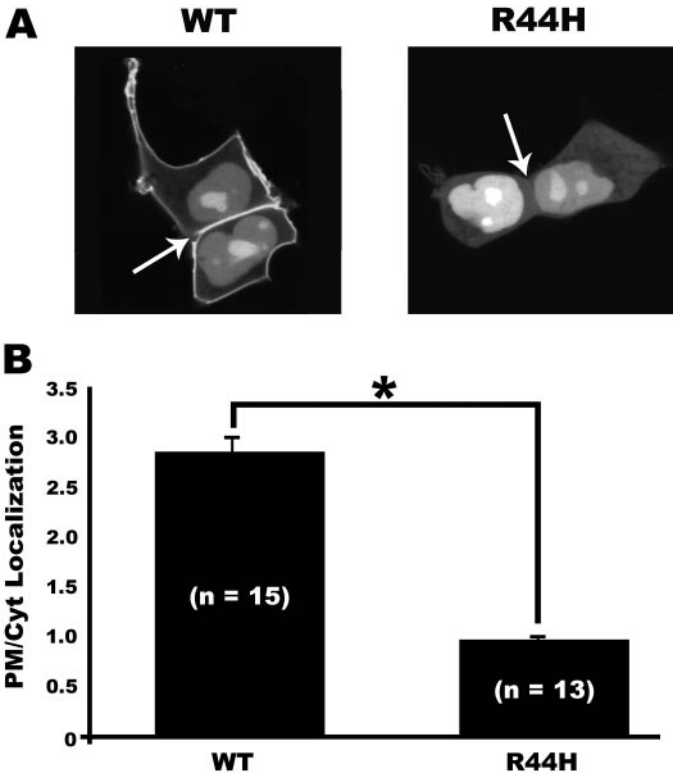

Fig. 3. R44H is not efficiently targeted to the PM. A, M1-HEK cells were transiently transfected with the indicated constructs and imaged 24 h after transfection with an Fluoview 1000 laser scanning microscope (Olympus, Tokyo, Japan). Shown are representative pictures of >50 live cells. B, the ratio of RGS2-YFP signal between the nucleus plasma membrane was analyzed by densitometry using ImageJ software. Shown are mean ratios of $n > 10$ cells ± S.E.M. *, $p < 0.05$.

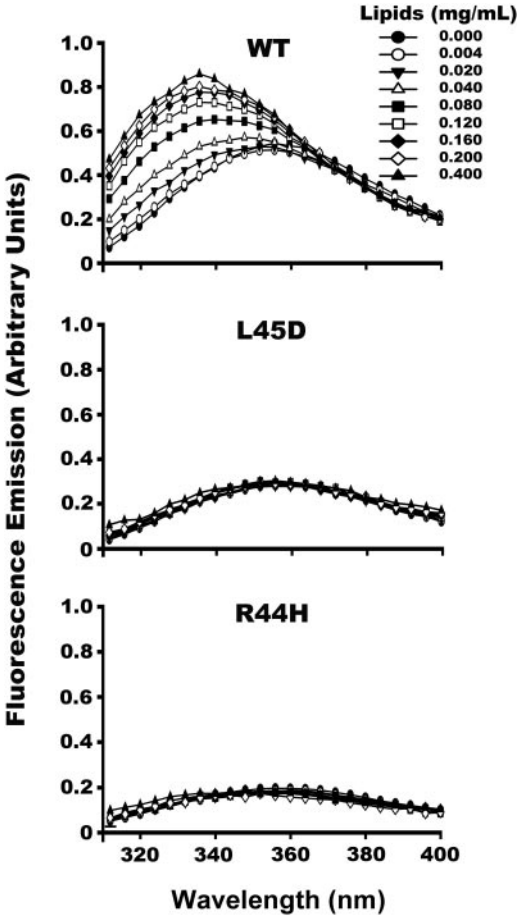

Fig. 4. R44H mutation results in decreased penetration of the hydrophobic face of the RGS2 helix into the lipid bilayer core. RGS2 helix domain peptides corresponding to residues 34 to 57 from WT, R44H, and L45D were incubated with increasing amounts of purified unilaminar liposomes from brain lipid extracts. Tryptophan fluorescence emission spectra of Trp41 on the hydrophobic face of the RGS2 amphipathic helix were collected with lipid concentrations ranging from 0 to 0.4 mg/ml. Shown is a representative experiment from three independent experiments.

changes of tryptophan observed in the presence of liposomes are due to its insertion into the hydrophobic core of the lipid bilayer.

R44H Mutation Does Not Disrupt the Helix Forming Capability of the RGS2 NTD Helix. The aspartic acid in the previously published mutant L45D is a known helix breaker and thus the L45D mutant does not associate with the PM because of its inability to form a helix. To test whether the R44H mutant's inability to associate with the PM is due to the same mechanism, we compared the secondary structure of RGS2, R44H, and L45D peptides in the presence and absence of anionic liposomes using circular dichroism. As expected, in the absence of lipids, all of the peptides show a disordered random coil CD signature. In the presence of anionic lipids, however, both RGS2 and R44H mutant peptides show characteristic α-helix formation with a molar ellipticity minima at 222 nm (Fig. 5), consistent with the non–helix-breaking nature of histidine. In contrast, CD spectra of L45D show a random coil spectrum even in the presence of liposomes (Heximer et al., 2001). Noncorrected, raw CD spectra are shown in Supplemental Fig. 3. Together, these data indicate that the R44H NTD, despite retaining the ability to form a proper α-helix, was unable to form a stable interaction with the lipid bilayer.

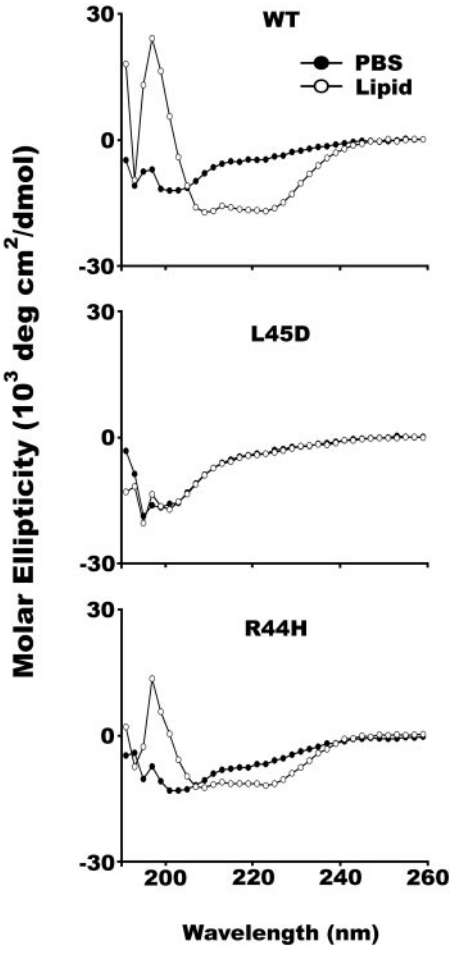

Fig. 5. R44H mutation shows normal helix forming potential in the presence of negatively charged phospholipids. Peptides corresponding to residues 34 to 57 in PBS were incubated either with or without lipids and CD spectra were collected as described under *Materials and Methods*. ●, control spectrum of peptides in PBS solution; ○, spectrum of peptide solution containing 1.4 mM lipid liposomes. Shown is a representative experiment from three independent experiments.

The R44H Mutant Does Not Behave As a Dominant-Negative Protein. Previously, it has been shown that the NTD of RGS2 can also act in a dominant-negative fashion to inhibit the function of wild-type (WT) RGS2 (Tang et al., 2003). Because the R44H allele was only found as a heterozygous mutation, we tested whether this mutant protein possessed a dominant interfering activity that would exaggerate its loss of function effects through its ability to interfere with the wild-type protein. We cotransfected increasing amounts of a triple-myc-tagged R44 clone with wild-type RGS2-YFP. The myc-tagged construct, RGS2(R44H)-myc, expressed a single protein band consistent with the predicted size (Fig. 6, inset). At no R44H:RGS2 ratio tested was there a change in RGS2-YFP localization or function (Fig. 6). Together, these data suggest that R44H mutant is functionally deficient but does not behave in a dominant interfering manner.

Discussion

Hypertension is a prevalent and growing health concern in industrialized countries. This condition increases the risk of stroke, myocardial infarction, as well as heart and renal failure, making it an important clinical research target. In a large number of hypertensive patients, the underlying etiology is unknown, and patients are often unresponsive to current therapeutic strategies. Thus, it is important to develop an improved understanding of the mechanisms underlying the development of hypertension so that new therapeutic approaches can be developed.

Our previous work has implicated the regulator of G-protein signaling, RGS2, as an important protein in the maintenance of normal blood pressure levels. Although the ubiquitous expression pattern of RGS2 has confounded efforts to

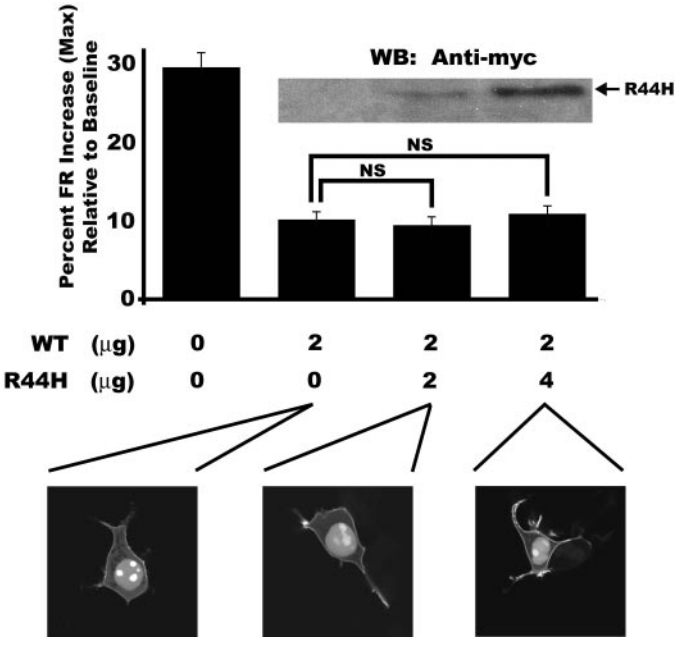

Fig. 6. R44H mutant does not behave as a dominant interfering mutation. M1-HEK cells were cotransfected with both RGS2-YFP and R44H-myc expression plasmid in the various microgram-to-microgram ratios shown. The relative activity of RGS2 to inhibit G_q signaling was measured using calcium imaging technique as described in the legend to Fig. 2. Confocal microscopy on similarly treated cells was used to assess the effect of R44H on RGS2-YFP localization. NS, not significant.

separate the contribution of vascular, kidney, and autonomic systems to the development of hypertension in RGS2 KO mice, it is clear that impaired RGS2 function makes these animals susceptible to altered homeostatic regulation of blood pressure. Yang et al. (2005) recently reported that a subset of hypertensive persons in the Japanese population had a single nucleotide polymorphism that produced a R44H missense mutation in RGS2. Previous work from our laboratory has demonstrated that plasma membrane localization is critical for the proper function of RGS2 as an inhibitor of G_q signaling; moreover, this efficient PM localization is dependent on the ability of the NTD to promote phospholipid bilayer interaction (Gu et al., 2007). Here, we show that the R44H mutation in RGS2 interferes with its lipid bilayer association and, as a result, G_q inhibitory function. These data implicate altered G_q signaling in effector tissues as a possible molecular explanation for the susceptibility of people who carry the R44H mutation to develop hypertension.

What is the mechanism by which R44H disrupts RGS2 function? Proper PM localization of amphipathic helix domain containing proteins has been shown to be dependent on the organization of hydrophobic and basic residues (Segrest et al., 1992). The R44H mutant does not stably bind to the lipid bilayer in cells or in biochemical assays despite its ability to form a proper helix. Thus, we looked for a molecular mechanism that would explain how a histidine for arginine replacement in the NTD could interfere with lipid bilayer interaction. Indeed, the R44 position is highly conserved within the NTD of R4/B subfamily members (Fig. 7a). In the case of several proteins targeted to the membrane by an amphipathic helix, arginine (and to a lesser extent lysine) has been shown to stabilize the interaction of the α-helix to lipid bilayers through "snorkeling"; the ability of long-chain basic amino acids to partition the hydrophobic and hydrophilic portions of their side chains within the lipid core and the membrane hydration shell, respectively (Segrest et al., 1990; Mishra et al., 1994). Thus, mutation of the long-chain arginine to the short-chain histidine may result in the loss of a critical snorkeling-capable residue and lead to the reduced ability of the helix to form a stable association with the lipid bilayer (de Planque et al., 2002). Although the vast majority of R44H-expressing cells do not show PM localization, a small subset of cells (<5%) showed some weak localization at cell-cell membrane junctions. This suggests either that R44H retains a weak residual level of membrane binding activity or that there is a weak interaction between RGS2 and another intracellular signaling partner. This weak level of PM localization may in fact explain why this mutant retains a small degree of G_q-inhibition. Based on these data, our model for helix-mediated targeting of RGS2 to the PM (Fig. 7b) involves a two-step membrane insertion process where negatively charged lipid head groups promote helix formation and subsequently the hydrophobic and snorkeling residues work together to facilitate penetration and subsequent entrenchment of the hydrophobic face of the helix deep into the lipid bilayer.

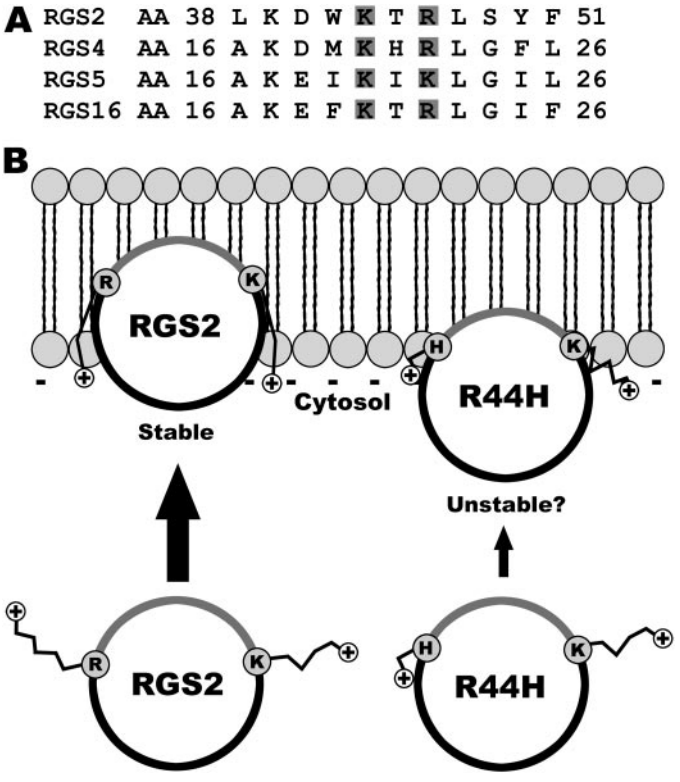

Fig. 7. Model for two-step membrane binding by the RGS2 NTD- impaired snorkeling capability in R44H reduces bilayer interaction without affecting helix formation. A, protein sequence for RGS2, -4, -5, and -16 are shown. Shown in gray background are two highly conserved residues that are within the amphipathic helix. B, proposed role of R44H in snorkeling-dependent stabilization of the RGS2-bilayer interaction. The arginine and lysine residues flanking the hydrophobic area (light gray) are able to arrange their side chains so that the positively charged ends are in the negatively charged region of the phospholipid head groups. The histidine that is replacing arginine has a short side chain that is unable to extend completely into the negatively charged head group region.

In the Japanese population, the R44H allele has thus far only been discovered in heterozygous persons. Our data suggest that the R44H mutant does not act as a dominant-negative mutation. Thus, proper expression from both wild-type *RGS2* loci may be required for its normal function as a regulator of blood pressure homoeostasis. This notion is supported by the fact that heterozygous RGS2-null mice showed a similar degree of hypertension compared with homozygous null animals (Heximer et al., 2003). The R44H mutation, however, was found in one normotensive person in the general Japanese population, reflecting the likelihood that other inheritable factors modulate the effect of RGS2 on blood pressure control. Thus, it will be of future interest to correlate copy number variation in the region of the *RGS2* locus with blood pressure phenotype data in larger patient cohorts as a means of determining whether partial loss of RGS2 activity is sufficient to cause an increase in blood pressure in affected persons.

Acknowledgments

We are grateful to the laboratory of Alan Davidson in the Department of Biochemistry at The University of Toronto. In particular, we would like to thank Zhou Yu and Maryna Gorelik for their help and expertise in performing the tryptophan spectroscopy and circular dichroism assays.

References

Bernstein LS, Grillo AA, Loranger SS, and Linder ME (2000) RGS4 binds to membranes through an amphipathic α-helix. *J Biol Chem* **275:**18520–18526.

Bodenstein J, Sunahara RK, and Neubig RR (2007) N-terminal residues control proteasomal degradation of RGS2, RGS4, and RGS5 in human embryonic kidney 293 cells. *Mol Pharmacol* **71:**1040–1050.

Burstein EA, Vedenkina NS, and Ivkova MN (1973) Fluorescence and the location of tryptophan residues in protein molecules. *Photochem Photobiol* **18:**263–279.
de Planque MR, Boots JW, Rijkers DT, Liskamp RM, Greathouse DV, and Killian JA (2002) The effects of hydrophobic mismatch between phosphatidylcholine bilayers and transmembrane alpha-helical peptides depend on the nature of interfacially exposed aromatic and charged residues. *Biochemistry* **41:**8396–8404.
Gross V, Tank J, Obst M, Plehm R, Blumer KJ, Diedrich A, Jordan J, and Luft FC (2005) Autonomic nervous system and blood pressure regulation in RGS2-deficient mice. *Am J Physiol Regul Integr Comp Physiol* **288:**R1134–R1142.
Gu S, Anton A, Salim S, Blumer KJ, Dessauer CW, and Heximer SP (2008) Alternative translation initiation of human regulators of G-protein signaling-2 yields a set of functionally distinct proteins. *Mol Pharmacol* **73:**1–11.
Gu S, He J, Ho WT, Ramineni S, Thal DM, Natesh R, Tesmer JJ, Hepler JR, and Heximer SP (2007) Unique hydrophobic extension of the RGS2 amphipathic helix domain imparts increased plasma membrane binding and function relative to other RGS R4/B subfamily members. *J Biol Chem* **282:**33064–33075.
Hercule HC, Tank J, Plehm R, Wellner M, Costa Goncalves AC, Gollasch M, Diedrich A, Jordan J, Luft FC, and Gross V (2007) Regulator of G protein signalling 2 ameliorates angiotensin II-induced hypertension in mice. *Exp Physiol* **92:**1014–1022.
Heximer SP, Knutsen RH, Sun X, Kaltenbronn KM, Rhee MH, Peng N, Oliveira-dos-Santos A, Penninger JM, Muslin AJ, Steinberg TH, et al. (2003) Hypertension and prolonged vasoconstrictor signaling in RGS2-deficient mice. *J Clin Invest* **111:**445–452.
Heximer SP, Lim H, Bernard JL, and Blumer KJ (2001) Mechanisms governing subcellular localization and function of human RGS2. *J Biol Chem* **276:**14195–14203.
Kozak M (1994) Features in the 5′ non-coding sequences of rabbit alpha and beta-globin MRNAs that affect translational efficiency. *J Mol Biol* **235:**95–110.
Mishra VK, Palgunachari MN, Segrest JP, and Anantharamaiah GM (1994) Interactions of synthetic peptide analogs of the class a amphipathic helix with lipids. Evidence for the snorkel hypothesis. *J Biol Chem* **269:**7185–7191.
Obst M, Tank J, Plehm R, Blumer KJ, Diedrich A, Jordan J, Luft FC, and Gross V (2006) NO-dependent blood pressure regulation in RGS2-deficient mice. *Am J Physiol Regul Integr Comp Physiol* **290:**R1012–R1019.
Salim S, Sinnarajah S, Kehrl JH, and Dessauer CW (2003) Identification of RGS2 and type V adenylyl cyclase interaction sites. *J Biol Chem* **278:**15842–15849.
Segrest JP, De Loof H, Dohlman JG, Brouillette CG, and Anantharamaiah GM (1990) Amphipathic helix motif: classes and properties. *Proteins* **8:**103–117.
Segrest JP, Jones MK, De Loof H, Brouillette CG, Venkatachalapathi YV, and Anantharamaiah GM (1992) The amphipathic helix in the exchangeable apolipoproteins: a review of secondary structure and function. *J Lipid Res* **33:**141–166.
Sinnarajah S, Dessauer CW, Srikumar D, Chen J, Yuen J, Yilma S, Dennis JC, Morrison EE, Vodyanoy V, and Kehrl JH (2001) RGS2 regulates signal transduction in olfactory neurons by attenuating activation of adenylyl cyclase III. *Nature* **409:**1051–1055.
Sun X, Kaltenbronn KM, Steinberg TH, and Blumer KJ (2005) RGS2 is a mediator of nitric oxide action on blood pressure and vasoconstrictor signaling. *Mol Pharmacol* **67:**631–639.
Tang KM, Wang GR, Lu P, Karas RH, Aronovitz M, Heximer SP, Kaltenbronn KM, Blumer KJ, Siderovski DP, Zhu Y, et al. (2003) Regulator of G-protein signaling-2 mediates vascular smooth muscle relaxation and blood pressure. *Nat Med* **9:**1506–1512.
Yang J, Kamide K, Kokubo Y, Takiuchi S, Tanaka C, Banno M, Miwa Y, Yoshii M, Horio T, Okayama A, et al. (2005) Genetic variations of regulator of G-protein signaling 2 in hypertensive patients and in the general population. *J Hypertens* **23:**1497–1505.

Address correspondence to: Scott P. Heximer, University of Toronto, Rm 3334 MSB, 1 King's College Circle, Toronto, ON, Canada M5S 1A8. E-mail: scott.heximer@utoronto.ca