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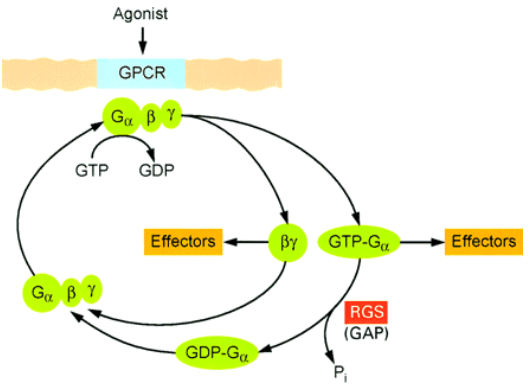

Figure 1. The GTPase cycle and role of RGS proteins in limiting the life span of GTP-bound Gα subunits.

RESEARCH LETTER

MOLECULAR CLONING AND EXPRESSION OF HUMAN REGULATORS OF G-PROTEIN SIGNALING 4, 5, AND 16

Dear *MJM*,

It has become clear, particularly over the past decade, that a relatively large family of heterotrimeric GTP-binding and hydrolyzing proteins play an essential transducing role in linking hundreds of cell surface receptors to effector proteins at the plasma membrane. These systems are widely utilized in nature, controlling processes ranging from mating in yeast to cognition in man. Receptors that activate G proteins are correspondingly diverse and encompass proteins that interact with hormones neurotransmitters, autacoids, odarants, tastants, pheromones, and photons. G-Protein Coupled Receptors (GPCRs) are septahelical integral membrane proteins that link to downstream signaling pathways through activation of heterotrimeric G-proteins; Gabg. Upon agonist binding, the receptor causes the associated Ga subunit to exchange GDP for GTP, thus activating the G-protein and causing dissociation into Ga and bg subunits. The Ga subunit has an inherent GTPase activity, which causes hydrolysis of GTP, reassociation with bg, and a return to the inactive state. The family of proteins known as Regulators of G-protein Signaling (RGS) accelerate the slow inherent GTPase activity on the Ga subunit, favoring the return of the G-protein to the inactive form (1 , Figure 1). The RGS proteins provide a mechanism by which cells can regulate both the duration and the magnitude of a signal generated through a heterotrimeric G protein. Such fine-tuning is undoubtedly essential for the orchestrated events that occur in response to chemokines, hormones and neuropeptides which signal through GPCRs.

Through understanding the biology of the signaling system, a number of diseases have been linked to altered expression of RGS proteins, including sepsis and cardiac hypertrophy (2,4,9). Sepsis and septic shock are systemic responses to infection, and afflicts more than half a million people annually in North America. Sepsis is characterized by severe systemic hypotension, peripheral vasodilation, and decreased tissue perfusion, despite elevated circulatory levels of vasoconstricting agents such as angiotensin II (AngII) and catecholamines. Since these vasoactive agonists function via GPCRs, hypotension induced by sepsis may partly be due to an increase in RGS gene expression. In fact, Panetta *et al*. (1999) have demonstrated that RGS1 and RGS16 were upregulated in the heart and aorta of septic pigs (2), while Grant *et al.* (2000) have identified that Ang II mediates RGS2 up-regulation (3). The discovery that RGS16 induction is mediated by PMA and TNF-a, a cytokine released in response to inflammation, further implicates the involvement of RGS in the pathophysiology of sepsis (4). In addition, it has been shown that RGS5 mRNA is expressed abundantly in the heart (5).

Heart failure is a condition that affects nearly five million North Americans of all ages and is responsible for more hospitalizations than all forms of cancer combined. Cardiac hypertrophy is an important adaptation in response to chronic heart failure, which in the longer term, leads to thickening of ventricular chambers and impaired contractility (6). Since decreased responsiveness of the β-adrenergic receptor (βAR) has been implicated in heart failure, it is possible that the elevated circulating levels of catecholamines that occur during heart failure may trigger an RGS mediated GPCR

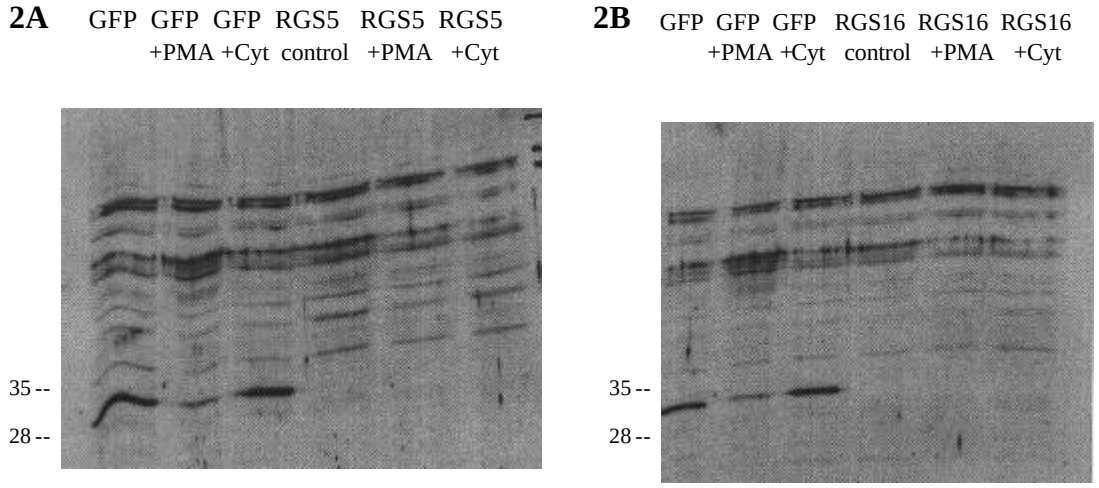

Figure 2. .Western Blot Analysis of RGS 5 (A) and RGS 16 (B) GFP fusion protein expression in SVEC-40 mammalian endothelial cells. Using anti-GFP antibody, it was determined that the RGS fusion proteins did not accumulate under both control (RGS control) and sepsis-like conditions (RGS+PMA, RGS+Cyt). As a control for transfection, the cells were transformed with the pGFP-N3 vector alone and expression of the GFP protein was measured under each of the three conditions (GFP, GFP +PMA, GFP + Cyt)

desensitization (7). Recent studies have demonstrated that RGS4 gene expression is up-regulated in cardiomyocyte hypertrophy (8), and in acutely failing donor hearts and end-stage heart failure (9).

In view of the fact that certain RGS proteins may be implicated in the inflammatory response that occurs during sepsis, the expression of green fluorescent protein (GFP) tagged RGS5 and RGS16 proteins was studied under normal and sepsis-like conditions in mammalian endothelial cells. PCR was used to amplify both RGS5 and RGS16, and primers were designed based on the coding sequences found at GenBank (10). For both RGS5 and RGS16, forward oligonucleotides contained a HindIII restriction endonuclease site and a consensus mammalian Kozak sequence 5' to the translational initiation codon. For reverse primers, a BamHI restriction endonuclease site was added to the 3'end of the RGS coding sequence, which lacked a stop codon. PCR using Taq polymerase was allowed to proceed for 20 cycles. RGS5 and RGS16 were subcloned into the pGFP-N3 vector to express the RGS proteins as GFP fusions in mammalian cells. Restriction mapping of pGFP-N3-RGS5 with HindIII/PstI and BamHI/PstI, and pGFP-N3-RGS16 with BglII and BamHI/ScaI restriction endonucleases produced bands of the expected size (not shown).

The mouse lymph node derived endothelial cell line SVEC-40 (ATCC no. CRL-2161) was transfected with the pGFP-N3-RGS5 and pGFP-N3-RGS16 plasmids using lipofectamine (Life Technnologies), and stably transfected cells were selected with 400µg/ml G418 (11). The SVEC-40 cells were grown in Dulbecco's modified Eagle's medium with 10% fetal bovine serum. Sepsis-like conditions were induced by stimulating the cells with a cytokine mix (4 hours) consisting of TNFa (10 ng/ml), IFN-g (100 µm/ml), LPS (100 µg/ml), and IL-1b (50 µm/ml). The expression of RGS was also verified under PMA treatment (0.1 mm/ml, 2 hours), which is an important mediator of the inflammatory response. Transfected cells were lysed in SDS sample buffer. Proteins were extracted by sonication and separated on 12% SDS-polyacrylimide gels. After transferring to nitrocellulose membranes, the expression of GFP tagged proteins was examined using anti-GFP monoclonal antibody as previously described (12). Western blot analysis revealed that the RGS-GFP proteins did not accumulate under either of these conditions (Figure 2). As a control for transfection, the cells were transformed with the pGFP-N3 vector alone and expression of the GFP protein was measured under each condition.

In addition, to investigate the role of RGS4 in the β-adrenergic receptor desensitization observed during cardiac hypertrophy, full length human RGS4 (618 bp) was isolated from a human fetal brain cDNA library (Clontech). The forward primer contained a BamHI restriction endonuclease site and a consensus yeast Kozak sequence linked 5' to the translation initiation codon. The reverse primers added a XhoI recognition site 3' to the translational stop codon. PCR using Taq polymerase was carried out for 35 cycles. RGS4 was subcloned as a BamHI/XhoI fragment into the polylinker of p423GAL1, placing its expression under the control of the GAL1 promoter (13).

Since a number of the mammalian RGS genes can functionally replace the endogenous SST2 gene (5,16),

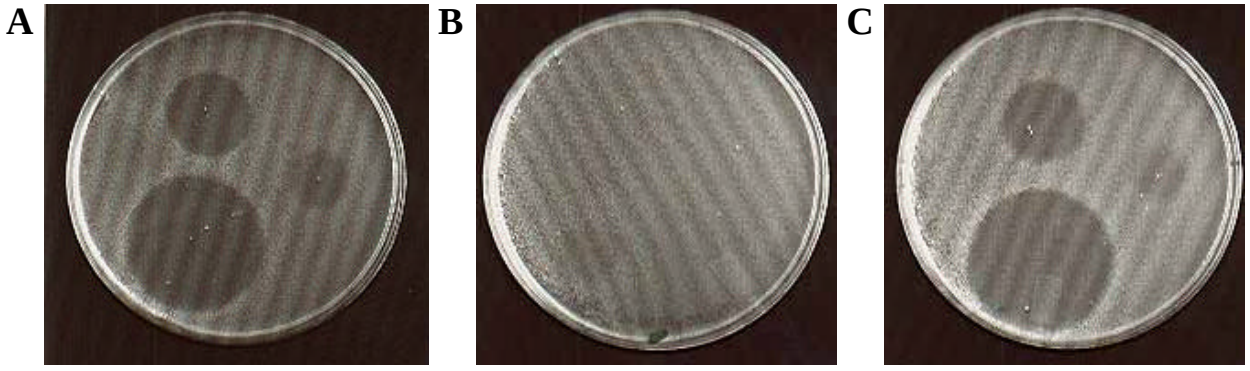

Figure 3. Yeast pheromone response halo assays. A p423GAL1construct containing full length RGS4 was transformed into BC-180 yeast cells and assessed for its ability to attenuate response to pheromone. A) p423GAL1 control plasmid. B) p423GAL1-RGS1, positive control plasmid. C) p423GAL1-RGS4. The assay was perfomed with 3 different concentrations of a-factor (10, 100, 1000 pmol, counterclockwise from right), Relative pheromone sensitivity is measure by the size of the clear area elicited in response to a given dose of α-factor.

the GAP activity of RGS4 was assessed using a yeast mutant lacking this RGS containing gene. These cells are hypersensitive to GPCR stimulation, and thus are hyper-responsive to growth inhibitory effects of a-factor pheromone (14). Thus, the Saccharomyces cerevisiae strain BC-180 (MATa, ade2-1, his 3-1D, leu 2-3, 112 ura 3-52, sst2-Δ2) was used to assess the ability of hRGS4 to complement its sst2 defect (14). Yeast cells were routinely grown on synthetic medium consisting of Yeast Nitrogen Base (YNB) containing 2% glucose supplemented with the appropriate nitrogen bases and amino acids. The construct (p423GAL1-RGS4) was introduced into yeast using lithium acetate as described ( ). The resulting transformants were selected by the omission of histidine. Negative control cells were transformed with p423GAL1 vector alone, whereas positive control cells were transformed with a p423GAL1-RGS1 construct that had been previously made (2). Replacing glucose with 2% galactose and 2% raffinose induced GAL1 dependant RGS expression. The halo assay was carried out essentially as described (16). Two μl of each of 3 concentrations of α-factor (10, 100, 1000 pmol) were spotted onto a lawn of yeast cells. The plates were incubated at 30^{o}C for 4 days. Results from the halo assay indicate that cells transformed with the plasmid p423GAL1 (negative control) showed a significant zone of no growth around the 3 concentrations of a-factor, whereas overexpression of RGS1, the positive control, significantly attenuated the pheromone response (Figure 3). However, the expression of RGS4 had little effect on the pheromone response, since p423GAL1-RGS4 transformed cells were phenotypically indistinguishable from negative control cells.

Heterotrimeric G-proteins are components of a complex membrane signaling system designed to transduce extracellular ligands into intracellular signals. RGS proteins increase the GTPase activity of Gα, thereby inhibiting its function. The expression of GFP tagged RGS proteins should permit studies of subcellular distribution in mammalian cells, since it has been demonstrated that RGS-GFP fusions maintain their GAP activity (17). Here, expression of RGS5-GFP and RGS16-GFP fusion proteins was studied in SVEC-40 cells under control and sepsis-like conditions. Since our experimental data suggests that fusion proteins did not accumulate in these cells, it is possible that the GFP-RGS fusion proteins are unstable. To address this issue, RGS5 and RGS16 will be tagged with the much smaller HA tag (9 aa), which may increase protein stability. Monoclonal anti-HA antibodies will be used to determine expression of the RGS-HA fusion proteins.

RGS4 was cloned into the yeast expression vector p423GAL1 and a pheromone response halo assay was performed as a functional assay for RGS GAP activity. Results indicated that RGS4 did not inhibit the pheromone response pathway. It is possible that the RGS4 protein may be unstable and rapidly degraded in yeast, or that the protein does not function with endogenous yeast G-proteins. However, western blot analysis to measure the level of RGS4 expression must await the availability of suitable antibodies directed to this protein. In order to generate specific antibodies directed against the RGS4 protein, an RGS4 cDNA was cloned into the TrcHisA vector. This will allow us to isolate milligrams of pure RGS4 protein that can be used as an antigen to generate RGS4 anti-sera in rabbits.

Through further studies, we would like to examine the possibility that RGS4 expression may be part of a negative feedback loop for the long-term regulation of cardiac hypertrophy. In fact, Rogers *et al*. have recently described that cardiac-specific co-expression of RGS4

in Gαq overexpressing mice, delays the cardiomyocyte hypertrophy of mice that overexpress Gαq alone (17). In addition, the laboratory of Dr. Greenwood has recently discovered that acute administration of the βAR agonist isoproterenol induces RGS5 expression in the heart.

There is increasing evidence that RGS plays a central role in the pathophysiology of sepsis and cardiac hypertrophy. Future studies examining the differential up-regulation of RGS proteins in a rodent model of catecholamine-induced myocardial hypertrophy, should clarify the mechanism that triggers βAR desensitization. It is clear that the RGS proteins will become the target for more intense investigation and pharmacological manipulation to treat critical illness.

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