The RGS2 gene product from a candidate hypertension allele shows decreased plasma membrane association and inhibition of Gq

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Running Title: Impaired function of the R44H mutation in human RGS2

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List of Abbreviations: G-protein; guanine nucleotide binding protein, GPCR; G-protein coupled receptor, GTP; guanosine triphosphate, RGS; regulator of G-protein signaling; NTD, amino terminal domain; YFP; yellow fluorescent protein; FR, fluorescent ratio; PM, plasma membrane

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 Gq signaling, is important for blood pressure regulation. Recently, several *RGS2* mutations in the Japanese population were found to be associated with hypertension. The product of one of these alleles, R44H, is mutated within the amino terminal amphipathic alpha 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. Here, we show that R44H is a weaker inhibitor of receptor-mediated Gq signaling than wild type RGS2. Confocal microscopy reveals that YFP-tagged R44H binds 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 show that the R44H mutation prevents 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 prevents proper lipid bilayer association and function of RGS2. Lastly, the R44H protein does 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 Gq-inhibition and contribute to the development of hypertension in affected individuals.

INTRODUCTION

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) in order 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 >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 Gq, the primary mediator of vasoconstrictive stimuli including norepinephrine, Ang II, and ET-1. RGS2 also inhibits some types of adenylyl cyclase (Salim *et al.*, 2003;Sinnarajah *et al.*, 2001), a pathway important for dopamine-mediated regulation of blood pressure in the kidneys.

Several studies from our laboratory and others have demonstrated 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;Hercule *et al.*, 2007;Gross *et al.*, 2005;Obst *et al.*, 2006). Moreover, RGS2 appears 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;Obst *et al.*, 2006;Sun *et al.*, 2005).

Miyata and colleagues 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 (Yang *et al.*, 2005). Indeed, Neubig and colleagues

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 (Bodenstein *et al.*, 2007). Amongst

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 individuals,

six of which were hypertensive. Notably, 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 (Gu *et al.*, 2007;Heximer *et al.*, 2001), we asked 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

individuals. Specifically, we set out to determine whether the R44H mutation in RGS2 affected

its ability to inhibit receptor-mediated Gq signaling.

MATERIALS AND METHODS:

Materials- The pEYFP-C1 plasmid (Clontech/BD Biosciences, Mississauga, ON) was used for

expression of all RGS protein constructs. The polyclonal anti-GFP antibody (cat #632376) was

also from Clontech/BD Biosciences. Fura2-AM and all tissue culture media and transfection

reagents were from Invitrogen (Burlington, ON). HEK293 cells stably expressing the M1

muscarinic receptor (M1-HEK) were a kind gift from P. Burgon (University of Ottawa Heart

Institute, Ottawa, ON) 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). Unless otherwise stated all other reagents and chemicals were from Sigma-Aldrich (Oakville, Ontario).

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 geneticin at 37 °C in a humidified atmosphere with 5% CO_2. Cells were transiently transfected using FuGENE 6 (Roche, Mississauga, ON) according to manufacturer's instructions.

RGS2 expression constructs- RGS2-YFP expression plasmids were generated in the Clontech Living Colors™ pEYFP-C1 vector 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 (GCCACC<u>ATG</u>GCG). 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 Quik-Change site-directed mutagenesis system (Stratagene, La Jolla, CA). Mutagenesis primers were designed to simultaneously introduce the R44H mutation and an Afl II restriction endonuclease site for screening purposes: FWD, 5'AAAGATTGGAAGACCCACTTAAGCTACTTCTTACAA 3'; REV, 5'TTGTAAGAAGTAGCTTAAGTGGGTCTTCCAATCTTT 3'. All plasmid constructs were purified using the Endofree Maxi kit (Qiagen, Mississauga, Ontario) and verified by sequencing of the complete protein-coding region.

Intracellular calcium imaging- The function of RGS2 as an inhibitor of Gq-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). Briefly, M1-HEK cells were seeded

on poly-L-lysine coated #1 glass coverslips and transiently transfected with the indicated

constructs using FuGENE 6. 24 hours post 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^oC with Ca1 buffer for 5 minutes. Baseline fluorescent ratio (FR)

values were collected for 5-10 seconds before the perfusate was changed to contain 200 µM of

carbachol. Peak relative percent FR increase above baseline =[(peak stimulated FR/ unstimulated

baseline FR) - 1] x 100%. FR values were converted to calcium concentrations using a standard

curve generated from standardized calcium solutions (Molecular Probes/Invitrogen) as described

previously(Gu *et al.*, 2007). Notably, 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 # 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 60x oil objective and processed post-capture with Adobe Photoshop 7.0. Shown

are pictures representative of at least 50 live cells. Where indicated, desitometric quantitation of

protein expression was performed using the gel analysis function of the ImageJ 1.32j software

package.

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, Alabaster, AL) and were diluted in PBS. Liposomes were added to peptide solution for 5 minutes before measurement to allow consistent lipid association. For each lipid concentration, fluorescence emission spectra following 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 non-peptide, background fluorescence emission. In experiments involving Trifluoroethanol (TFE), solutions with peptides were thoroughly mixed and incubated for 5 minutes prior to measurement.

Circular Dichroism (CD)- 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). Briefly, a 3:2 solution of DPPC:DPPG (Avanti Polar Lipids, Alabaster, AL) in chloroform was dried under nitrogen and resuspended in PBS. Lipids in solution were sonicated for 5 min with 20 sec 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 190-260 nm in 1 nm increments averaged over 4 seconds after a 5 minute incubation period. The spectra of lipids and PBS alone were subtracted from sample measurements to account for non-peptide, 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 alpha 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. Recently, we showed that unique features of the RGS2 alpha helical domain mediate its constitutive association with the PM and increased relative function as an inhibitor of Gq 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 alpha helical NTD to result in less RGS2 function as a Gq inhibitor.

Previously, we reported a fura-2 ratiometric calcium signaling assay for studying relative RGS-YFP protein inhibition of Gq-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 similar levels of expression and SDS PAGE migration patterns to wild type RGS2-YFP (Fig. 2a). To assess relative Gq 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. Fig 2b shows the

kinetics of a typical carbachol-induced response of M1-HEK cells transfected with YFP, RGS2

and R44H. Notably, cells transfected with RGS2 show a blunted response to the carbachol

stimulus compared to 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 to YFP controls while 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). Since potent Gq-inhibitory activity of RGS2 is dependent on its ability to associate

with the PM (Gu *et al.*, 2007) and the R44H mutation occurs within the membrane targeting

domain, we next tested whether this mutation affected subcellular localization. Indeed, PM

association of R44H is greatly reduced compared to that of wild type RGS2 (Fig. 3a), whereas

nuclear and nucleolar localization appear 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 as it is

sometimes detectable in small amounts within the membrane interface of cell-cell junctions

(*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. Since 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 alpha 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. Non-corrected 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 alpha 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 Figure 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 R44 residue contributes to the hydrophobic environment of the nearby W41, such an interaction cannot explain the profound blue-shift observed in the presence of liposomes. Accordingly, these data suggest that the spectral 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 due to 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 alpha 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 shows a random coil spectrum even in the presence of

liposomes (Heximer *et al.*, 2001). Non-corrected, raw CD spectra are shown in Supplemental

Figure 3. Together, these data indicate that despite retaining the ability to form a proper alpha

helix, the R44H NTD is unable to form a stable interaction with the lipid bilayer.

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 WT RGS2 (Tang *et al.*, 2003). Since 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 co-transfected 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 individuals 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. While the ubiquitous expression pattern of RGS2 has confounded efforts to 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. It was recently reported that a subset of hypertensive individuals 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 Gq-signaling and moreover, that 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, Gq inhibitory function. These data implicate altered Gq signaling in effector tissues as a possible molecular explanation for the susceptibility of individuals 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 alpha helix to lipid bilayers

through 'snorkeling'; the ability of long chained 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-chained

arginine to the short-chained 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 still

retains a small degree of Gq-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.

In the Japanese population, the R44H allele has thus far only been discovered in heterozygous individuals. 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 when compared to homozygous null animals (Heximer *et al.*, 2003). The R44H mutation was, however, found in one normotensive individual 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 increased in blood pressure in affected individuals.

ACKNOWLEDGEMENTS

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Figure Legends

Figure 1. Single nucleotide polymorphism in *RGS2* results in missense mutation (R44H).
A. Alignment of wild type (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 *grey*. *Arrows* denote the amino acids affected by the R44H mutation

Figure 2. R44H mutation shows decreased Gq-inhibitory function. *A*. YFP, RGS2 and R44H were transiently transfected into M1-HEK cells and harvested after 24 hours. RGS protein expression was evaluated by SDS PAGE 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 in Materials and Methods. Cells with similar levels of YFP fluorescence (3500 < relative YFP fluorescence units < 12000) 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 percent fluorescent ratio (FR) increase above baseline +/- S.E.M. for n > 50 cells. All data are representative of at least three independent experiments. *$p<0.001$.

Figure 3. R44H is not efficiently targeted to the PM. *A*. M1-HEK cells were transiently transfected with the indicated constructs and imaged 24 hours post transfection with an Olympus Fluoview 1000 laser scanning microscope. 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.

Figure 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 34-57 from WT, R44H and L45D were incubated with increasing amounts of purified unilaminar liposomes from brain lipid extracts. Tryptophan fluorescence emission spectra of W41 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 3 independent experiments.

Figure 5. R44H mutation shows normal helix forming potential in the presence of negatively charged phospholipids. Peptides corresponding to residues 34-57 in PBS were incubated either with or without lipids and CD spectra were collected as described in Materials and Methods. Control spectrum of peptides in PBS solution are shown as filled circles while spectrum of peptide solution containing 1.4 mM lipid liposomes are shown as empty circles. Shown is a representative experiment from 3 independent experiments.

Figure 6. R44H mutant does not behave as a dominant interfering mutation. M1-HEK cells were co-transfected with both RGS2-YFP and R44H-myc expression plasmid in the various µg:µg ratios shown. The relative activity of RGS2 to inhibit Gq signaling was measured using

calcium imaging technique as described in the legend to Figure 2 above. Confocal microscopy on similarly treated cells was used to assess the effect of R44H on RGS2-YFP localization. *ns = not significant

Figure 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 grey 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 grey) are able to arrange their side chains so that the positively charged ends are in the negatively charged region of the phospholipids head groups. The histidine that is replacing arginine has a short side chain that is unable to extend completely in to the negatively charged head group region.

Figure 1

A

| AA | 41 | W | K | T | R/H | L | S | Y | 47 |

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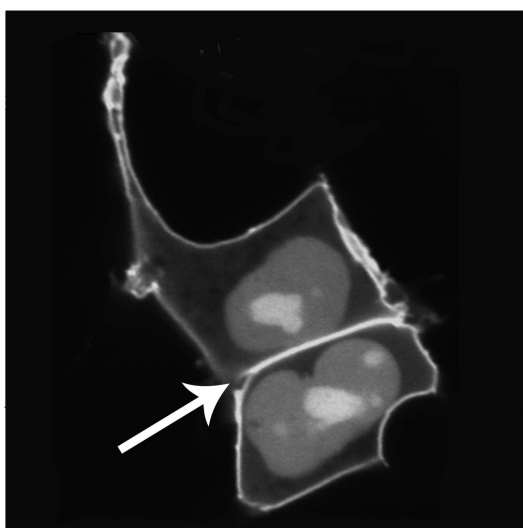
R44H NT 120 tggaagacccatttgagctac 141

B
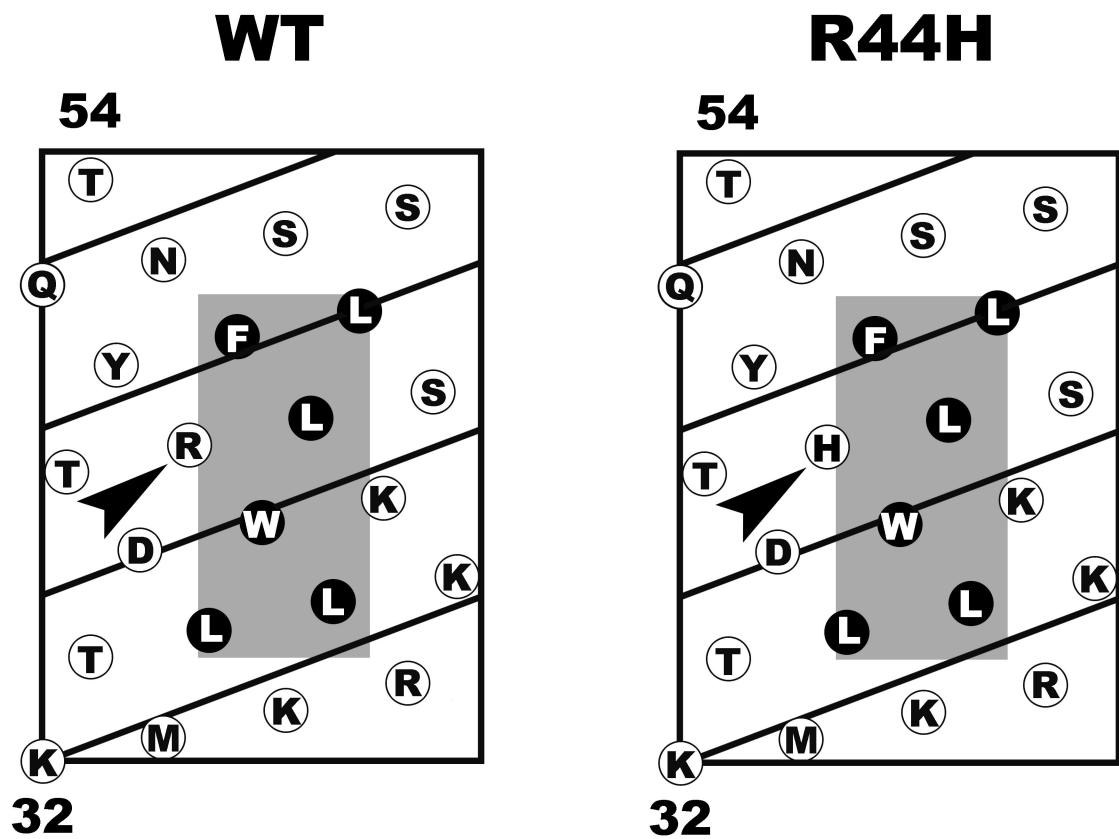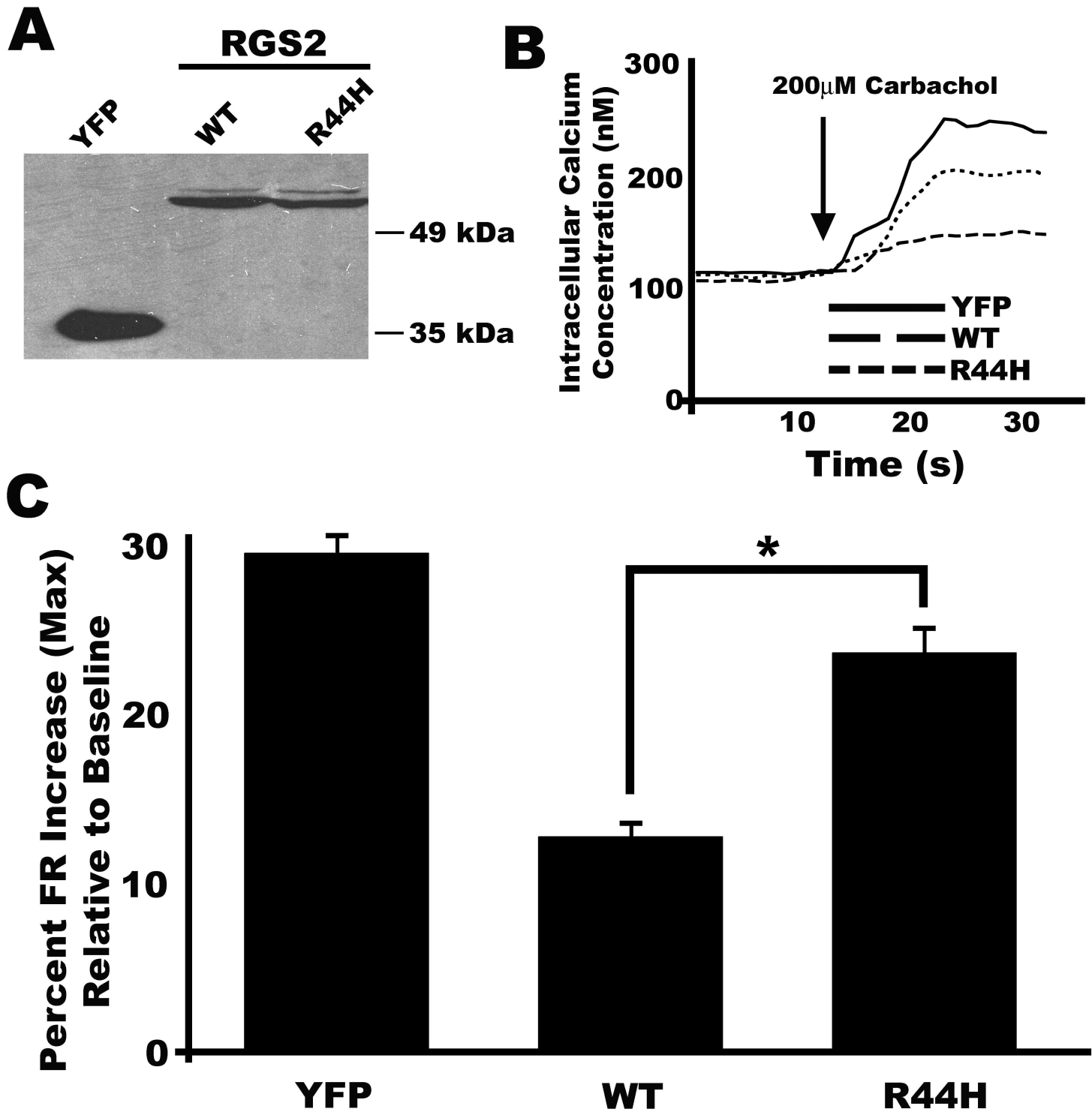
WT   R44H

Figure 2

Figure 3

A

WT   R44H
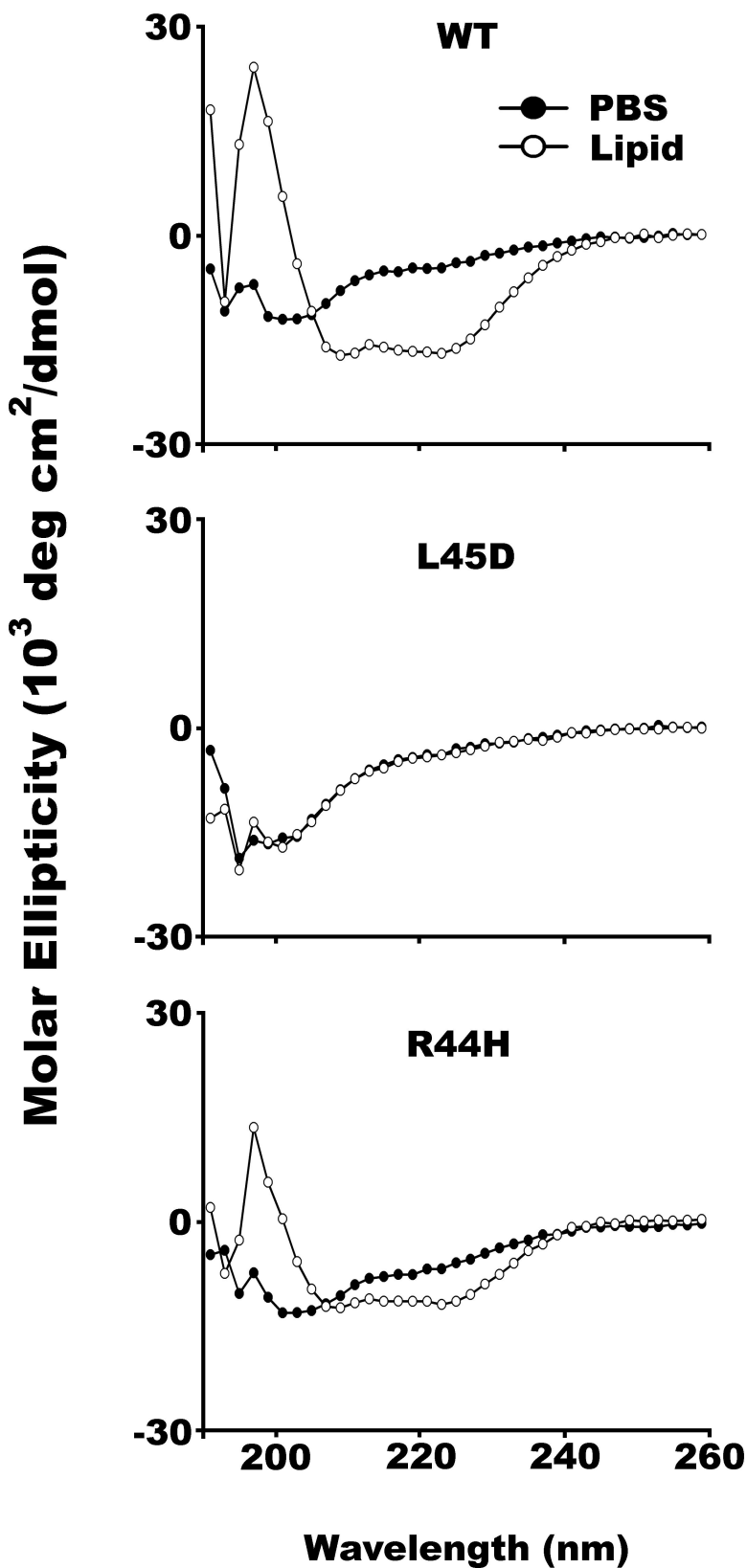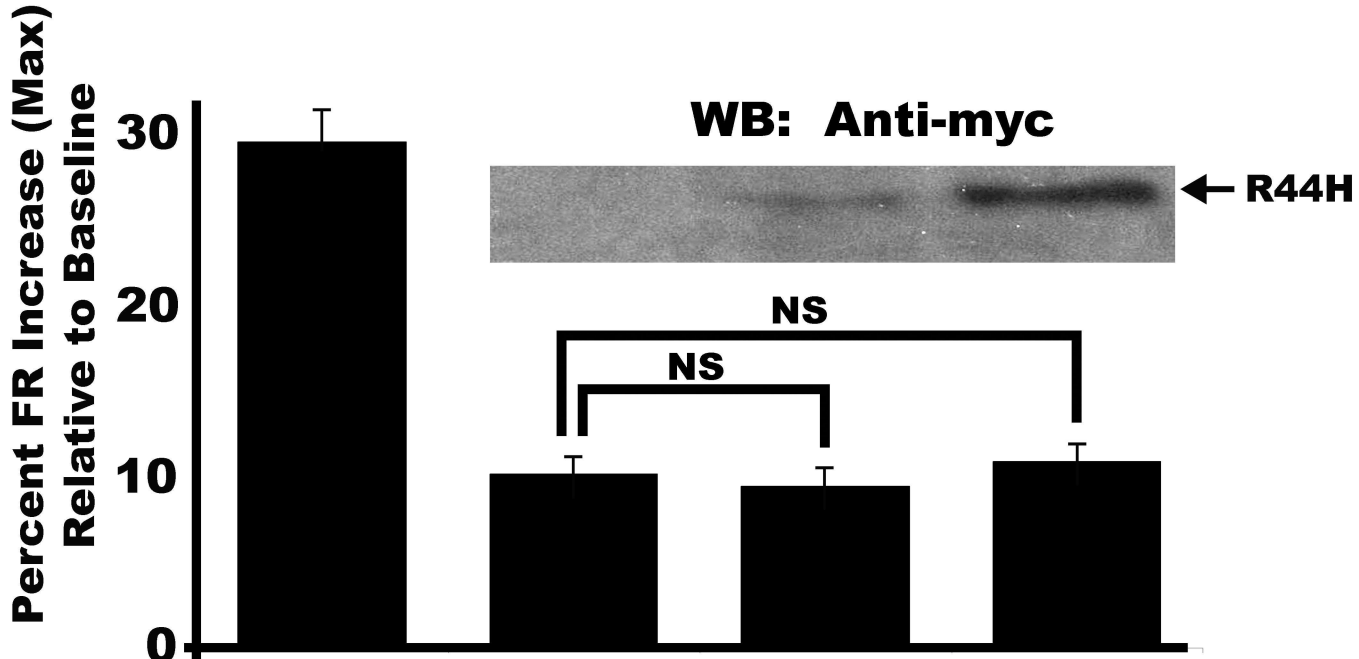
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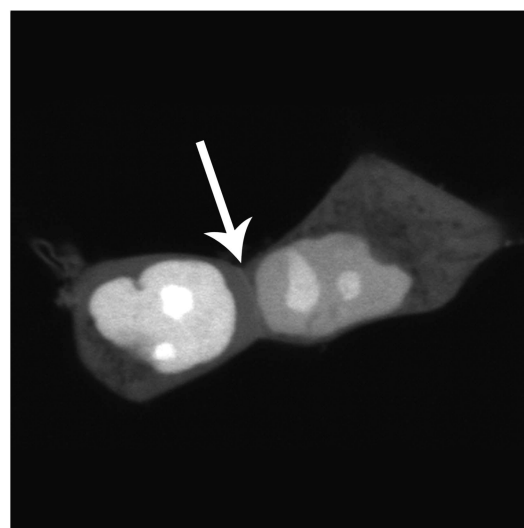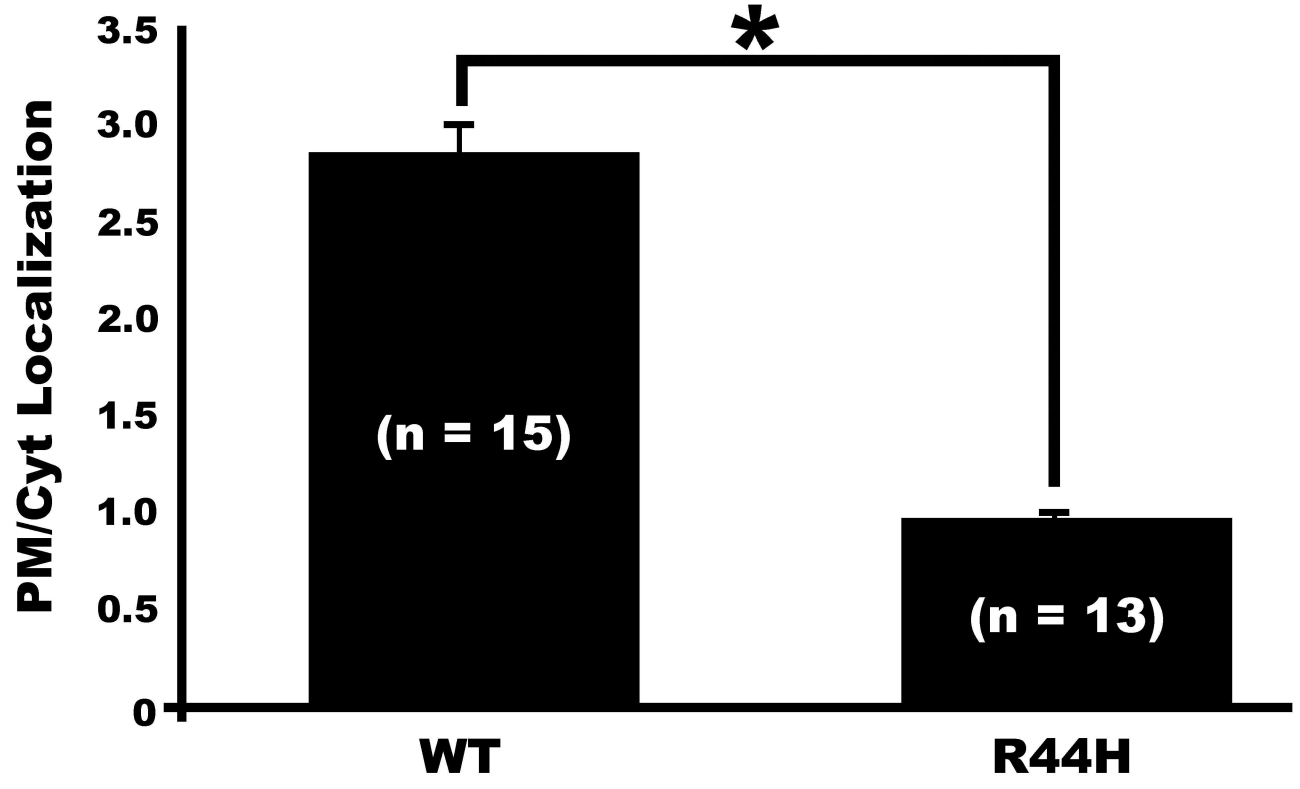
Figure 4
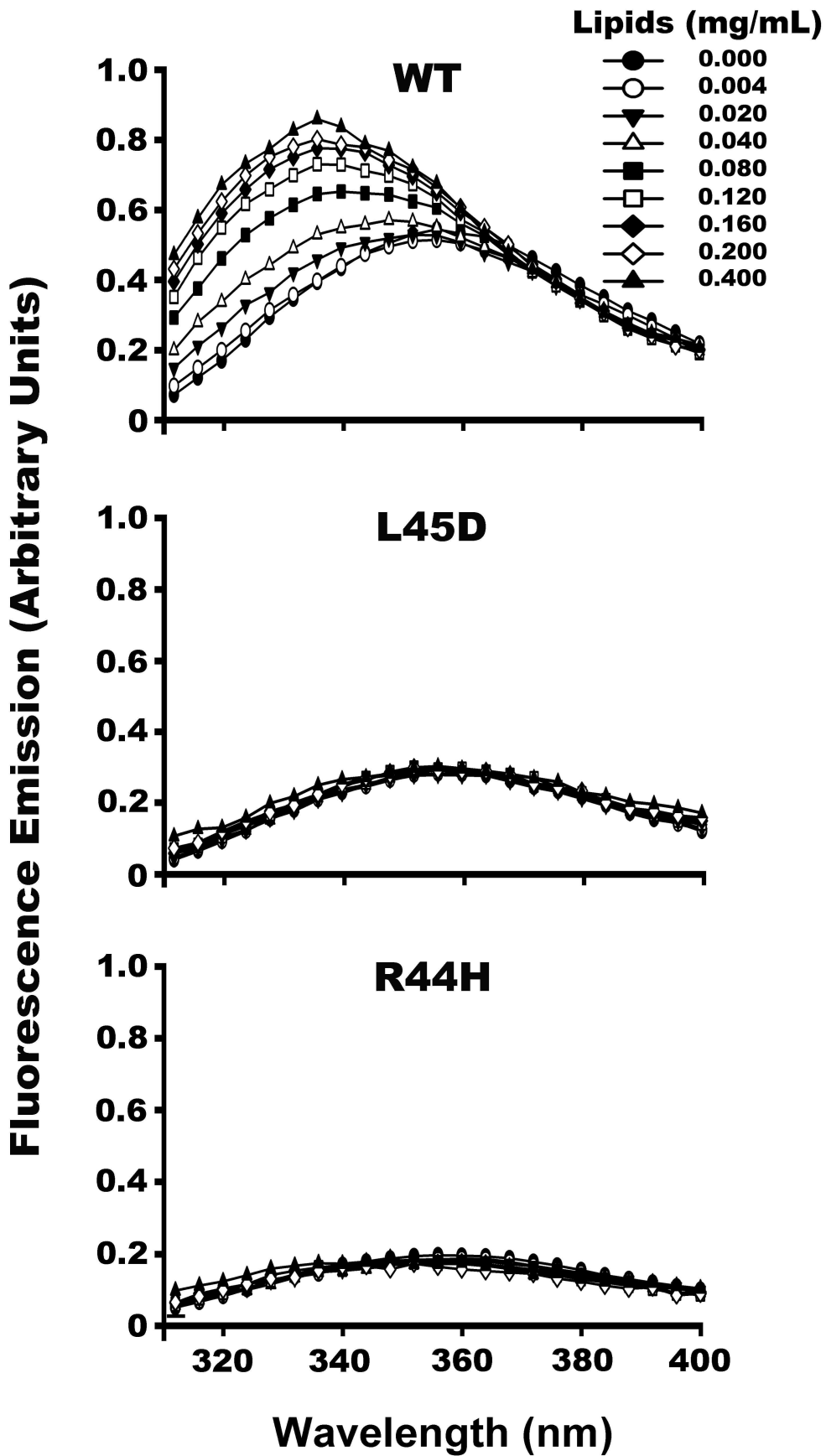
Figure 4

Figure 5

Figure 6

Figure 7

A

```
RGS2   AA 38  L K D W K T R L S Y F 51
RGS4   AA 16  A K D M K H R L G F L 26
RGS5   AA 16  A K E I K I K L G I L 26
RGS16  AA 16  A K E F K T R L G I F 26
```
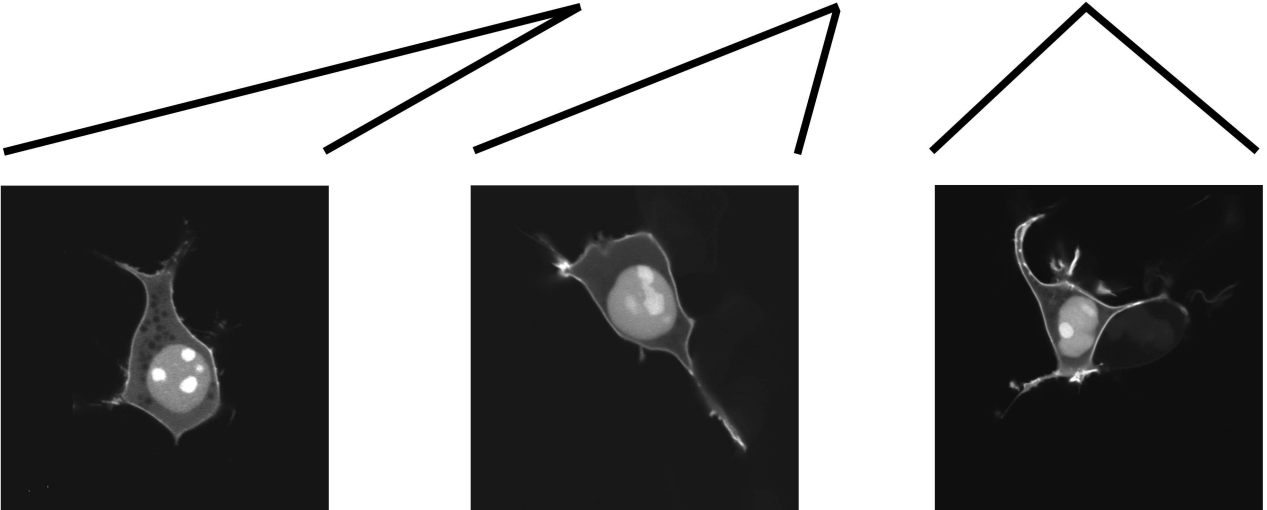
B