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Analysis of chimeric RGS proteins in yeast for the functional evaluation of protein domains and their potential use in drug target validation

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Abstract

For the identification of regulators of G-protein signaling (RGS) modulators, previously, we developed a luciferase based yeast pheromone response (YPhR) assay to functionally investigate RGS4 (K.H. Young, Y. Wang, C. Bender, S. Ajit, F. Ramirez, A. Gilbert, B.W. Nieuwenhuijsen, in: D.P. Siderovski (Ed.), Meth. Enzymol. 389 Regulators of G_protein Signaling, Part A, 2004.). To extend the diversity of this assay, additional RGS proteins were evaluated for functional complementation in a RGS (*sst2*Δ) knockout yeast strain. For RGS proteins that did not function in their native form, a series of chimeric constructs were generated with the N terminus of RGS4 fused in frame with the partial or full-length RGS cDNA of interest. RGS4 N terminus fused to either full-length or the C terminus of RGS7 successfully complemented *sst2*Δ. On the contrary, the RGS7N/RGS4C chimera (N terminus of RGS7 in frame with RGS domain of RGS4) was not effective, showing that N terminus of RGS4 helps in targeting. RGS10 exists as two splice variants, differing only by 8 amino acids (aa) in the N terminus, being either 168 aa (RGS10S), or 174 aa (RGS10). While RGS10 was functional in yeast, RGS10S required the presence of the N terminus of RGS4 for its activity. Although the same RGS4 N terminus domain was present in chimeras generated, the GTPase accelerating protein (GAP) function observed was not similar, suggesting differences in the RGS domain function. In conclusion, the use of RGS4 N terminus chimeric constructs enabled us to develop a selectivity assay for different RGS proteins.

Keywords: RGS4; RGS7; RGS10; Chimeric RGS proteins in yeast

1. Introduction

Signal transduction is the fundamental biological process of converting extracellular information into changes in intracellular functions. One important class of signal-transduction pathways with profound clinical significance is that controlled by the heterotrimeric guanine–nucleotide-binding proteins (G proteins). Many marketed therapeutics target G-protein-coupled receptor (GPCR) function at the extracellular ligand–GPCR interface. The proven clinical utility of modulating GPCR signal transduction has sustained formidable efforts in the pharmaceutical industry to identify new GPCR–ligand pairs that impact clinically relevant signaling pathways [1].

Established models supported that hormones and neurotransmitters use a GPCR, a G-protein and a target effector to transmit signals across the plasma membrane. Regulators of G-protein signaling (RGS) proteins now represent a newly appreciated fourth component in G-protein signaling that have the potential to be new drug discovery targets. RGS proteins are a large family of highly diverse, multifunctional signaling proteins, which share a conserved signature domain (RGS domain) that binds directly to activated Gα subunits to modulate G protein signaling. RGS proteins differ widely in their overall size and amino acid (aa)

Abbreviations: RGS, regulator of G-protein signaling; GAP, GTPase accelerating protein; GPCR, G-protein-coupled receptor; DEP domain, disheveled/egl-10/pleckstrin domain; GGL domain, G-protein gamma subunit-like domain; RGS4N/RGS7, chimeric N-terminal of RGS4 (aa 1–57) and full-length RGS7 (aa 1–470); RGS4N/RGS7C, chimeric N-terminal of RGS4 (aa 1–57) and C-terminal of RGS7 (aa 255–470); RGS7N/RGS4C, chimeric N-terminal of RGS7 (aa 1–332) and C-terminal of RGS4 (aa 58–206); RGS4N/RGS10S, chimeric N-terminal of RGS4 (aa 1–57) and full-length RGS10S (aa 1–168).

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identity, and many family members possess a remarkable variety of structural domains and motifs that regulate their actions and/or enable them to interact with protein binding partners with diverse cellular roles [2].

The availability of specific chemical inhibitors will be the first step towards the development of RGS inhibitor drugs. The 'gold standard' assays for RGS function are single turnover [^{32}P] GTPase assays that are not amenable to high-throughput or automated approaches. *Saccharomyces cerevisiae* is a useful model system to study G-protein signaling pathway because almost all the component of the signaling cascade have a human equivalent, many of which are functionally interchangeable with the corresponding yeast protein [3,4]. Negative regulation of RGS proteins in GPCR signal termination was first appreciated in studies of yeast [5]. To address the need for a stable and non-radioactive assays to permit high-throughput screening (HTS) for inhibitors of the GTPase activating protein (GAP) function of RGS proteins, we developed a pheromone-responsive yeast-based screening platform [6]. To extend the diversity of this assay to investigate additional RGS proteins and identify tools, different short and long RGS proteins were evaluated for functional complementation in a RGS (sst2) knockout yeast strain using pheromone responsive luciferase and halo assays. The potential of Gβ5 to augment the function of longer RGS proteins was also evaluated. The assay utilized the yeast pheromone response (YPhR) pathway and used a luciferase reporter, which provides a rapid and robust quantitative signal in yeast. To address the need for finding inhibitors/modulators for RGS proteins that were not functional in yeast when expressed in their native forms, a series of chimeric constructs were made (Fig. 1). These chimeric genes were made with the N terminus of RGS4 fused in frame with partial or full-length RGS cDNA of interest. The RGS4 N-terminal contains a 33-amino acid cationic amphipathic α helix that drives RGS4 membrane attachment [7]. The RGS4 N-terminal amphipathic domain confers similar membrane binding behavior on the RGS domains of either RGS10 or RGSZ1 [8]. Our studies showed that the N terminus of RGS4 fused in frame with full-length RGS7, the RGS domain of RGS7 or RGS10S enabled complementation of the sst2 knockout strain. Although the N terminus of RGS4 was the same for the functional chimeras, the resulting complementation observed was at different levels for the RGS proteins, which could be due to the differences in the RGS core domain itself.

Chimeric RGS proteins described here can thus be used not only to identify chemical entities that could modulate RGS function but also could be used to investigate RGS-subtype specificity of compounds. Studies on the effect of different N-terminal and C-terminal combinations of RGS proteins also proved to be useful in the functional evaluation of protein domains.

2. Materials and methods

2.1. Cloning and chimeric gene construction

RGS7 pcDNA 3.1 [9] (Accession number AF090116) was digested with *Pme*1 to release the RGS7 insert and subcloned into the *Sma*1 sites of p426tef vector (ATCC, Manassas, VA). The RGS4N/RGS7C construct was made as follows: RGS4N region comprised of 171 bp which encoded amino acids 1–57 was obtained by PCR using rat RGS4 cDNA (Accession number NM_017214) as template and forward primer (primer 1) *Bam*H1 CGC GGA TCC

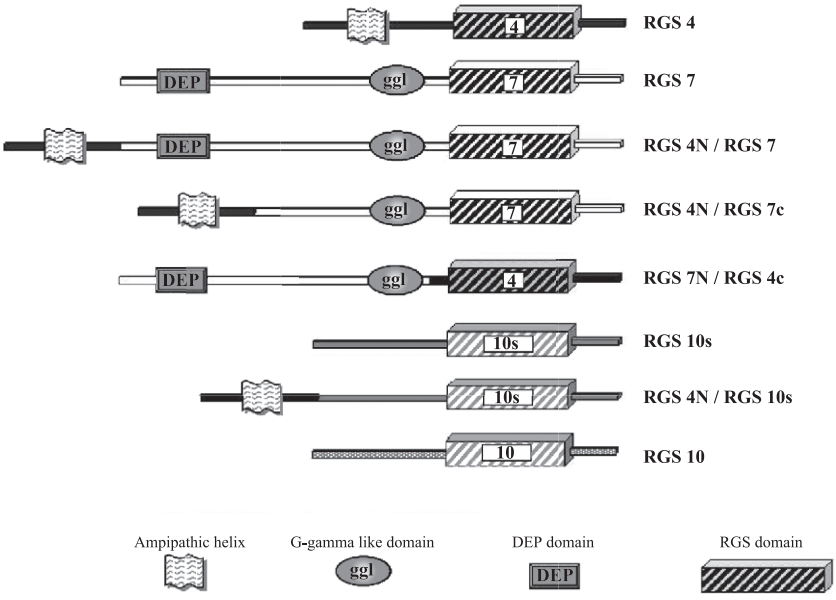

Fig. 1. Schematic representation of RGS constructs.

ATG TGC AAA GGG CTT GCA and the reverse primer (primer 2) *Sma*I TCC CCC GGG CTT GAC TTC CTC TTG GCT. The RGS7C region begins with the G-protein gamma subunit-like domain (GGL domain) of full-length human RGS7 through the last amino acid was amplified to produce a 645-bp product encoding amino acids 255–470. The forward primer (primer 3) *Sma*1 TCC CCC GGG GAT GAG TTA CAA CAA CAG and reverse primer (primer 4) *Cla*1 CCC ATC GAT TTA GTA AGA CTG AGC were used with RGS7 cDNA template. The two PCR products were gel-purified, restriction enzyme digested with *Sma*I and then ligated. The ligation mixture was used as template to produce the full-length chimeric 816-bp PCR product using primers 1 and 4 as forward and reverse primers, respectively. The resulting chimeric gene was gel purified, reamplified, digested with appropriate enzymes, and was ligated into the *Bam*H1 and *Cla*1 site of p426 TEF vector with N terminus HA tag.

For RGS4N/RGS7 full-length construct, the RGS4N region was obtained as described above. The cDNA encoding full-length RGS7 was amplified using human RGS7 plasmid [9] as template to produce a 1410-bp PCR product encoding 1–470 amino acids using the forward primer (primer 5) *Sma*I 5′TCC CCC GGG ATG GCC CAG GGG AAT and the reverse primer (primer 6) *Cla*I 5′CCC ATC GAT TTA GTA AGA CTG AGC. The two PCR products were gel-purified, digested with *Sma*I and then ligated. The ligation mixture was then used as PCR template with primers 1 and 6 to obtain the RGS4N/RGS7 full-length chimeric cDNA. The resulting chimeric cDNA was gel-purified, reamplified, and cloned into the *Bam*H1 and *Cla*1 site of p426 TEF vector with N terminus HA tag.

For RGS7N/RGS4C construct, the RGS7N region had the GGL domain of RGS7 and was amplified with the forward primer (primer 7) *Bam*HI CGC GGA TCC ATG GCC CAG GGG AAT and the reverse primer (primer 8) *Sma*1 TCC CCC GGG AAA ACC CCA TCG TTT using human RGS7 cDNA as template to produce a 996 bp PCR product encoding amino acids 1–332. The RGS4C region contains only the RGS4 core domain and was amplified using forward primer (primer 9) *Sma*1 5′-TCC CCC GGG AAA TGG GCT GAA TCA CTG, reverse primer (primer 10) *Cla*1 5′-CCC ATC GAT TTA GGC ACA CTG AGG GAC and rat RGS4 cDNA as a template to produce a 447-bp PCR produce encoding amino acids 58–206. The two PCR products were gel-purified, restriction enzyme digested with *Sma*1 and then ligated. The ligation mixture was used as template in PCR with primers 7 and 10 for the amplification of the chimeric gene product of 1443 bp. The resulting chimeric gene was gel-purified, reamplified, cut with appropriate enzymes, and cloned into the *Bam*H1 and *Cla*1 site of p426 tef vector with N terminus HA tag.

The plasmid p426 RGS10S (Accession number XM_049797) was constructed by PCR amplification of human brain library (Clonetech, Palo Alto, CA) as template using forward primer (primer 11) *Hind*III 5′-CCC AAG CTT ATG GAA CAC ATC CAC GAC AGC and the reverse primer (primer 12) *Xho*I 5′-CCG CTC GAG TCA TGT GTT ATA AAT TCT GGA. The PCR product of 504 bp encoding the full-length RGS10S was gel purified and cloned into *Hind*III and *Xho*I sites of p426 TEF vector. This plasmid was used as a template for amplifying RGS10S for cloning into pcDNA3.1 (Invitrogen, Carlsbad, CA) with C terminus myc-His tag using *Not*1 5′-AAGGAAAAAA GCG GCC GC ATG GAA CAC ATC CAC GAC A as forward and *Bam*H1 5′-CGC GGA TCC TGT GTT ATA AAT TCT GGA A as reverse primer. The restriction enzyme digested, gel-purified PCR product was cloned into *Not*1 and *Bam*H1 sites of pcDNA3.1 vector.

For RGS4N/RGS10S construct the RGS4N region is identical to that described for the RGS4N/RGS7C chimera. RGS10S for this chimera was obtained by restriction digest of p426 RGS10S with *Sma*1 and *Xho*1. The RGS4N region PCR product was restriction enzyme-digested with *Sma*1 and ligated with the gel-purified *Sma*I and *Xho*I restriction-digested fragment of RGS10S. The ligation mixture was used as template for PCR to obtain the RGS4N/RGS10S chimeric gene. The forward and reverse primers used were primers 1 and 14, respectively. Resulting chimeric gene was gel purified, re-amplified and cloned into the *Bam*H1 and *Xho*1 site of p426 tef vector with an N terminus HA tag.

RGS10 (Accession number AF368902) was obtained by PCR amplification of human brain library (Clonetech, Palo Alto, CA) using forward primer (primer 13) *Hind*III 5′CCC AAG CTT ATG CAG TCT GAA CTT TGC TTT and reverse primer (primer 14) *Xho*I 5′-CCG CTC GAG TCA TGT GTT ATA AAT TCT GGA. The 522 bp PCR product encoding the full-lengthfull-length RGS10 was gel purified and cloned into *Hind*III and *Xho*I sites of p426tef vector. This plasmid was used as a template for amplifying RGS10 for cloning into pcDNA3.1D/V5-His-TOPO vector (Invitrogen, Carlsbad, CA) using forward primer 5′-CACC ATG CAG TCT GAA CTT TGC TTT and reverse primer 5′-TGT GTT ATA AAT TCT GGA A. The gel-purified PCR product was ligated with the vector to obtain the plasmid pcDNA3.1 RGS10 with C terminus V5 tag.

The cloning of human Gβ5 cDNA (Accession number AF017656) into p425tef vector (ATCC, Manassas, VA) has been described before [10].

2.2. Strain generation

Yeast strains were produced to evaluate the ability of the various RGS chimeric proteins to complement an Sst2 deletion strain. The RGS chimeric plasmids were transformed [6] into the base strain yKY113 {MATa ura3–52 lys2–801a ade2–101o trp1-D63 his3-D200 leu2-D1 sst2 YDM400 (sst2)}. Strains expressing RGS7 and or RGS7 chimeras were generated with the presence or absence of human Gbeta5 plasmid. All strains contained either an empty vector control or an expression plasmid for RGS and firefly luciferase reporter gene to enable investigation under

similar media conditions. Transformed yeast was plated on appropriate dropout plates. Six independent yeast colonies were picked, grown overnight in 5 ml SC-ULT dropout media, and used for luciferase or halo assay.

2.3. Luciferase assay and halo assay

Yeast pheromone response assay is activated in haploid cells by GPCR (Ste2) upon addition of mating pheromone a-factor. A mating response is mediated by free βγ, activation of the MAP kinase pathway, and can be measured quantitatively by FUS1-luciferase reporter gene. RGS complementation of sst2 knockout strains was studied using luciferase reporter gene and halo assays. Luciferase assays was done as described before [6]. For the halo assay, yeast cells (0.4 OD) were added to 5 ml melted appropriate dropout top agar and plated on same dropout plates. Alpha factor was spotted at 1 mmol, 100, 10, and 1 μmol concentrations and plates were incubated at 30 °C for ~48 h.

2.4. Western blot

Proteins were extracted from yeast strains using the Urea/SDS method as described in Clontech's yeast protocol handbook (BD Biosciences, Palo Alto, CA). Proteins were separated using SDS-PAGE, and transferred to PVDF membranes (Amersham Biosciences, Piscataway, NJ). Following incubation with the appropriate secondary antibody, proteins were visualized using enhanced chemiluminescent detection (PerkinElmer Life Sciences, Boston, MA). RGS7 antibody has been described before [11]. Anti HA peroxidase monoclonal antibody was purchased from Roche Applied Science (Indianapolis, IN).

2.5. Immunofluorescence

COS cells were maintained in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 0.1 mM MEM nonessential amino acids solution. Cell lines were maintained at 37 °C in a humidified atmosphere containing 5% CO_2. All cell culture reagents were purchased from Invitrogen Life Technologies (Carlsbad, CA). CHOK1 cells were seeded at a density of 9×10^5 cells. The following morning, cells were transiently transfected using Lipofectamine Plus (Invitrogen Carlsbad, CA) according to the manufacturer's protocol. Forty-eight hours after transfection, cells were rinsed with PBS and fixed in freshly prepared 3% paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.4) for 30 min. Cells were rinsed with PBS with 0.1% Triton X-100, and blocked in PBS containing 4% nonfat dried milk and 0.1% Triton X-100 for 30 min at room temperature. After five more rinses with PBS (5× for 5 min), cells were then incubated with a monoclonal antibody to V5 and Myc (Invitrogen, Carlsbad, CA; 1:1000 dilution) for 16–18 h at 4 °C. Omission of primary antibodies was used as a negative control. The following day, cells were washed 5×5 min with blocking buffer, then incubated with Alexa Fluor 594 goat anti-rabbit IgG (H+L) secondary (for RGS10) and Alexa Fluor 488 goat anti-mouse IgG (H+L) secondary (for RGS10S; Molecular Probes, Eugene, OR, USA), each at a dilution of 1:100, for 1 h at room temperature. Cells were washed 3×5 min with blocking buffer, then 2×5 min with PBS at room temperature. Cells were incubated with DAPI stain for 3 min and after 3×5 min rinse in PBS were mounted using the ProLong Antifade Kit (Molecular Probes). Cells were viewed in indirect immunofluorescence on a Zeiss Axiovert 135 TV microscope (Carl Zeiss, Thornwood, NY, USA) using either a 63× oil/1.40 numerical aperture Plan-APOCHROMAT objective or a 100× oil/1.30 numerical aperture Plan-NEOFLUAR objective. Fluorescent images were captured by a Sony DXC-970MD 3CCD 24-bit color video camera using MCID Elite 6.0 software (Imaging Research, St. Catharines, Ontario, Canada).

3. Results

Yeast lacking Sst2 (*sst2*Δ) fail to resume growth after exposure to pheromone. Strains were tested for functional complementation in the pheromone response assay, using a pheromone responsive luciferase gene. Strains were also tested in qualitative halo assay in response to 48-h exposure to alpha factor for pheromone response. RGS4 is highly effective in complementing the Sst2 knockout phenotype (Fig. 2). The halo size is a good indication of the GAP activity of the RGS domain being tested. In a halo assay, RGS4 is more effective than the endogenous yeast RGS, Sst2, as observed by a lack of halo in strains expressing RGS4 (data not shown).

3.1. Effect of RGS10 splice variants and RGS4N/RGS10S chimera

RGS10 exists as two splice variants distinguished by overall aa length of 168 aa and 174 aa. The 168-aa-long RGS10 is distinguished from 174 aa RGS10 by designating

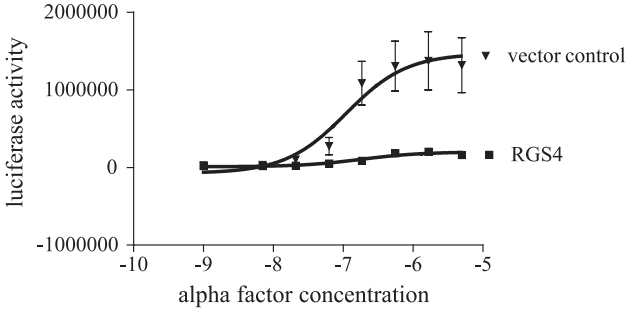

Fig. 2. Effect of RGS4 on pheromone luciferase activity. RGS4 complements *sst2*Δ.

the shorter form as RGS10S. The short and long form differs only in the N terminus with RGS10S having MEH (aa 1–3) and RGS10 having MQSELCFAD (aa 1–9) sequence. RGS10S when expressed in its native form, does not functionally complement *sst2*Δ strain in the luciferase assay, but had a small effect in halo assay (Fig. 3a and c, respectively). The RGS4N/RGS10S chimera however, restored the RGS function, suggesting the N terminus of RGS4 facilitate functional regulation of G-protein function. Interestingly RGS10 which is longer by 8 aa in the N terminus was functional in both luciferase and halo assays. This result is comparable to the effects reported for splice variants of RGS8 [12]. When RGS10 and RGS4N/RGS10S chimera are compared, the latter seems to be more efficient. We did not test the effect of RGS4N/RGS10 chimera, as RGS10 was functional in its native form.

3.2. Effect of native and chimeric RGS7 proteins

RGS7 is the prototypical member of the R7 family of RGS proteins [13] and contains an N-terminus dishevelled/egl-10/pleckstrin (DEP) domain [14], as well as a G protein γsubunit-like (GGL) domain [15] located between the DEP and RGS domains. The GGL domain interacts with Gβ5, a unique G protein β subunit predominantly expressed in brain [16]. The binding of RGS7 to Gβ5 increases the stability of RGS7 (Ref. [17], and references therein). Thus, we investigated the ability of RGS7 to complement *sst2*Δ and the impact of Gβ5 to influence the activity of RGS7. RGS7 wild type did not differ from the control, both in the presence and absence of Gβ5 (Fig. 4). Addition of the N terminus of RGS4 to full-length RGS7 improved the regulation of G-protein signaling by RGS7 in yeast (Fig. 4b). This effect was also observed since halo size was diminished in RGS4N/RGS7 chimera (Fig. 4c). Coexpression of Gβ5 with RGS4N/RGS7 did not influence the halo assay. In the luciferase assay, Gβ5 seems to slightly decrease the responsiveness of RGS chimera to alpha factor. A similar trend was observed over several experiments (see Fig. 5a also). The role of Gβ5 in augmenting endogenous Ste4 (yeast Gbeta) has been discussed elsewhere [10].

Domain swapping experiments were performed (1) to determine if the RGS4N terminus is helping in the targeting of nonfunctional RGS proteins in yeast and (2) to see if the ineffectiveness of wild type RGS7 in the assays was due to the lack of function of RGS domain of RGS7 in yeast. Results from studies using two chimeric proteins, one with RGS4 N terminus fused in frame with the C terminus of

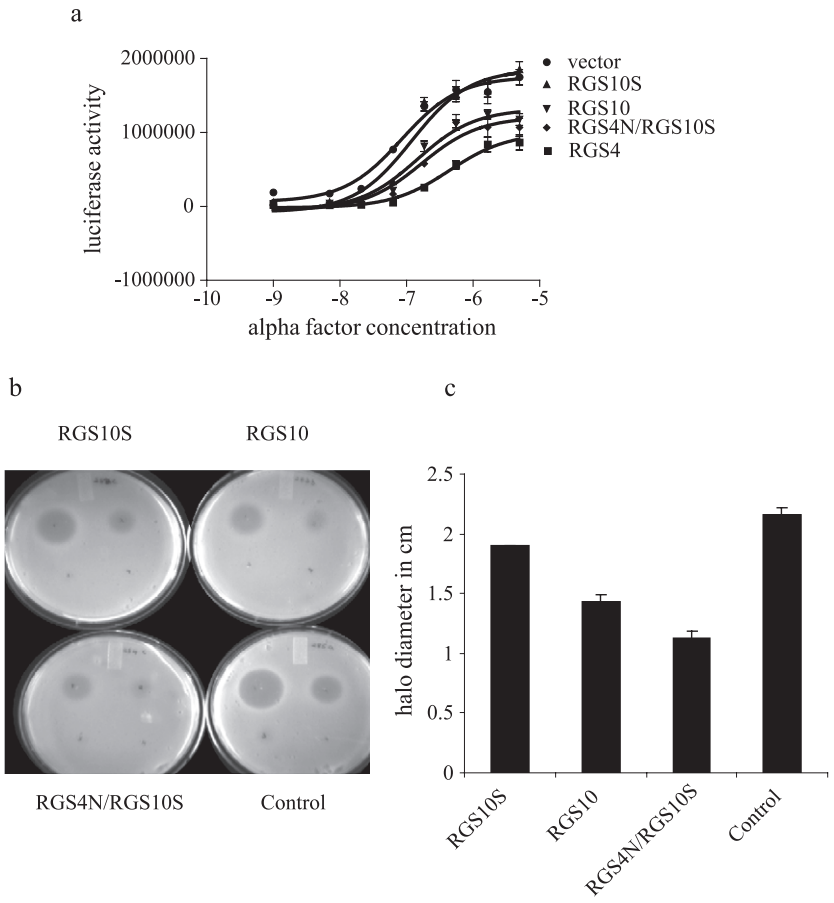

Fig. 3. Effect of RGS10S, RGS4N/RGS10S and RGS10 in luciferase reporter gene and halo assays. (a) Having N terminus which differ by seven amino acids conferred RGS10 with good GAP activity. Similar results were obtained with halo assay (b). The size of the halo of growth inhibition of yeast grown on agar plates (1 mM of factor) was calculated by measuring its diameter (n=6 for luciferase assay and n=3 for halo assay; c).

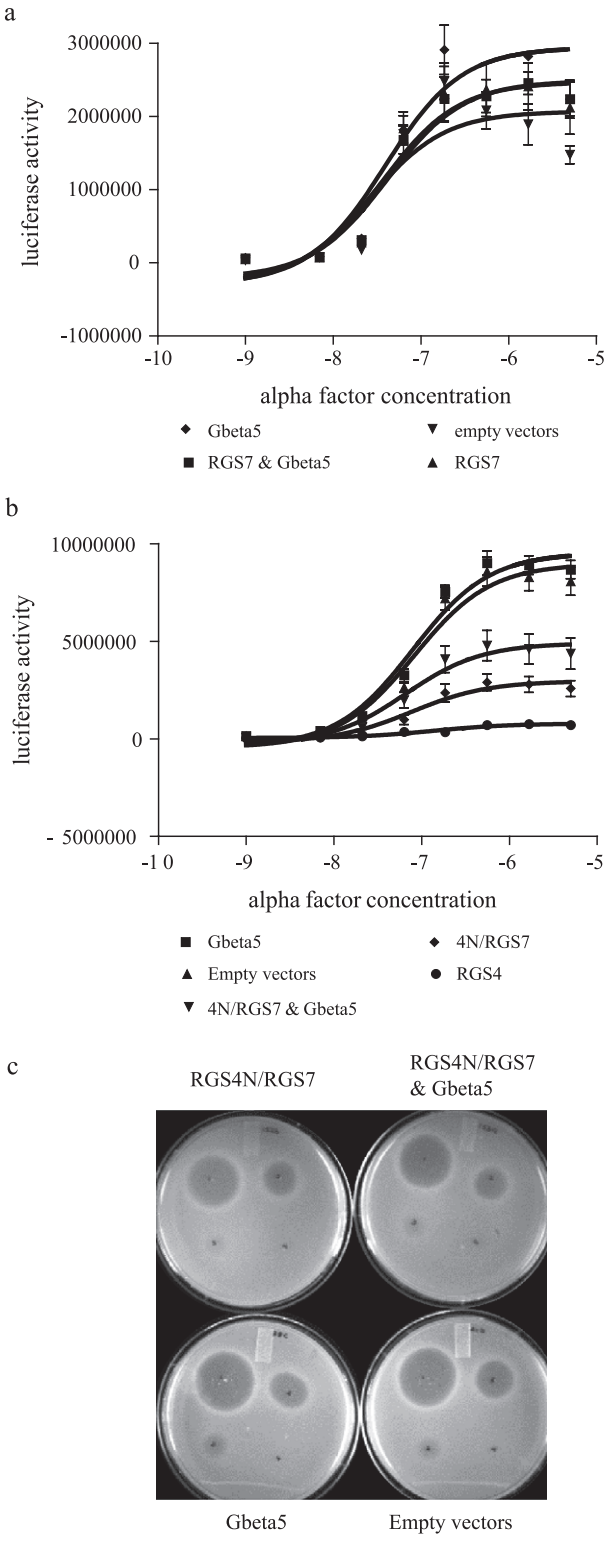

Fig. 4. Effect of RGS7 (a) wild type and RGS4N/RGS7 full-length chimera on pheromone response luciferase assay and halo assays. The N terminus of RGS4/full length RGS7 improved the GAP activity of RGS7 (b and c). Gbeta5 when present along with RGS4N/RGS7 slightly decreased the responsiveness of RGS chimera to alpha factor in the luciferase assay.

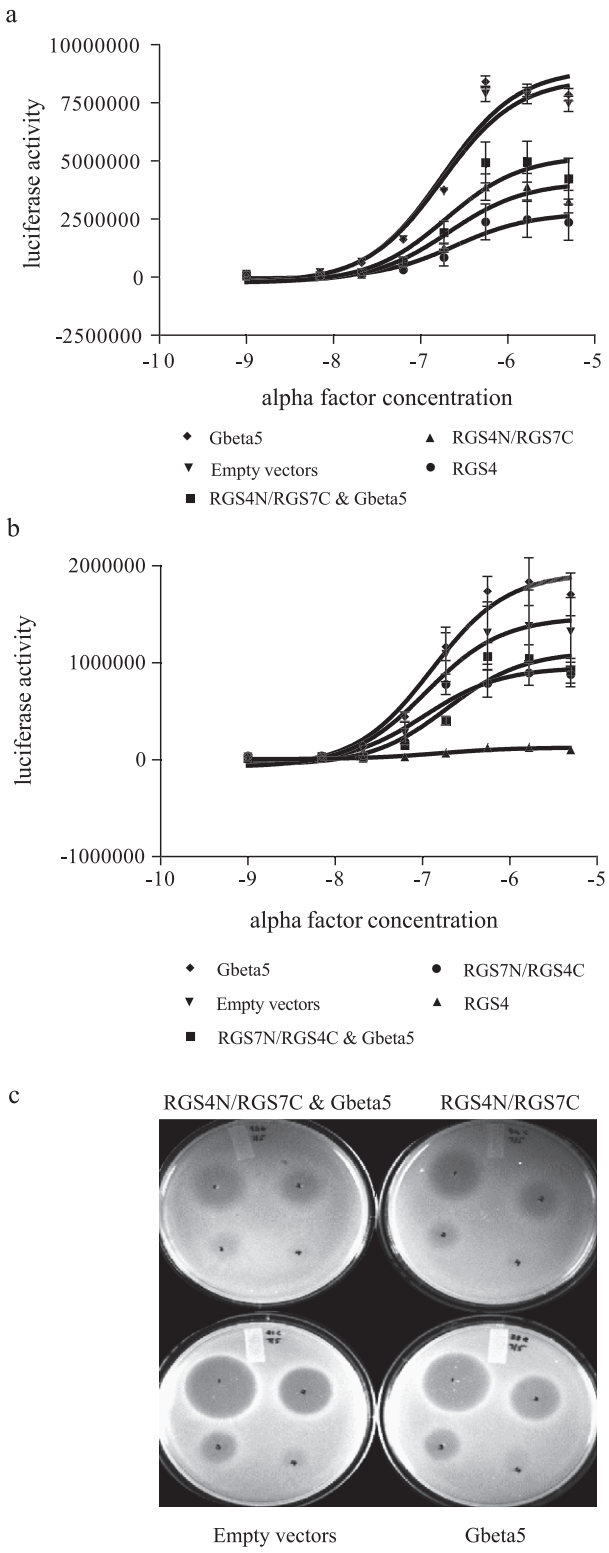

Fig. 5. Effect of RGS4N/RGS7C and RGS7N/4C chimeras on pheromone responsive luciferase reporter gene assay and halo assays. Having N terminus of RGS4 with C terminus of RGS7 (including GGL domain) improved the GAP activity of RGS7 (a and c). Coexpression of Gbeta5 with RGS4N/RGS7 caused a very slight decrease in the responsiveness of RGS chimera to alpha factor in the luciferase assay. On contrary RGS7N/RGS4C chimera, which has the RGS domain of RGS4 was similar to control (b).

RGS7 and the other with the N terminus of RGS7 in frame with RGS domain of RGS4 are shown in Fig. 5. For RGS4N/RGS7C chimera, the C terminus had the GGL domain of RGS7 (see Fig. 1). Fig. 5a shows that RGS4N/RGS7C chimera was able to successfully complement *Sst2* although not to the extent observed for RGS4. This result was confirmed in halo assay as seen by the diminished halo size (Fig. 5c). Figure for RGS4 halo assay where there is a complete absence of a halo is not shown. Thus, the presence of RGS4 N terminus enabled RGS7 C terminus and to a lesser extend full-length RGS7, to complement *sst2*Δ as seen by decreased size of the halo in response to alpha factor stimulation. On the contrary, RGS7N/RGS4C chimera had no effect in modulating the pheromone response (Fig. 5b). This data clearly shows that N terminus of RGS4 plays a role in targeting the RGS proteins and supports the concept that a strong RGS box (as in the case of RGS7N/RGS4C chimera) is not sufficient as observed by the lack of GAP activity. The shorter version of RGS7 (RGS7C which lacks the DEP domain but has the GGL domain and RGS domain) was more effective than the full-length RGS7 when fused to RGS4 N terminus.

3.3. Western blot analysis of RGS chimeric proteins

The chimeric RGS7 proteins showed that RGS7N/RGS4C (N terminus HA tag) is expressed at the expected size (~58 kDa; Fig. 6a). However, two bands were observed for strains expressing RGS4N/RGS7C. The expected 33-kDa protein was the prominent band but a slightly smaller band, which may represent a cleaved chimeric protein was also present.

3.4. Immunofluorescence

Immunoflorescence microscopy of COS cells transfected with V5 tagged RGS10 and Myc tagged RGS10S showed that both the protein had predominant localization in the cytoplasm in a diffuse manner similar to what has been reported earlier [18]. There was no difference in the distribution of RGS10 and RGS10S in the transfected cells (data not shown).

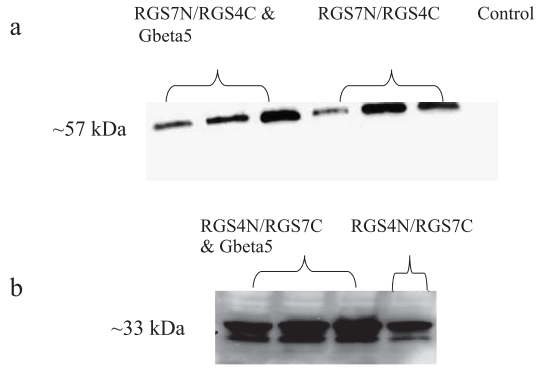

Fig. 6. Western blot analysis of chimeric RGS7 proteins. RGS7N/RGS4C chimeric proteins blotted with HA antibody (a). RGS4N/RGS7C chimeric proteins blotted with RGS7 antibody (b). Analysis shows that RGS7N/RGS4C is at the expected size (~58 kDa) but two bands were observed for strains expressing RGS4N/RGS7C. The expected 33-kDa protein was the prominent band but a slightly smaller band, which may represent a cleaved chimeric protein, was also present.

4. Discussion

Identification of small molecules for new drugs that regulate either Gα or RGS function or both requires reproducible and nonradioactive assays that permit high-throughput screening for inhibitors. A potential site of modulation is the RGS domain. RGS-box inhibitors should also have specificity among the RGS-subtypes. Here we report the extension of an HTS screen design for RGS box modulators utilizing the yeast pheromone response pathway. The described luciferase reporter gene provides a simple, quantitative, and robust assay that is amenable to HTS [6] and that could be extended to all RGS proteins.

RGS4 was among the first RGS proteins characterized [19,20] and its biochemical and cellular properties have been studied extensively. RGS4 is the prototypical member of the R4 family [13], is composed of an RGS domain flanked by minimal N and C termini, and lacks additional protein motifs. Structural features on RGS4 responsible for its membrane attachment have been identified. The N terminus of RGS4 contains a 33-amino acid cationic amphipathic α helix that drives RGS4 membrane attachment ([7,8]. RGS4 N-terminal amphipathic domain confers similar membrane binding behavior on the RGS domains of either RGS10 or RGSZ1 [8]. RGS4 is also reversibly palmitoylated near its N terminus at Cys2 and, to a lesser extent, Cys12 [7]. N-terminal palmitoylation also targets RGS4 to specialized cholesterol and glycosphingolipid-rich vesicles in vitro, and it has been suggested that reversible acylation may target RGS4 and other RGS proteins to specialized lipid rafts within the plasma membrane [21]. Studies utilizing the pheromone response assay showed that deletion of the N-terminal 33 aa of RGS4 yielded a nonfunctional protein that could be due to loss of plasma membrane localization in yeast. These functions were restored by addition of a C-terminal membrane-targeting sequence to RGS4. Thus, plasma membrane localization is tightly coupled with the ability of RGS4 to inhibit signaling [22].

Although no direct evidence has been reported demonstrating RGS4 physically binding to receptors, several lines of indirect evidence support the idea that RGS4 assembles related signaling proteins, perhaps as a stable complex with receptors [23]. The RGS4 N-terminal domain appears to function autonomously for lipid binding [7] and functions independently of the RGS domain to which it is attached. The N-terminal region of RGS4 acts primarily to support adsorption to the bilayer, the first step in positioning a GAP for regulating the receptor-stimulated, steady-state GTPase

reaction. The N-terminal domain may also be involved with subsequent reorientation of RGS4, but such an effect cannot readily be distinguished [8]. In the process of developing assays for different RGS proteins, we discovered that some RGS proteins were not functional when expressed in their native forms. To overcome this obstacle, a series of chimeric genes were developed, in which the N terminus of RGS4 was fused in frame with either full-length or RGS domains of the other subfamily members, resulting in RGS modulation of G-protein signaling.

Additionally, the pheromone response assay also was instrumental in determining that the two splice variants of RGS10 indeed function differently in yeast and thus possibly in mammalian cells. The two RGS10 splice variants differ only in the N terminus. Almost all studies published to date have investigated the 174 aa RGS10. Studies on RGS10 has shown that PKA phosphorylation at the C terminus caused its translocation to the nucleus and thus makes it unavailable to limit GPCR signals at the plasma membrane [18]. Like RGS4, RGS10 is also palmitoylated at a conserved cysteine residue in their RGS box, which inhibits the interaction of both proteins with Gα subunits [24]. However, in reconstituted receptor assays, palmitoylated RGS10 is actually a more effective GAP than the unpalmitoylated form. Authors suggest that the hydrophilic RGS10, which lacks an amphipathic helix, is not recruited to the membrane to associate with Gα in the absence of palmitate [8]. In our studies, the N terminus of RGS4 enhanced the GAP function of RGS10S, while native RGS10 was functional. Palmitoylation is unlikely to have caused the difference in the function of two RGS10 isoforms studied because palmitoylation has been reported to be occurring at aa 66 in the RGS domain [24] which is identical in both RGS10 splice variants studied. Our results showed that, in yeast, the N terminus may be playing a critical role in membrane targeting. The possibility that the unique N terminus of RGS10 showing preference for a particular receptor (in this case, Ste2 receptor of yeast) similar to that of RGS8, cannot be ruled out. Further studies using different receptors are required for definitive answer. The 9 aa in the N terminus of RGS8 contributes to functional inhibition of Gq-coupled signaling in a receptor-type-specific manner. RGS8 decreased the amplitude of the response upon activation of M1 muscarinic or substance P receptors, but did not remarkably inhibit signaling from M3 muscarinic receptors. In contrast, RGS8S (shorter RGS8) showed much less inhibition of the response of either of these Gq-coupled receptors. The subcellular distribution of RGS8 and RGS8S did not differ significantly in transfected cells [12]. We also did not see significant differences in subcellular distribution of the two RGS10 splice variants in transfected COS7 cells. Coexpression of constitutively active mutant Gαi or Gαo, along with either of RGS10 splice variants also did not make a difference (data not shown). In vitro assays with purified proteins have shown that RGS10 increased potently and selectively the GTP hydrolytic activity of several members of the G alphai family, including G alphai3, G alphaz, and G alphao [25]. Thus the other possibility of one RGS10 splice variant not being an effective GAP for yeast G alpha (Gpa1), which is more like Gαi, is less likely. Further studies with the two splice variants with different receptors and G alpha proteins would address the difference in activity observed for the two RGS10 splice variants. RGS2 and RGS4 recruitment was specific for receptors functionally linked to the target G protein and was independent of the activation state of either receptor or G protein [26]. These findings suggest that GPCRs, either alone or in coordinated effort with their linked G proteins, can selectively recruit certain RGS proteins to the plasma membrane to determine their signaling functions [27]. RGS4N/RGS10 chimera was not constructed and used in this study since native RGS10 was functional. The effectiveness of RGS10 GAP activity may be further enhanced by the addition of N terminus of RGS4 if the assay window required for HTS need to be improved further.

In the process of developing a screen for RGS blockers/modulators, we also investigated the effect of N-terminal and C-terminal combinations of RGS7 protein. Thus, domain swapping experiments were done to study if RGS4N terminus is helping in the targeting and the ineffectiveness of RGS7 observed in the assays were not due to the inability of RGS domain of RGS7 not functioning in yeast. Addition of the RGS4 N terminus to either full-length RGS7 or its C terminus (encoding amino acids 255–470) enabled functional complementation. GAP activity for the truncated version of RGS7 (RGS4N/RGS7C), which lacks the DEP domain, was better than the full-length protein. Smaller chimeric protein may have been processed and transported more efficiently to the plasma membrane than the longer full-length chimeric protein and hence more effective in yeast. RGS7 and other R7 family members are highly degraded when ectopically expressed without Gβ5, indicating that complex formation is necessary for protein stability ([17] and references there in). To study the effect of coexpression of Gβ5 on RGS7, and its influence on GAP activity, all the RGS7 constructs were tested with the presence and absence of Gβ5. Gβ5 coexpression with RGS4N/RGS7 had no effect on the halo size; however, a slight decrease in responsiveness of RGS chimera to alpha factor was seen. This same trend was observed in many experiments (see Fig. 5a also). The role of Gβ5 in augmenting endogenous Ste4 (yeast Gbeta) has been discussed elsewhere [10].

Western blot analysis of chimeric RGS7 proteins shows RGS7N/RGS4C, which has N terminus HA tag, is at the expected size (~58 kDa) but RGS4N/RGS7C showed two bands. The prominent band is of the expected size but a slightly smaller protein was also detected. This could be a cleaved product of the chimeric protein. Only further experiments, beyond the scope of current studies, can provide a definitive answer. The presence of Gβ5 did not

alter expression level, presence, or absence of double bands in the chimeras tested.

Thus, by utilizing the membrane targeting property of RGS4 N terminus, we developed a series of chimeric constructs that enabled us to develop assay that is specific for different RGS proteins. While RGS4 is the most effective RGS protein in the functional complementation of the *sst2*Δ, RGS7 and RGS10S were nonfunctional. However, addition of the RGS4 N-terminal to either the RGS7 C-terminal or the full-length RGS7 protein enabled functional complementation, possibly due to correct targeting. Similar results were observed for RGS10S. Moreover, results obtained in luciferase assay were confirmed in a halo assay. Localization is an important aspect of RGS core domain ability to regulate G-protein signaling in yeast. Thus, the N terminus of RGS4 enhanced complementation and thus GAP activity all RGS proteins investigated although to different levels which could be due to the difference in the RGS core domain itself. Further manipulation of this system will assist in understanding the functional differences of the various RGS proteins using a highly malleable system.

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