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Isolation and characterization of a novel human RGS mutant displaying gain-of-function activity

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Abstract

Regulator of G protein signaling (RGS) proteins play a crucial role in the adaptation of cells to stimulation by G protein-coupled receptors via heterotrimeric G proteins. Alterations in RGS function have been implicated in a wide range of disease states, leading to many researchers focusing on controlling the action of these regulatory proteins. Previous studies have centered on reducing or inhibiting the action of RGS proteins, utilizing inactive mutants or small molecular RGS inhibitors. Here we describe the isolation and characterization of a novel human RGS4 mutant which displays enhanced or gain-of-function (GOF) activity. $RGS4^{S30C}$ demonstrates GOF activity both in an in vivo yeast-based signalling pathway and in vitro against the $G\alpha_{o1}$ subunit contained in an α_{2A}-adrenoreceptor-$G\alpha_{o1}^{C351I}$ fusion protein. Mutational analysis of serine 30 identified a number of alternative substitutions that result in GOF activity. GOF activity was retained upon transposition of the serine 30-cysteine mutation to the equivalent serine residue in human RGS16. As with previously identified GOF mutants, $RGS4^{S30C/S30F/S30K}$ demonstrate increased steady state protein levels, however these mutants also demonstrate enhanced GAP activity through an additional mechanism distinct from the increased protein content. The identification of human RGS mutants with GOF activity may provide novel therapeutic agents for the treatment of signaling-based diseases and the ability to transpose these mutations to other human RGS proteins extends their application to multiple pathways.

Keywords: RGS; Gain-of-function; GPCR; G protein

1. Introduction

A vast array of cellular responses is mediated by the stimulation, and subsequent downstream signaling, of G protein-coupled receptors (GPCRs). A key component in signal transduction through GPCRs is the heterotrimeric G protein, consisting of a Gα, a Gβ and a Gγ subunit. Stimulation of the GPCR results in exchange of GDP for GTP on the Gα subunit, generating active Gα-GTP which interacts with effectors to propagate the signal. Deactivation of Gα-GTP is achieved through the intrinsic GTPase activity of the Gα subunit. This activity is accelerated by the presence of GTPase activating proteins (GAPs) including regulator of G protein signaling (RGS) proteins [1].

RGS proteins comprise a substantial family with over 30 mammalian members [1]. These proteins, characterized by a conserved 130 residue RGS-fold, can be divided into 6 subfamilies on the basis of sequence identity and the additional domains and motifs they contain [2]. The simplest RGS proteins, comprising little more than the RGS-fold, are contained within the B/R4 family and are characterized by a conserved 33 residue N-terminal amphipathic helix implicated in membrane localization [3–5]. The prototypical member of the B/R4 family, RGS4, is a 205 residue protein with short N- and C-termini in addition to the RGS-fold, and the simplicity of this protein has led to its use in numerous studies to investigate the mode of action of RGS proteins.

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Alterations in RGS function have been implicated in a number of disease states, including cardiac disease [6], cancer [7] and schizophrenia [8]. The wide ranging effects of RGS proteins have led to many attempts to either increase or decrease their GAP activity. The majority of these studies have focused on reducing the activity of RGS proteins by isolating mutants with either reduced or absent GAP activity [9,10], and also identifying molecules capable of inhibiting RGS action [11–13]. Mutants of the human proteins RGS4 and RGS5 have been identified which demonstrate gain-of-function (GOF) activity as a result of increased protein expression/stability [14,15]. However, only one study, involving the prototypical RGS protein SST2 from *Saccharomyces cerevisiae*, has isolated an RGS mutant with enhanced activity but unaltered expression levels [16]. Here we report isolation of a mammalian RGS4 mutant which has both increased steady state protein levels and enhanced GAP activity through an additional, distinct mechanism.

Historically, the genetic amenability and tractability of yeast have played a major role in the understanding of RGS function (reviewed [17]). The simplicity and plasticity of the yeast has facilitated large-scale mutagenic screens which would not have been possible in mammalian systems [12,13]. The pheromone-response pathway of the fission yeast *Schizosaccharomyces pombe* consists of a GPCR signaling cascade containing the three main components of the signaling unit, a GPCR, a Gα subunit and a single RGS protein [18]. Each of these components can be functionally replaced with their mammalian homologues, thus allowing their actions to be studied in isolation [17,19,20].

The present study describes the isolation of a GOF mutant of the human RGS4 protein, $RGS4^{S30C}$, through a random genetic screen in *S. pombe*. Subsequent characterization utilizing both *S. pombe* and a mammalian in vitro assay demonstrated GOF activity against $G\alpha_{o1}$. Furthermore, this study also illustrates the ability to transpose the identified mutation to RGS16, another member of the B/R4 family of RGS proteins, thus allowing additional pathways to be targeted. RGS proteins are crucial regulators of GPCR signaling pathways and the ability to influence the action of these proteins utilizing GOF mutants may provide a novel basis for future therapeutic strategies.

2. Materials and methods

2.1. Yeast strains, reagents and general methods

General yeast procedures were performed as described previously [21,22]. Culture media used were yeast extract (for routine cell growth) and a defined minimal medium (for selective growth). Plates utilized were supplemented amino acid plates lacking leucine (AA), AA plates lacking uracil, and AA plates incorporating 5-fluoro-orotic acid (FOA).

2.2. Random mutagenesis of RGS4

RGS4 mutants were generated by multiple rounds of error-prone polymerase chain reaction (PCR) using *Thermus aquaticus* (*Taq*) DNA polymerase (Invitrogen) as described in Allen et al., [23]. Oligonucleotide primers (Alta Bioscience, University of Birmingham) utilized to amplify the RGS4 open reading frame (ORF) were JO917 (**ATG**TGCAAAGGGCTTGCAGGTC) with the ATG start codon shown in bold, and JO918 (**TTA**GGCACACTGAGGGACCAG) with the TTA termination anti-codon shown in bold. Mutant RGS4 ORFs were integrated into the yeast expression vector pREP3x [24] (containing the thiamine repressible *nmt1* promoter) by gap repair [25] utilizing a protocol described previously [23].

2.3. Plasmid DNA extraction from yeast

Plasmids were isolated from yeast using a protocol from Hoffman and Winston, 1987 [26].

2.4. Assay of β-galactosidase activity

β-galactosidase assays were performed utilizing a method modified from Dohlman et al., [16] as described previously [19,27]. Cell concentrations were determined using a Coulter Channelyser (Beckman Coulter). β-galactosidase activity was calculated as optical density at 420 nm (OD_{420}) per 10^6 cells [27].

2.5. Generation of RGS4 constructs containing mutations at position 30

Conversion of the serine 30 residue to cysteine, phenlyalanine, proline, lysine, alanine, glutamic acid or methionine was performed by inverse PCR utilizing a common anti-sense oligonucleotide JO2275 (TTTTTGCAGCAGGAAACCTAGCCGATGTTTCATATC), and sense oligonucleotides for the individual mutants as follows: cysteine — JO2276 (TgTGATTCCTGTGAACACAATTCTTCCCACAACAAG); phenylalanine — JO2283 (TtcGATTCCTGTGAACACAATTCTTCCCACAACAAG); proline — JO2281 (cCgGATTCCTGTGAACACAATTCTTCCCACAACAAG); lysine — JO2282 (aagGATTCCTGTGAACACAATTCTTCCCACAACAAG); alanine— JO2285 (gCgGATTCCTGTGAACACAATTCTTCCCACAACAAG); glutamic acid — JO2286 (gagGATTCCTGTGAACACAATTCTTCCCACAACAAG); and methionine — JO2284 (atgGATTCCTGTGAACACAATTCTTCCCACAACAAG). All subcloning and PCR amplifications were carried out using standard techniques and constructs were sequenced before use.

2.6. Immunoblotting of S. pombe cell lysates

Cell extracts containing RGS4 proteins were prepared from duplicate cultures of *S. pombe* cells as described previously [28]. Samples were separated by polyacrylamide gel electrophoresis (PAGE) in the presence of sodium dodecyl sulphate (SDS) under standard conditions. Separated proteins were transferred to polyvinyl difluoride (Bio-Rad). Immunoblotting was performed using a primary antibody against RGS4 (1:500 dilution; Santa Cruz, California, USA), and horseradish peroxidase-conjugated secondary antibodies (1:5000 dilution; Sigma-Aldrich Company Ltd (Poole, Dorset, UK)).

2.7. Analysis of protein expression

Protein expression levels were determined by analysis of band intensity on immunoblots utilizing TotalLab 2003 ImageQuant. Coomassie Blue stained gels of whole cell lysates were utilized as loading controls and band intensity was adjusted to account for variable loading and calculated as percentage expression compared to RGS4 which was given a value of 100%. Normalization of GOF activity was achieved by determining the fold-decrease in signaling assuming each protein was expressed at 100%.

2.8. Fluorescence microscopy of S. pombe

Cell cultures were grown in the relevant medium to a density of 5×10^6 cells ml^{-1}. 1 ml of culture was harvested and the cells were washed in growth medium. Cells were then resuspended in 50 μl of Vectashield® (Vector Laboratories). Cell suspension (3 μl) was transferred to polylysine-coated slides (Sigma-Aldrich Company) and viewed using a True Confocal Scanner Leica TCS SP2 microscope (Leica Microsystems).