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Peishen Zhao, *The University of Western Ontario*

Supervisor: Dr. Peter Chidiac, *The University of Western Ontario*
A thesis submitted in partial fulfillment of the requirements for the Doctor of Philosophy degree in Pharmacology and Toxicology
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Regulation of G Protein Signaling by GoLoco Motif Containing Proteins

(Spine Title: Regulation of G protein signaling by GoLoco motif containing proteins)

(Thesis Format: Integrated-Article)

by

Peishen Zhao

Graduate Program
in
Pharmacology and Toxicology

A thesis submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy

School of Graduate and Postdoctoral Studies
The University of Western Ontario
London, Ontario, Canada

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Thesis by

Peishen Zhao

Entitled:

Regulation of G Protein Signaling by GoLoco Motif Containing Proteins

is accepted in partial fulfilment of the
requirements for the degree of
Doctor of Philosophy

Date _____July 26 2011________________

____
Dr. R. A. Ventresca
Chair of the Thesis Examination Board

ABSTRACT

Signal transduction via heterotrimeric G proteins in response to transmembrane G protein-coupled receptors plays a central aspect in how cells integrate extracellular stimuli and produce biological responses. In addition to receptor-mediated activation of heterotrimeric G proteins, during the last few decades, accessory proteins have been found to regulate G protein activity via different mechanisms. Several proteins have been identified that contain multiple G protein regulatory domains. Using various molecular and biochemical approaches, we have characterized the effects of two such proteins, G18 and RGS14, on G protein activity. Both proteins contain a second G protein binding domain in addition to the GoLoco domain, which primarily acts as a guanine nucleotide dissociation inhibitor (GDI) on Gi/o proteins. Our results identified that the N-terminal region of G18 is a novel G protein-interacting domain, which may have distinct regulatory effects within the Gi/o subfamily and could potentially play a role in differentiating signals between these related G proteins. In addition, we characterized the tissue and cellular distribution of G18. We found that G18 is expressed in primary isolated rat aortic smooth muscle cells and endothelial cells. A protein-protein interaction assay indicated that G18 is able to directly interact with RGS5, an RGS protein that is also highly expressed in vascular tissue. This interaction results in an increase in RGS5 GTPase accelerating protein (GAP) activity with little or no effect on G18 activity. In Chapter 4 of the thesis, we identified a novel GAP enhancing domain located at the Ras-binding (RB) region of RGS14. This enhancement may be due to the intramolecular interaction between the RB domain and the RGS domain. Furthermore, this interaction may also result in an inhibitory effect on the GDI activity of the RGS14 GoLoco motif.

Overall, my work suggests that GoLoco motif containing proteins G18 and RGS14 are organizers of G protein signaling that also modulate RGS protein function.

Key words

G protein-coupled receptors (GPCR)

Heterotrimeric G proteins

Regulator of G protein signalling proteins (RGS proteins)

Gi/o-Loco interacting motif (GoLoco motif)

GTPase accelerating proteins (GAPs)

Guanine nucleotide dissociation inhibitors (GDIs)

Guanine nucleotide exchange factors (GEFs)

Protein-protein interaction

Signaling complex

CO-AUTHORSHIP

Chapter 2 of this thesis is published in the Journal of Biological Chemistry. 2010 Mar 19;285(12):9008-17. Epub 2010 Jan 22, Chapter 3 and Chapter 4 of this thesis are either submitted for publication or in preparation for submission. All cDNA constructs used were created in our laboratory except where noted in the Materials and Methods section of each Chapter. Specifically, GST-G18 constructs were provided by Dr. David Siderovski (University of North Carolina). I performed all of the experiments in Chapter 2 and Chapter 3. In Chapter 4, I performed all of the experiments except Figures 4.2, and 4.3, which were contributed by Dr. Caroline Nunn and Wendy Cladman. All the experiments were carried out in the Chidiac Laboratory in the Department of Physiology and Pharmacology at The University of Western Ontario. Dr. Chidiac provided supervision and funding for all the studies presented in this thesis.

DEDICATION

This Thesis is dedicated to my family

Heng Zhao, Ying Shen, and Bonan Liu

For always encouraging me and supporting me to become a scientist

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Throughout my PhD study, I have been encouraged and supported by many people who placed their confidence in me. I would like to express my sincere gratitude to my supervisor, Dr. Peter Chidiac, who has been a great mentor and friend over the past 5 years, and who always cheered me up during all the stressful moments. I will always be grateful for all your advice, guidance and patience. I am very thankful to my advisory committee, Dr. David Freeman, Dr. Stephen Ferguson and Dr. Shawn Li, for their continuous support and good advice. Also, I would like to acknowledge a former member in Chidiac lab, Dr. Chau Nguyen, who shared his knowledge with me, taught me many laboratory techniques and moreover, helped me to become an independent researcher. I would like to thank all the other members from Chidiac lab, Dr. Caroline Nunn, Dr. Min-xu Zou, Dr. Lylia Nini, Alina Sobiesiak and William Xue, as well as all labs and students on the 2nd floor of the Medical Sciences Building. It has been a great fun to work with all of you and thanks for all the helpful assistance and enjoyable company.

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TABLE OF CONTENTS

Certificate of Examination ... ii
Abstract and Keywords ... iii
Co-Authorship ... v
Dedication ... vi
Acknowledgements ... vii
Table of Contents ... ix
List of Figures ... xv
List of Tables ... xvii
List of Appendices ... xviii
List of Abbreviations ... xix

CHAPTER 1

1 INTRODUCTION ... 2
1.1 G protein signaling ... 2
1.1.1 Classical receptor dependent G protein signaling ... 2
1.1.2 Diversity of G proteins ... 4
1.1.3 Structural basis of Gα protein activation ... 6
1.2 Regulator of G protein signaling (RGS) proteins ... 12
1.2.1 Structural basis of RGS protein-G protein interface ... 14
1.2.2 Mechanisms of RGS protein GAP activity ... 16
1.2.3 Selective regulation of RGS proteins ... 18
1.2.4 The RGS-like subfamily of RGS proteins ... 20
1.2.5 Non-canonical function of RGS proteins ... 24
1.2.6 Overview of RGS14 ... 26
1.3 GDI proteins ... 28
1.4 GoLoco motif containing proteins ... 29
1.4.1 Identification and characterization of GoLoco motif containing proteins ... 29

1.4.2 Diversity of GoLoco motif containing proteins 31
1.4.3 Molecular basis for the GDI activity of GoLoco motif containing proteins 33
1.4.4 Regulation of GoLoco motif function 34
1.4.5 Cellular functions of GoLoco motif containing proteins 37
1.4.6 Overview of G18 41
1.5 Receptor-independent GEFs 42
1.6 Regulation of G protein signaling by proteins containing multiple G protein binding domain containing proteins. 45
1.6.1 Proteins that contain more than one molecular domain that regulates heterotrimeric G protein function. 45
1.6.2 Cross-talk between heterotrimeric G protein signaling and monomeric G protein signaling 48
1.7 Kinetic regulation of G protein activity 49
1.7.1 GEF-mediated activation of G proteins 50
1.7.2 GDI-mediated inhibition of G protein activation 51
1.7.3 GAP-mediated GTP hydrolysis 52
1.8 Experimental approaches to study the function of G protein accessory proteins 53
1.8.1 Sf9 cell/ baculovirus system 53
1.8.2 Measuring RGS protein GAP activity using GTPase assays 54
1.8.3 Measuring GEF and GDI activities using nucleotide exchange assays 55
1.9 Research goals and significance 57
1.10 References 59

CHAPTER 2

2 Chapter 2 78
2.1 CHAPTER SUMMARY 78
2.2 INTRODUCTION 79
2.3 MATERIALS AND METHODS 82
2.3.1 RNA preparation and Reverse Transcription PCR 82
2.3.2 Preparation of recombinant epitope tagged-G18 fusion proteins 82
2.3.3 Protein purification 84

2.3.4 Protein-protein interaction assay 85
2.3.5 Immunoblotting 85
2.3.6 Pre-steady state GTPase assay 86
2.3.7 GTPγS binding assay 87
2.3.8 Solution-based steady state GTPase assay 87
2.3.9 Receptor- and agonist-stimulated GTPase assay 88
2.4 RESULTS 89
2.4.1 Tissue distribution of G18 in mice 89
2.4.2 Purified G18 can interact with both inactive and fluoroaluminate-activated Gα proteins. 89
2.4.3 The N-terminal domain of G18 can interact with fluoroaluminate-activated Gα proteins 93
2.4.4 G18 has no effect on G protein GTPase activity in pre-steady state GTPase assays. 93
2.4.5 The N-terminal domain of G18 acts as a guanine nucleotide exchange factor (GEF) on Gαi1 97
2.4.6 The N-terminal domain of G18 inhibits nucleotide exchange on Gαo. 100
2.4.7 Effects of G18 on receptor- and agonist- stimulated G protein GTPase activity. 103
2.5 DISCUSSION 111
2.6 References 116

CHAPTER 3

3 Chapter 3 122
3.1 CHAPTER SUMMARY 122
3.2 INTRODUCTION 123
3.3 MATERIALS AND METHODS 127
3.3.1 Generation of G18 antibody 127
3.3.2 Cell culture and transfection 127
3.3.3 RNA Preparation and Reverse Transcription PCR 128
3.3.4 Constructs and protein purification 128

3.3.5 Purified protein pull-down assay 128
3.3.6 Protein co-immunoprecipitation from cell lysates 129
3.3.7 GTPγS binding assay 129
3.3.8 Pre-steady state GTPase assay 130
3.3.9 Receptor- and agonist-stimulated steady-state GTPase assay 130
3.4 RESULTS 132
3.4.1 Characterization of G18 antibody 132
3.4.2 Expression and localization of G18 in aortic smooth muscle cell 132
3.4.3 Protein-protein interaction between G18 and RGS proteins 135
3.4.4 Effect of G protein on G18 and RGS5 interaction 138
3.4.5 Effect of G18 and on RGS5 GAP activity 138
3.4.6 Effect of RGS5 on G18 GDI activity 141
3.4.7 Combined effects of G18 and RGS5 on steady-state GTPase activity 146
3.5 DISCUSSION 148
3.6 References 154

CHAPTER 4

4 Chapter 4 158
4.1 CHAPTER SUMMARY 158
4.2 INTRODUCTION 159
4.3 MATERIALS AND METHODS 163
4.3.1 Protein expression and purification 163
4.3.2 Receptor- and agonist-stimulated GTPase assay 163
4.3.3 GTPγS binding assay 165
4.3.4 Protein-protein interaction assay 166
4.3.5 Immunoblotting 166
4.4 RESULTS 168
4.4.1 RGS14 stimulates M2 muscarinic receptor-activated G protein GTPase activity 168
4.4.2 R14-RBD/GL increases receptor-dependent GAP activity of RGS proteins. 168

4.4.3 The enhancement of RGS protein GAP activity by RGS14 is not due to its GoLoco motif. 169
4.4.4 The RB domain region of RGS14 can directly interact with RGS proteins. 175
4.4.5 The RB domains of RGS14 are sufficient to enhance RGS protein GAP activity 180
4.4.6 Removal of the RGS domain enhances RGS14 GDI activity.......... 183
4.5 DISCUSSION 186
4.6 References 192

CHAPTER 5

5 General Discussion 196
5.1 Summary of research projects 196
5.2 Effects of GoLoco proteins on receptorstimulated G protein signaling.......... 200
5.3 Regulation of RGS GAP activity by adapter domains 203
5.4 Alternative splicing in multi-G protein binding domain containing proteins .. 205
5.5 Contributions of this thesis to the field of G protein regulation research 209
5.5.1 Physiological relevance of G18 on regulating receptor-dependent and independent G protein signaling 209
5.5.2 Physiological relevance of RGS14 on regulation of RGS protein GAP activity 214
5.6 Future directions 215
5.6.1 Further characterizing the interaction between N-terminus of G18 and G proteins 216
5.6.2 Cellular function of G18 and its splice variants 217
5.6.3 Characterizing RGS14 GAP adapter domain and enhancing domain 219
5.7 General conclusion 219
5.8 References 224

APPENDICES 228

CURRICULUM VITAE 242

LIST OF FIGURES

Figure	Title	Page
1.1	Receptor-mediated activation of G proteins	3
1.2	Regulation of G protein cycle	5
1.3	Regulation of systemic functions by signaling through G protein pathways	7
1.4	Structural features of heterotrimeric G proteins	9
1.5	Heterotrimeric G protein activation by G-protein-coupled receptors	11
1.6	Mechanisms of GTP hydrolysis by Gα	13
1.7	Structures and Classification of mammalian RGS protein	15
1.8	RGS-domain interactions with the Gα all-helical domain	17
1.9	Diversity of GoLoco motif containing proteins	32
1.10	The GoLoco region of RGS14 interacts with Gαi1	35
1.11	Comparable signal transduction complexes, centered around Pins family members, regulate asymmetric cell divisions and mammalian cell division.	40
2.1	Amino acid sequence and tissue distribution of G18	91
2.2	Constructs and purified proteins	92
2.3	Protein-protein interaction between G proteins and G18	94
2.4	Protein-protein interaction between G proteins and the N-terminus of G18	96
2.5	The effects of G18 on G protein GTPase activity under pre-steady state conditions	99
2.6	The effects of G18 on G protein nucleotide exchange	102
2.7	The effects of G18 on Gα protein GTPase activity under steady state conditions	105
2.8	The effects of G18 on receptor- and agonist- stimulated G protein GTPase activity	109
2.9	The effects of G18 mutants on receptor- and agonist-stimulated G protein GTPase activity	110

3.1	Generation and characterization of G18 antibody	134
3.2	Expressions of G18 in primary aortic smooth muscle cells and endothelial cells	137
3.3	Protein-protein interaction between G18 and RGS5 proteins	139
3.4	Effect of G protein on G18-RGS5 interaction	140
3.5	Effect of RGS5 on G18 GDI and GEF activity	143
3.6	Effects of G18 on RGS5 GAP activity	145
3.7	Combine effects of G18 and RGS proteins on agonist- and receptor-dependent Gαi1 GTPase activity	147
4.1	Diagram of constructs used in this study	164
4.2	Effect of RGS14 on M2 muscarinic receptor stimulated GTPase activity	171
4.3	Effect of R14-RBD/GL on RGS4 GAP activity	173
4.4	Effect of R14-RBD/GL on steady-state GTPase activity	174
4.5	R14-RB domains enhance the GAP activity of RGS proteins on Gα proteins	177
4.6	Protein-protein interaction between R14-RB domains and RGS proteins	179
4.7	Effect of isolated RGS14 RB domains on RGS4 GAP activity	182
4.8	Effect of the RGS domain on RGS14 GDI activity	185
5.1	Co-localization between G18 and Gi1	198
5.2	Amino acid sequence of G18 and potential splice variant	207
5.3	The effect of G18-short (G18-s) on nucleotide exchange	208
5.4	Expression of G18 in WKY and SHR cells	211
5.5	Co-localization between G18 and β-tubulin during interphage	220
5.6	Co-localization between G18 and β-tubulin during Mitosis	222

LIST OF TABLES

Table	Title	Page
1.1	Classes of Gα subunits, their expression patterns and effectors	8
1.2	Selectivity of interactions between mammalian RGS proteins and G proteins	21
2.1	Primers used for making His-G18 and its mutants	83

LIST OF APPENDICES

Title	Page
Appendix A	
Certificate of Radiation Safety Training	229
Certificate of WHIMS Training	230
Appendix B	
Permissions to reprint Figures	231

LIST OF ABBREVIATIONS

Abbreviation	Full Name
AC	Adenylyl cyclase
ACD	Asymmetric cell division
AGS	Activator of G protein signaling
AR	Adrenergic receptor
BRET	Bioluminescence resonance energy transfer
cAMP	Cyclic adenosine monophosphate
DAG	Diacylglycerol
DEP	Dishevelled, worm EGL-10, and mammalian Pleckstrin
eIF	Eukaryotic translation initiation factor
ERK	Extracellular signal-regulated protein kinase
GAIP	G alpha interacting protein
GAP	GTPase accelerating protein
GDI	Guanine nucleotide dissociation inhibitor
GDP	Guanosine 5’-diphosphate
GEF	Guanine nucleotide exchange factor

GGL	G gamma like
GIRK	G protein-activated inwardly rectifying K^+ channels
GoLoco	Gi/o-Loco interacting
GOS	G0/G1 switch genes
GPCR	G protein-coupled receptor
GPR	G protein regulatory
GPSM	G-protein signaling modulator
GRK	G protein-coupled receptor kinases
GST	Glutathione-S-transferase
GTP	Guanosine-5'-triphosphate
HEK	Human embryonic kidney
IBMX	Isobutyl-methyl-xanthine
IP3	Inositol trisphosphate
LKB	Serine/threonine kinase 11
M_2R	M2-muscarinic acetylcholine receptor
Ngb	Neuroglobin
NMR	Nuclear magnetic resonance

PDZ	PSD95, Dgl and ZO-1/2
PKA	cAMP-dependent protein kinase
PKC	Protein kinase C
PLC	Phospholipase C
PTB	Protein tyrosine binding
RBD	Ras binding domain
RGS	Regulator of G protein signaling
RH	RGS homology
Ric	Resistance to inhibitors of cholinesterase

Chapter 1

INTRODUCTION

1 INTRODUCTION

1.1 G PROTEIN SIGNALING

1.1.1 CLASSICAL RECEPTOR DEPENDENT G PROTEIN SIGNALING

G protein-coupled receptors (GPCRs) comprise the largest family of cell surface receptors (Lagerstrom and Schioth, 2008). Over 60% of drugs currently available on the market target GPCRs, acting to mimic or block endogenous ligands or to modulate ligand levels (Leurs *et al.*, 1998;Gesty-Palmer and Luttrell, 2008). GPCRs transduce extracellular signals into the cell via heterotrimeric GTP binding proteins (G proteins). Heterotrimeric G proteins consist of the three subunits α, β, γ. The Gα subunit contains the nucleotide binding site, and in the inactive state, Gα is associated with GDP and Gβγ subunits which normally form a complex. GPCRs can be activated by different stimuli such as hormones, neurotransmitters and chemokines (Neves *et al.*, 2002). Binding of an activating ligand or agonist to the receptor triggers a conformational change which leads to nucleotide exchange and activation of the Gα subunit of the G protein (GTP for GDP). This also is thought to cause dissociation of the Gβγ dimer from the Gα subunit. Both activated Gα (GTP-bound) and Gβγ are able to activate downstream effectors including adenylyl cyclase (AC) and phospholipase Cβ (PLC-β) (Neves *et al.*, 2002;Smrcka, 2008). G protein activation is terminated by the intrinsic GTPase activity of Gα, which hydrolyzes GTP to GDP, returning Gα to its inactive (GDP-bound) state and reforming the Gαβγ heterotrimer *(Figure 1.1)*. After activation, GPCRs can undergo internalization, and many can signal from endosomal compartments via G protein-independent mechanisms. This process appears to be regulated primarily by phosphorylation and arrestin binding (Marchese *et al.*, 2008).

Figure 1.1

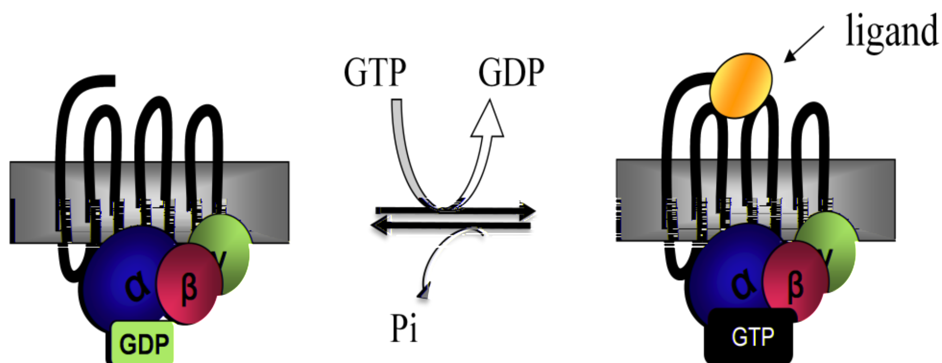

Fig. 1.1. Receptor-mediated activation of G proteins. The binding of the extracellular ligand to the receptor causes a conformational change of the receptor, which leads to the activation of the Gα subunit. This activation promotes the exchange of GTP for GDP and is thought to cause the dissociation of Gβγ dimer from the complex. Both the GTP-bound Gα and free Gβγ are capable of initiating signals by interacting with downstream effectors. The process is terminated by the GTPase activity of the Gα subunit which can hydrolyze GTP to GDP, returning the Gα subunit to its inactive form and reforming the Gαβγ complex.

Diverse mechanisms exist to precisely regulate the magnitude and duration of G protein signaling. Nucleotide exchange can be modulated by guanine nucleotide exchange factors (GEFs) and guanine nucleotide dissociation inhibitors (GDIs), whereas the GTPase activity of Gα subunit can be enhanced by GTPase accelerating proteins (GAPs) (Siderovski and Willard, 2005). Regulation of G protein signaling by these accessory proteins will be discussed below (sections 1.2, 1.3, 1.4 and 1.5) *(Figure 1.2).*

1.1.2 Diversity of G proteins

To date, 23 α subunit, 7 β subunit and 12 γ subunit isoforms have been identified (McIntire, 2009). Based on their sequence similarities and effector selectivities, Gα subunits can be divided into four subfamilies (Gs, Gi/o, Gq, G12/13) (Neves *et al.*, 2002). Gs proteins (Gαs, Gαolf) can stimulate AC activity, increasing cyclic adenosine monophosphate (cAMP) production. This second messenger in turn can regulate activities of cAMP-dependent protein kinase (PKA) or exchange proteins directly activated by cAMP (Epac). In addition to these two major effectors, cAMP is also able to activate cyclic nucleotide-gated ion channels and certain phosphodiesterases (Weinstein *et al.*, 2004). Gi/o family members (Gαi1, Gαi2, Gαi3, Gαo, Gαz, Gαt) exhibit inhibitory effects on AC activity. Gi/o proteins may also regulate c-Src activity and Rap pathways, however, the physiological consequences of these functions still remain to be established (Weissman *et al.*, 2004;He *et al.*, 2005). The primary effector for Gq (Gαq, Gα11, Gα14, Gα15, Gα16) is PLC-β, the activation of

Figure 1.2

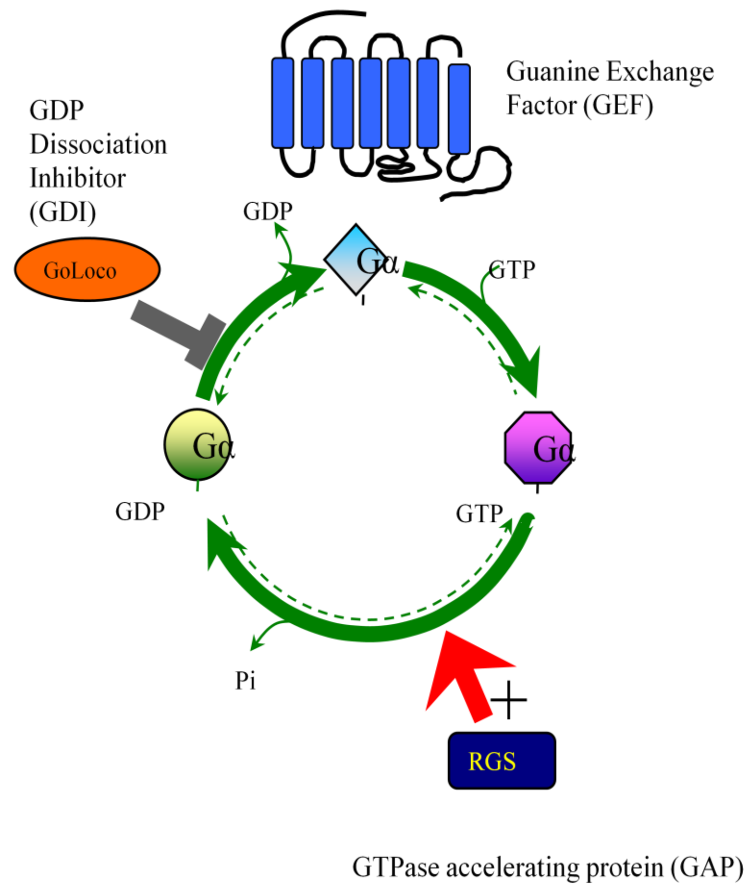

Fig. 1.2. Regulation of G protein cycle. The rate of nucleotide exchange can be altered by guanine nucleotide dissociation inhibitors (GDIs) and guanine nucleotide exchange factors (GEFs). GTP hydrolysis can be regulated by GTPase accelerating proteins (GAPs).

which leads to the production of inositol trisphosphate (IP3) and diacylglycerol (DAG), and further regulates Ca^{2+} release as well as protein kinase C (PKC) activity (Mizuno and Itoh, 2009). The cellular targets for G12/13 (Gα12, Gα13) are not yet fully established. It has been shown that G12/13 can regulate Rho/Rho-kinase activation via RhoGEFs (e.g. p115RhoGEF) and further play a role in cell migration (Bian *et al.*, 2006;Suzuki *et al.*, 2009). There are 7 β subunits and 12 γ subunits. Upon the activation of GPCRs and rearrangement of G protein subunits, Gβγ is able to activate a large number of its own effectors; however, the mechanism of Gβγ interaction with its effectors is not well understood (McCudden *et al* 2005) *(Figure 1.3, Table 1.1)*.

1.1.3 STRUCTURAL BASIS OF GALPHA PROTEIN ACTIVATION

All Gα proteins have structures composed of two domains, a GTPase domain and a helical domain *(Figure 1.4)* (Oldham and Hamm, 2006a). The GTPase domain shares homology with the family of monomeric G proteins, and the three flexible loops (Switches I, II and III) within this domain undergo dramatic structural changes during the nucleotide exchange and hydrolysis cycle (Lambright *et al.*, 1994;Mixon *et al.*, 1995). The GTPase domain also contains binding sites for Gβγ, receptors and effectors. The helical domain of the Gα subunit contains an α-helical lid over the nucleotide-binding site, burying the bound nucleotide in the core of the protein (Lambright *et al.*, 1994;Warner *et al.*, 1998). Since this domain is the most divergent among the four Gα subfamilies, it may also regulate coupling of specific G proteins to receptors, and other regulators (Liu and Northup, 1998).

Figure 1.3

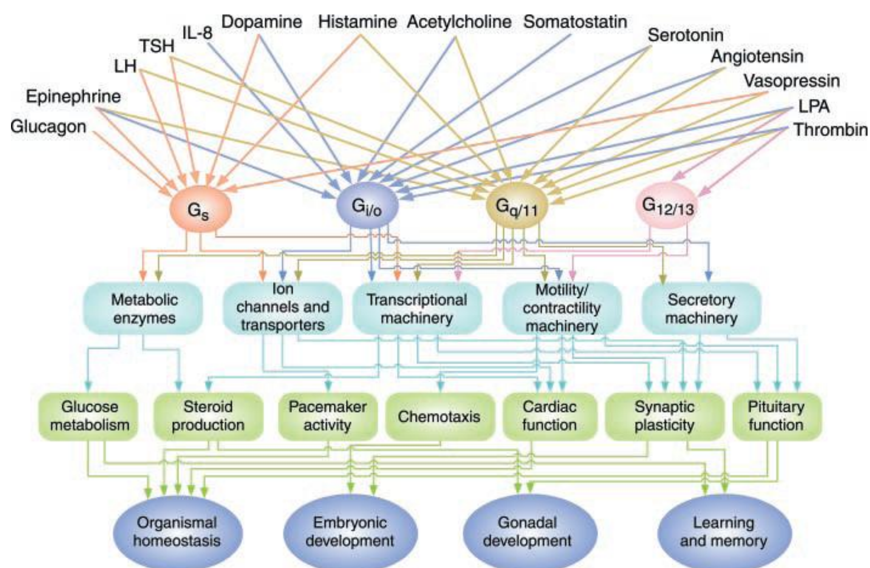

Fig. 1.3. Regulation of systemic functions by signaling through G protein pathways. A schematic representation of how signaling through G protein pathways can regulate systemic functions. Reproduced from Neves *et al.*, 2002.

Table 1.1

Members of the Gα family, their expression pattern and their signaling model			
Gα class		expression	effector proteins
Gαs	Gαs	Ubiquitous	stimulation of adenylyl cyclase
	Gαolf	Olfactory neurons	
Gαi	Gαi1/2/3	Ubiquitous	inhibition of adenylyl cyclase
	GαoA/B	Brain	
	Gαt1/2	retina	stimulation of cGMP-phosphodiesterase
	Gαz	Brain/platelets	K^+ channel closure
Gαq/11	Gα15/16	Hematopoietic cells	stimulation of PLCβ
	Gαq/11	Ubiquitous	
	Gα14	Lung, kidney, liver	
Gα12/13	Gα12/13	Ubiquitous	Rho GEF

Table 1.1. Classes of Gα subunits, their expression patterns and effectors.

Figure 1.4

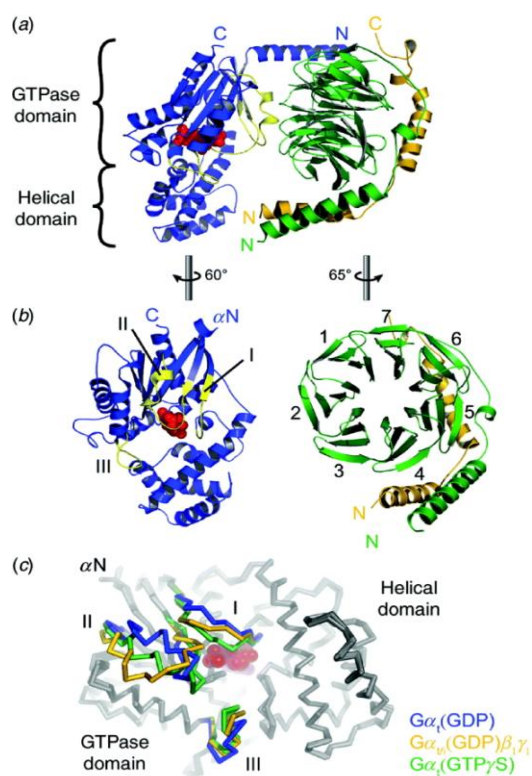

Fig. 1.4. Structural features of heterotrimeric G proteins. (a) Ribbon model of $G\alpha_{t/i}(GDP)\beta_1\gamma_1$ heterotrimer (1GOT). (b) The subunits have been rotated to show the intersubunit interface. (c) When the GDP-bound (1TAG), GTPγS-bound (1TND) and heterotrimeric (1GOT) structures of Gα are aligned. Reproduced from Oldham *et al.*, 2006a.

Although receptor-stimulated GDP release is not fully understood, several potential mechanisms have been proposed (Oldham *et al.*, 2006b) *(Figure 1.5)*. These mechanisms involve the α5-helix (Marin *et al.*, 2000;Ceruso *et al.*, 2004), β6-strand (Onrust *et al.*, 1997) and intramolecular contacts within the N-terminus of Gα (Natochin and Artemyev, 2000). It has been suggested that upon activation, the receptor uses the N-terminus helix of Gα to "pull" the Gβγ complex and switches I and II away from the nucleotide binding pocket, resulting in GDP release (Iiri *et al.*, 1998). Due to the high cellular GTP concentration (both absolute concentration and relative concentration compared to GDP), Gα will most likely exist in a very transient nucleotide-free state before the binding of GTP. This binding causes a structural rearrangement of the heterotrimeric G protein which exposes the effector binding site and leads to signal transduction. In addition, the binding of GTP to Gα can be facilitated and stabilized by Mg^{2+}, which has been suggested to act as a keystone locking the Gα in a conformation that favors effector binding. Binding of Mg^{2+}-GTP upon the release of GDP is followed by the clamping of the Mg^{2+}-GTP complex into place where Mg^{2+} is coordinated with Ser and Thr residues in the switch I region and the α1 helix of the GTPase domain of Gα (Birnbaumer and Zurita, 2010).

Structural studies on the mechanism of GTP hydrolysis have demonstrated the importance of the three switch regions (Sondek *et al.*, 1994;Coleman *et al.*, 1994) *(Figure 1.6)*. Inspection of the crystal structure of AlF_4^--activated Gα reveals the

Figure 1.5

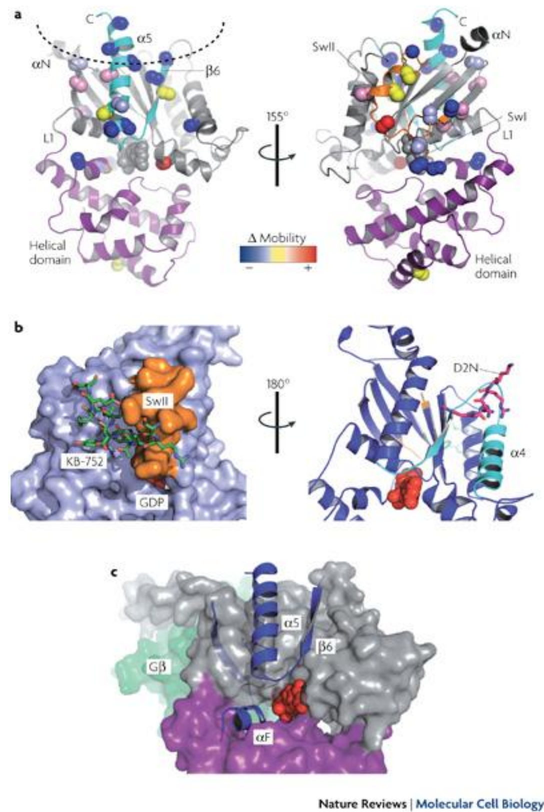

Fig. 1.5. Heterotrimeric G protein activation by Gprotein-coupled receptors. (a) Several regions in the Gα subunit show receptor-mediated changes in mobility on receptor binding. (b) Peptides may mimic receptor-mediated conformational changes in Gα, as shown in the model of $G\alpha_{i1}$ bound to the KB-752 and D2N peptides (Protein Data Bank ID 2HLB). (c) Transparent surface model of the Gi heterotrimer with the α5–β6 and SwI–αF motifs shown as ribbons. Reproduced from Oldham and Hamm, 2008.

functional role of conserved glutamine and arginine residues within the nucleotide binding pocket (Noel *et al.*, 1993;Coleman *et al.*, 1994). The intrinsic GTP hydrolysis activity varies among different Gα proteins (WM Oldham, HE, Hamm 2006). The mechanisms of these differences have not been studied in detail, however, the relatively slow GTPase activity of Gα can be stimulated by GAPs.

1.2 Regulator of G protein signaling (RGS) proteins

The duration of G protein signaling is determined by the length of time that the Gα subunit is in its GTP-bound state (Ross and Wilkie, 2000). Originally, GTP hydrolysis was considered to be an unregulated function of the Gα subunit, which has intrinsic GTPase activity (Brandt and Ross, 1985). However, several groups have identified the inconsistency between the rapid G protein signal inactivation rates *in vivo* and relatively slow GTP hydrolysis rate *in vitro* (Breitwieser and Szabo, 1988;Vuong and Chabre, 1991). This suggests the existence of a mechanism *in vivo* that can stimulate the GTPase activity of Gα and accelerate GTP hydrolysis. So far, regulator of G protein signaling (RGS) proteins are one of the most well understood proteins that serve such a role.

SST2, Egl-10 GOS8 (renamed RGS2), and GAIP (RGS19), were among the first RGS proteins identified in the mid 90s (Siderovski *et al.*, 1994;De Vries *et al.*, 1995;Koelle and Horvitz, 1996;Apanovitch *et al.*, 1998). Since then, more RGS proteins have joined the family, and so far, 20 distinct genes in mammals have been found encoding RGS proteins (Abramow-Newerly *et al.*, 2006). They can be grouped into four

Figure 1.6

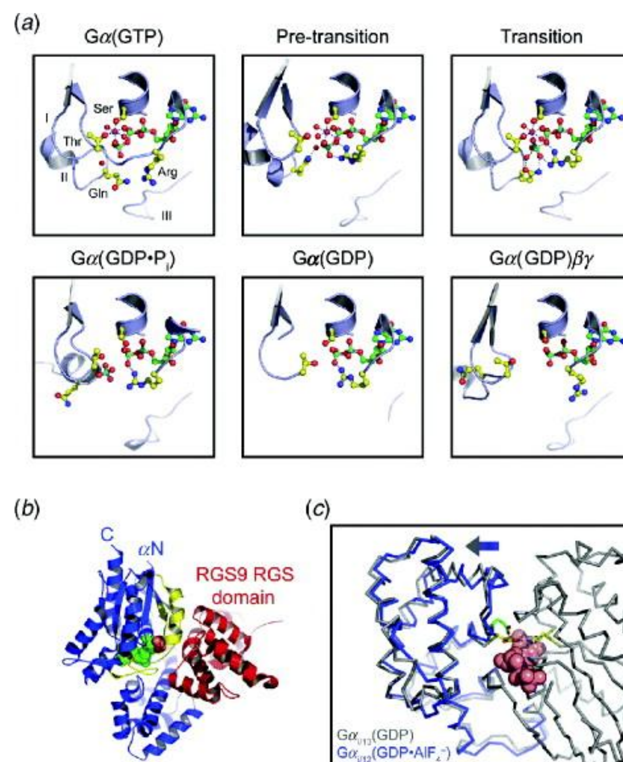

Fig. 1.6. Mechanisms of GTP hydrolysis by Gα. (a) Crystallographic snapshots of GTP hydrolysis. Four important residues for stabilizing the transition state are shown (b) Structure of the RGS9 RGS domain binding to $G\alpha_t(GTP{\cdot}AlF_4^-)$ (1FQK). The RGS proteins enhance the basal GTP hydrolysis rate of Gα subunits by stabilizing the transition state. (c) The recently solved structures of $G\alpha_{i/12}(GTP{\cdot}AlF_4^-)$ (1ZCA) and $G\alpha_{i/13}(GDP)$ (1ZCB) suggest that GTP hydrolysis leads to an 8.5° rotation of the helical domain away from the GTPase domain in this family of Gα subunits (arrow). Reproduced from Oldham *et al.*, 2006a.

subfamilies based on their sequence similarity in the RGS domain (R4/B, RZ/A, R7/C, and R12/D). In addition, there are also a similar number of "RGS like" proteins, and some of these proteins exhibit GAP activity (Ross *et al.*, 2000;Abramow-Newerly *et al.*, 2006) *(Figure 1.7)*. All the RGS proteins share a signature RGS domain (120 amino acids). Although many RGS proteins are relatively simple proteins that contain little more than a RGS domain, there are also some RGS proteins that contain signaling domains other than the RGS domain, thus RGS proteins may also have non-canonical functions distinct from inactivating Gα subunits (Sethakorn *et al.*, 2010).

1.2.1 Structural basis of RGS protein-G protein interface

RGS proteins bind to the active/transition state of Gα proteins, and increase the rate of GTP hydrolysis up to 2000 fold (Mukhopadhyay and Ross, 2002). Thus, the GTPase activities of Gα measured in the presence of RGS proteins *in vitro* correspond well to or exceed the cellular deactivation rate (Ross *et al.*, 2000). The molecular and structural mechanism of RGS protein GAP activity has been well studied. The classic RGS domain consists of 9 α-helices bundled into two lobes, one formed by helices αI, αII, αIII, αVIII, and αIX. The other lobe consists of the αIV, αV, αVI, and αVII helices. (Tesmer *et al.*, 1997;Soundararajan *et al.*, 2008). This canonical structure is shared by most RGS domains, however, it has also been suggested that members from the R12 subfamily (RGS10, RGS12, RGS14) may have an extended αV-αVI loop compared with the other three families. Furthermore, they do not contain a complete αVI helix, which shows some flexibility (Soundararajan *et al.*, 2008).

Figure 1.7

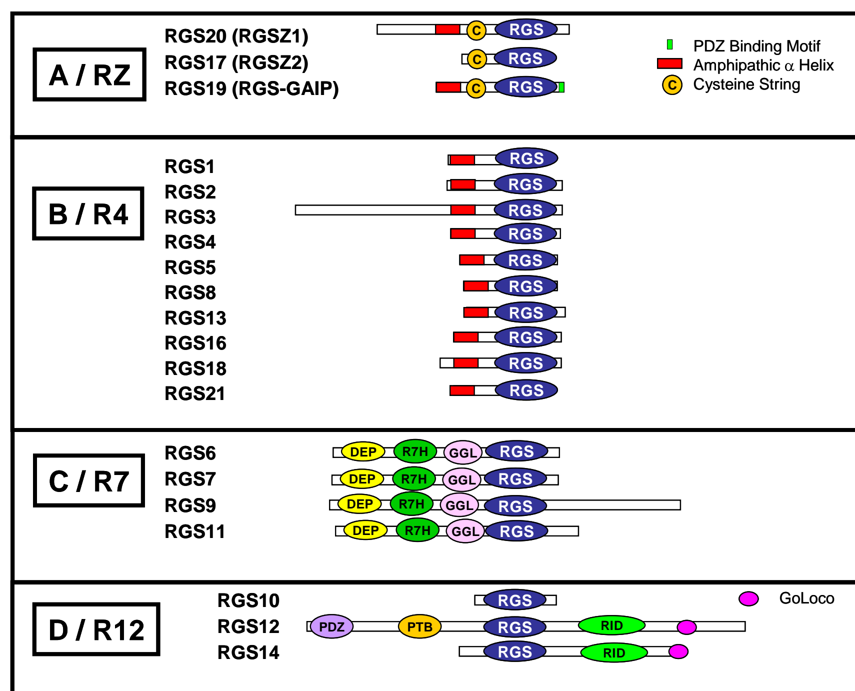

Fig. 1.7. Structures and classification of mammalian RGS protein. RGS proteins are classified into subfamilies A-D based on alignment of RGS domain amino acid sequences. Note that RGS-like proteins are not listed here. Abbreviations: RGS: regulator of G protein signaling; DEP: Dishevelled, worm EGL-10, and mammalian Pleckstrin; R7BP: R7 binding proteins; GGL: G gamma like; PDZ: PSD95, Dgl and ZO-1/2; PTB: protein tyrosine binding; RID: Ras interaction domain. Adapted from Hollinger and Hepler, 2002.

The typical G protein-RGS protein interface has also been solved by both NMR and crystallography (Tesmer *et al.*, 1997;Moy *et al.*, 1999). These studies highlighted interactions between the RGS domain and G proteins that are important for the GAP activity. For example, the αIII-αIV loop interacts with the switch II region of Gα, while the αV-αVI loop, and αVI helix interact with all three switch regions of Gα, and the αVII, αVIII helices and transition region interact with switch I of Gα (Soundararajan *et al.*, 2008). However, due to the different structure of R12 RGS domains, their interactions with G proteins are likely to vary. For example, the amino acids within αVI helix of RGS10 are disordered, thus it does not retain the typical interactions between the switch III region of the G protein and the αVI helix of the RGS domain (Soundararajan *et al.*, 2008). The selectivity between different RGS proteins and G proteins is likely determined by the interaction between the αVII and αVIII helices of RGS domains and the Gα all-helical domain (Skiba *et al.*, 1999;Soundararajan *et al.*, 2008) *(Figure 1.8).*

1.2.2 MECHANISMS OF RGS PROTEIN GAP ACTIVITY

The mechanism of RGS protein GAP activity was first studied using RGS4-Gαi1 as a prototype (Tesmer *et al.*, 1997;Srinivasa *et al.*, 1998). From these studies, the authors concluded that RGS4 stimulates GTP hydrolysis primarily, if not exclusively, by binding to and stabilizing the transition state conformation of the Gα subunit that is most likely to hydrolyse GTP. Moreover, the crystal structure of RGS4 bound to transition state Gαi1 (i.e. with GDP and AlF_4^- in the binding pocket) provided more detailed information regarding the interaction between RGS protein and G protein. These results indicated that RGS4 does not directly interact with either GDP or AlF_4^-, instead, RGS4 catalyzes

Figure 1.8

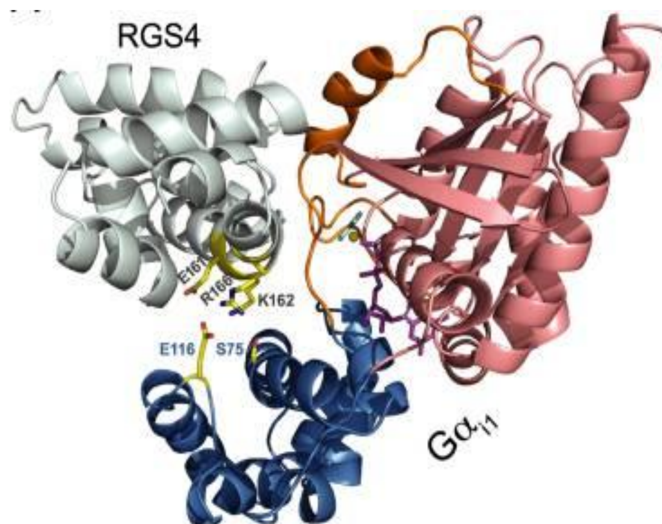

Fig. 1.8. RGS-domain interactions with the Gα all-helical domain. RGS4/Gα_{i1} complex (PDB ID 1AGR). Glu-161, Lys-162, and Arg-166 in the RGS4 αVII helix are within 4.0 Å of the Gα_{i1} all-helical domain residues Ser-75 or Glu-116. Reproduced from Soundararajan *et al.*, 2008.

GTP hydrolysis by reducing the energy of the transition state of the Gα subunit and destabilizing the Gα-GTP complex (Tesmer *et al.*, 1997). Several studies have highlighted the importance of an asparagine residue (Asn-128) within the RGS domain of RGS4. This is the only residue positioned at the active site of Gα, and it interacts with a glutamine residue (Gln-204) of Gαi1 that polarizes the attacking water molecule in the GTPase reaction (Tesmer *et al.*, 1997;Srinivasa *et al.*, 1998;Xie and Palmer, 2007). Subsequent studies using other RGS-Gα complexes such as RGS16/Gαt and RGS9/Gαt (McEntaffer *et al.*, 1999;Slep *et al.*, 2001), p115-RhoGEF/Gα13/i1 (Chen *et al.*, 2005) and RGS1, GAIP/Gαi/o (Watson *et al.*, 1996) also confirmed that the RGS domains bind to and stabilize the flexible (switch) regions of Gα during the transition state of GTP hydrolysis.

1.2.3 Selective regulation of RGS proteins

The selectivity of RGS proteins is dependent on sequence elements both within and outside of the RGS domain as well as the helical domain of the Gα protein (De Vries *et al.*, 2000). Most RGS proteins studied so far are GAPs for the Gαi/o and Gαq subfamilies of G proteins. However, their affinity toward different G proteins varies. For example, RGS19 binds with high affinity to Gαi1, Gαi3 and Gαo, very weakly with Gαi2, and it does not appear to interact at all with Gαs and Gαq (De Vries *et al.*, 1996). On the other hand, RGS4 interacts strongly with both Gαi/o and Gαq (Xu *et al.*, 1999). Comparison between the crystal structures of these two RGS proteins indicated that RGS19 has a Ser (156) at the position corresponding to the Asn128 position in RGS4, which may contribute to the observed difference in G protein selectivity (Tesmer *et al.*,

1997;De Alba *et al.*, 1999). So far, there is only one RGS protein that has been reported to selectively interact with Gαs, RGS-PX1, however, this remains to be confirmed independently by other laboratories (Zheng *et al.*, 2001) *(Table 1.2)*.

Besides the amino acid residues located within the RGS domain, the N-terminal regions of RGS proteins also serve as important determinants for their selectivity. Members from different RGS subfamilies contain N-terminal regions with diverse structural features. Members of the B/R4 subfamily of RGS proteins each have an amphipathic α-helix of about 30 amino-acid residues in length with several palmitoylation sites (Somerville *et al.*, 2003), the A/RZ subfamily RGS proteins have a cysteine-rich domain referred to as a cysteine string motif (Nunn *et al.*, 2006), whereas in other RGS subfamilies, molecular domains such as the DEP domain (C/R7 subfamily) or PDZ domain (RGS12) are near the N-terminus of the protein. The N-terminal domain of RGS proteins regulates selectivity by either mediating RGS protein sub-cellular localization or making direct contact with specific GPCRs or effector proteins (Xie *et al.*, 2007). For example, deletion of the N-terminus of RGS2 (1-67 aa) greatly reduces its plasma membrane and nuclear localization, as well as its biological activity (Heximer *et al.*, 2001). A selective interaction between the N-terminus of RGS4 and the muscarinic acetylcholine receptor also determines RGS4 selectivity on the muscarinic acetylcholine receptor over the cholecystokinin receptor (Zeng *et al.*, 1998).

The biological function and selectivity of RGS proteins are also regulated by their tissue distribution and alternative gene splicing. Although all RGS proteins share a similar

RGS domain, there are very different tissue expression patterns among different RGS proteins. For example, RGS2 has a relatively ubiquitous expression pattern, which suggests a more general function (De Vries *et al.*, 2000). In contrast, RGS9-1, is selectively expressed in retina, while its splice variant RGS9-2 is expressed in brain (He *et al.*, 1998;Kim *et al.*, 2005). RGS5 is highly expressed in vascular tissues especially pericyte and endothelial cells, with lower expression in skeletal muscle and kidney tissues (Jin *et al.*, 2009). RGS21 is the newest member of the RGS protein family, it is expressed in the taste bud cells and selectively interacts with G gustducin (Von Dannecker et al., 2004;Li et al., 2005) *(Table 1.2)*. The relatively narrow tissue expression of these RGS proteins hints that they may have specialized roles.

1.2.4 THE RGS-LIKE SUBFAMILY OF RGS PROTEINS

Besides proteins that contain a typical RGS domains that function as a GAP, there is another group of proteins that contains a RGS-like (RGL, or RH (RGS homology)) domain, which is more distantly related to the RGS domains (Hollinger *et al.*, 2002;Abramow-Newerly *et al.*, 2006;Tesmer, 2009). Proteins containing an RGL domain include Axins, D-AKAPs (dual specificity Akinase anchoring proteins), p115RhoGEFs, RGS-PX1, GRKs (G protein receptor kinases) and PLC-β (Ross *et al.*, 2000;Hollinger *et al.*, 2002). Only a few of these homologous domains have been shown to interact with Gα proteins, for example, GRK2, GRK3 and RhoGEFs, but the GAP activities of most of these proteins are modest (Ross *et al.*, 2000;Tesmer, 2009). The fact that RGL domains have been found in proteins that are involved in different signaling

Table 1.2

RGS protein	**Tissue**	**G protein Targets**	**Reference**
RGS1	Retina B cell;Rods	Go	(Hoffmann *et al.*, 2001) (Bansal *et al.*, 2007)
RGS2	Ubiquitous	Gq, Gi/o, Gs	(Xu *et al.*, 1999) (Kimple *et al.*, 2009) (Gu *et al.*, 2008) (Roy *et al.*, 2006)
RGS3	Ubiquitous	G11, Gi3	(Dulin *et al.*, 1999)
RGS4	CNS, Heart	Gi/o, Gq, Gz	(Xu *et al.*, 1999) (Inanobe *et al.*, 2001) (Cavalli *et al.*, 2000) (Berman *et al.*, 1996) (Tu *et al.*, 2003)
RGS5	Vascular	Gi/o, Gq	(Gu *et al.*, 2009)
RGS6	Brain, Spinal cord	Gi/o	(Anderson *et al.*, 2009) (Posner *et al.*, 1999)
RGS7	Brain	Gi/o	(Anderson *et al.*, 2009) (Posner *et al.*, 1999)
RGS8	Brain, Testis, Thyroid gland	Gi/o, Gq	(Kurogi *et al.*, 2009) (Bansal *et al.*, 2007) (Benians *et al.*, 2004) (Saitoh *et al.*, 2002)
RGS9	Retina, Rods	Gi/o, Gt	(Anderson *et al.*, 2010)

			(Skiba *et al.*, 2000) (He *et al.*, 2000)
RGS10	Leukocyte Platelet	Gi3/o/,Gz	(Wu *et al.*, 2005)
RGS11	Brain, Pancreas, Retina	Go	(Anderson *et al.*, 2009) (Masuho *et al.*, 2010)
RGS12	Brain	Gi/o	(Snow *et al.*, 1998)
RGS13	T lymphocytes, B lymphocytes Mast cells	Gi/o, Gq	(Shi *et al.*, 2002)
RGS14	Brain, Lymphocyte	Gi/o, G12/13	(Hepler *et al.*, 2005) (Cho *et al.*, 2000)
RGS16	B cells	Gi/o, Gq	(Hoffmann *et al.*, 2001) (Slep *et al.*, 2008) (Anger *et al.*, 2004)
RGS17	Brain	Gi/o, Gq, Gz	(Mao *et al.*, 2004) (Nunn *et al.*, 2006)
RGS18	Brain, Spleen		
RGS19	Atrial myocytes	Go, Gi3, Gq	(Tu *et al.*, 2003) (Woulfe and Stadel, 1999) (Hepler *et al.*, 1997) (Huang *et al.*, 1997) (Doupnik *et al.*, 2001)
RGS20	Beta-cells	Gz, Gi/o	(Nunn *et al.*, 2006) (Maurice *et al.*, 2010)

			(Pagano *et al.*, 2008)
RGS21	Taste bud cells	Gi/o, Ggustducin	(Li *et al.*, 2005) (Von Dannecker *et al.*, 2004)

Table 1.2. Selectivity of interactions between mammalian RGS proteins and G proteins.

pathways suggests that they may function as integrators linking other signaling pathways to heterotrimeric G protein pathways (further discussed below in section 1.6).

1.2.5 Non-canonical function of RGS proteins

RGS proteins were first identified as negative regulators of G protein signaling via their GAP activities. However, some RGS proteins contain other molecular domains outside of the RGS domain that have binding partners other than Gα subunits of G proteins, and thus further regulate either their subcellular localization, GAP activity or receptor coupling (Sethakorn *et al.*, 2010). In addition, these additional domains may enable RGS proteins to serve non-canonical functions and limit signaling via GAP-independent mechanisms such as effector antagonism and guanine nucleotide dissociation inhibition (Abramow-Newerly *et al.*, 2006;Sethakorn *et al.*, 2010).

In addition to their binding to G proteins, RGS proteins bind to many different effector proteins such as ACs, PLC-β, and GIRK channels (Abramow-Newerly *et al.*, 2006). The interaction between RGS proteins and the effectors may have both positive and negative effects on signal transduction. In some cases, RGS proteins can bind to either the effectors or the active G protein and interfere with the productive interaction between these two proteins. Thus, RGS proteins function as effector antagonists (Dowal *et al.*, 2001). On the other hand, RGS proteins may also serve as anchors and create RGS-G protein-effector complexes, resulting in rapid transduction of the G protein signal (Chidiac and Roy, 2003).

The C/R7 subfamily of RGS proteins contains a conserved G gamma-like (GGL) domain, which forms a requisite stable complex with the G protein subunit Gβ5 (Witherow and Slepak, 2003). It has been suggested that the GGL domain has a scaffolding role in forming the complex between GPCRs and RGS proteins. However, the existence of this complex has not been identified (Abramow-Newerly *et al.*, 2006). In addition to C/R7 RGS proteins, RGS3 has been suggested to interact with Gβ1γ2 through a region N-terminal to the RGS domain (Shi *et al.*, 2001). Overexpression of RGS3 inhibits Gβ1γ2-mediated production of IP3, and activation of extracellular signal- regulated protein kinase (ERK) (Shi *et al.*, 2001).

Regulation of non-G protein signaling by RGS proteins has also been investigated. Both RGS13 and RGS16 have been found to interact with the p85α subunit of PI3 kinase in a G protein-independent way, and inhibit signaling events downstream of PI3 kinase (Bansal *et al.*, 2008;Liang *et al.*, 2009). In addition, the interaction between RGS3 and Smad family proteins interferes with TGF-β-induced dimerization of Smad3 with Smad4, thereby inhibiting Smad-mediated gene transcription (Yau *et al.*, 2008). Work done by our lab has identified a novel inhibitory role of RGS2 on global protein synthesis, wherein it interacts with the ε subunit of eIF2B to inhibit its GEF activity on eIF2 (Nguyen *et al.*, 2009). In addition, RGS proteins or RGL proteins have been suggested to play a role in regulating nuclear signaling, for example by modulating gene transcription (Sethakorn *et al.*, 2010).

1.2.6 OVERVIEW OF RGS14

The D/R12 subfamily RGS proteins contain three members, RGS10, RGS12 and RGS14. Besides RGS10, which is similar in size to the B/R4 proteins, RGS12 and RGS14 are large multi-domain proteins. In addition to the RGS domain, both proteins contain a second Gα binding domain, the GoLoco motif (discussed in detail below), as well as two small G protein binding domains (Ras binding domains or RB domains) located between the RGS domain and the GoLoco motif. The longest isoform of RGS12 also contains a PDZ domain and a PTB (protein tyrosine binding) domain N-terminally to the RGS domain, whereas RGS14 only has a relatively short N-terminus. The biochemical activities of the two heterotrimeric G protein binding domains of RGS14 have been studied previously (Cho *et al.*, 2000;Mittal and Linder, 2004;Hepler *et al.*, 2005;Mittal and Linder, 2006). The GAP activity of RGS14 has been studied both in solution and in receptor-stimulated membrane-based assays (Cho *et al.*, 2000;Hepler *et al.*, 2005). To date RGS14 has been found to act as a GAP solely on the Gi/o subfamily of G proteins (Cho *et al.*, 2000;Chidiac *et al.*, 2002). The GoLoco domain of RGS14 inhibits GDP release from isolated Gαi1 and Gαi3, but not Gαi2 and Gαo (Mittal *et al.*, 2004). On the other hand, the RGS domain of RGS14 exhibits similar GAP activity toward all four members of the Gi/o subfamily of G proteins in solution-based assays (Mittal *et al.*, 2004;Hepler *et al.*, 2005). Interestingly, the affinity of full-length RGS14 for Gαi/o subunits is apparently greater than that of the isolated RGS domain (Hollinger *et al.*, 2001).

The RB domains of RGS12 and RGS14 were originally identified through their similarity to the RB domains found in Raf-1 proteins. Similar to other RB domain containing proteins such as RalGDS, phosphoinositide-3 kinase and the Raf kinases, the RB domains of RGS12 and RGS14 selectively interact with active Ras/Rap family members. However, apparently, the RB domains of RGS12 and RGS14 each have a few distinct binding partners. RGS12 preferentially interacts with H-Ras to form a signaling complex with Ras/Raf/MEK proteins and regulates nerve growth factor-mediated differentiation. The RGS domain of RGS12 has low binding affinity for K-Ras, M-Ras, R-Ras and Rap proteins (Willard *et al.*, 2007b). On the other hand, RGS14 was thought to selectively to interact with Rap, but not Ras proteins *in vitro* (Traver *et al.*, 2000;Mittal *et al.*, 2006). Recent studies have, however, shown that both H-Ras and, Raf-1 can bind in a positively cooperative manner to the RB domains of RGS14 and modulate signaling through Ras/Raf/MAP kinase cascades (Shu *et al.*, 2010). Since both RGS12 and RGS14 contain two distinct Gα binding sites as well as two small G protein binding sites, it has also been proposed that they may act as scaffolding proteins that integrates heterotrimeric G protein and small G protein pathways (Willard *et al.*, 2009;Shu *et al.*, 2010).

The tissue distribution of RGS14 implies that it is highly expressed in brain and spleen, at a modest level in lung and at very low levels in various other tissues (Snow *et al.*, 1997). Different G protein-interacting domains of RGS14 play important roles in regulating RGS14 subcellular localization. Several studies have shown that RGS14 shuttles between the nucleus and plasma membrane. Its localization to the nucleus depends on the RGS and RBD domains, whereas its translocation to the plasma membrane depends on its

GoLoco motif (Shu *et al.*, 2007). The cellular and physiological functions of RGS14 have also been studied to some extent, but most studies have focused on its roles in brain function and cell division. By associating with microtubules, RGS14 is able to regulate microtubule polymerization and spindle organization (Martin-McCaffrey *et al.*, 2005). It has also been pointed out that RGS14 may play an important role in hippocampal-based learning and memory by acting as a natural suppressor of synaptic plasticity in CA2 neurons (Lee *et al.*, 2010).

1.3 GDI PROTEINS

Nucleotide exchange and hydrolysis are the two steps that control the lifetime of a G protein-mediated signal. GTP hydrolysis can be regulated by RGS proteins. Similarly, nucleotide exchange can also be regulated by factors including GEF and GDI proteins. GDI proteins directly interact with and stabilize the inactive (GDP-bound) Gα subunit and inhibit GDP dissociation, which will in turn slow down the activation of Gα. In heterotrimeric G proteins, the association between Gα subunit and Gβγ dimer favors the GDP-bound state of Gα, and the protein complex undergoes major conformational changes upon binding of GTP to Gα. Therefore the Gβγ dimer is considered to act as a GDI that stabilizes Gα in its inactive state, suppressing spontaneous Gα activation and facilitating its coupling with the receptors (Neer, 1995;Tang *et al.*, 2006). The effect of Gβγ on nucleotide exchange has been studied in detail with Gαs (Brandt *et al.*, 1985) and Gαo. In the absence of Mg^{2+}, the affinity of GDP for Gαo is increased markedly by Gβγ (K_d from ~40 nM to 0.1 nM), which results from both an increase in the rate of association of GDP and a decrease in the rate of dissociation (Gilman, 1987).

Besides $G\beta\gamma$, other proteins have also been identified as GDIs for $G\alpha$ subunits. One example is human neuroglobin (Ngb), a heme protein that is expressed in the brain and can bind reversibly to oxygen. Oxidized neuroglobin binds exclusively to the GDP-bound form of $G\alpha i$ (Wakasugi *et al.*, 2003). Single-turnover GDP dissociation and GTPγS binding experiments suggested that ferric Ngb serves as a GDI for both $G\alpha i$ and $G\alpha o$, and it does this apparently by blocking GDP release. Mutagenesis experiments also imply that residues around Cys46 of Ngb are important for GDI activity (Wakasugi *et al.*, 2003). Interestingly, a BLAST search of the Ngb protein reveals that it shares 25%-35% amino acid sequence homology with the RGS domain of GPCR kinases (GRKs), however, it acts as a GDI rather than a GAP. Moreover, ferrous ligand-bound Ngb, which is the predominant form under normoxia, does not have GDI activity. Thus Ngb may act as an oxidative stress-responsive sensor for signal transduction in the brain.

1.4 GoLoco motif containing proteins

1.4.1 Identification and characterization of GoLoco motif containing proteins

Among all the GDIs identified for heterotrimeric G proteins, the best studied proteins are those containing the GoLoco motif. The first GoLoco motif identified was in *loco*, a RGS12 homologue found in *Drosophila melanogaster*. A second G protein interaction site was identified C-terminally to the RGS domain of the protein (Granderath *et al.*, 1999). This observation led to the discovery of several other proteins that share a highly

conserved 19 amino acid motif (Siderovski *et al.*, 1999). The GoLoco motif was also named G protein regulatory (GPR) domain by Cismowski and colleagues, who identified a receptor-independent activator of Gβγ signaling (Cismowski *et al.*, 1999;Takesono *et al.*, 1999).

The GoLoco motif has a much higher binding affinity for GDP-bound Gα relative to either nucleotide-free or GTP-bound Gα (Kimple *et al.*, 2002a;Kimple *et al.*, 2002b). The interaction between the GoLoco motif and Gα stabilizes the latter in its inactive form, and slows down spontaneous nucleotide exchange (Siderovski *et al.*, 2005). Binding of the GoLoco motif results in a significant conformational change of the switch region of the Gα subunit, and impairs the binding of Gβγ. Thus, Gα-GDP-Gβγ and Gα-GDP-GoLoco complexes are mutually exclusive (Natochin *et al.*, 2001;Bernard *et al.*, 2001;Siderovski *et al.*, 2005).

It has been suggested that the GoLoco motif and Gβγ are able to compete for binding to the Gα proteins (Takesono *et al.*, 1999). Thus, the GoLoco-Gα interaction may either promote heterotrimer dissociation or interfere with subunit re-association. A GoLoco motif consensus peptide derived from AGS3 has the ability to inhibit Gα binding to Gβγ 10 times greater than the Gβγ hot spot-binding peptide (SIGK) (which also interferes with the binding between Gβγ and Gα) with an IC_{50} of 250 nM. In addition, this GoLoco peptide was able to cause a rapid dissociation of the G protein βγ subunits from the Gα subunit about 13 fold higher than the intrinsic off rate of Gα (Ghosh *et al.*, 2003).

However, full-length RGS14 failed to disrupt pre-formed G protein heterotrimers either *in vitro* or in cells (Mittal *et al.*, 2006). Still, GoLoco peptide derived from RGS14 seems to be able to prevent the reformation of the Gαβγ heterotrimer (Webb *et al.*, 2005). Overall, the ability of the GoLoco motif to promote G protein subunit dissociation may depend on the experimental or cellular context and the particular proteins in question.

1.4.2 DIVERSITY OF GOLOCO MOTIF CONTAINING PROTEINS

To date, the GoLoco motif has been found to exist in four well-defined families of proteins. i) A single GoLoco motif is present in the RGS and RBD domain containing proteins RGS12, RGS14, and Drosophila Loco. ii) Pcp-2/GPSM4 and G18/GPSM3/AGS4 are relatively small proteins that contain two and three GoLoco motifs, respectively. iii) A single GoLoco motif is present at the N-terminus of RapGAP domain containing proteins (Rap1GAP1a, -b and Rap1GAP2b,-c). iv) GoLoco motifs are found in multiple arrays in the tetratricopeptide-repeat (TPR) domain containing proteins Pins, GPSM2/LGN and GPSM1/AGS3 (Figure 1.9).

Most of the GoLoco motifs identified so far selectively interact with Gαi, but not other subfamilies of G proteins (Blumer *et al.*, 2005). The relative affinities of the GoLoco motif for Gαi and Gαo vary among different GoLoco containing proteins (Mittal *et al.*, 2004;Willard *et al.*, 2006;Willard *et al.*, 2007a;Zhao *et al.*, 2010). Some GoLoco motif containing proteins also interact with G proteins other than Gi or Go. For example,

Figure 1.9

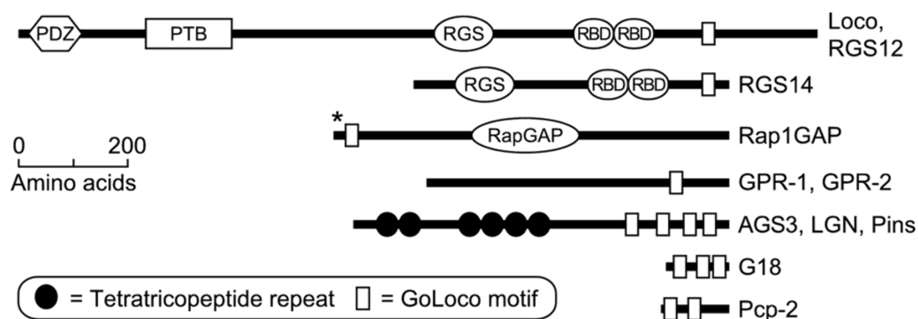

Fig. 1.9. Diversity of GoLoco motif containing proteins. The $G\alpha_{i/o}$-Loco interaction, or GoLoco motif, is found singly, or in tandem arrays, in a number of different proteins. Domain abbreviations are PDZ, PSD-95/Discs large/ZO-1 homology domain; PTB, phosphotyrosine-binding domain; RGS, regulator of G protein signaling box; RBD, Ras-binding domain; and RapGAP, Rap-specific GTPase-activating protein domain. Asterisk denotes N-terminal variation in GoLoco motif sequence between isoforms I and II of Rap1GAP. Reproduced from Willard *et al.*, 2004.

AGS3 interacts with Gαt and blocks rhodopsin-induced dissociation of GDP (Natochin *et al.*, 2000). It has been suggested that the selectivity between GoLoco motif containing proteins and G protein subtypes is influenced by amino acid residues outside of the core domain of the GoLoco motif, as well as the all-helical domain of Gα (Kimple *et al.*, 2002a).

1.4.3 Molecular basis for the GDI activity of GoLoco motif containing proteins

The crystal structure of the GoLoco motif region of RGS14 coupled with Gαi1 highlights the importance of GoLoco's highly conserved Asp/Glu-Gln-Arg triad and the switch II region of the Gα subunit (Kimple *et al.*, 2002a). The amino terminus of the GoLoco peptide forms an α-helix that inserts between the switch II and the α3 helix of the Gα, displacing the switch II away from the α3-helix. The side chain of the arginine finger within the Asp/Glu-Gln-Arg triad of the GoLoco motif reaches into the nucleotide binding pocket and makes direct contact with the α and β phosphates of the bound GDP molecule. Mutation of this Arg to Phe leads to a complete loss of GDI activity (Peterson *et al.*, 2000;Kimple *et al.*, 2002a). The binding of the GoLoco motif to Gα proteins also displaces Arg178 within the switch I region of the Gαi1, which makes contact with the phosphate group, resulting in the formation of a new contact with GDP ribose. This newly formed contact is believed to be the molecular basis of GoLoco GDI activity (Kimple *et al.*, 2002a) (Figure 1.10).

The selectivity between different GoLoco motif containing proteins and G proteins is determined by the all-helical domain of the Gα subunit and the amino acid C-terminal to the core GoLoco motif. A Gαo-insensitive GoLoco peptide derived from RGS14 and AGS3 exhibited GDI activity on a chimeric Gαo subunit containing the all-helical domain of Gαi1 (Kimple *et al.*, 2002a). In addition, replacement of the C-terminal domain of RGS14 with the corresponding region in Pcp-2 (a GoLoco protein sensitive to Gαo) leads to a gain of function similar to wild type Pcp-2 (Kimple *et al.*, 2002a).

1.4.4 REGULATION OF GOLOCO MOTIF FUNCTION

Structural studies provide a basic foundation for studying the mechanisms of GoLoco motif-regulated G protein activation. However, little is known regarding the regulation of GoLoco motif function. Most GoLoco proteins identified so far contain one or more phosphorylation sites either located within or N-terminally to the core GoLoco motif, which may potentially affect their function. Phosphorylation of Thr-494, a cAMP-dependent protein kinase (PKA) substrate just N-terminal to the start of the GoLoco motif in RGS14, enhances its GDI activity up to three fold (Hollinger *et al.*, 2003). However, whether or not this effect is due to a direct contribution of the phosphorylation to the interaction between the GoLoco motif and Gα protein remains unclear (Willard *et al.*, 2004). On the contrary, phosphorylation of the GoLoco motifs of AGS3 by LKB1, the mammalian homologue of serine-threonine kinases in *C. elegans* (PAR-4), reduces its ability to interact with G proteins (Blumer *et al.*, 2003). LGN is a homologue of AGS3, and it contains 4 GoLoco motifs. Phosphorylation of the Thr450 site N-terminal to the first GoLoco motif by

Figure 1.10

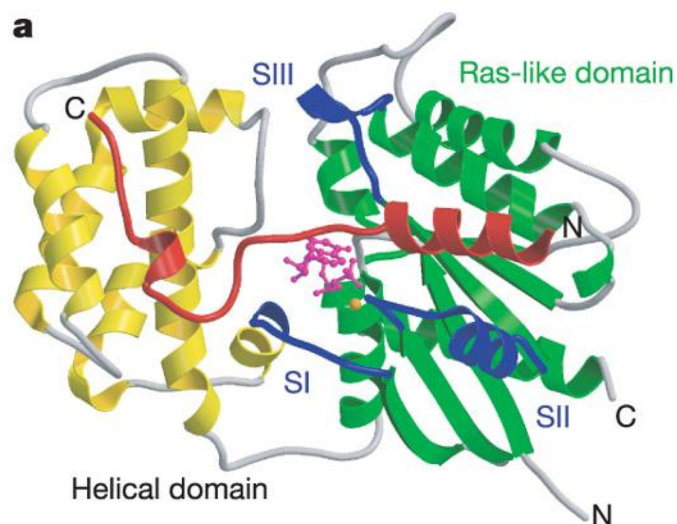

Fig. 1.10. The GoLoco region of RGS14 interacts with Gαi1. Ribbon drawing of R14GL peptide (red) in contact with the Ras-like (green) and all-helical (yellow) domains of G_{i1}. Also shown are the three switch regions of G_{i1} (blue), GDP (magenta) and Mg^{2+} (orange). Adapted from Kimple *et al.*, 2002a.

PBK/TOPK leads to an enhancement of cell growth; however, it is unknown whether this effect is related to G protein signaling (Fukukawa *et al.*, 2010). Thus, phosphorylation may serve as a general mechanism regulating the interaction and function of GoLoco motifs. However, post-translational modifications may result in opposite effects and lead to increased or decreased G protein signals depending on the cellular context and the specific GoLoco proteins in question.

Besides phosphorylation, GoLoco-Gα protein complexes can also be regulated by GPCRs and Gβγ. In an overexpression system, AGS3 and Gαi were found to exhibit a specific bioluminescence resonance energy transfer (BRET) signal. Activation of the α2 adrenergic receptor (α2-AR) or μ-opioid receptor, but not the M3 muscarinic receptor, greatly diminished this signal. Apparently, this decrease in BRET signal is Gβγ independent, but is inhibited by the co-expression of RGS4, implying that both nucleotide exchange and hydrolysis play a role in this regulatory effect (Oner *et al.*, 2010a). Similarly, the coupling between G18 and Gαi is also reduced upon activation of the α2 adrenergic receptor (Oner *et al.*, 2010b). The functional consequences of these effects remain unknown.

From a biochemical point of view, GoLoco proteins are able to inhibit receptor-catalyzed guanine-nucleotide exchange in both single-turnover GTPγS binding assays (Natochin *et al.*, 2000;Kerov *et al.*, 2005) and steady-state GTPase assays (Natochin *et al.*, 2000;Kerov *et al.*, 2005;Zhao *et al.*, 2010). However, depending on the particular

receptor and GoLoco protein being tested, the GDI activity may be masked by the relatively slow GTP hydrolysis, which is the rate limiting step in a receptor-stimulated system (discussed in section 1.7). In the presence of RGS protein, which speeds up the rate of GTP hydrolysis, and thus changes the rate limiting step back to nucleotide exchange, it again becomes possible to detect GoLoco GDI activity. This allows us to measure GoLoco protein activity in a receptor-stimulated system, which is thought to be closer to physiological conditions compared to solution-based assays.

1.4.5 Cellular functions of GoLoco motif containing proteins

Functional studies of GoLoco motif containing proteins suggest a wide range of physiological roles, including involvement in cell division, neuronal outgrowth, and ion channel regulation (Blumer *et al.*, 2007). The influence of GoLoco motif containing proteins on G protein-regulated ion channels also been investigated in HEK293 cells and *X. laevis* oocytes expressing G protein-regulated inwardly rectifying K^+ (GIRK) channels. Full-length GPSM2 and GoLoco peptides, based on the GoLoco region of this protein, activated basal Gβγ-dependent K^+ currents, while siRNA knockdown of GPSM2 decreased basal K^+ currents in primary neuronal cultures (Wiser *et al.*, 2006). GPSM2 also modulated receptor regulation of Cav2.1 calcium channels expressed in *X. laevis* oocytes, but has no effect on the basal current (Kinoshita-Kawada *et al.*, 2004). The complexes between Gαi-GDP and the GoLoco regions of AGS3, Pins, and GPSM2 have been found to regulate both Drosophila and mammalian asymmetric cell division (ACD). In addition, such interactions may also affect receptor- and nucleotide-independent

activation of Gβγ-dependent ACD (McCudden *et al.*, 2005). From a broader perspective, different proteins tend to use their GoLoco motifs for different functional outcomes.

1.4.5.1 Involvement of GoLoco proteins in cell division

The most well established cellular function of GoLoco proteins is their role in regulating ACD. Mitotic cell division can be divided into two basic categories, symmetric cell division and asymmetric cell division. Conventional cell division produces two identical daughter cells, whereas asymmetric cell division results in a different fate of the daughter cells. In ACD, The mother cell will establish an axis of polarity followed by unequal distribution of cell fate determinants, as well as unequal orientation of the mitotic spindle along the axis. Finally the cell will asymmetrically divide into two daughter cells (Crouch *et al.*, 2000;Gonczy, 2008). ACD is used by many species during development to generate cellular diversity, such as Drosophila neuroblasts and sensory organ precursor.

The first report to demonstrate the potential role of GoLoco motif in ACD found that Pins (a four GoLoco motif containing protein,) is required for the asymmetrical localization of the cell fate determinant inscuteable in Drosophila neuroblasts and it is critical in the orientation of neuroblast mitotic spindles (Schaefer *et al.*, 2000). Similarly, deletion of the GoLoco protein GPR1/GPR2 in *C. elegans* embryos leads to a spindle positioning defect that results in a symmetric P0 division and the production of equal sized AB and P1 blastomeres, a phenotype indistinguishable from that of deletion of endogenous G proteins in the embryos (Gonczy *et al.*, 2000;Gonczy, 2008) (*Figure 1.11*).

Increasing evidence has revealed that the role of GoLoco proteins may not be limited to ACD processes, as such proteins may also regulate symmetric cell division by influencing mitotic spindle poles, microtubule dynamics, etc. (Kaushik *et al.*, 2003;Martin-McCaffrey *et al.*, 2005;Blumer *et al.*, 2006). Increasing expression levels of LGN have been observed at the metaphase of mammalian cell division. Furthermore, subcellular localization studies indicate that LGN is important for the cortical positioning of the spindle pole, which likely reflects stronger pulling or pushing forces on the spindle pole (Blumer *et al.*, 2006). RGS14 has also been suggested to be a microtubule-associated protein and a component of the mtotic spindle that regulates microtubule polymerization and spindle organization (Martin-McCaffrey *et al.*, 2005).

Many studies have implied that the coupling between GoLoco proteins and G proteins is important for the proper functioning of the latter during cell division. First, Gαi proteins share similar sub-cellular localization with GoLoco proteins during mitosis (Cho and Kehrl, 2007). Secondly, the interaction between GoLoco domains and G proteins influences the sub-cellular localization of GoLoco proteins during both interphase and mitosis (Cho *et al.*, 2007;Shu *et al.*, 2007). Thirdly, blocking the normal interactions between GoLoco proteins and G proteins leads to abnormal exaggerated mitotic spindle rocking in kidney epithelial cells (Willard *et al.*, 2008), as well as cytokinesis defects (Cho *et al.*, 2007). In contrast, overexpression of LGN and Gαi causes aberrant metaphase chromosome alignment and a repositioning of the spindle poles closer to the cell cortex (Du and Macara, 2004;Blumer *et al.*, 2006).

Figure 1.11

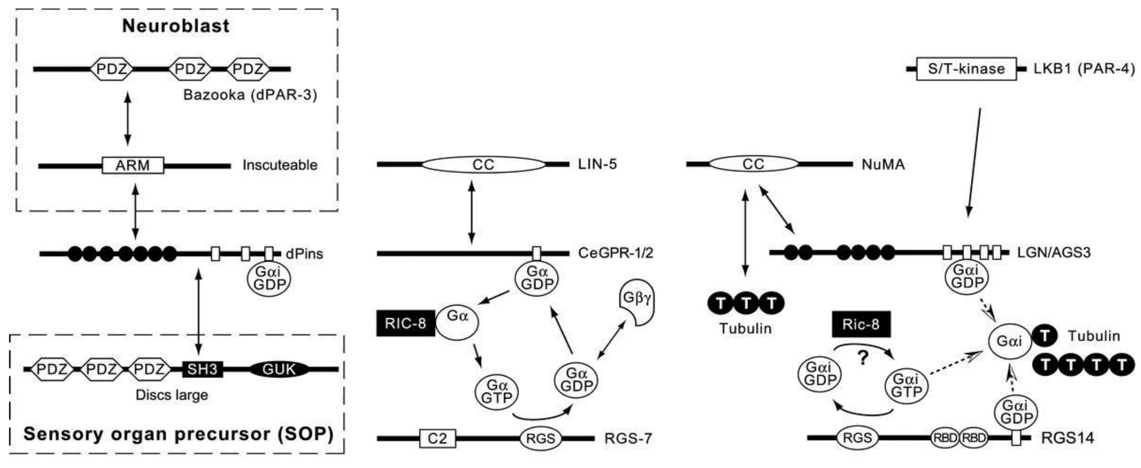

Fig. 1.11. Comparable signal transduction complexes, centered around Pins family members, regulate asymmetric cell divisions. Interactions between signaling components are abstracted from genetic and direct biochemical evidence. Domain abbreviations are ARM, weakly predicted Armadillo repeats; C2, homology to conserved region 2 of protein kinase C; CC, coiled-coil region; GUK, membrane-associated guanylate kinase homology domain; PDZ, PSD-95/Discs large/ZO-1 homology domain; RBD, Ras-binding domain; RGS, regulator of G protein signaling box; SH3, Src homology-3 domain; and S/T-kinase; serine/threonine kinase domain. Reproduced from Willard *et al.*, 2004.

As mentioned earlier, interactions between GoLoco motifs and Gα proteins also influence proper coupling between Gα subunits and Gβγ dimers, the latter of which is required for the mitotic-spindle orientation of neural progenitors in the developing neocortex. Silencing AGS3 leads to a phenotype similar to that of the impairment of Gβγ (Sanada and Tsai, 2005).

1.4.6 Overview of G18

G18 is encoded by a gene within the major histocompatibility complex class III region of chromosome 6 (Cao *et al.*, 2004;Kimple *et al.*, 2004). It contains three GoLoco motifs at its C-terminus, while its short N-terminus contains multiple proline residues (14 out of 60 amino acids in total). The mRNA of G18 is expressed in a variety of tissues such as heart, placenta, lung and liver (Cao *et al.*, 2004). Only two previous studies have been published focusing on the biochemical function of G18. These studies found that the GoLoco motifs of G18 can interact with GDP-bound Gαi1 both *in vitro* and following co-transfection, act as a GDI to the Gi subfamily of G proteins. However, in a celluar context, overexpression of G18 does not seem to alter the activation level of PLC-β, which is a direct effector of Gβγ. This latter observation does not support the idea that GoLoco motif is able to promote subunit dissociation and further activate Gβγ-mediated cell signaling. Thus, there are many aspects of G18 remaining to be determined, such as its cellular localization, binding properties with other partners and effects on GPCR-mediated cell signaling.

Recently, the effect of receptor activation on coupling between G18 and G protein has also been examined. The specific signal between G18 and Gαi1 obtained from bioluminescence resonance energy transfer (BRET) assays is reduced upon activation of the receptor. In addition, in the presence of G proteins, BRET signals are observed between G18 and the α2 adrenergic receptor, suggesting that G18-Gαi complex may serve as substrate for agonist-induced G protein activation (Oner *et al.*, 2010b).

1.5 RECEPTOR-INDEPENDENT GEFS

It is well known that receptor-mediated activation of G protein pathways is very important in regulating cellular processes. However, increasing evidence suggests that alternative modes of regulation of G proteins may exist. A functional yeast-based screen has identified a number of non-receptor proteins that could influence the activation state of G proteins (Cismowski *et al.*, 1999;Takesono *et al.*, 1999). In parallel with these observations came the realization that some signal processing through G protein pathways is independent of receptor function. For example, heterotrimeric G protein signaling is likely to be involved in regulating spindle pole orientation, and microtubule dynamics during asymmetric cell division in many different species (Bellaiche and Gotta, 2005;Izumi and Matsuzaki, 2005). However, to date, there is no evidence for such processes of receptor activation. It has been proposed that during cell division, nucleotide exchange on G protein is stimulated by a receptor-independent GEF (Ric-8) (Wang *et al.*, 2005;Woodard *et al.*, 2010). Unlike GEFs for monomeric G proteins, there are only a few known non-GPCR GEFs for heterotrimeric G proteins. These proteins

include AGS1(Gαi/o), Ric-8 (Gαi/o, Gs, Gq), GIV (Gαi), and GAP-43 (Gαo) (Strittmatter *et al.*, 1991;Nakamura *et al.*, 1998).

Activator of G protein signaling 1 (AGS1 or DexRas) is a dexamethasone-inducible-Ras-related gene in AtT20 cells. A yeast-based functional screen indicates that Gαi2, and Gαi3 activation can be regulated by AGS1 (Cismowski *et al.*, 1999;Cismowski *et al.*, 2000). *In vitro* biochemical assays also suggest that AGS1 acts as a GEF for Gαi1, Gαi2 and purified brain G protein heterotrimer (Cismowski *et al.*, 2000). Overexpression of AGS1 blocks receptor-mediated heterologous desensitization of AC1 (Nguyen and Watts, 2006), which is consistent with a regulatory role for AGS1 in Gαi signaling. The molecular basis of the potential GEF activity of this protein has yet to be determined. It has been suggested, however, that the cationic regions in AGS1, similar to those found in the activation loops of GPCRs, may function to directly facilitate GDP release (Cismowski *et al.*, 2000).

Resistance to inhibitors of cholinesterase (Ric-8) is another direct activator of heterotrimeric G proteins that is of great interest. Ric-8 was originally identified as a conserved protein that is required for Gαq signaling in *C. elegan*s (Miller *et al.*, 2000). It has two mammalian homologs Ric-8A and Ric-8B. Biochemical characterization of Ric-8A revealed that it is a potent GEF for Gαq, Gαi1 and Gαo, but not Gαs (Tall *et al.*, 2003). Interestingly, Ric-8B has been found to interact with Gαs and Gαq. It serves as a GEF for Gs, Gαolf and Gαq (Von Dannecker *et al.*, 2005;Von Dannecker *et al.*,

2006;Kerr *et al.*, 2008;Chan *et al.*, 2011), and it may also positively regulate Gs signaling via a GEF-independent, but ubiquitination-related mechanism (Nagai *et al.*, 2010). In contrast to AGS1, Ric-8 seems unable to promote nucleotide exchange when Gα is coupled to Gβγ (Tall *et al.*, 2003). Thus, the GEF effect of Ric-8 has to be initiated after Gβγ dissociation or before the reassociation of Gβγ. Recent studies on asymmetric cell division suggest a potential model for Ric-8-regulated G protein signaling. This model proposes that the interaction between a GoLoco motif containing protein and Gα promotes dissociation of Gβγ dimer from the Gα subunit. This GoLoco-Gα protein complex then serves as a substrate for Ric-8 binding, which will further stimulate nucleotide exchange on Gα (Afshar *et al.*, 2004;Hess *et al.*, 2004;Tall and Gilman, 2005;Thomas *et al.*, 2008).

Although different receptor-independent GEF proteins have been discovered, unlike RGS protein and GoLoco motif containing proteins, they share limited sequence homology and molecular domain similarity, suggesting that they may regulate G protein activation via different mechanisms. The G protein interacting domain of GIV (also called Girdin 12, a selective GEF for Gαi subunits) has been studied. GIV interacts with the Gαi3 subunit through two independent domains and the GIV-C-tail is responsible for the state-dependent interaction and its GEF activity (Garcia-Marcos *et al.*, 2009). The important residues that contribute to the GIV-Gαi interface have been also identified. However, this GEF domain shares no significant homology with other GEFs or GDIs. Indeed, it will be helpful to determine the structure of the interface between these GEFs and Gα

proteins, which will provide more information to unmask the different mechanisms of receptor-independent GEF regulation of G protein activation.

1.6 REGULATION OF G PROTEIN SIGNALING BY PROTEINS CONTAINING MULTIPLE G PROTEIN BINDING DOMAINS.

1.6.1 PROTEINS THAT CONTAIN MORE THAN ONE MOLECULAR DOMAIN THAT REGULATES HETEROTRIMERIC G PROTEIN FUNCTION.

Many proteins discussed above bear complex, multidomain structures that suggest an ability to actively orchestrate interdependent signaling events. For instance, RGS12, RGS14, G18 and GRK family proteins all contain extra molecular domains that can regulate GPCR-mediated G protein signaling via different mechanisms.

Members of the R7 RGS protein subfamily contain a conserved G protein gamma-like (GGL) domain that specifically interacts with Gβ5 (type 5 G protein β subunit) protein (Witherow *et al.*, 2000). Gβ5 is essential for the stability of the R7 RGS proteins, thus R7 RGS-Gβ5 complexes exhibit enhanced GAP activity (Kovoor *et al.*, 2000). It has also been suggested that, unlike traditional Gβγ subunits that form dimers, the major binding partner of Gβ5 is the GGL domain of the R7 RGS proteins (Posner *et al.*, 1999;Skiba *et al.*, 2001). Thus, the interaction between R7 RGS proteins and Gβ5 may negatively regulate Gβγ-stimulated cellular signaling.

For many GPCRs, short term regulation is initiated by phosphorylation of the receptor by GPCR kinases (GRKs). All GRKs share an RGS homology (RGL) domain at their amino-terminal region (Siderovski *et al.*, 1996). The RH domain of GRK2 has been suggested to interact selectively with Gαq/11 but not other G protein subfamilies (Carman *et al.*, 1999;Sallese *et al.*, 2000). Unlike other RGS proteins, GRK2 only weakly stimulates GTP hydrolysis when Gαq is coupled with M1 muscarinic receptor (Carman *et al.*, 1999). However, GRK2 is able to inhibit Gαq-stimulated PLC-β both *in vivo* and *in vitro*. The underlying mechanism still remains to be established. It has been shown that the binding site between the RH domain of GRK2 and Gαq is different than that of other RGS proteins (Tesmer *et al.*, 2005), which may account for the weak GAP activity observed *in vitro* (Sterne-Marr *et al.*, 2003). The role of GRK2 in regulating Gα signaling remains to be fully elucidated. Besides phosphorylation-dependent deactivation of G protein signaling via its kinase domain, the RH domain maybe involved in phosphorylation-independent attenuation of signaling (Pao and Benovic, 2002;Dhami *et al.*, 2005). It has been suggested that the binding of GRK2 to both the receptor and G protein is required for phosphorylation–independent desensitization to occur (Sterne-Marr *et al.*, 2004).

As mentioned before, G18 was originally identified as a 3 GoLoco motif containing protein that acts as a GDI on Gαi but not Gαo (Cao *et al.*, 2004;Kimple *et al.*, 2004). Studies described in this thesis have revealed novel functions of G18 including its

interactions with Gαi/o proteins which produces a GEF effect on Gαi1, but yields an overall decrease in steady-state GTP turnover on Gαo (further discussed in **Chapter 2**); Moreover, we have examined the possible cross-talk between G18-regulated and RGS protein-regulated G protein activity (further discussed in **Chapter 3**).

Similarly, RGS12 and RGS14 both contain a GoLoco motif in addition to an RGS domain. Biochemical activities of these two domains have been studied individually. However, the net combined effect of the RGS domain and GoLoco domain on regulating G protein activity still remains poorly understood. It appears that both the GoLoco and RGS domains are required for RGS14 to maximally inhibit carbachol-stimulated ERK activation (Traver *et al.*, 2004). However, work from our lab identified a novel "RGS enhancing" activity of the Ras-binding region of RGS14. By binding to the RGS domains of different proteins, it appears that the RB domains may not only restore the relatively weak GAP activity of the R14-RGS domain, but also increase the GAP activity of other RGS proteins such as RGS4 and RGS5 (Chapter 4). These observations provide an alternative interpretation, which involves intramolecular interaction and potentially also splice variants (further discussed in **Chapter 4**).

1.6.2 Cross-talk between heterotrimeric G protein signaling and monomeric G protein signaling

Proteins containing multiple molecular domains can regulate single signaling pathways via different mechanisms under certain condition. In addition, these proteins may have a

scaffolding role that integrates signals from different pathways and regulates signal cross-talk. Such mechanisms have been found to coordinate signaling between heterotrimeric G protein signaling and monomeric G protein pathways.

Dbl homology RhoGEF proteins (including p115RhoGEF, PDZ-RhoGEF and LARG) contain a DH/PH domain, which can stimulate exchange of GDP for GTP on Rho GTPases (Rossman and Sondek, 2005). Several studies indicate that GPCRs are key upstream regulators of Rho GTPases (Sah *et al.*, 2000). A link between GPCR signaling and Rho activation was uncovered by the identification of an RGS-like domain located at the N terminus of RhoGEF, which stimulates the intrinsic GTP hydrolysis activity of Gα12/13 (Fromm *et al.*, 1997) and Gαq (Booden *et al.*, 2002). Reciprocally, activation of Gαq or Gα12/13 activates the RhoGEF function of these proteins and thus leads to a synergistic activation of RhoA (Booden *et al.*, 2002;Chikumi *et al.*, 2002).

In some cases, scaffolding proteins may link heterotrimeric G proteins to the inactivation of small G proteins. Yeast two hybrid screening to identify potential binding partners for Gαz resulted in the discovery of another small G protein regulator Rap1GAP, which stimulates the GTPase activity of Rap1 (Meng *et al.*, 1999). The N-terminus of Rap1GAP encodes a GoLoco motif that binds to Gαi1/2 (Rap1GAPI) or Gαz (Rap1GAPII) (Mochizuki *et al.*, 1999;Meng and Casey, 2002). Activation of G protein recruits Rap1GAP to the plasma membrane which attenuates Rap1 signaling, resulting in

a decrease or an increase in ERK activation depending on the specific operative Rap1-effector pathways in the cell lines at hand (Mochizuki *et al.*, 1999;Meng *et al.*, 2002).

Finally, the two tandem Ras binding domains (RBD) in RGS12 and RGS14 have also been shown to integrate G protein and Ras/RafMAPkinase signaling pathways (Ponting, 1999;Willard *et al.*, 2009;Shu *et al.*, 2010). The inhibitory effect of RGS14 on Erk phosphorylation is reversed by co-expression of Gαi1, which recruits RGS14 to the plasma membrane and inhibits Raf binding to RGS14. Overall, these RGS proteins may act as molecular switches that organize cellular signal transduction via different pathways. Moreover, the RB domains of RGS14 may also regulate the GAP activity of the RGS domain and also the GDI activity of the GoLoco domain, which will be discussed in **Chapter 4**.

1.7 Kinetic regulation of G protein activity

The kinetics of the G protein activation and deactivation reactions are very important in understanding how G protein-mediated signaling occurs. GDP dissociation is the first step in the G protein activation/deactivation cycle. The relatively high concentration of GTP in the cell leads to a rapid association of GTP after GDP dissociation with relatively little association of GDP. Thus, the rate of nucleotide exchange mainly depends on the rate of GDP dissociation. Although the association of GTP is reversible, GTP dissociation is slow. As a result, GTP typically is hydrolyzed by the G protein even before it dissociates and the cycle starts over. Overall, nucleotide exchange and GTP

hydrolysis are two key reactions that determine the duration of G protein in its GTP-bound form which provokes downstream signaling. The kinetics of G protein activity is tightly regulated by GEFs, GDIs and GAPs. The output of G protein signaling in response to a stimulus reflects the balance of these various regulatory mechanisms which may co-exist in the cell.

1.7.1 GEF-MEDIATED ACTIVATION OF G PROTEINS

From a kinetic point of view, the fractional activation of G protein reflects the balance between GEF-promoted activation (*i.e.*, GDP dissociation and GTP binding) and GAP-promoted deactivation (Ross, 2008). GEFs such as GPCRs dramatically increase the rate of GDP dissociation, resulting in an increase in GTP association and G protein activation.

The mechanism of Ric-8-mediated G protein activation shares a similar but not identical mechanism with that of the receptor. Ric-8 favors binding with high affinity to the open conformation of the G protein (Chan *et al.*, 2011). Thus, the effect of Ric-8B on fractional activation of G protein may be nucleotide concentration dependent. Ric-8 promotes dissociation of both GDP and GTP, and at lower concentrations of GTP (< 500 nM) this favors the nucleotide-free state of the G protein and leads to a decrease in the overall rate of GTP turnover. At higher GTP concentrations, such as those that are found intracellularly (~150 μM), GTP association is greater than dissociation and the Gα-GTP form predominates (Chan *et al.*, 2011).

In addition to promoting nucleotide dissociation, Ric-8 has also been reported to increase cellular G protein levels apparently by stabilizing G proteins in a nucleotide-free conformation and preventing them from denaturing. This observation has profound implications with respect to the kinetics of Gq activation. Gq tends to have a fast denaturation rate when it is in the nucleotide-free state (Chidiac *et al.*, 1999). As the binding of GTP is competing with the denaturation of unliganded Gq, increasing the concentration of GTP decreases denaturation and thus enables the formation of Gq-GTP. The fact that Ric-8 is able to decrease the denaturation rate of the G protein will further benefit the formation of the active GTP-bound G protein.

1.7.2 GDI-MEDIATED INHIBITION OF G PROTEIN ACTIVATION

Nucleotide exchange is limited by the relatively slow rate of nucleotide dissociation. The GDI activity of the GoLoco motif, which decreases the GDP dissociation, may also lead to an overall reduction in G protein activation. It has been shown that the rate of GTPγS binding is decreased up to 80% in the presence of GoLoco proteins or peptide derived from the Goloco motifs of RGS12 and RGS14 (Kimple *et al.*, 2001;Zhao *et al.*, 2010). However, the effects of GoLoco proteins on G protein–mediated signaling outputs still remain to be established. This is further complicated by the tendency of the GoLoco motif to compete with Gβγ. The Gβγ dimer is also able to slow down the rate of intrinsic GDP dissociation from Gα subunit up to 50 fold, depending on the specific G protein in question (Gilman, 1987). On the other hand, Gβγ is required for receptor-stimulated Gα activation since it can stabilize Gα-receptor coupling.

1.7.3 GAP-MEDIATED GTP HYDROLYSIS

The GTP hydrolysis (k_{hyd}) rates of G proteins can be increased up to 2000 fold by GAPs such as RGS proteins (Mukhopadhyay and Ross, 1999). As a result, RGS proteins negatively regulate the G protein cycle both by dampening signaling output and by rapidly terminating G protein activation upon removal of the stimulus. Interestingly, kinetic characterization indicated that the rate of the overall steady-state GTPase reaction measured in the presence of PLC-β is 10 times faster than the rate of GTP binding to G protein in the absence of PLC-β, which is the first step to form Gα-GTP (Biddlecome *et al.*, 1996). Moreover, even though the k_{hyd} is dramatically increased by GAPs, the fact that the fractional activation of G protein still remains high suggests that either the activation rate (k_{on}) is also increased or the GAP activity is inhibited while the receptor is activated.

One theory that supports the idea that RGS proteins are able to potentiate receptor-mediated G protein activation is a proposed kinetic scaffolding mechanism (Biddlecome *et al.*, 1996;Zhong *et al.*, 2003). In this model, GAPs are able to reduce depletion of local Gα-GDP levels to permit rapid recoupling to receptor and sustained G protein activation. In combination with the kinetic scaffolding mechanism, there is another model based on the idea of physical scaffolds, where RGS proteins may directly or indirectly interact with the receptor and facilitate receptor-G protein coupling and promote signal onset. Thus RGS proteins may act as a scaffold to assembly different signal components (Popov *et*

al., 2000;Lambert *et al.*, 2010). The mechanisms described above provide many insights into how G protein signaling is regulated by different factors. Our next challenge will be to determine among those different mechanisms, which one, if any, is predominant in the cell, and how these co-existing mechanisms regulate G protein signaling dynamics, as well as how these interrelated reactions combine to determine output in response to stimuli.

1.8 EXPERIMENTAL APPROACHES TO STUDY THE FUNCTION OF G PROTEIN ACCESSORY PROTEINS

Traditional analysis of GPCR signaling has relied on changes in the activity of downstream effectors as a readout of G protein function. However, the receptor pharmacology is often system-dependent. That is, many variants such as receptor-G protein coupling, selectivity, and specific downstream effectors measured, can contribute to the final readout (Windh and Manning, 2002). Thus, direct assessment of G protein activation by G protein-coupled receptors is perhaps best accomplished by measuring either the nucleotide exchange step or GTP hydrolysis during G protein cycle.

1.8.1 SF9 CELL/ BACULOVIRUS SYSTEM

Heterotrimeric G proteins and receptors can be overexpressed in mammalian cells and GTPase activity can be measure using isolated cell membranes or intact cells. However, mammalian cells often express endogenous receptors or G proteins that may interfere with these measurements (Schneider and Seifert, 2010). Thus, in order to obtain specific

signal with reasonable signal to noise ratio, we need a system that has a relatively "clean background". Insect cells derived from *Spodoptera frugiperda* pupal ovarian tissue fulfill these requirements to a large extent. Only one G protein from the Gαi family has been detected in Sf9 cells (Knight and Grigliatti, 2004). However, this insect G protein does not couple appreciably to mammalian GPCRs. This advantage makes Sf9 cells a virtually Gαi free environment. Functionally reconstituted signaling in Sf9 cells is similar to the receptor expressed in a mammalian system, and Sf9 cells also carry out most of the post-translational modifications, which makes this system similar to mammalian systems (Asmann *et al.*, 2004).

Besides Sf9 cells, Sf21 and High-five insect cells are also used to study G protein functions. They have advantages and disadvantages, for example, compared to Sf9 cells, the expression level of particular receptors may be higher in Sf21 or High-five cells. However, both may carry some form of endogenous G protein subtypes (Wehmeyer and Schulz, 1997).

1.8.2 Measuring RGS protein GAP activity using GTPase assays

Two general approaches are used to measure GAP activity. The most basic method is to measure the rate of GTP hydrolysis in a single enzymatic turnover. Single turnover refers to the hydrolysis of a single molecule of GTP by each molecule of Gα. Since Mg^{2+} is required for GTP hydrolysis, but not nucleotide exchange, radiolabeled GTP can be pre-loaded to G protein in the absence of Mg^{2+} and the addition of Mg^{2+} with or without

GAPs will stimulate GTP hydrolysis (Krumins and Gilman, 2002). The level of free P_i generation is then measured and intrinsic GTP hydrolysis is subtracted. The second general method to measure GAP activity is via steady-state GTPase assays. In the absence of the receptor, nucleotide exchange is the rate limiting step in G protein cycle. Thus the increase in GTP hydrolysis by GAPs may be masked by the slow rate of nucleotide exchange (Zhao *et al.*, 2010). Stimulation is significant only if the nucleotide exchange rate is sufficiently fast. To observe GAP activity under steady-state conditions, GDP/GTP exchange must be accelerated by a GEF (either GPCRs or receptor-independent GEFs). The GAP-independent hydrolysis during the assay is subtracted as background. The advantage of a steady-state GTPase assay is that it is presumably closer physically to signals in cells compared to single-turnover assays.

1.8.3 Measuring the activities of GEFs, GDIs using nucleotide exchange assays

1.8.3.1 GDP dissociation assays

Nucleotide exchange consists of two steps, GDP dissociation and GTP binding. As mentioned earlier, the rate limiting step in this process is typically GDP dissociation. Due to the abundance of GTP in the cell, GTP will occupy the empty nucleotide pocket once GDP has dissociated. Thus, in most cases, the observed rates of GDP dissociation and nucleotide exchange are similar.

Gα protein alone or in combination with Gβγ subunits can be pre-coupled with radiolabeled [α-^{32}P]GDP, [α-^{3}H]GDP or [α-^{32}P]GTP (the intrinsic GTPase activity of Gα proteins catalyze the conversion of [α-^{32}P]GTP to [α-^{32}P]GDP). Excess GDP with or without regulators in question may be added to trigger GDP release from the G protein. Similar to the GTPγS binding assay discussed below (section 1.8.3.2), GDP dissociation assays can be carried out either in solution using purified components or in membranes to study the receptor-stimulated release of nucleotide.

1.8.3.2 [^{35}S]GTPγS binding assays

Under some conditions, nucleotide association might also be affected by G protein regulators, thus only looking at GDP dissociation might not present a complete picture and might lead to false conclusions. GTPγS binding assays are also used to further study nucleotide exchange. Since the GTP analog guanosine 5'-O-[gamma-thio] triphosphate (GTPγS) dissociates slowly and cannot be hydrolyzed to GDP, the binding of GTPγS to Gα proteins leads to an irreversible reaction where the level of radioactive isotope can be measured. Unlike GTPase assays which can be performed at steady-state or pre-steady-state, GDP- GTPγS exchange assays can only be performed under single-turnover conditions (Windh *et al.*, 2002). GTPγS binding assays can be performed either in solution or using recombinant cell systems. Depending on the purpose of the experiments, different assays should be considered. For example, solution-based assays using purified protein components can provide a relatively simple system with low background levels. It is optimal for looking at G protein activity in the presence and

absence of its regulators. On the other hand, membrane-based, agonist-simulated assays should be used when looking at receptor-G protein coupling.

1.9 RESEARCH GOALS AND SIGNIFICANCE

Originally, the fundamental components of GPCR-mediated cell signaling were recognized to be the receptors, heterotrimeric G proteins and effectors. However, more and more studies suggest that signaling processes are not as simple as once imagined. First of all, an increasing number of factors have been found to regulate a single signaling pathway via different mechanisms. Secondly different signaling pathways are able to link together and form signaling networks that alter a cellular response to a stimulus. Multi-domain proteins play a significant role in signaling pathway cross-talk. Therefore, the purpose of my thesis is to identify and characterize the biochemical and cellular activities of proteins that contain multiple G protein regulatory domains, and to understand how these different G protein binding domains contribute to the net effect of these proteins on G protein activity. My studies have focused on two such proteins, G18 and RGS14. The overall objective of this research is to elucidate the mechanisms by which G18 and RGS14 regulate G protein activity. The **specific aims** of this study are:

1. **To characterize the effects of G18 on Gi/o protein activities and examine the potential contribution of its amino terminal domain and carboxyl terminal GoLoco motifs.**
2. **To investigate the subcellular localization of G18 and its potential effect on RGS protein GAP activity.**

3. **To characterize the effect of the non-RGS domains of RGS14 on RGS proteins GAP activities.**

The proposed studies should help to elucidate how the different G protein binding domains of G18 and RGS14 work in combination to selectively regulate G protein mediated GPCR signaling. The study of how G protein activation and deactivation steps are regulated by multi-G protein binding domain containing proteins will help us further understand the basic aspects of G protein function, It will also give us a better idea of how G proteins can regulate cell signaling under normal and pathological conditions, as well as how GPCR-targeted drugs produce their effects.

1.10 References

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Chapter 2

THE PROLINE-RICH N-TERMINAL DOMAIN OF G18 EXHIBITS A NOVEL G PROTEIN REGULATORY FUNCTION

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2 CHAPTER 2

2.1 CHAPTER SUMMARY

The protein G18 (also known as AGS4 or GPSM3) contains three conserved GoLoco/GPR domains in its central and C-terminal regions which bind to inactive Gαi, while the N-terminal region has not been previously characterized. We investigated whether this domain might itself regulate G protein activity by assessing the abilities of G18 and mutants thereof to modulate the nucleotide binding and hydrolytic properties of Gαi1 and Gαo. Surprisingly, in the presence of fluoroaluminate (AlF_4^-), both G proteins bound strongly to full length G18 (G18wt) and to its isolated N-terminal domain (G18ΔC), but not to its GoLoco region (ΔNG18). Thus it appears that its N-terminal domain promotes G18 binding to fluoroaluminate-activated Gαi/o. Neither G18wt nor any G18 mutant affected the GTPase activity of Gαi1 or Gαo. In contrast, complex effects were noted with respect to nucleotide binding. As inferred by the binding of [^{35}S]GTPγS to Gαi1, the isolated GoLoco region as expected acted as a guanine nucleotide dissociation inhibitor (GDI), whereas the N-terminal region exhibited a previously unknown guanine nucleotide exchange factor (GEF) effect on this G protein. On the other hand, the N-terminus inhibited [^{35}S]GTPγS binding to Gαo, albeit to a lesser extent than the effect of the GoLoco region on Gαi1. Taken together, our results identify the N-terminal region of G18 as a novel G protein-interacting domain that may have distinct regulatory effects within the Gi/o subfamily, and thus it could potentially play a role in differentiating signals between these related G proteins.

2.2 INTRODUCTION

The classical model of G protein signaling includes three major components: G protein-coupled receptor (GPCR), heterotrimeric G protein and effector. The inactive Gα subunit binds with high affinity to GPCR, Gβγ and GDP. The binding of an agonist to the receptor promotes its guanine nucleotide exchange factor (GEF) activity towards the G protein that results in the exchange of GDP for GTP. This activates the G protein and is thought to cause the dissociation of Gα and Gβγ. Both GTP-bound Gα and free Gβγ are capable of initiating signals by interacting with downstream effectors such as adenylyl cyclase, phospholipase Cβ, and various ion channels and kinases (Neves *et al.*, 2002). Signaling is terminated by the intrinsic GTPase activity of the Gα subunit, thereby returning the latter to its inactive form and regenerating the inactive Gαβγ complex. It is now recognized that heterotrimeric G protein signaling is more complex than originally proposed, with a number of factors having been identified that can modulate G protein activity. These include the regulator of G protein signaling (RGS) proteins that accelerate G protein deactivation, and the receptor-independent activator of G protein signaling (AGS) proteins that modulate G protein signals through several distinct mechanisms. The Gi/o-Loco (GoLoco)/G protein regulatory (GPR) motif of the Group II AGS proteins can alter the activities of both Gα and Gβγ (Blumer *et al.*, 2007).

The GoLoco/GPR motif was originally identified in the Drosophila RGS12 homologue, Loco (Siderovski *et al.*, 1999;Cao *et al.*, 2004;Willard *et al.*, 2004). The GoLoco motif is

a 19 amino acid sequence that can bind to the Gα subunit of $G_{i/o}$ proteins in their inactive state (Gα-GDP) to inhibit the exchange of GDP for GTP. This biochemical activity serves as the basis for its function as a guanine nucleotide dissociation inhibitor (GDI) (De Vries *et al.*, 2000;Kimple *et al.*, 2001;Natochin *et al.*, 2001;Kimple *et al.*, 2002a;Cao *et al.*, 2004;McCudden *et al.*, 2005;Willard *et al.*, 2006) to impede Gα activation. Several important contact points have been identified between the GoLoco motif and Gα subunits, the most notable being the extension of its highly conserved Asp/Glu-Gln-Arg triad into the nucleotide-binding pocket of Gα that interacts directly with the α- and β-phosphates of GDP (Kimple *et al.*, 2002a;Kimple *et al.*, 2002b). This interaction between the GoLoco motif and Gα subunits has been shown to promote the dissociation of the Gβγ dimer from Gα *in vitro* (Kimple *et al.*, 2002b). In this way, the GoLoco motif may act as a receptor-independent activator of Gβγ signaling (Bernard *et al.*, 2001;Schaefer *et al.*, 2001;Ghosh *et al.*, 2003;Yu *et al.*, 2005). The Gα-GoLoco complex may also affect physical coupling between Gα and the receptor (Natochin *et al.*, 2000;Sato *et al.*, 2004) and this may further impact the control of G protein function.

The modulation of G protein activities by GoLoco motif-containing proteins has been implicated in multiple physiological processes. In *C. elegans* and drosophila, GPR1/2 and Pins GoLoco motifs, respectively, play essential roles in asymmetric cell division (Willard *et al.*, 2004;Izumi *et al.*, 2006;Siller and Doe, 2009) and analogous mechanisms appear to exist in mammalian systems. For example, the Pins homologue LGN recently was shown to be critical for cell polarization during oocyte meiosis in mice (Guo and Gao, 2009). Emerging evidence also points to a role in GPCR signaling. Endogenously

expressed LGN, for example has been found to regulate G protein-dependent GIRK channel function in hippocampal neurons (Wiser *et al.*, 2006), while another mammalian GoLoco protein, Pcp2, is able to modulate receptor regulation of Cav2.1 calcium channels expressed in *X. laevis* oocytes (Kinoshita-Kawada *et al.*, 2004).

The current study examines the effects of G18 (AGS4/GPSM3) on G protein activity in a variety of experimental contexts. G18 is a 160 amino acid protein that is composed of three tandem GoLoco motifs interspersed through its middle and C-terminal area, plus an uncharacterized N-terminal segment that is rich in proline (14 out of 60 residues). Previous biochemical analyses have shown that at least two GoLoco motifs of G18 can interact with GDP-bound Gαi1 both *in vitro* and in overexpressed cell systems (Cao *et al.*, 2004;Kimple *et al.*, 2004). However, the overall physiological function of G18 still remains unknown. In the current study, we further examine the interactions between G18 and heterotrimeric G proteins and moreover we identify its N-terminus as a novel G protein-interacting domain.

2.3 MATERIAL AND METHODS

2.3.1 *RNA PREPARATION AND RT (REVERSE TRANSCRIPTION) PCR*

Tissues from three month old C57BL/6 mice were collected and homogenized. Total RNA was extracted using trizol reagent (Invitrogen) and further purified using RNeasy mini columns (Qiagen). 2 μg of total RNA was used for reverse transcription with the High Capacity Reverse Transcription kit (Applied Biosystems). Primers specific for the open reading frame of G18 were used in PCR reactions to examine the tissue distribution of G18. The level of GAPDH was used as loading control.

2.3.2 *PREPARATION OF RECOMBINANT EPITOPE TAGGED-G18 FUSION PROTEINS*

GST-tagged G18wt (full length G18), ΔNG18 (a N-terminal 60 amino acid truncation mutant, containing only the three GoLoco motifs) and ΔNG18-mGL (lacking the N-terminal 60 amino acid and containing point mutations at the last amino acid of each GoLoco motif from R to F) were kindly provided by Dr. David P. Siderovski (The University of North Carolina, Chapel Hill NC. U.S.A). G18-mGL (containing R to F mutations at the last amino acid of each GoLoco motif) was generated using the Site-Directed Mutagenesis kit (Stratagene). G18ΔC (the N-terminal domain of G18) which contains only the first 60 amino acids of G18 was generated by inserting a stop codon at the appropriate position. The PCR product was subcloned into the pET-19b or pGEX4T2 vector to generate a His-tagged or GST-tagged fusion protein, respectively. All other constructs of G18 were further subcloned into the pET-19b vector using primers listed in Table 1. Proteins were expressed and purified as described below.

Table **1.** Primers used for making His-G18 and its mutants

Wild type G18	**sense primer:**	**-gcactccatatgtcgatggaggctgagagaccccaggaag**
	antisense primer:	**-cgctttaagcttgctcaagtctcagcaggtgtgtgtgg**
ΔNG18	**sense primer:**	**-gcactccatatggccatgcagactgaactccttctggac**
	antisense primer:	**-cgctttaagcttcgactactagtctcagcaggtgtgtgtgg**

2.3.3 *PROTEIN PURIFICATION*

N-terminally His_{10}-tagged G18 and mutants thereof were purified from *Escherichia Coli* BL21 (DE3) strain as follows. Six liters of LB medium containing 100 μg/ml ampicillin was inoculated with previously transformed cells and grown with vigorous shaking at 37°C to an OD600≥0.5. Expression of the proteins was induced by the addition of isopropyl-β-D-thiogalacto pyranoside (IPTG) to a final concentration of 500 μM, and incubated for an additional 4 hours before harvesting the bacteria by centrifugation. Bacteria were resuspended in 70 mL buffer A (25 mM Tris pH8.0, 500 mM NaCl, 1% tween 20, 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 1 μg/ml leupeptin, 10 μg/ml aprotinin, and 5 mM imidazole) and incubated on ice with 0.2 mg/ml lysozyme for 30 minutes. After a further 20 minute incubation with 25 μg/ml DNaseI and 0.5 mM $MgCl_2$, 3 ml of a 50% slurry of Ni-NTA affinity resin (Qiagen) pre-equilibrated in buffer A was added and the mixture was gently rocked at 4°C for 2 hours. The resin was subsequently loaded onto a 30 ml column and washed with buffer B (25 mM Tris pH 8.0, 500 mM NaCl, 1% tween20, 0.1 mM PMSF, 1 μg/ml leupeptin, 10 μg/ml aprotinin, 20 mM imidazole) followed by buffer C (25 mM Tris, pH 8.0, 500 mM NaCl, 0.2 mM PMSF, 2 μg/ml leupeptin, 20 μg/ml aprotinin, 40 mM imidazole). Proteins were eluted from the Ni-NTA beads by adding 800 μl buffer D (final concentrations: 25 mM Tris, pH 8.0, 500 mM NaCl, 0.2 mM PMSF, 1 μg/ml leupeptin, 10 μg/ml aprotinin, 250 mM imidazole) following a 30 minutes incubation at 4ºC. This process was repeated a total of six times. This procedure yielded proteins that were approximately 60% pure as determined by Coomassie Blue staining. Samples were further purified by FPLC using a Superdex 75 column (Pharmacia) to yield proteins that were > 90% pure. Peak fractions were pooled, and stored in aliquots at −80 °C.

GST-tagged G18 proteins were purified using a previously established glutathione-Sepharose 4B affinity purification method (Abramow-Newerly *et al.*, 2006). His-tagged Gαi1 and Gαo were grown in enriched medium (2% tryptone, 1% yeast extract, 0.2% glycerol, 0.5% NaCl, 50 mM KH_2PO_4), induced with 30 μM IPTG and purified as described previously (Mao *et al.*, 2004).

2.3.4 PROTEIN-PROTEIN INTERACTION ASSAY

Purified His_6-Gαi1 or His_6-Gαo (500 nM) was preincubated for 1 hour in binding buffer (50 mM Tris (pH 7.5), 0.6 mM EDTA, 150 mM NaCl, 1 mM DTT, 0.1% Triton-X 100, PMSF 2 μg/ml leupeptin, and 20 μg/ml aprotinin) at 30ºC in the presence of either 10 μM GDP or GDP+AMF (10 mM NaF, 10 mM $MgCl_2$, 20 μM $AlCl_3$). An equimolar amount (500 nM) of GST-tagged G18WT, G18-mGL, ΔNG18, ΔNG18-mGL or G18ΔC was added to the Gα mixture and incubated on a rotating platform at 4ºC for 2 hours. Glutathione Sepharose 4B beads or Ni-NTA agarose beads (20 μL bed volume) were then added into the protein mixture and incubate overnight. The protein mixture was washed three times with binding buffer in the presence of GDP±AMF and the beads were resuspended in 2X Laemmli buffer. Eluted proteins were separated on a 12% SDS gel and transferred to a Polyvinylidene Fluoride Transfer (PVDF) membrane (Pall Corporation) for immunoblotting.

2.3.5 IMMUNOBLOTTING

Membranes were incubated with blocking buffer (Tris-Buffered Saline Tween-20 (TBST) with 5% skim milk) for 1 hour and then probed with anti-His or anti-GST antibody (1:1000) (Santa Cruz biotechnology) diluted in blocking buffer overnight on a rotating platform at 4ºC. Blots were subsequently washed 3 times with TBST and then incubated with HRP-conjugated secondary antibody (1:2000) (Promega) diluted in TBST for 1 hour at room temperature. After another 3 washes with TBST, the blot was visualized by LumiGLO Reserve Chemiluminescence substrate (KPL, Inc) using a FluorChem 8000 imaging system.

2.3.6 Pre-steady state GTPase assay

Pre-steady state GTPase activity of purified G proteins was measured as described earlier (Mao *et al.*, 2004). Purified His_6-Gαi1 or His_6-Gαo (1 μM) was incubated with [γ-^{32}P]-GTP (1×10^6 cpm/assay) plus 1 μM nonradioactive GTP for 15 minutes at 30ºC or 20ºC in GTP binding buffer (50 mM Hepes (pH 7.5), 0.05% Lubrol, 1 mM DTT, 10 mM EDTA, 5 μg/ml BSA) and then kept on ice. The GTP binding reaction was stopped by the addition of 0.25 volumes of mix buffer (50 mM Hepes (pH 7.5), 10 mM $MgCl_2$, 500 μM GTP) and a single round of GTP hydrolysis was initiated by adding 10 mM of Mg^{2+} in the presence or absence of 1 μM G18, one of its mutants, RGS4 (300 nM in Figure 2.5, 100nM in Figure 2.8) or both RGS4 and G18. Aliquots were taken at the indicated time points and quenched with ice-cold 5% (w/v) Norit in 0.05 M NaH_2PO_4. The level of radioactive $^{32}P_i$ in the supernatant was detected by liquid-scintillation counting on a Packard Tri-Carb 2900TR liquid scintillation counter (Perkin Elmer).

2.3.7 *GTPγS BINDING ASSAY*

Purified His_6-Gαi1 (100 nM) or His_6-Gαo (100 nM) was incubated for 1 hour at 4°C in binding buffer (20 mM Hepes (pH 8.0), 1 mM EDTA (pH 8.0), 100 mM NaCl, 1 mM DTT, 2 mM $MgCl_2$, 0.1 mg/ml BSA, 0.1% Lubrol, PMSF and 1 μg/ml leupeptin, 10 μg/ml aprotinin) in the presence or absence of 1-2 μM G18 or its mutants. Binding assays were initiated by adding 0.5 μM [^{35}S]-GTPγS (1.25×10^5 cpm/pmol). The incubation continued for 30 minutes at 30ºC (Gαi1) or 60 minutes at 20ºC (Gαo). The assay was terminated by adding ice-cold stop buffer (20 mM Tris (pH 8.0), 10 mM $MgCl_2$, 100 mM NaCl, 0.1% Lubrol, 1 mM GTP and 0.1 mM DTT). Samples were filtered through nitrocellulose membranes (Millipore) followed by washing four times with 2 mL ice-cold wash buffer (20 mM Tris (pH 8.0), 100 mM NaCl, 10 mM $MgCl_2$). Radioactivity was measured using liquid-scintillation counting. The nonspecific binding was determined in the presence of 100 μM unlabeled GTPγS, and these values were subtracted to yield specific binding.

2.3.8 *SOLUTION-BASED STEADY STATE GTPASE ASSAY*

Purified His_6-Gαi1 or His_6-Gαo (250 nM) was incubated with 3 μM G18 or one of its mutants for 30 minutes at 4ºC in assay buffer (50 mM NaHepes (pH 8.0), 1 mM EDTA, 2 mM, DTT, 0.1%Triton-X 100, 6 mM $MgSO_4$, 1 μg/ml leupeptin, 10 μg/ml aprotinin, and PMSF). [γ-^{32}P]GTP (1×10^6 cpm/assay) plus 5 μM nonradioactive GTP was then added and the protein mix was further incubated for 1 hour at 30ºC (Gαi1) or 20ºC (Gαo). The reaction was stopped by adding ice-cold 5% (w/v) Norit in 0.05 M NaH_2PO_4. After

centrifugation, the level of radioactive $^{32}P_i$ in the supernatant was determined by liquid-scintillation counting.

2.3.9 RECEPTOR- AND AGONIST-STIMULATED GTPASE ASSAY

Sf9 membranes overexpressing M2 muscarinic receptor and heterotrimeric G proteins were prepared as indicated previously (Cladman and Chidiac, 2002). These Sf9 cell membranes (8 µg/tube) were assayed for 100 µM carbachol-stimulated GTP hydrolysis at 30ºC for 5 minutes in the absence or presence of the indicated purified proteins in the reaction buffer (20 mM Hepes, pH 7.5, 1 mM EDTA, 1mM DTT, 0.1 mM PMSF, 10 mM NaCl, 2 mM $MgCl_2$ (7.5 mM calculated free Mg^{2+}) 1 µM GTP, 1 mM ATP, [γ-^{32}P] GTP (1×10^6 cpm per assay) and protease inhibitors) in a total reaction volume of 50 µL. The assay was stopped by adding 950 µL of ice-cold 5% (w/v) Norit in 0.05 M NaH_2PO_4. The reaction mixture was centrifuged and the level of $^{32}P_i$ in the resulting supernatant was determined by liquid-scintillation counting. The nonspecific GTPase activity was defined as that in the presence of the inverse agonist tropicamide (10 µM), and these values were subtracted from the total counts per minute to yield the agonist- and receptor-dependent GTP hydrolysis.

2.4 RESULTS

2.4.1 Tissue distribution of G18 in mice

G18 is a 160 amino acid protein encoded by a 1472 bp mRNA, with 88% similarity between humans and mice at the amino acid level (Cao *et al.*, 2004). To determine the tissue distribution of G18 in mice, total RNA was extracted from different tissues of three month old C57BL/6 mice and primers specific for the open reading frame of G18 were used to probe for G18. As shown in *Figure 2.1*, full-length G18 was detected at approximately 500 bp, corresponding well to the open reading frame of 480bp. We found that G18 was highly expressed in spleen and lung, moderately expressed in heart, kidney, liver, brain, and adipose tissue. These results are consistent with a previous report (Cao *et al.*, 2004) by *Cao et. al* using a human RNA blot thus indicating a similar tissue distribution between human and mouse.

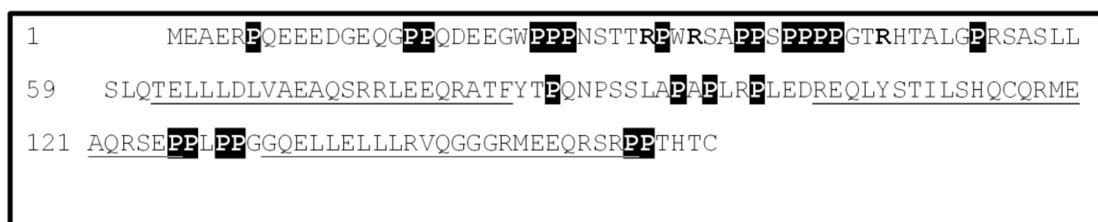
2.4.2 Purified G18 can interact with both inactive and fluoroaluminate-activated Gα proteins.

Previous studies have shown that the GoLoco motifs of G18 have higher binding affinity toward Gαi-GDP compared to Gαo-GDP (Cao *et al.*, 2004;Kimple *et al.*, 2004). To extend these findings, we tested the binding between purified G18 and purified Gαi1 or Gαo in both their inactive GDP-bound and fluoroaluminate-activated states. Three G18 mutants were examined (*Figure 2.2*) which contain an N-terminal truncation (ΔNG18), inactivating point substitutions within each GoLoco motif (G18-mGL), or a combination of both modifications (ΔNG18-mGL). Consistent with previous studies (Cao *et al.*, 2004;Kimple *et al.*, 2004), G18wt and ΔNG18 interacted with Gαi1-GDP whereas G18-

Fig. 2.1. (A) Amino acid sequence of G18. The three GoLoco motifs are underlined. The proline residues are shown in white on a black background, and arginines that could potentially contribute to the N-terminal effects are indicated in bold type. (B) Tissue distribution of G18. Various tissues from 3 month old C57BL/6 mice were isolated, total RNA was extracted, and RT-PCR followed by PCR was performed using primers specific for the open reading frame of G18. GAPDH was used as loading control. Control lane indicates the reference length of G18 using the PCR product from the plasmid.

Figure 2.1

A



B

Tissue Distribution of G18

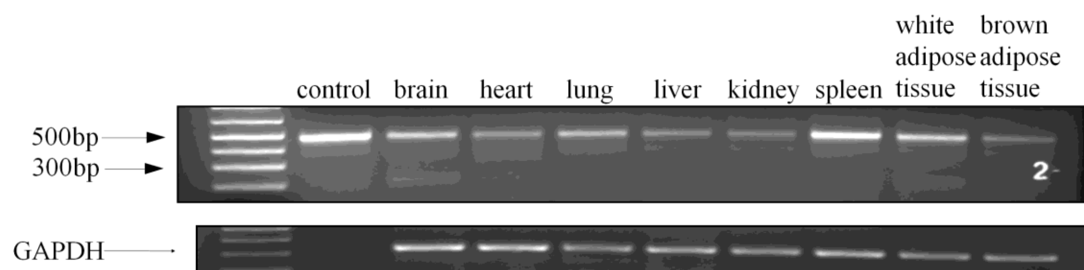


Figure 2.2

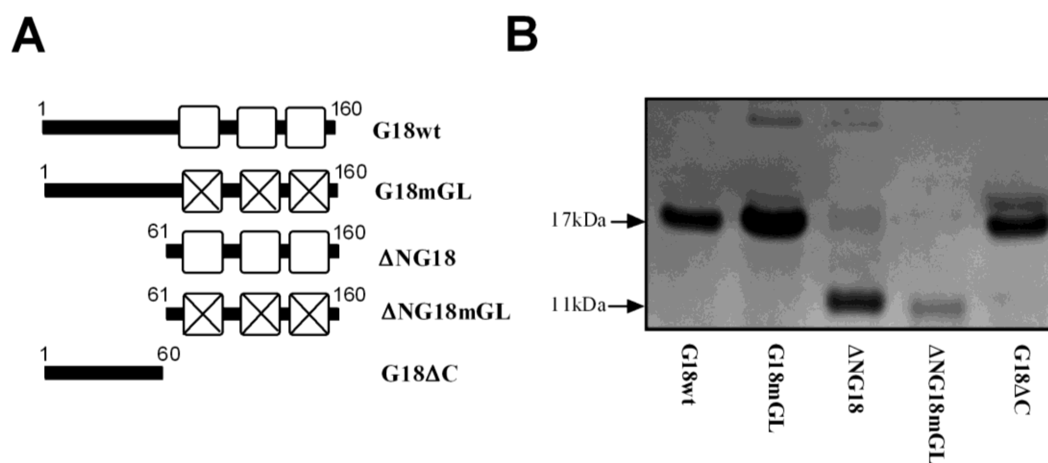


Fig. 2.2. Constructs and purified proteins. (A) Domain architecture of different constructs used in the study. (B) His-tagged proteins were purified from *E.Coli* strain BL21 (DE3) using Ni-NTA affinity purification followed by FPLC. Protein purity was estimated by Coomassie staining. The correct molecular size of G18ΔC (which may have run anomalously due to its high proline content) was verified by mass spectrometry.

mGL and ΔNG18-mGL did not (*Figure 2.3*). None of the purified G18 proteins displayed any detectable binding to the GDP-bound form of Gαo under the conditions employed in our studies (*Figure 2.3*).

To determine whether the observed G18 interactions were specific for inactive G protein, we performed parallel *in vitro* pull-down assays in the presence of AlF_4^- to mimic the transition state of G protein. Surprisingly, in the presence of AlF_4^-, both Gαi1 and Gαo interacted with G18wt (*Figure 2.3*). Moreover, removal of the G18 N-terminal domain diminished its binding to G proteins (*Figure 2.3*). These results suggest that whereas the GoLoco motifs of G18 are responsible for its interaction with inactive Gαi, the N-terminal segment of G18 may serve to bind the fluoroaluminate-activated $G\alpha_{i/o}$.

2.4.3 THE N-TERMINAL DOMAIN OF G18 CAN INTERACT WITH FLUOROALUMINATE-ACTIVATED Gα PROTEINS

We also generated and tested an additional truncation mutant of G18 containing only the first 60 residues (G18ΔC) to confirm the binding between the N-terminus of G18 and transition state of G proteins. Indeed, this segment of G18 was sufficient to bind to fluoroaluminate-activated forms of both Gαi1 and Gαo (*Figure 2.4*).

2.4.4 G18 HAS NO EFFECT ON G PROTEIN GTPASE ACTIVITY IN PRE-STEADY STATE GTPASE ASSAYS.

We examined the effects of G18wt, ΔNG18 and G18ΔC on the various stages of the G protein guanine nucleotide-binding cycle to determine the biochemical significance of the

Figure 2.3

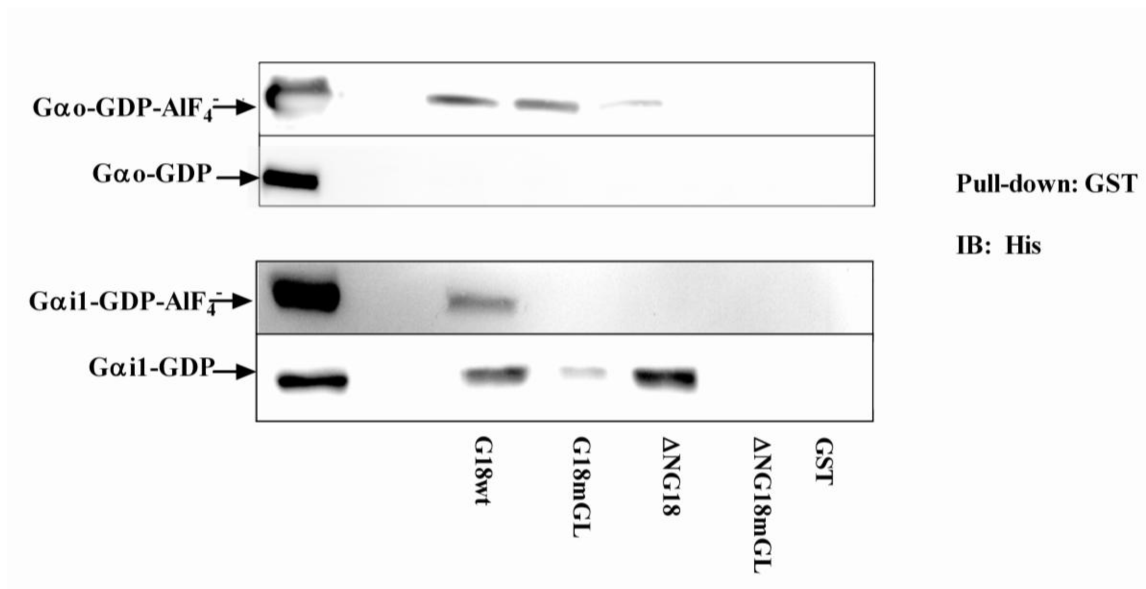
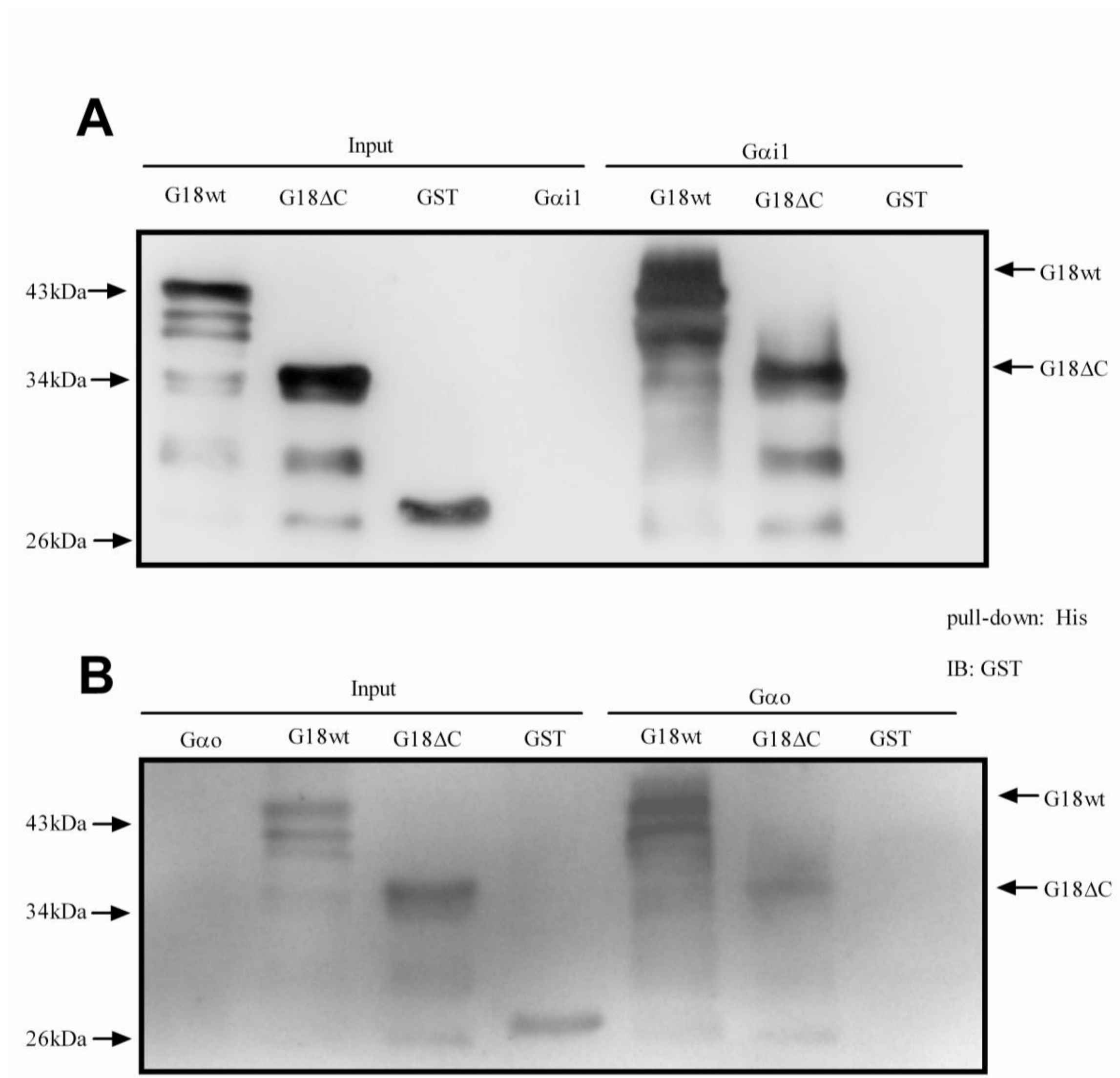


Fig. 2.3. Protein-protein interaction between G proteins and G18. Purified His_6-Gαi1 or Gαo was incubated with excess GDP±AlF_4^- for 30 minutes at 4°C, purified GST-tagged G18 or one of its mutants was added to the solution, and the incubation was continued for another 2 hours before adding glutathione-Sepharose 4B beads. After overnight incubation at 4°C on a rotating platform, the mixture was centrifuged, washed and the resulting pellet was retained for immunobloting analysis. Membranes were probed with anti-His antibody. Input represents 10% of the protein used in the pull-down assay. A representative blot of 3 independent experiments is shown.

Fig. 2.4. Protein-protein interaction between G proteins and the N-terminus of G18. Purified His_6-Gαi1 or Gαo was incubated with excess GDP+AlF_4^- for 30 minutes at 4°C, purified GST-tagged G18 or its isolated N-terminus (G18ΔC) was added to the solution, and the incubation was continued for another 2 hours before adding Ni-NTA agarose beads. The protein mix was further incubated overnight at 4°C on a rotating platform, samples were centrifuged and the resulting pellet was retained for immunobloting analysis. Membranes were probed with anti-GST antibody. Input represents 10% of the protein used in the pull-down assay. A representative blot of 3 independent experiments is shown.

Figure 2.4



interaction between the G18 N-terminus and Gα subunits. Since GTPase activating proteins (GAP) tend to have high affinity for fluoroaluminate-activated G proteins (Berman *et al.*, 1996;Watson *et al.*, 1996), we first investigated the possibility that the G18 N-terminus might have GAP activity towards Gαi/o subunits using a solution-based pre-steady state GTPase assay.

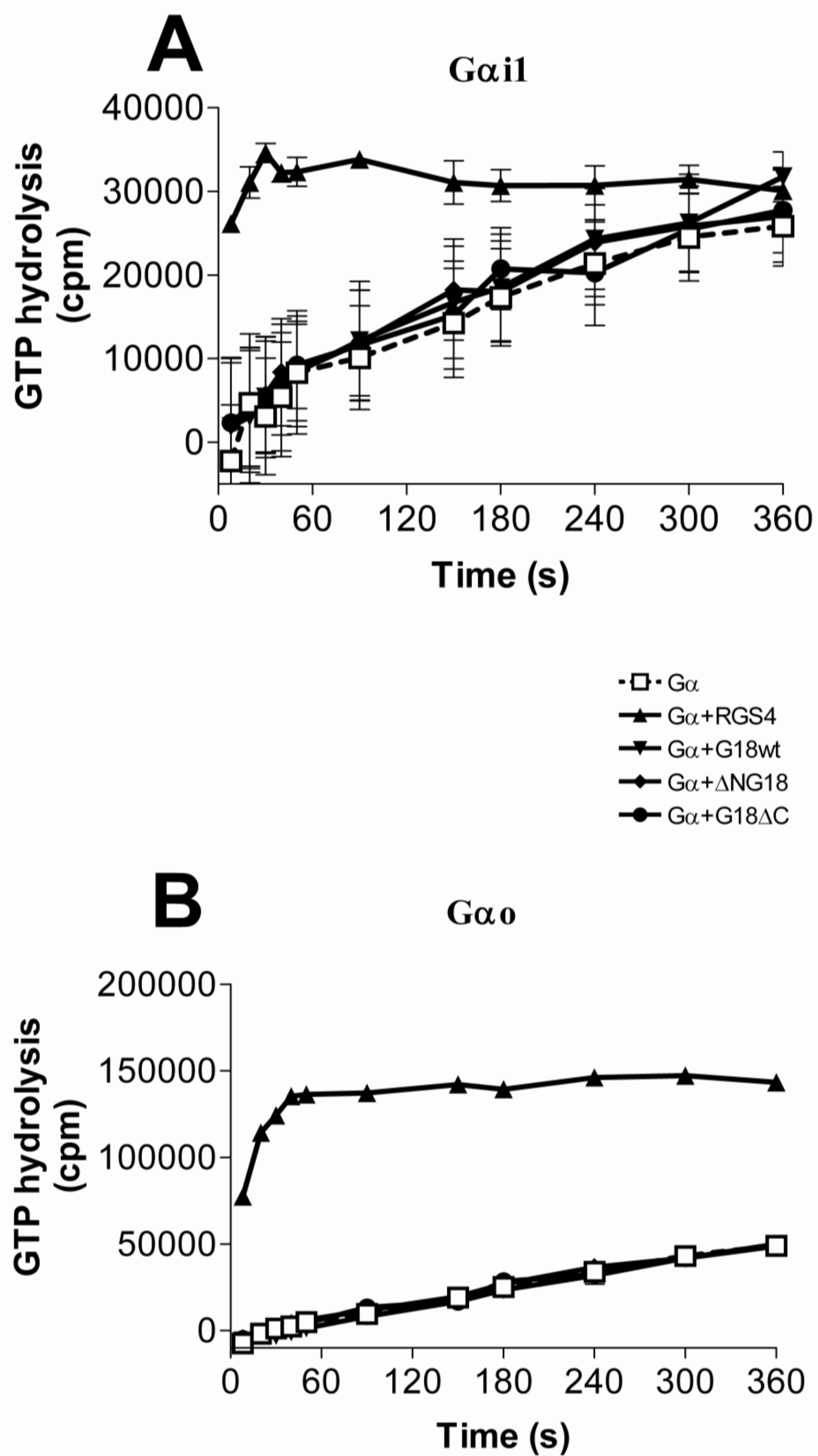
Our results revealed that none of the purified G18 proteins tested had any impact on the rate of GTP hydrolysis by Gαi1 or Gαo (*Figure 2.5*). RGS4, serving as a positive control, exhibited robust GAP activity on both Gαi1 and Gαo (*Figure 2.5*). These results indicate that G18 does not serve as a GAP towards free Gαi/o subunits.

2.4.5 The N-terminal domain of G18 acts as a guanine nucleotide exchange factor (GEF) on Gαi1

We next assessed whether the N-terminus of G18 might have any effects on nucleotide exchange, distinct from the established GDI activity of its GoLoco motifs on Gαi proteins. Changes in the rate of GDP dissociation from Gα proteins were inferred from changes in the rate of GTPγS binding using a solution-based pre-steady state assay. As expected, the GoLoco region of G18 (ΔNG18) acted to inhibit GDP dissociation from Gαi1, as revealed by an 85% decrease in GTPγS binding to the latter (*Figure 2.6A*). In contrast, the isolated N-terminal segment of G18 (G18ΔC) *increased* GTPγS binding to Gαi1 by approximately 60%. Interestingly, full-length G18 had essentially no effect on the observed rate of GTPγS binding to Gαi1, suggesting that the opposing functions of

Fig. 2.5. The effects of G18 on G protein GTPase activity under pre-steady state conditions. Purified (A) His_6-Gαi1 or (B) His_6-Gαo was incubated with γ^{32}P]-GTP (1×10^6cpm/assay) for 15 minutes at 30ºC (Gαi1) or 20ºC (Gαo). A single round of GTP hydrolysis was measured at 0ºC in the presence of 10 mM Mg^{2+} and RGS4, G18, or one of its mutants as indicated. Data points shown are means ± S.E.M from 3 independent experiments.

Figure 2.5



the two domains balance out under these experimental conditions. These results suggest that G18 can serve as a bifunctional regulator of Gαi1, whereby its GoLoco region functions as a GDI and its N-terminal domain acts as a GEF.

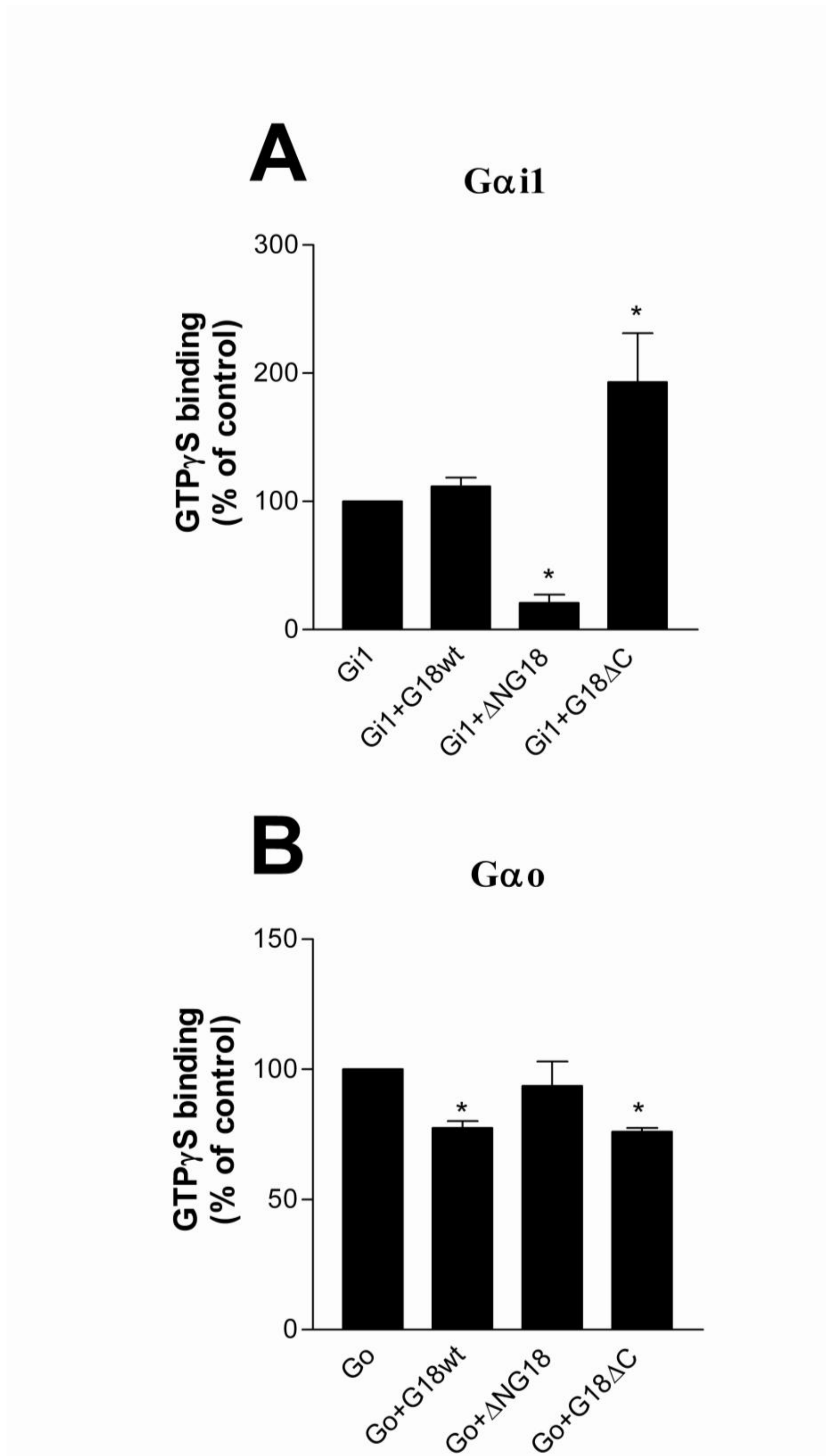
We further used a solution-based, steady state GTPase assay to corroborate the putative GEF activity of the G18 N-terminal region on Gαi1. Interestingly, full length G18 significantly promoted GTP turnover (*Figure 2.7A*). In contrast, the N-terminal deletion mutant ΔNG18 decreased GTPase activity (*Figure 2.7A*), which is consistent with its GDI activity. In agreement with the results obtained from pre-steady state GTPγS binding assays (*Figure 6A*), there was a trend towards an increase with G18ΔC (*Figure 2.7A*) and this reached statistical significance when the concentration was raised to 10 μM (data not shown). These results suggest that under steady state conditions with free Gαi1, the GEF activity of the N-terminal domain of G18 predominates over the GDI function of its GoLoco region.

2.4.6 The N-terminal domain of G18 inhibits nucleotide exchange on Gαo.

The effects of G18 and its mutants on Gαo activity were also examined. Surprisingly, G18wt inhibited nucleotide exchange on free Gαo by approximately 25% (*Figure 2.6B*). The GoLoco region of G18 (ΔNG18) had no effect on GDP dissociation from Gαo, which is consistent with its poor binding to Gαo-GDP. In contrast, G18ΔC inhibited

Fig. 2.6. The effects of G18 on G protein nucleotide exchange. Purified (A) His_6-Gαi1 or (B) His_6-Gαo was preincubated with G18 at 4ºC. Binding assays were initiated by adding 0.5 μM [^{35}S] GTPγS (1.25×10^5 cpm/pmol) at 30ºC (Gαi1) or 20ºC (Gαo). The binding of GTPγS to Gα proteins was measured after 30 min (Gαi1) or 60 min (Gαo) of incubation. Nonspecific binding was estimated in the presence of excess unlabeled GTPγS, and these values were subtracted from the results. The data are presented as the mean ± S.E.M. of 3-5 independent experiments performed in duplicate. * $P<0.05$, compared to G protein alone (One way ANOVA with Tukey's Multiple Comparison Test).

Figure 2.6



GTPγS binding to Gαo to the same level as G18wt. These results indicate that the effect of G18 on Go nucleotide exchange is primarily attributable to its N-terminal domain.

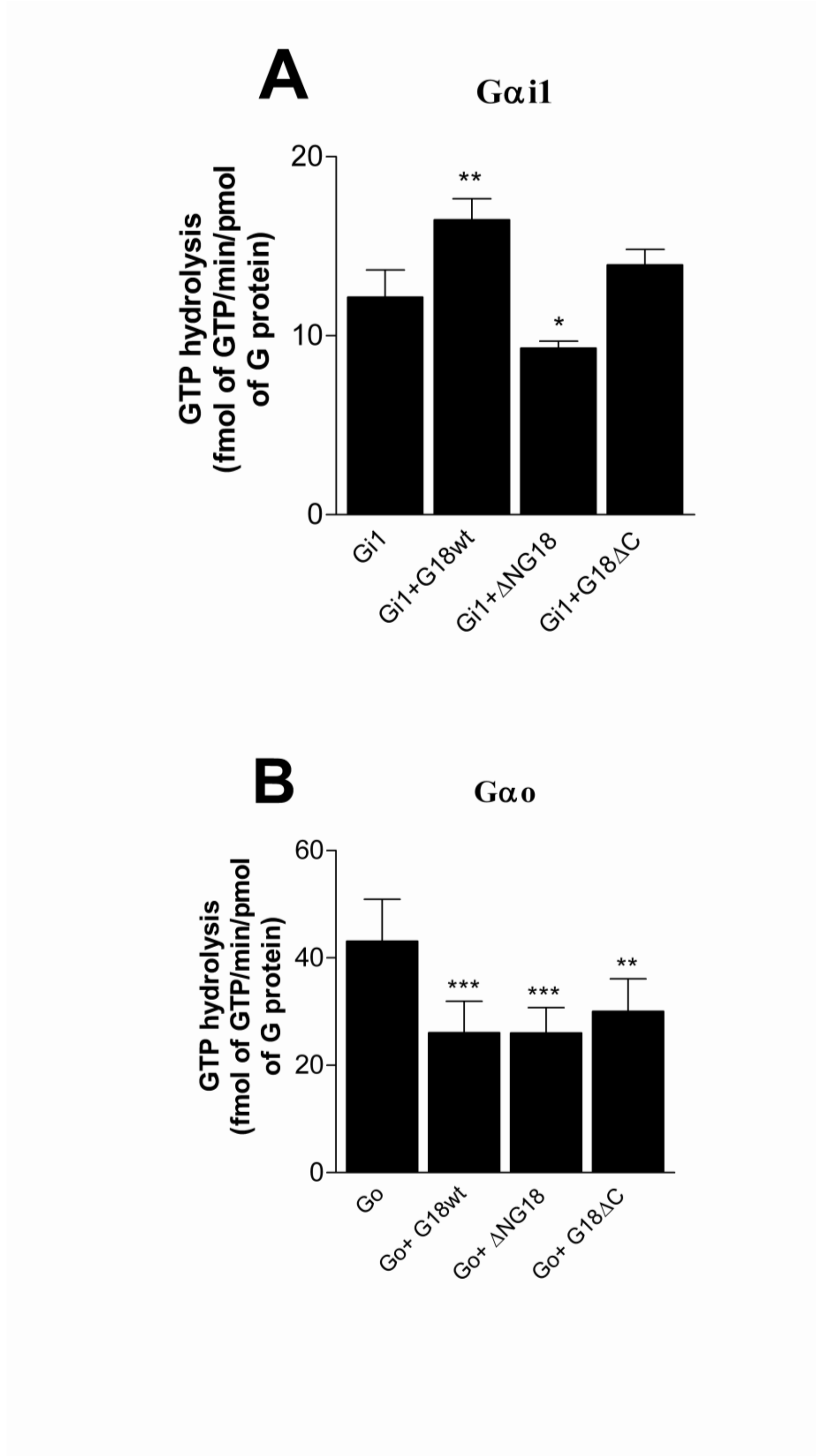
Further, in the solution-based steady state GTPase assay, both G18wt and G18ΔC decreased the GTP hydrolysis of free Gαo, consistent with the observed inhibitory effects of the full length protein and the isolated N-terminal domain (*Figure 2.7B*). ΔNG18 also inhibited the GTPase activity of Gαo under these conditions. The reason for the apparent discrepancy regarding the effects of ΔNG18 in *Figure 2.6B* versus *Figure 2.7B* is not clear. Overall our results suggest that the function of the N-terminal domain of G18 may depend on which G protein is involved, *i.e.*, promoting nucleotide exchange at Gαi1 but decrease overall exchange at Gαo.

2.4.7 Effects of G18 on receptor- and agonist- stimulated G protein GTPase activity.

The foregoing observations indicate that the activity of G18 is not limited to its GoLoco motifs, as its N-terminal domain also modulates G protein-nucleotide interactions. In addition, these results clearly identify Gαo as a novel interacting partner of G18. However, little is known regarding the activity of G18 (and GoLoco motif-containing proteins in general) within the context of receptor-stimulated G protein function. Therefore, we used a receptor- and agonist-dependent steady state GTPase assay to study the effects of G18 on GTP turnover by overexpressed Gαi1 or Gαo in membranes from Sf9 cells also co-expressing exogenous M2 muscarinic receptor and Gβγ subunits.

Fig. 2.7. The effects of G18 on Gα protein GTPase activity under steady state conditions. Purified (A) His_6-Gαi1 or (B) His_6-Gαo was mixed with G18 at 4ºC. The protein mixture was incubated with [γ-^{32}P]-GTP (1×10^6 cpm/assay) in the presence of 6 mM Mg^{2+} at 30ºC (Gαi1) or 20ºC (Gαo). Free $^{32}P_i$ level was measured after 60 minutes of incubation. The data are presented as the mean ± S.E.M. of 3 independent experiments performed in duplicate. * $P<0.05$, ** $P<0.01$, *** $P<0.001$ compared to G protein alone (One way ANOVA with Tukey's Multiple Comparison Test).

Figure 2.7



The addition of G18wt to carbachol-activated M2+Gi1 or M2+Go membranes yielded little or no change in agonist-dependent GTPase activity (*Figure 2.8A,B*), notwithstanding its demonstrated effects in solution-based assays. Mutant forms of G18 similarly lacked activity under these conditions (data not shown). This apparent lack of effect could reflect a masking of changes in nucleotide exchange rates by the relatively slow intrinsic hydrolytic activities of Gαi1 and Gαo in the presence of the receptor. To ensure that GTP hydrolysis *per se* was not rate limiting, cyclical GTP turnover was also measured in the presence of purified RGS4, which accelerates the hydrolytic step (*Figure 2.5*). Indeed, the inclusion of RGS4 in these assays revealed effects of G18wt on both Gi1 and Go, which were inhibited respectively by approximately 60% and 80% at the maximally obtainable concentration of G18wt (*Figure 2.8A, B*). Another conceivable explanation is that this observation may reflect an effect of G18 on RGS4 activity. We used a pre-steady state GTPase assay to test this possibility, and found that G18 had little or no effect on the GAP activity of RGS4 on either Gαi1 or Gαo (*Figure 2.8C, D*).

To determine which regions of G18 might contribute to its effects on receptor-stimulated GTP turnover by Gi1 and Go, mutants bearing truncations and/or inactivating GoLoco point substitutions were also evaluated. Compared to full length G18wt, N-terminally truncated G18 (ΔNG18) produced a similar inhibitory effect on receptor-activated Gi1 (*Figure 2.9A*) but a greatly reduced effect on Go (*Figure 2.9B*). In contrast, mutation of the GoLoco motifs (G18-mGL) substantially reduced activity on Gi1 (*Figure 2.9C*) but caused only a minor change in the inhibitory effect of G18 on Go GTPase activity (*Figure 2.9D*). Despite the evident GEF effect of G18ΔC on isolated Gαi1 in solution

(*Figure 2.6A*), such activity was not observed in membranes in the presence of agonist-activated receptor plus Gβγ (*Figure 2.9E*), suggesting that the GEF activity of the receptor may exceed that of the N-terminal domain of G18. G18ΔC instead produced a marginal inhibitory effect on receptor-activated Gi1 (*Figure 2.9E*), and a more pronounced inhibitory effect in corresponding experiments with Go (*Figure 2.9F*). The latter observation reinforces the notion that G18ΔC has the potential to inhibit nucleotide turnover by inhibiting nucleotide exchange towards Gαo. Overall, the inhibitory effect of full length G18 on M2+Gi1 GTPase activity is attributable primarily to its GoLoco motifs, whereas the effect on M2+Go seems to derive mostly from its N-terminal domain.

Fig. 2.8. The effects of G18 on receptor- and agonist- stimulated G protein GTPase activity. (A, B) Sf9 cell membranes overexpressing M2 Muscarinic acetylcholine receptor and heterotrimeric Gαi1 or Gαo were prepared as indicated in Experimental Procedures. Carbachol was used to activate M2 receptor. Steady-state GTPase activities of G proteins were measured in the presence (solid line) or absence (dashed line) of RGS4 and the indicated concentrations of G18wt. Nonspecific signal was determined in the absence of added purified proteins and in the presence of tropicamide. The data are presented as the mean ± S.E.M. of 3-4 independent experiments. (C, D), Purified His_6-Gαi1 or His_6-Gαo was incubated with [γ-^{32}P]-GTP (1×10^6cpm/assay) for 15 minutes at 30ºC (Gαi1) or 20ºC (Gαo). A single round of GTP hydrolysis was measured at 0ºC in the presence of 10 mM Mg^{2+} (□) and RGS4 (▲) or RGS4+G18 (▼). Data points shown are means ± S.E.M from 3 independent experiments.

Figure 2.8

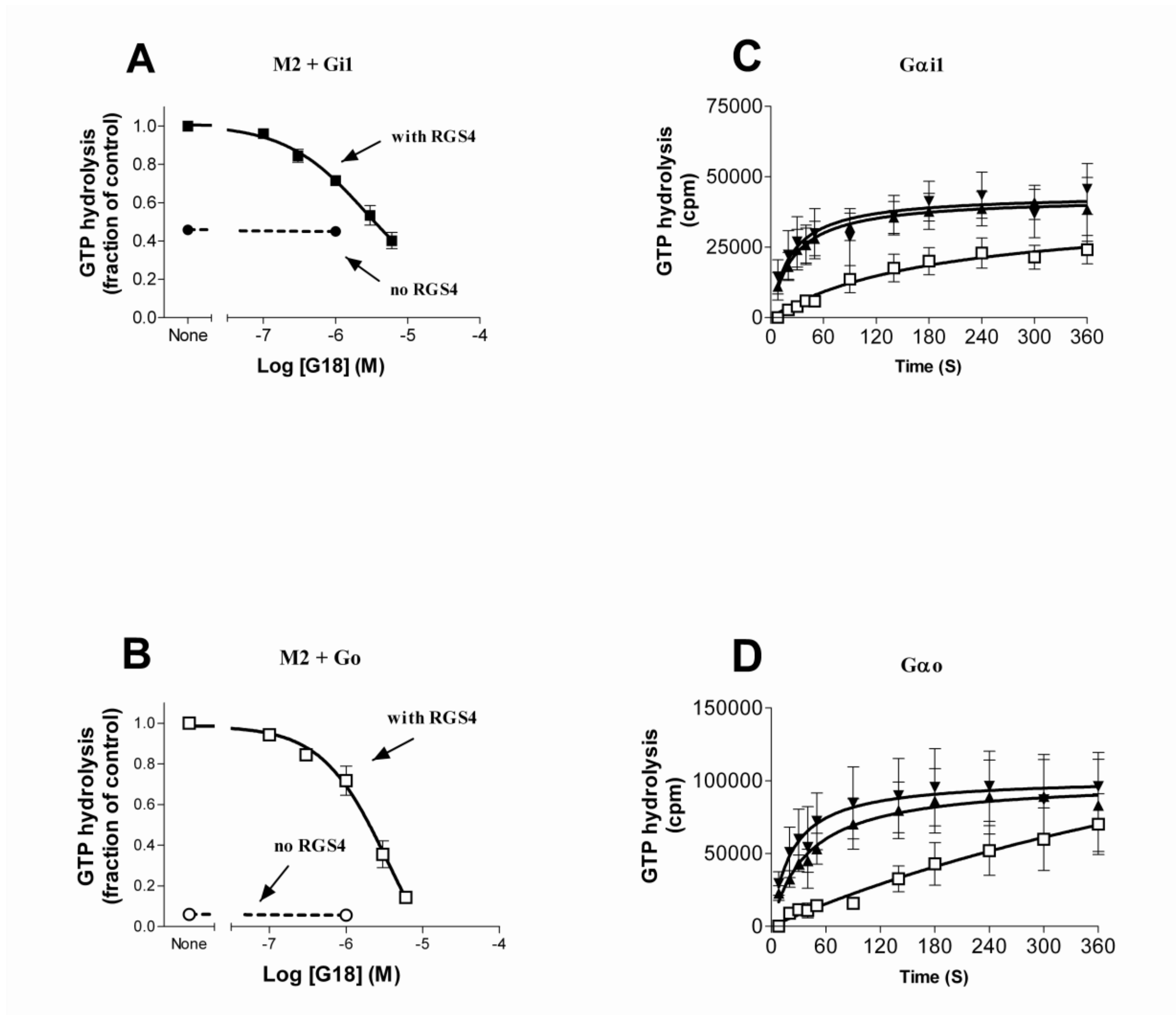


Figure 2.9

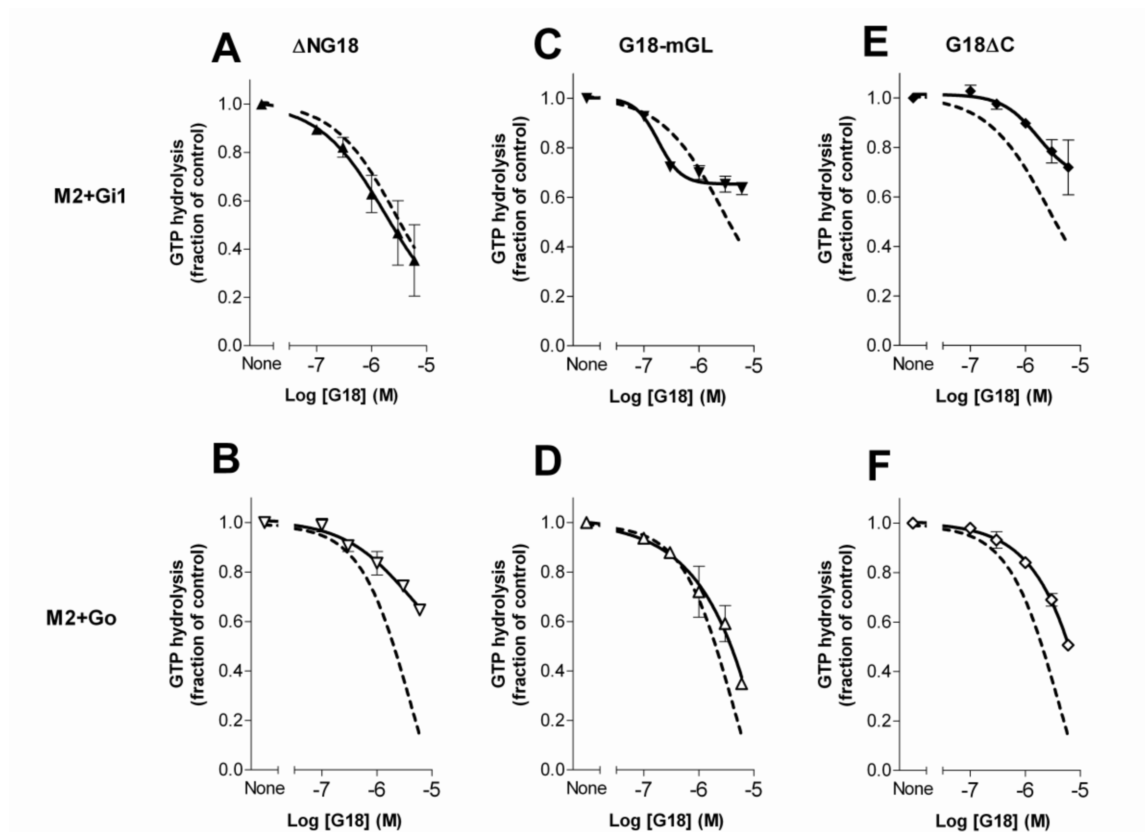


Fig. 2.9. The effects of G18 mutants on receptor- and agonist-stimulated G protein GTPase activity. M2-Gi1 and M2-Go membranes from sf9 cells were assayed for agonist-stimulated steady-state GTPase activity in the presence of RGS4 and the indicated concentrations of G18 mutants, as described in Figure 2.7. G18wt activity (Figure 2.7) is shown as a dashed line for comparison in each panel. The data points shown are means ± S.E.M. from 3-4 independent experiments.

2.5 DISCUSSION

G18 was first identified within the major histocompatibility complex class III region on chromosome 6, and thus may be involved in the control of host immune defense and inflammatory responses (Gruen and Weissman, 2001;Moulds, 2001). Such a role is further suggested by its relatively high expression levels in the spleen (*Figure 2.2*) and other immune tissues (Cao *et al.*, 2004), although overall it appears to be fairly widely distributed. Little is known about the biological function of G18, and a clear understanding of this is difficult without accurate knowledge of its biochemical behavior. The most significant finding described herein is the identification of the N-terminal region of G18 as a novel binding partner of Gαi/o proteins. Surprisingly, this domain promotes nucleotide exchange on Gαi1 but seemingly inhibits nucleotide exchange on Gαo. To our knowledge, this is the first example of a single domain that has distinct regulatory effects toward different Gα proteins. Another unusual property of G18 is that it contains multiple G protein binding domains that produce dissimilar effects on the activity of a common target, and the ability of G18 both to promote and to impede GDP dissociation from Gαi1 respectively via its N-terminal and GoLoco regions appears to be unique. A comparable enigma exists with the R12 subfamily of RGS proteins, most of which contain a GoLoco motif that can produce GDI effects on Gαi and also an RGS domain that accelerates GTPase activity (Kimple *et al.*, 2001).

The most widely recognized GEF effects on heterotrimeric G proteins are those produced by agonist-activated GPCRs, but beyond this classical paradigm a variety of non-receptor

GEFs have also been identified including Ric-8A (Tall and Gilman, 2005;Thomas *et al.*, 2008), Ric-8B (Kerr *et al.*, 2008), CSPα (Natochin *et al.*, 2005), GIV (Garcia-Marcos *et al.*, 2009), AGS1/Dexras1 (Cismowski *et al.*, 2000), GAP-43/neuromodulin /B-50 (Strittmatter *et al.*, 1991), and the yeast protein Arr4 (Lee and Dohlman, 2008). The primary amino acid composition of the N terminal domain of G18 does not resemble any of the previously identified GEFs, however, there are structural attributes of G18 that could conceivably contribute to interactions with G proteins. For example, the N terminal segment of G18 is highly enriched in proline (14 out of 60 residues), which has a special role in protein function due to its unique side chain structure and its effects on overall protein conformation. Proline residues tend to disrupt both α-helical and β-sheet structures and two or more residues in a row typically promote left-handed PPII (polyproline type II) helices containing three residues per turn (Williamson, 1994;Li, 2005). PPII helices can readily adopt different conformations and thus bind to a variety of proline recognition domains, such as SH3 and WW domains (Li, 2005). Proline-rich motifs have been found within several effectors of monomeric G proteins, such as Son of sevenless (Garbay *et al.*, 2000;Gureasko *et al.*, 2008), Sprouty 2 (Garbay *et al.*, 2000;Lao *et al.*, 2006), and POB1 (Garbay *et al.*, 2000;Oshiro *et al.*, 2002). Our results suggest that a proline-rich motif may also serve as a binding partner for heterotrimeric G proteins. The mechanism by which G18 confers GEF activity on free Gαi1 requires further study but the presence of multiple arginine residues, particularly those at positions 31, 34 and 46 (which would line up in a PPII helix) could conceivably provide the cationic interface needed to promote nucleotide exchange (Higashijima *et al.*, 1990).

Consistent with the present results, previous studies have shown the GoLoco region of G18 to selectively bind to and impede GDP dissociation from inactive Gαi (Cao *et al.*, 2004;Kimple *et al.*, 2004). However, the GoLoco motifs in proteins such as Pcp2 and Rap1GAP appear not to be selective between Gαi and Gαo (Jordan *et al.*, 1999;Luo and Denker, 1999;Natochin *et al.*, 2001). Also it is not obvious that the potential effects of activating agents have necessarily been tested in all studies on GoLoco-Gα interactions. The present results indicate that the binding of the GoLoco region of G18 to Gαo can be induced, albeit modestly, by AlF_4^- (*Figure 3, lane 4*). Nothing analogous to this observation could be found in the literature, however, the drosophila GoLoco protein Pins has been shown to bind to both active and inactive Gαo (in this case drosophila Gαo purified from bacteria) and to regulate Gαo-dependent GPCR signaling (Kopein and Katanaev, 2009). Although we and Kimple *et al.* (Kimple *et al.*, 2004) were unable to show binding between non-activated mammalian Gαo and the GoLoco region of G18, Cao and co-workers did observe binding to Gαo-GDP (Cao *et al.*, 2004). The latter study used Gαo purified from insect cells rather than *E. coli* suggesting that co- and/or post-translational modification of G proteins may affect their GoLoco interactions.

All of the G protein binding domains of G18 appear to be sensitive to the activation states of their Gα targets (*Figure 3*). N-terminal domain binding seems to be selective for the transition state of both Gαi1 and Gαo. These interactions appear to be of primary importance for the binding of full length G18wt in the presence of AlF_4^-, as G protein binding was greatly reduced or eliminated in the absence of the N-terminal domain. However, the effects of the N-terminal domain of G18 on G protein activity must

ultimately be viewed within the context of the entire protein, including its three GoLoco motifs. Important considerations include 1) which, if any, domain has a predominant effect on a particular G protein either as it signals at the plasma membrane or performs other functions in the cell interior, 2) whether G protein binding is mutually exclusive or can occur simultaneously to both the N-terminal domain and one of the GoLoco motifs, and 3) whether an individual G protein can bind to different G18 domains at different points within its GTPase cycle.

The effect of G18 on a G protein may depend on its cellular localization and/or other binding partners. We observed that the N-terminal GEF effect negates (*Figure 2.6A*) or overrides (*Figure 2.7A*) the GoLoco GDI effects on Gαi1 in solution, whereas the ability of G18 to inhibit receptor-stimulated Gi1 activity is unaffected by the removal of the N-terminal domain (*Figure 2.9A*). This suggests that perhaps free intracellular Gαi1 would be activated by the N-terminal GEF function of G18 while the GoLoco motifs would inhibit receptor-dependent Gαi1 activation at the plasma membrane.

Together, the four G protein binding domains within G18 have the potential to produce complex effects on G protein activity. It is unclear whether the N-terminal and GoLoco domains might either impede or facilitate the other's binding to Gα, or whether the different domains can act sequentially as Gα goes through its GTP cycle. If they act independently, then multiple G proteins could be affected at the same time. Kimple *et. al* (Kimple *et al.*, 2004) have shown that the first and third GoLoco motifs within N-

terminally truncated G18 can simultaneously bind individual G proteins and thus function as independent GDIs, although this could potentially differ in the presence of other G protein regulators. The idea that the N-terminal domain might be able to access GoLoco-associated Gα is suggested by evidence that Ric-8A can exert its GEF activity on Gαi while the latter is coupled to the GoLoco region of AGS3. Analogously, GPCRs and Gβγ must act in concert for agonist-stimulated nucleotide exchange to occur (Bourne, 1997;Cabrera-Vera *et al.*, 2003;Mahon *et al.*, 2006). While it is an intriguing possibility, the present results do not directly speak to whether the N-terminal and GoLoco domains of G18 might bind simultaneously to either Gαi1 or Gαo (or alternatively inhibit one another), and thus further studies will be required to address this issue.

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Chapter 3

REGULATION OF RGS5 GAP ACTIVITY BY THE GOLOCO MOTIF CONTAINING PROTEIN G18

A version of this chapter is in preparation for publication: Zhao P, Chidiac P (2011) Regulation of RGS5 GAP activity by the GoLoco motif containing protein G18

3 CHAPTER 3

3.1 CHAPTER SUMMARY

RGS5 is an R4 subfamily RGS protein that negatively regulates GPCR signaling by promoting G protein GTP hydrolysis. The tissue distribution of RGS5 indicates that it is highly expressed in the vascular system. RGS5 also serves as a pericyte marker at sites of physiologic and pathologic angiogenesis. Here, we show that the three GoLoco motif containing protein G18/AGS4 is also expressed in vascular smooth muscle cells. GoLoco motif containing proteins are thought to negatively regulate Gα protein activity by acting as guanine nucleotide dissociation inhibitors (GDIs) that impede nucleotide exchange on Gαi/o proteins. The objective of the current study is to examine the combined effect of RGS5 and G18 on the G protein GTPase cycle. Surprisingly, G18 potentiated RGS5 GAP activity up to two fold. On the other hand, in pre-steady state GTPγS binding assays, RGS5 exhibited little or no effect on G18 GDI activity. More interestingly, *in vitro* pull-down assays indicated that G18 directly interacts with RGS5, which may help to account for the observed increase in RGS5 GAP activity. The underlying mechanism of this enhancement in RGS5 activity by G18 is unclear, but our observations suggest it is possible that the GoLoco proteins and RGS proteins co-exist in the same cell type and potentially regulate each other's activity, and collectively modulate G protein activity.

3.2 INTRODUCTION

Nucleotide exchange and GTP hydrolysis are two steps that control the duration of G protein activation (Siderovski and Willard, 2005). Many factors have been identified that can modulate G protein activity by altering the rate of either nucleotide exchange or GTP hydrolysis (Koelle, 1997). Regulators of G protein Signaling (RGS) proteins can accelerate GTP hydrolysis by binding to the activated/transition state of Gα subunit via their RGS domain (~120 amino acids), and acting as GTPase accelerating proteins (GAPs) (Siderovski *et al.*, 2005;Blumer *et al.*, 2007). The receptor-independent activator of G protein signaling (AGS) proteins are another family of proteins that can regulate G protein activity via other mechanisms different from those of RGS proteins (Seki *et al.*, 1998). The members of the Group II AGS protein all contain a single or multiple Gi/o-Loco interaction (GoLoco/GPR) motifs (Blumer *et al.*, 2005). Each conserved GoLoco motif contains ~19 amino acids which are able to bind to the inactive (GDP-bound) state of Gαi/o proteins and inhibit the dissociation of GDP from Gα. The direct interaction between the conserved E/DQR triad of each GoLoco motif with the nucleotide binding pocket of Gα serves as the foundation for its function as a guanine nucleotide dissociation inhibitor (GDI) (Kimple *et al.*, 2002).

G18 (a.k.a. AGS4 or GPSM3) is a 160 amino acid protein that contains three tandem GoLoco motifs at its C-terminus with a relatively short N-terminal domain that contains multiple prolines (Chapter 2, (Cao *et al.*, 2004)). The GoLoco motifs of G18 are thought

to act as GDIs on Gαi, but not Gαo (Kimple *et al.*, 2004). Recent work from our own lab has demonstrated that the N-terminus of G18 acts as a novel Gi/o protein binding partner. The N-terminus exhibits a previously unknown guanine nucleotide exchange factor (GEF) activity on Gαi, but limits GTPγS binding to Gαo. Thus, the net effect of G18 activity may vary depending on the G proteins in question (Chapter 2). Furthermore, the presence of a G protein-coupled receptor (GPCR) also seems to influence which functional domain of G18 predominates with respect to its effects on G protein activity. For example, the GEF activity of G18 was not observed in a membrane-based steady-state GTPase assay, as GPCR-stimulated G protein activation did not appear to be further enhanced by the N-terminus of G18. This suggested that G18 may function as a GPCR-independent GEF. In addition, the function of G18 may also vary depending on the experimental or cellular context (Chapter 2).

Gene expression studies revealed that the mRNA of G18 is highly expressed in immune and cardiovascular system tissues such as spleen, lung and heart (Chapter 2, Cao *et al.*, 2004). In the current study, we first report the expression of G18 in primary smooth muscle cells isolated from both rat and mice. G18 expression was detected at both the mRNA and protein levels. Interestingly, the expression level of G18 is elevated in smooth muscle cell isolated from spontaneously hypertensive rat (SHR) compared to normotensive rat (WKY), suggesting a potential role for G18 in the regulation of vascular functions and/or blood pressure.

Here, we have examined the combined effect of G18 with another G protein regulatory protein that has been reported specifically expressed in vascular system, RGS5, on receptor-mediated G protein activation. RGS5 is a relatively short RGS protein that belongs to the R4/B subfamily of RGS proteins. It is highly expressed in vascular tissues (Seki *et al.*, 1998;Cho *et al.*, 2008;Nisancioglu *et al.*, 2008). It has been shown to play an important role in regulating vascular maturation and remodeling (Manzur and Ganss, 2009b). The role of RGS5 in regulating other aspects of cardiovascular function has also been studied. For example, two individual studies have demonstrated that both systolic and diastolic blood pressure were significantly lower in RGS5-deficient mice compared with wild type (Manzur and Ganss, 2009a). However, the mechanisms underlying this phenomenon are not clear since *in vitro* studies have suggested that the GAP activity of RGS5 is mainly linked to Gi and Gq proteins, both of which are mediators of vasoconstriction (Brinks and Eckhart, 2010). Obviously, more studies are required to fully understand how RGS5 works in a cellular/tissue context. It is possible that regions outside the RGS box serve a role in regulating RGS5 activity on G proteins, and other regulatory proteins also interact with RGS5 in a signaling complex and regulate its function.

Mechanisms of RGS and GoLoco regulation of G protein activities have been investigated individually. However, increasing evidence suggests that these proteins may co-regulate G protein activities in cells. Interestingly, RGS12 and RGS14 are two relatively large RGS proteins that also possess a GoLoco domain at their C-terminal

region (Kimple *et al.*, 2001). Since both G18 and RGS5 are expressed in smooth muscle cells and they both can regulate G protein activity, we examined the possibility that they may do so in a coordinated manner. The combined effects of RGS5 and G18 on G protein activity as well as the influence of one protein on the binding and biochemical function of the other protein thus were studied. Surprisingly, in membrane-based, agonist/receptor-stimulated steady-state GTPase assays, G18 failed to inhibit the GTP hydrolysis promoted by RGS5, which is expected due to its GDI activity, but on the other hand, it did inhibit RGS4 GAP activity in a dose-dependent manner. *In vitro* pull-down assays using purified proteins showed that G18 and RGS5 are able to form a protein complex by direct interaction. Furthermore, we also found that by interacting with RGS5, G18 can potentiate RGS5 GAP activity on $G\alpha i1$ in pre-steady state GTPase assays. Overall, this study provides a new evidence of how RGS5 function can be regulated by other G protein regulatory proteins.

3.3 MATERIAL AND METHODS

3.3.1 GENERATION OF G18 ANTIBODY

A 12 amino acid peptide was designed based on the N terminus of G18 (amino acids 1-12). Peptides were synthesized by the GeneScript Co., and rabbits were immunized through the GeneScript Co. antibody synthesis facility. Each antiserum was characterized using both purified His-G18 fusion protein and transiently transfected CHO and HEK293 cells overexpressing Flag-tagged G18 to verify specificity and optimize the conditions for immunoblotting and immunoflorescence experiments.

3.3.2 CELL CULTURE AND TRANSFECTION

Wistar-Kyoto rats (10 to 12 weeks of age; Charles River) were utilized in our studies as a source of VSMCs. They were cared for in accordance with the Canadian Council on Animal Care guidelines. The protocol for their use was approved by the Animal Use Subcommittee, University of Western Ontario. Isolation of rat aortic VSMCs was performed as described previously (Gros *et al.*, 2006).

Chinese hamster ovary (*CHO*) cells were seeded onto 10 cm dishes (7×10^5 cells/plate) or 35 mm dishes (0.5×10^5 cells/plate), the day before transfection, the cells were transiently transfected with pcDNA Flag-tagged G18 or HA-tagged RGS5 constructs, using lipofectamine. Control cells were mock-transfected. Two days post transfection, cells were harvested for co-immunoprecipitation or fixed for immunofluorescence studies.

3.3.3 RNA PREPARATION AND REVERSE TRANSCRIPTION PCR

Tissues from 3-month-old C57BL/6 mice were collected and homogenized. Total RNA was extracted using Trizol reagent (Invitrogen) and further purified using RNeasy mini columns (Qiagen). 2 μg of total RNA was used for reverse transcription with the High Capacity Reverse Transcription kit (Applied Biosystems). Primers specific for the open reading frame of G18 were used in PCR reactions to examine the expression of G18 (Zhao *et al.*, 2010).

3.3.4 CONSTRUCTS AND PROTEIN PURIFICATION

G18, its mutants and RGS5 were subcloned into pET19b or pGEX4T2 vectors to make His-tag or GST-tag fusion proteins, which were expressed in *E. coli* and purified by affinity chromatography followed by size exclusion FPLC as described in Chapter 2. Protein concentrations were determined by Bradford assay and purity was estimated by Coomassie staining.

3.3.5 PURIFIED PROTEIN PULL-DOWN ASSAY

GST-G18 or its truncated mutants were incubated in binding buffer with purified RGS protein (RGS4, RGS5, or RGS16) in the presence or absence of increasing concentration of Gαi1, which had been preincubated with 10 mM GDP. Glutathione 4B beads were added to the solution and the incubation was continued overnight at 4°C. Beads were pelleted by centrifugation and washed with binding buffer, and proteins were separated by SDS-page and transferred to PVDF membrane for immunoblotting.

3.3.6 *PROTEIN CO-IMMUNOPRECIPITATION FROM CELL LYSATES*

48 h after transfection, cells were rinsed with PBS, treated with Trypsin-EDTA (Gibco), collected by centrifugation, resuspended in lysis buffer (final concentrations: 50 mM Tris–HCl, pH 7.6, 1 mM EDTA, pH 8.0, 0.4 M NaCl, 1% Triton X-100, 10% glycerol, 0.5 mM NaF, 0.2 mM Na_3VO_4, 0.2 mM PMSF, 1 μg/ml leupeptin, 10 μg/ml aprotinin). The samples were centrifuged at 14000 rpm for 15 min and the supernatants were transferred to new microcentrifuge tubes. 500 μl of supernatant was incubated with 50 μl of a 50% slurry of IgG agarose beads (equilibrated in lysis) for 1 h and subjected to centrifugation and then transferred into fresh tubes. Pre-cleared cell lysates (500 μl) were incubated with 10 μg anti-G18 antibody and 50 μl of a 50% slurry of lgG agarose beads overnight at 4°C, with gentle rotation. Cell lysates were then subjected to centrifugation and agarose beads were washed by resuspension and centrifugation three times in lysis buffer. Proteins were released from the beads by heating at 99 °C for 5 min, subsequent to the addition of loading buffer, followed by SDS-PAGE and transfer to PVDF membrane for immunoblot analysis. For negative controls, each lysate was incubated with buffer-equilibrated agarose beads as appropriate to determine non-specific binding. To verify protein expression, 5% of cell lysate taken prior to the pull-down experiment was assessed by immunoblotting in parallel with immunoprecipitated samples.

3.3.7 *GTPγS BINDING ASSAY*

Purified His_6-Gαi1 was preincubated for 1 hour at 4 °C in the absence or presence of purified His-G18 ± RGS5. Binding assays were initiated by adding 0.5μM [^{35}S]-GTPγS

($1.25*10^5$ cpm/pmol). The combined proteins were further incubated at 30 °C for 30 min. The assay was terminated by the addition of cold stop buffer (Tris (pH 8.0) 20mM, $MgCl_2$ 10mM, NaCl 100mM, Lubrol 0.1%, GTP 1mM, DTT 0.1mM) and samples were filtered through nitrocellulose membranes followed by washing with ice-cold wash buffer. The level of radioactive ^{35}S binding to G protein was measured by liquid-scintillation counting. The nonspecific binding was measured in the presence of 100 µM unlabeled GTPγS, and these values were subtracted to yield specific binding.

3.3.8 Pre-steady state GTPase assay

Purified His_6-Gαi1 (500nM) was incubated with 10^6cpm of [γ-^{32}P]-GTP for 15 min. The binding reaction was stopped by the addition of 500 µM cold GTP and a single round of GTP hydrolysis was initiated by adding 10 mM of Mg^{2+} in the presence or absence of RGS proteins ± WT G18. Aliquots were taken at indicated time points and the assay was quenched with ice cold activated charcoal. The level of radioactive ^{32}Pi in the supernatant was measured by liquid-scintillation counting.

3.3.9 Receptor- and agonist- stimulated steady-state GTPase assay

Sf9 membranes (from cells expressing N-terminal c-myc-tagged M2 muscarinic receptor, Gαi1, Gβ1, and Gγ2) were incubated with γ[^{32}P]GTP in the presence of purified RGS5 at indicated concentration with or without G18. Nonspecific membrane GTPase signal was estimated by adding 1mM of unlabeled GTP to the above assay mix, and this value was subtracted from the total counts per minute. Reactions were stopped by activated

charcoal and the level of ^{32}Pi in the resulting supernatant was determined by liquid-scintillation counting. Agonist-dependent GTPase activity was determined by subtracting the signal measured in the presence of the inverse agonist tropicamide.

3.4 RESULTS

3.4.1 Characterization of G18 antibody

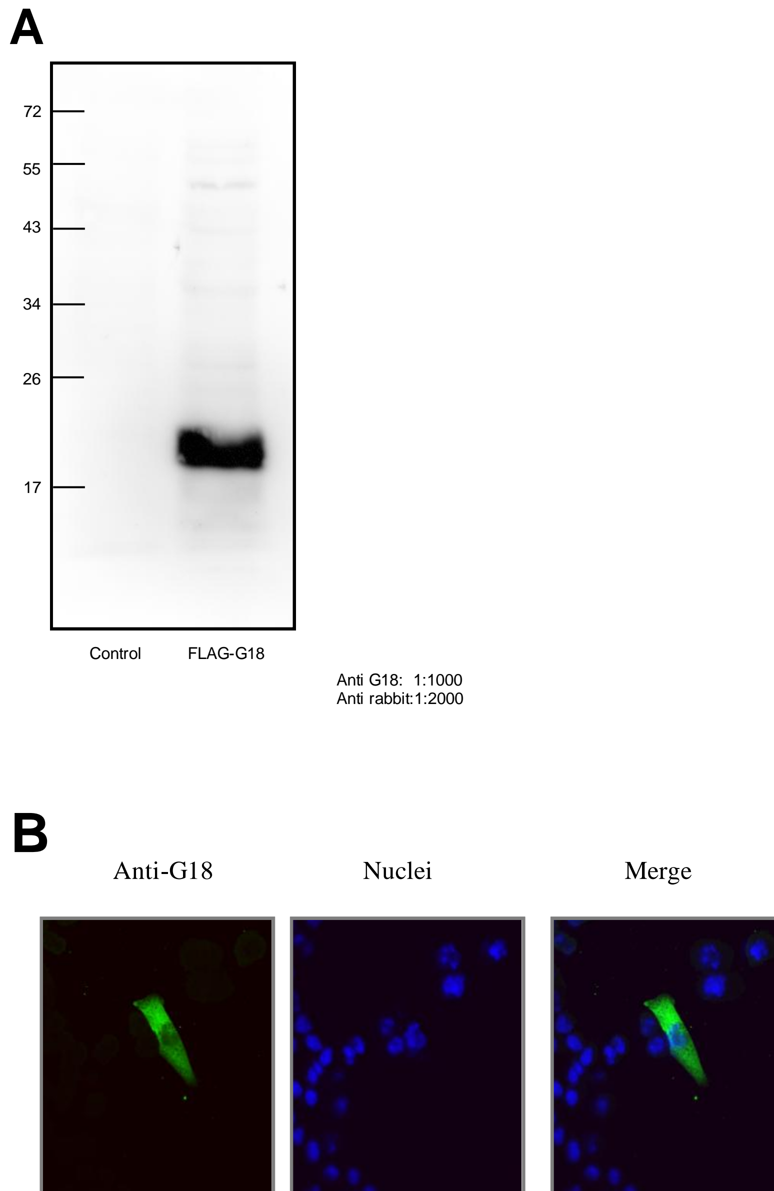
To determine the specificity of our new G18 antibody, CHO cells were transiently transfected with flag-tagged G18, and 48 hours after transfection, cells were either subjected to immunofluorecence or lysed and the lysate was harvested for immunoblot analysis. As indicated in *Figure 3.1A*, and consistent with a previous report (Cao *et al.*, 2004), no endogenous G18 was detected in CHO cells. When G18 was overexpressed, an immune reaction band occurred at the expected molecular weight. Immunofluorescence experiments showed that only cells that are overexpressing G18 were stained by the G18-antibody, with primarily cytosolic expression (*Figure 3.1B*).

3.4.2 Expression and localization of G18 in aortic smooth muscle cell

We examined the endogenous expression of G18 mRNA using different cell lines and primary cell cultures. G18 is absent from both of the established cell lines that we examined including HEK293 and CHO cells, however, to our surprise, the mRNA of G18 is detected in primary smooth muscle cells isolated from both mouse and rat aorta (*Figure 3.2A*). Interestingly, the expression of G18 at the protein level was detected using G18 antibody in both smooth muscle cells and endothelial cells with relatively greater expression in the former (*Figure 3.2B*). Furthermore, cells isolated from spontaneously hypertensive rats (SHR) express elevated levels of G18 compared to those isolated from normotensive rats (WKY) (*Figure 5.3*).

Fig. 3.1. Characterization of G18 antibody. CHO cells were seeded in 10 cm plates and transiently transfected with plasmid encoding Flag-G18 or pcDNA3.1 vector (control lysate). 48 hours after transfection, cells were either lysed and the cell lysate were separated by SDS-PAGE and transferred to PVDF membrane for immunoblotting (A) or fixed and subjected to immunofluorescence study (B). Blots or fixed cells were probed with anti-G18 antibody (1:1000) and anti rabbit secondary antibodies (1:1000 (western blot), or 1:500 (immunoblotting)). Data are representative of three independent experiments.

Figure 3.1



Immunocytochemistry-based assays showed that, consistent with a previous report and our own earlier results using an overexpression system, endogenous G18 was localized mainly in the cytosolic fraction (*Figure 3.2C*). Interestingly, G18 was also seems to be evident at the lamellipodia region of most of the motile smooth muscle cells.

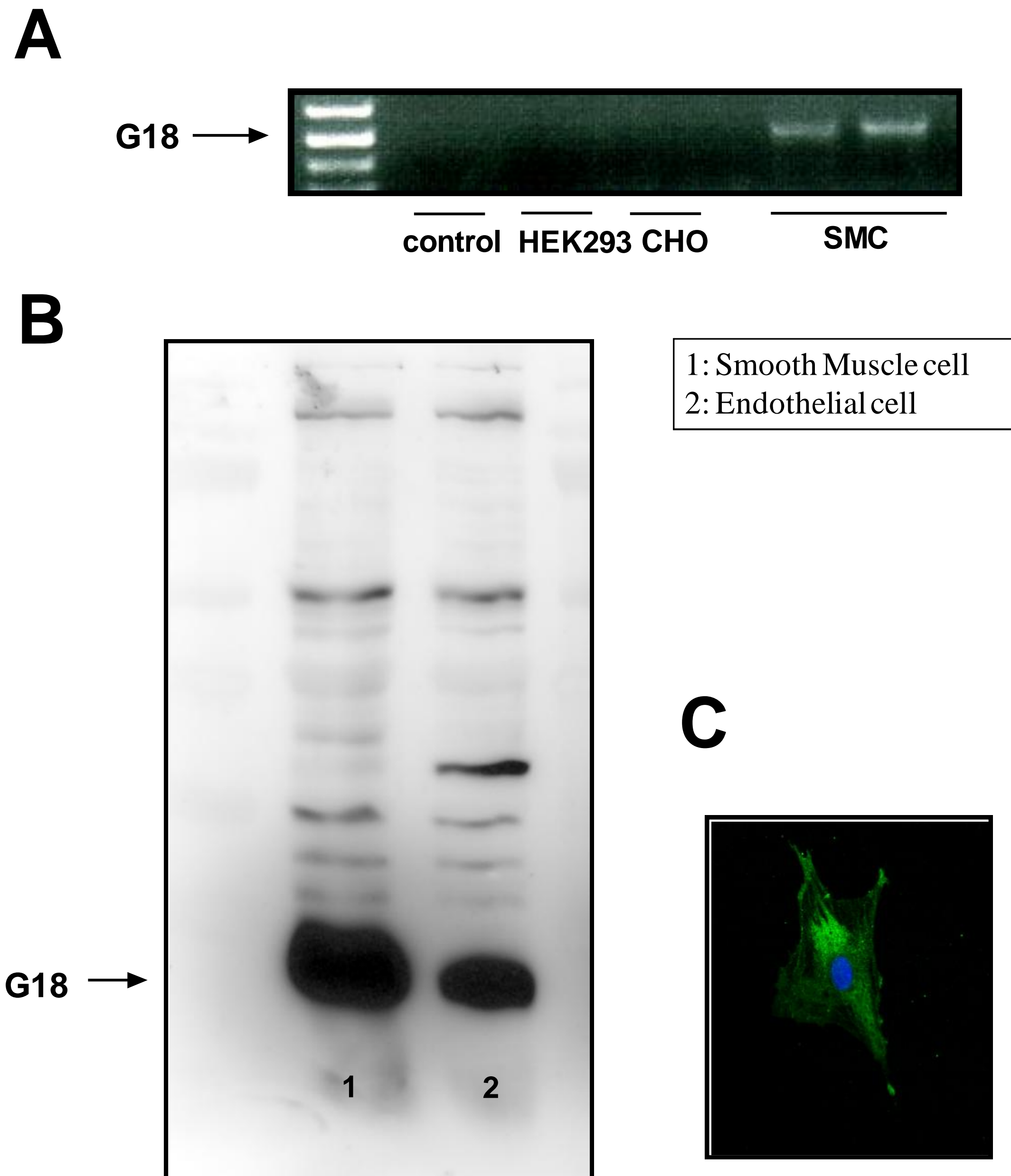
3.4.3 INTERACTION BETWEEN G18 AND RGS PROTEINS

RGS5 is highly expressed in vascular tissues and is considered to play a very important role in regulating vascular function (Mitchell *et al.*, 2008), thus RGS5 may coexist in aortic smooth muscle cells with G18 *in vivo*. Therefore, we studied the combined effect of G18 and RGS5 on G protein activity. First, we sought to confirm the expression of RGS5 in isolated smooth muscle cells, and RGS5 mRNA was indeed detected using RT-PCR in those cells.

As noted, we have identified that both RGS5 and G18 are expressed in the aortic smooth muscle cells. Since both G18 and RGS5 are negative regulators of Gi/o signaling, it is possible that one protein's effect on G protein activity may be influenced by the presence of the other protein, thus, the overall duration of G protein signaling may be altered. To examine this possibility, first we determined if G18 and RGS5 can form a complex, we used *in vitro* purified protein pull-down assay to address this issue. As shown in *Figure 3.3A*, His-RGS5 directly interacts with GST-G18, with no noticeable interaction with the GST tag or the GSH beads. In contrast to its interaction with RGS5, G18 does not appear to interact with RGS2, RGS4 (data not shown) or RGS16 (*Figure 3.3A*).

Fig. 3.2. Expression of G18 in primary aortic smooth muscle cells and endothelial cells. (A) Total RNA was isolated from cultured cell lines and primary cells, reverse transcribed to cDNA and PCR was performed using primers designed to specifically probe for G18. (B) Lysates from cultured primary aortic smooth muscle cells and endothelial cells were separated by SDS-PAGE and transferred to PVDF membrane for immunoblotting. (C) Primary aortic smooth muscle cells were fixed and subjected to immunoflurescence study. Blots or fixed cells were probed with anti-G18 antibody (1:1000). Data are representative of three independent experiments.

Figure 3.2



Since G18 contains three GoLoco motifs at its C-terminus as well as a short proline-rich N-terminus which may also play an important role in protein-protein interaction (Chapter 2), we next tried to elucidate which part of G18 is responsible for its interaction with RGS5. Constructs encoding either the isolated N-terminus (from 1-60 amino acids) or the C-terminus of G18 (from 61-160 amino acids) were used *in vitro* pull-down assays. *Figure 3.3B* shows that full length G18 as expected is able to interact with RGS5, however, little or no binding was observed when either terminus of the protein is removed. These data suggest that the two termini of G18 may both contain elements that are necessary for its interaction with RGS5.

3.4.4 Effect of G protein on G18 and RGS5 interaction

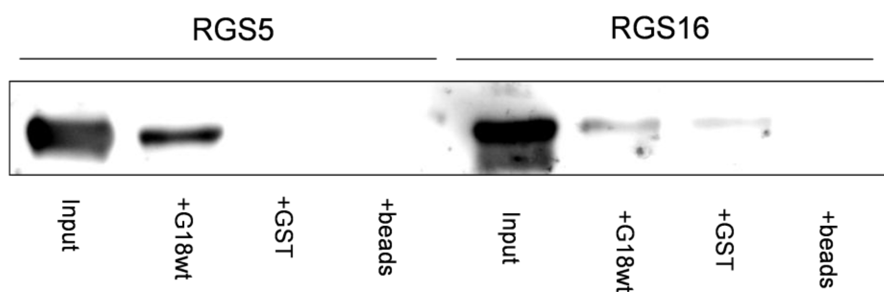
Next, we examined if G protein is able to compete with RGS5 for binding to G18, or alternatively whether the three proteins can form a complex. To address this issue, we performed a G18-RGS5 binding assay in the presence of increasing concentrations of Gαi1-GDP from 150 nM to 1.2 μM and constant concentrations of G18 (300 nM) and RGS5 (300 nM). As shown in *Figure 3.4*, with increasing concentration of Gαi1, the binding between RGS5 and G18 was decreased.

3.4.5 Effect of G18 on RGS5 GAP activity

To study the functional consequences of the interaction between G18 and RGS5, solution-based single-turnover assays were performed. These assays used all purified

Figure 3.3

(A)



(B)

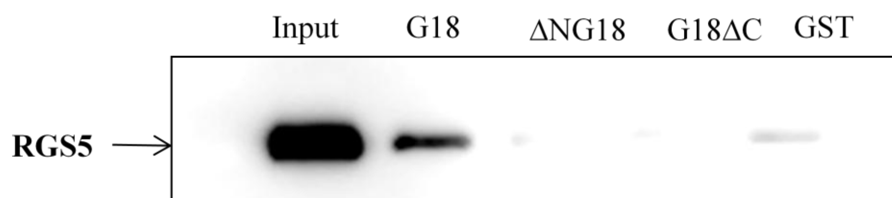


Fig. 3.3. Protein-protein interaction between G18 and RGS5 proteins. Purified His-tagged RGS proteins (300 nM) were incubated with GST-tagged wild type G18 (300 nM) (A), different truncated mutants of G18 (ΔNG18 or G18ΔC) (B). *In vitro* pull-down assays were performed as indicated in Materials and Methods. Briefly, GST-tagged G18 and associated proteins were isolated using glutathione-sepharose beads, which were precipitated by centrifugation and washed three times with buffer. The protein complex was separated using 12% SDS-PAGE and transferred to PVDF membrane, which was probed using and anti-polyhistidine antibody. The blots shown are representative of three independent experiments with similar results.

Figure 3.4

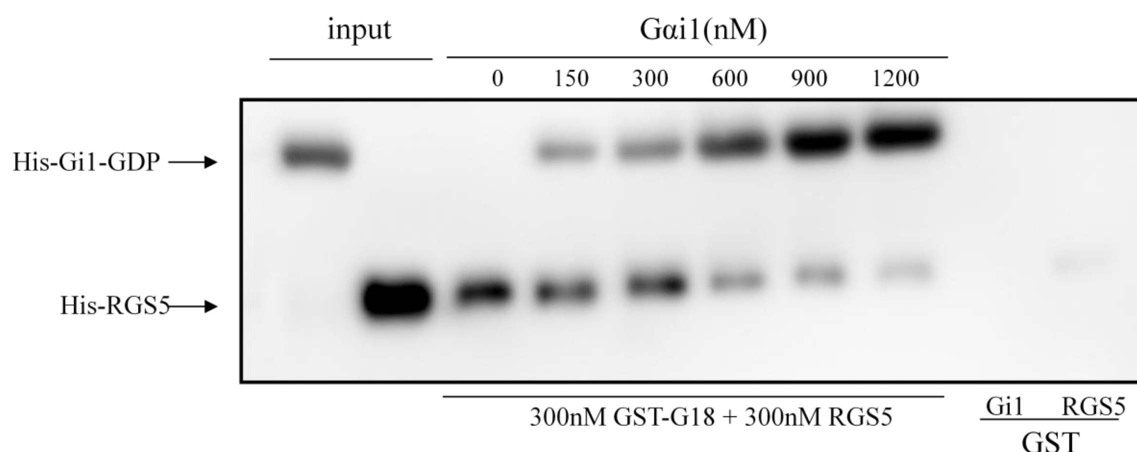


Fig. 3.4. Effect of G protein on G18-RGS5 interaction. Purified His-tagged RGS5 (300nM) proteins were incubated with GST-tagged wild type G18 (300nM), and increasing concentrations of purified Gαi1 as indicated. *In vitro* pull-down assays were performed as described in Material and Methods. Briefly, GST-G18 was pulled down using glutathione-sepharose beads, the pellet was washed three times by resuspension and centrifugation, and the final pellet was and resuspended in 2X protein loading dye and heated to 99˚C for 5 min. The protein complex was separated using 12% SDS-PAGE, transferred to a PDVF membrane and probed for His-tagged proteins. 10% proteins were used as input. The blots shown are representative of three independent experiments with similar results.

protein components, allowing us to examine a single cycle of activation (nucleotide exchange) or deactivation (hydrolysis), and also provided a more simplified system of analysis compared to membrane-based assays or cellular systems. Using a solution-based single-turnover GTPase assay, we examined the effect of G18 on RGS5 GAP activity. In the absence of G18, RGS5 as expected greatly enhanced GTP hydrolysis from Gαi1 compare to agonist alone, exhibiting GAP activity (*Figure 3.5A*). Interestingly, in the presence of both G18 and RGS5, the GAP activity of RGS5 was increased up to 2 fold (*Figure 3.5A*). On the other hand, G18 showed no effect on RGS4 GAP activity, which does not interact with G18 (*Figure 3.5B*). These results suggest that the binding between G18 and RGS5 is able to potentiate RGS5 GAP activity.

3.4.6 Effect of RGS5 on G18 GDI activity

In another set of experiments, we used a GTPγS binding assay to test whether RGS5 is able to alter the biochemical activities of G18, specifically its GDI, and GEF activities. As shown in *Figure 3.6A*, RGS5 alone has no effect on nucleotide exchange. In addition, *Figure 3.6B* shows that the GoLoco motifs of G18 exhibit GDI activity on free Gα, and the presence of RGS5 has no effect on G18 GDI activity. A previous study from our lab identified that the N-terminus of G18 exhibits a novel GEF activity toward Gαi1, and the net effect of the full length protein on nucleotide exchange shows a balance between the GDI activity and the GEF activity. Here, *Figure 3.6 D* showed that RGS5 also has no effect on activity of full-length G18. The simplest explanation for this is that the binding of RGS5 to full-length G18 does not affect either the GEF or GDI functions.

Fig. 3.5. Effects of G18 on RGS5 GAP activity. Purified His-Gαi1 (250 nM) was incubated with [γ-^{32}P]-GTP (1X10^6cpm/assay) for 15 min at 30ºC. A single round of GTP hydrolysis measured at 0ºC in the presence of 10 mM Mg^{2+}, RGS5 (A), or RGS4 (B) (100 nM) with or without G18 (1 μM). The graphs are presented as the mean ± S.E.M. of 3 independent experiments. * $P<0.05$ ** $P<0.01$, compared to RGS protein alone (student t-test).

Figure 3.5

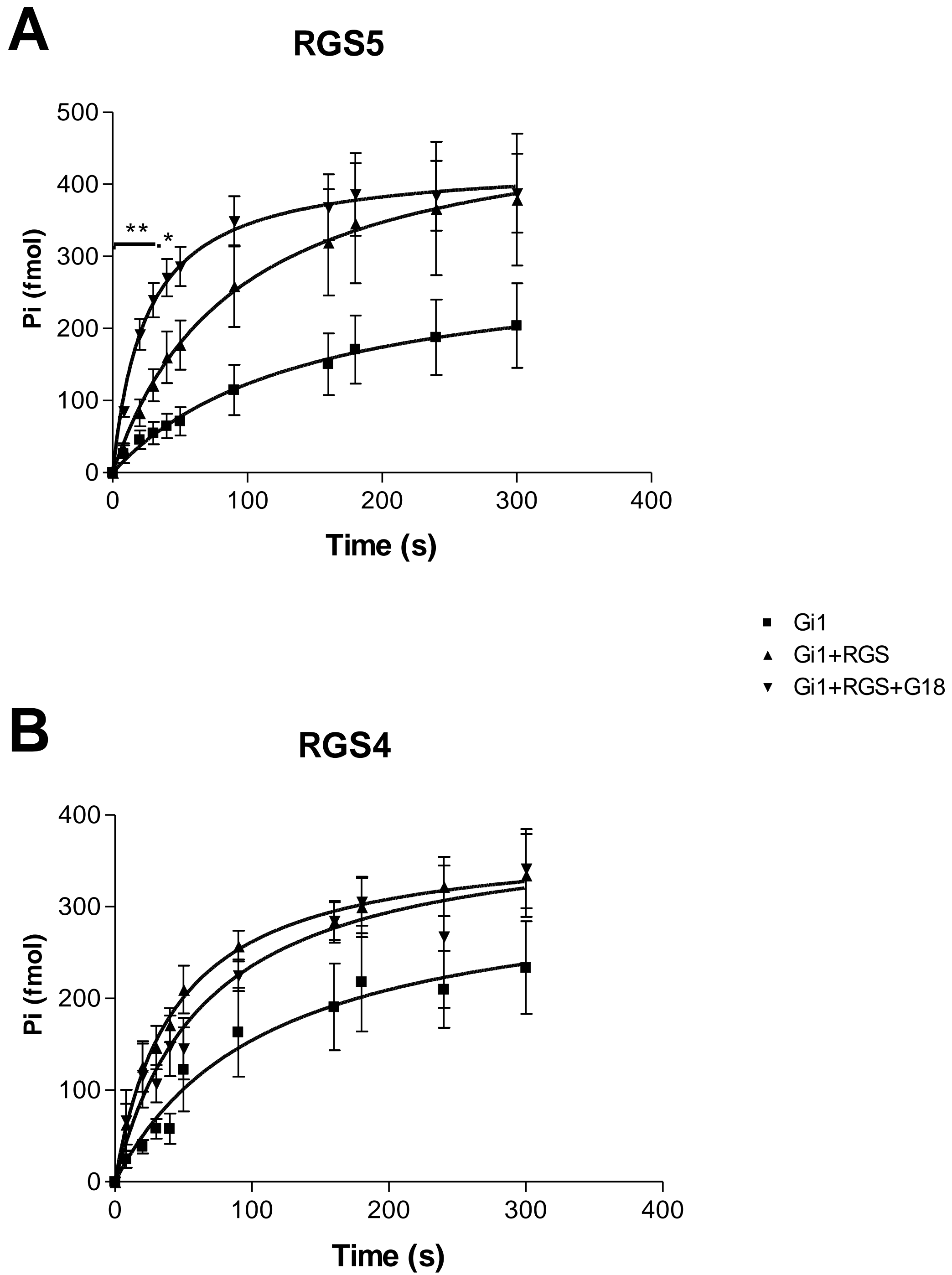
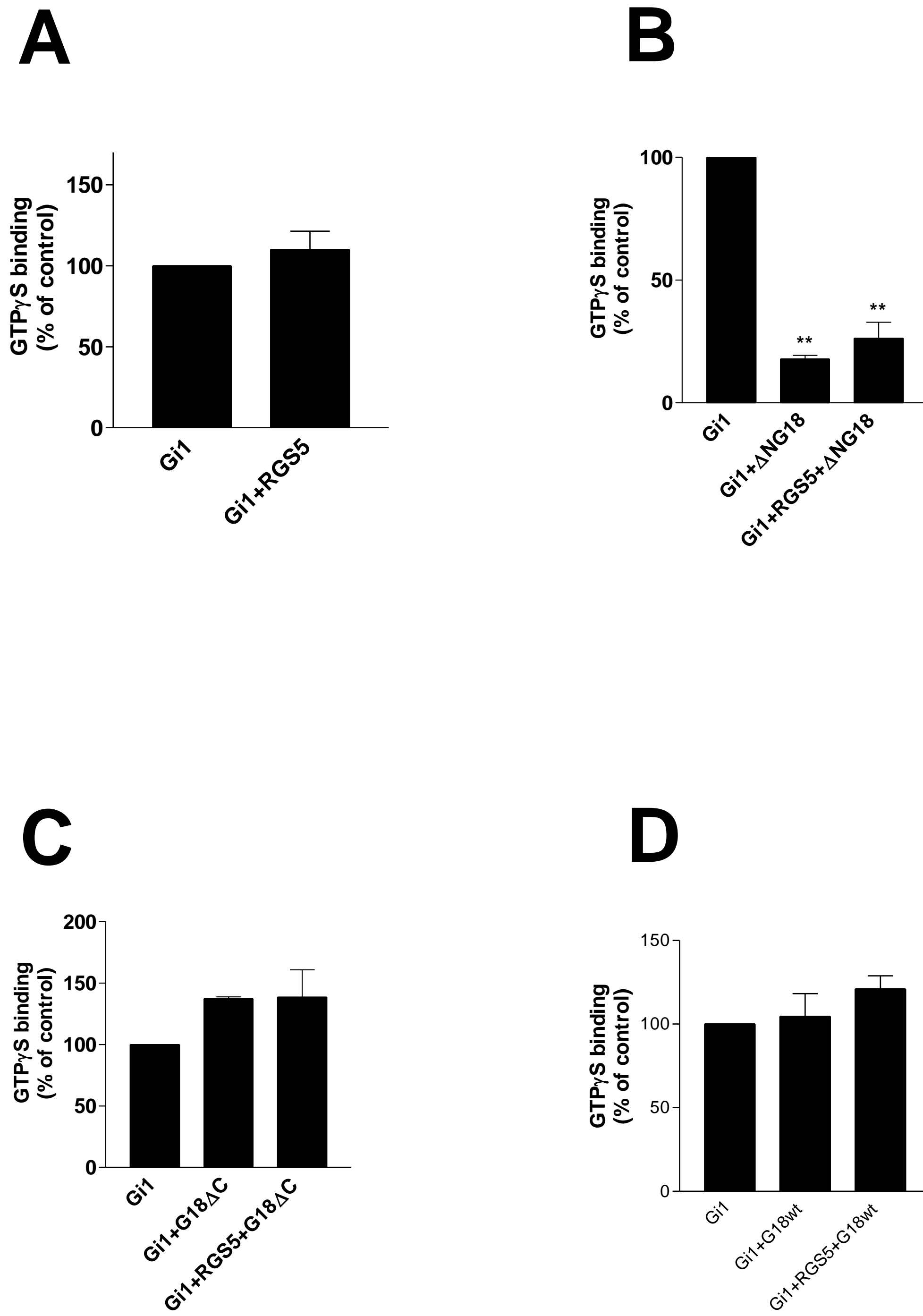


Fig. 3.6. Effect of RGS5 on G18 GDI and GEF activity. Purified His-Gαi1 (100 nM) was incubated alone or in the presence of RGS5 (A), ± the GoLoco region of G18 (G18ΔN) (B), the N-terminus of G18 (G18ΔC) (C), or full-length G18 (D) at 1 μM for 1 hour at 4 ºC followed by addition of 0.5μM [^{35}S]GTPγS (1.25 × 10^5 cpm/pmol) and the incubation continued at 30 °C (Gαi1). The binding of GTPγS to Gα proteins was measured after 30 min (Gαi1) of incubation. Nonspecific binding was estimated in the presence of excess unlabeled GTPγS, and these values were subtracted from the results. The data are presented as the mean ± S.E. of three to five independent experiments performed in duplicate. ** $P<0.01$, compared to G protein alone (One way ANOVA with Tukey's Multiple Comparison Test).

Figure 3.6



3.4.7 *Combined effects of G18 and RGS5 on steady-state GTPase activity*

Using pre-steady-state assays, we studied the effects of the interaction between RGS5 and G18 on each other's biochemical activities, and found that this interaction leads to an enhancement in the GAP activity of RGS5 without altering the effects of G18 on nucleotide turnover. Finally, we used a membrane-based steady-state assay, which is thought to be more close to physiological conditions, to examine the net effect of this interaction on G protein activity. We also used RGS4, which does not bind to G18 as a negative control. Consistent with the Chapter 2, *Figure 3.7B* shows that compared to RGS4 alone, combination of RGS4 and G18 leads to a decrease in the maximum GTP hydrolysis without changing the EC_{50}. Since the rate limiting step in the presence of RGS protein is nucleotide exchange, this decrease most likely reflects an inhibition in nucleotide exchange which is caused by the GDI activity of G18. Interestingly, this inhibitory effect was not observed with RGS5. In the presence or absence of G18, there is no difference in GTP hydrolysis (*Figure 3.7A*). Thus, under these experimental conditions, either the access for G18 to G protein is blocked by RGS5, which leads to a loss of GDI activity of G18, or the GDI activity of G18 and the increase in the GAP activity of RGS5 by G18 balance out each other and result in little or no net effect on GTP turnover.

Figure 3.7

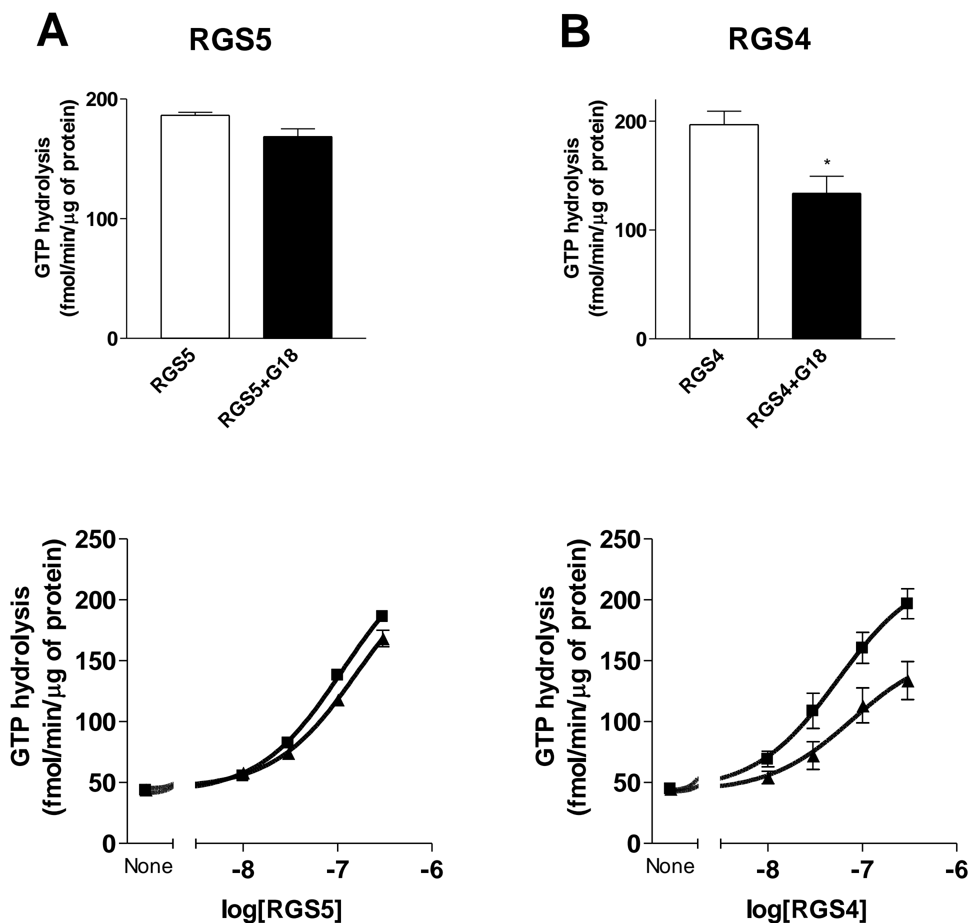


Fig. 3.7. Combined effects of G18 and RGS proteins on agonist- and receptor-dependent Gαi1 GTPase activity. Membranes derived from Sf9 cells coexpressing the M2 muscarinic acetylcholine receptor plus heterotrimeric Gi1 were assayed for GTPase activity with the agonist carbachol (100 µM) or the inverse agonist tropicamide (10 µM), in the presence of increasing concentration of RGS5 (A) or RGS4 (B) without or with G18 (1 µM) at the indicated concentrations. Data shown represent the means ± S.E.M. taken from 3 independent experiments carried out in triplicate. * $P<0.05$ (student t-test).

3.5 DISCUSSION

In the present study, we have developed a specific polyclonal G18 antibody, and reported the endogenous expression of G18 in primary isolated rat-aortic smooth muscle cells and endothelial cells. We have also identified a novel protein-protein interaction between G18 and RGS5. In addition, this binding has a functional consequence where G18 is able to enhance RGS5 GAP activity. On the other hand, RGS5 has negligible effects on G18 GDI or GEF activity. Moreover, Gαi1-GDP inhibits and may possibly compete with RGS5 for binding to G18, as increasing concentrations of Gαi1-GDP lead to a decrease in RGS5 binding to G18.

The tissue distribution of G18 mRNA indicates that its expression is relatively restricted (Cao *et al.*, 2004, Chapter 2). In the current study, we examined the expression of G18 in both established cell lines as well as primary cell cultures. Consistent with its limited expression pattern, G18 is absent from both of the established cell lines examined (*Figure 3.1*). Interestingly, G18 was found in primary smooth muscle cells isolated from adult rat and mouse aorta. The fact that the expression level of G18 exhibits variability between different cell types or cells isolated from different backgrounds, such as smooth muscle cells or endothelial cells, normotensive animals or hypertensive animals, suggests that its expression can be regulated, however, the mechanism of this regulation remains to be studied.

Sub-cellular localization studies reveal a portion of G18 is localized at the lamellipodia region of the smooth muscle cells. Lamellipodia are a characteristic feature at the front, leading edge, of motile cells. Promigratory stimuli activate signal transduction cascades that trigger remodeling of the cytoskeleton, change the adhesiveness of the cell to the matrix, and activate motor proteins. At the same time, many signaling pathways become activated during this process, for example, small G proteins (Rho, Rac, and Cdc42) regulate actin-binding proteins such as WAVE, WASP to promote actin nucleation (Song *et al.*, 2006). Interestingly, it has been suggested that WAVE1 protein also contains a GoLoco motif (Knoblich, 2001). Thus, we speculate that our data may reflect a potential function of G18 in regulating cell migration and actin polymerization.

Many studies have focused on the role of G proteins in regulating vascular function. For example, high blood pressure is often associated with increased signaling via G protein-coupled receptors (Gu *et al.*, 2009). Most of the regulators of G protein-mediated cell signaling in the vascular system are members of the RGS protein superfamily. Among these proteins, RGS5 is of particular interest due to its specific expression pattern and activity regulation (Mittmann *et al.*, 2003). RGS5 has been detected in all isolated cardiovascular tissues with the highest expression level detected in aorta (Adams *et al.*, 2000). However, RGS5 expression was dramatically decreased in cultured vascular smooth muscle cells from the rat aorta (Adams *et al.*, 2000). Here, we report the expression of another G protein regulatory protein, G18, in the vascular system. Functionally, RGS5 serves as a GAP on both Gi/o and Gq subfamilies of G protein (Zhou

et al., 2001), whereas G18 may serve as a GEF or GDI depending on the cellular context and the G proteins in question (Zhao *et al.*, 2010). Since both proteins are expressed in smooth muscle cell and regulate G protein activity, we examined the combined effects of RGS5 and G18 on G protein activity.

Only a few studies have focused on investigating the binding partners of GoLoco proteins other than Gα proteins. AGS3 and LGN are relatively large proteins that contain seven TPR motifs which can interact with mammalian Inscuteable (Insc) protein and regulate spindle orientation and microtubule dynamics during asymmetric cell division (Sanada and Tsai, 2005;Vural *et al.*, 2010). However, the effects of these interactions on the GDI activity of AGS3 or LGN remain unknown. Using GTPγS binding assays, we examined the effect of RGS5 on G18 GEF, GDI and net activity on nucleotide exchange from Gαi1. RGS5 does not seems to have any effect on G18 GDI as well as GEF activity; however, a limitation of these experiments is that we used the truncated G18 mutants lacking either the N-terminus or the C-terminus domain, which appear to have greatly reduced binding affinity for RGS5. On the other hand, RGS5 has no effect on full-length G18 activity, which reflects a combined effect of its GEF and GDI domains (Chapter 2). Overall, our results suggested that although G18 can directly interact with RGS5, this binding does not alter G18 activity. An alternative explanation is that RGS5 may produce proportionally similar increases or decreases in both the GDI and GEF activities of full-length G18.

Interactions between RGS proteins and other signaling proteins via RGS domain-dependent or -independent mechanisms have been demonstrated before. For example, we found that 14-3-3 directly binds to RGS4, RGS5 and RGS16. However, 14-3-3 only inhibited RGS4 and RGS16 GAP activity, but had little or no effect on RGS5 GAP activity (Abramow-Newerly *et al.*, 2006). In another study, coupling between PDEγ and RGS9 was found to enhance RGS9 GAP activity (He *et al.*, 1998). Similarly, work from our lab indicates that the Ras binding domains of RGS14 contain a "GAP-enhancing" acitivity which can increase GAP activity of many B/R4 RGS proteins (Zhao P, Chidiac P manuscript under revision, Chapter 4). Interestingly, the interaction between RGS5 and G18 also leads to a positive effect on RGS5 GAP activity. This is evident in the single-turnover GTPase assay, which directly measures GTPase activity of Gα subunit using purified protein components. The rate of GTP hydrolysis was significantly increased in the presence of G18, suggesting that this enhancement is probably due to an increase in the binding affinity between RGS protein and G protein. G18 itself has no effect on G protein GTPase activity (Zhao *et al.*, 2010).

The fact that G18 has little or no effect on RGS5 GAP activity under steady-state conditions in the presence of activated receptor can be viewed from different aspects. One possible explanation of this lack of effect is that activation of the receptor or the presence of Gβγ subunits leads to a steric effect or a decrease in the affinity between G18 and G protein, which reduces its GDI function. In support of this hypothesis, a recent study reported that the interaction between G protein and G18 (as determined by an

intermolecular BRET signal) was decreased up to 40% upon activation of an associated GPCR (Oner *et al.*, 2010). A conformational change upon the activation of Gα thus may contribute to the observed lack of GDI activity. However, the foregoing explanation for the lack of an effect of G18 in the presence of RGS5 may be unlikely, since in otherwise identical experiments G18 is clearly able to inhibit receptor- and RGS4-promoted GTP hydrolysis (Figure 3.7). The latter finding suggests that G18 actually does have the ability to slow down nucleotide exchange at receptor-activated G proteins, so that the overall GTP hydrolysis is decreased. Alternatively, the interaction between G18 and RGS5 may interfere with the binding between G18 and the receptor-activated G protein, and as a result G18 fails to act as a GDI. Yet another possibility is that (as we observed in single-turnover GTP hydrolysis assays) there is an increase in RGS5 GAP activity in the presence of G18 but that this increase is masked by the GDI activity of G18. As a result, the increase in GTPase activity and decrease in nucleotide exchange cancel each other out and together produce little or no change in Pi production.

Another interesting observation in the current study is that we saw a pattern consistent with competitive binding between Gαi1-GDP, RGS5 and G18, where increasing G protein concentration leads to a decrease in RGS5 binding to G18 (*Figure 3.4*). However, in our GTPγS binding assays, we did not observe altered G18 activity in the presence of RGS5 (*Figure 3.6*). These results suggest that G18 may have higher affinity for Gαi1-GDP compared to that for RGS5 and thus RGS5 seems to be unable to cause the dissociation of G18 from G protein. On the other hand, the G protein is able to dissociate

G18 from, RGS5. When the G protein is activated, both G18 and RGS5 are able to interact with Gα-GTP (Zhao *et al.*, 2010), thus it remains possible that the three proteins may form a complex which results in an increase in RGS5 GAP activity (*Figure 3.5*). However, experimental evidence is required to confirm this hypothesis.

The potential mechanism and binding site between G18 and RGS5 remains to be elucidated. However, elements of both the amino and carboxy terminal domains of G18 are apparently required for this interaction, since truncated mutants missing either the N-terminal GEF domain or the C-terminal GoLoco motifs showed little or no binding. Overall, our results are consistent with the idea that the binding site between RGS5 and G18 is overlapping with the binding site between G18 and inactive Gαi1, which would suggest that the conserved D/EQR triad may play a role in this binding (Kimple *et al.*, 2002).

Protein-protein interactions play an important role in signal transduction and integration. Data from the current study identified a novel protein-protein interaction between G18 and RGS5, and we have also investigated the potential effects of this interaction on each protein's respective biochemical function. Both RGS5 and G18 are found to be expressed in vascular tissue, thus, if is possible that these two proteins are functioning in a common complex. Thus, this will help us to better understand the cellular function of these two proteins.

3.6 References

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Chapter 4

RGS14 REGULATES GAP ACTIVITY VIA ITS RAS-BINDING REGION

A version of this chapter is in submission: Zhao P, Nunn C, Ramineni S, Hepler J, Chidiac P; RGS14 regulates GAP activity via its ras-binding region (2011).

4 CHAPTER 4

4.1 CHAPTER SUMMARY

RGS14 is a 60 kDa protein that contains a regulator of G protein signalling (RGS) domain near its N-terminus, a central pair of tandem Ras binding (RB) domains, and a Gi/o-Loco binding (GoLoco) motif near its C-terminus. The RGS domain of RGS14 exhibits GTPase accelerating protein (GAP) activity toward Gi/o proteins, while its GoLoco domain acts as a guanine nucleotide dissociation inhibitor (GDI) toward Gi1 and Gi3. We previously showed that the C-terminal half of RGS14 can enhance RGS4 GAP activity in membrane based steady-state GTPase assays. Here we show that this novel "RGS enhancing" function correlates to an ability to bind directly to RGS proteins, and that these properties map to a region roughly equivalent to the two RB domains. We also examined the effect of the RGS14 RGS domain on its GoLoco domain function, and found that removal of the RGS domain greatly enhanced GDI activity. In conclusion, our data suggest a mechanism wherein intramolecular interactions between the RB domain and RGS domain of RGS14 may influence its effects on heterotrimeric G proteins, favoring the GAP activity of its RGS domain while disfavoring the GDI activity of its GoLoco domain.

4.2 INTRODUCTION

Heterotrimeric G proteins are involved in many important cellular processes. Binding of activating ligands to the receptor leads to the exchange of the nucleotide on the alpha subunit of G protein, which further regulates many downstream effectors, such as adenylyl cyclase and ion channels (Neves *et al.*, 2002). Nucleotide exchange and GTP hydrolysis are two major events that control the duration of G protein signalling. Signal termination *in vivo* tends to be more rapid than observed rates of GTP hydrolysis *in vitro* (Dohlman and Thorner, 1997). There are many factors that can regulate G protein signalling, such as GTPase accelerating proteins (GAPs), guanine nucleotide exchange factors (GEFs) and guanine nucleotide dissociation inhibitors (GDIs) (Siderovski and Willard, 2005). Several proteins such as G18 (Zhao *et al.*, 2010) and RGS14 (Hollinger *et al.*, 2001;Mittal and Linder, 2006) have been found to contain more than one G protein regulatory domain. The net effects of these complex proteins on signalling events still remain poorly understand.

RGS14 is a relatively large RGS protein (~60 kDa) that belongs to the *D/R12* subfamily. Two members of this subfamily, RGS12 and RGS14, are multidomain proteins. Besides the RGS domain, each contains a second Gα binding region (GoLoco motif), as well as a pair of Ras-binding (RB) domains; RGS12 and RGS14 are among the largest RGS proteins, while the remaining D/R12 member, RGS10, is similar in size to the B/R4 subfamily of RGS proteins (Ross and Wilkie, 2000). Most studies on the physiological function of RGS14 have focused on its roles in the brain and in cell division (Martin-McCaffrey *et al.*, 2004a;Martin-McCaffrey *et al.*, 2004b;Martin-McCaffrey *et al.*,

2005;Rodriguez-Munoz *et al.*, 2007;Lee *et al.*, 2010). For example, RGS14 is a mitotic spindle protein that associates with microtubules (Martin-McCaffrey *et al.*, 2004a;Martin-McCaffrey *et al.*, 2004b;Martin-McCaffrey *et al.*, 2005). It has also been pointed out that RGS14 may play an important role in hippocampal-based learning and memory by acting as a natural suppressor of synaptic plasticity in CA2 neurons (Lee *et al.*, 2010).

The individual biochemical activities of the two heterotrimeric G protein binding domains of RGS14 have been well studied (Kimple *et al.*, 2001;Hollinger *et al.*, 2003;Mittal and Linder, 2004;Shu *et al.*, 2007). The RGS domain exhibits GAP activity in single-turnover GTPase assay in solution, whereas the GoLoco domain inhibits GDP release from isolated Gαi1 and Gαi3. Interestingly, the affinity of full-length RGS14 for Gαi/o subunits was apparently greater than that of the isolated RGS domain (Hollinger *et al.*, 2001). Thus it is possible that amino acid residues outside the RGS domain may help promote its interactions with G protein. Either the RGS or GoLoco domain effect of RGS14 on heterotrimeric G proteins may predominate under a given set of circumstances (Hollinger *et al.*, 2001;Traver *et al.*, 2004;Vellano *et al.*, 2011), although how this happens is not known.

As noted above, both RGS12 and RGS14 contain two tandem binding domains for activated Ras-like monomeric G proteins (RB domains), which were identified through

their similarity to RB domains found in Raf-1 proteins (Ponting, 1999). Recent studies have shown that both H-Ras and, surprisingly, Raf-1 can bind in a positively cooperative manner to the RB domains of RGS14 and modulate signalling through Ras/Raf/MAP kinase cascades (Willard *et al.*, 2009;Shu *et al.*, 2010). Since RGS14 contains two distinct Gα binding sites as well as two Ras binding sites, it has also been proposed that RGS14 may act as a scaffolding protein that integrates heterotrimeric G protein and small G protein pathways (Willard *et al.*, 2009;Shu *et al.*, 2010). Indeed, the binding of Gαi1 to RGS14 appears to modulate its ability to govern H-Ras signalling (Shu *et al.*, 2010). Given the complexity of its structure, other interdomain effects could potentially occur between the various domains of RGS14.

Besides the full-length protein, various splice variants of RGS14 have been tentatively identified (see Discussion), although no specific activities have yet been attributed to them (Martin-McCaffrey *et al.*, 2004a). In several RGS14 variants, the RGS domain is missing or incomplete, suggesting that there may be functions yet to be uncovered. In a previous study, we identified an unexpected effect of an experimentally truncated form of RGS14 on Goα and Giα function distinct from its RGS domain GAP and GoLoco domain GDI activities (Hepler *et al.*, 2005). We showed that the C-terminal half of RGS14 can enhance RGS protein GAP activity, apparently by increasing RGS affinity toward the G protein (Hepler *et al.*, 2005). However, the specific determinants that underlie this activity remain unknown. In the current study, we further narrow down the RGS enhancer region to amino acid residues 300-444 which includes the two RB domains.

We also examined whether the functionality of the C-terminal half of RGS14 might in turn be affected by the RGS domain, and indeed we found that removal of the latter corresponded to an increase in the GDI activity of the GoLoco domain. Overall, our findings suggest that both of these observations may be due to the internal interaction between the RGS domain and the RB domains which on one hand increases the affinity between RGS domain and G protein, and on the other hand interferes with the binding of the GoLoco domain to the G protein.

4.3 MATERIAL AND METHODS

4.3.1 *PROTEIN EXPRESSION AND PURIFICATION*

Hexahistidine (H6)-tagged thioredoxin (Tx), Tx- and H6-tagged full-length RGS14 (TxH_6-RGS14), truncated versions of the protein which contain the RB domains (aa205-490, H_6-R14-RBD), RB domains with active or inactive GoLoco domain (aa299-544, R14-RBD/GL, R14-RBD/GL(LLAA)), the active or inactivated GoLoco domain (aa444-544, R14-GL or R14-GL(LLAA)), were constructed and expressed (Figure 4.1). Proteins were purified from BL21/DE3 bacterial cells as described (Hepler *et al.*, 2005). Where indicated, fusion of RGS protein with Tx was necessary to generate an intact, stable protein. The cells were grown to mid-log phase, and protein production was induced with 1 mM IPTG for 2 h. Cells were lysed using the French Press method, and the supernatant was recovered, loaded to a Ni^{2+} HiTrap affinity column (Amersham Pharmacia, NJ), and purified by FPLC. Proteins were eluted with an imidazole gradient from 20 to 200 mM imidazole in 50 mM HEPES at pH 7.4 and 150 mM NaCl. For TxH_6-R14, the cell supernatant was loaded to Ni-NTA agarose beads, washed and eluted using 200 mM imidazole, and further purified by FPLC using a superdex-200 column (Pharmacia-Biotech). Histidine-tagged RGS4, Gi1α, and were grown in *Escherichia coli* and purified as described previously (Zhao *et al.*, 2010).

4.3.2 *RECEPTOR- AND AGONIST-STIMULATED GTPASE ASSAY*

Sf9 membranes overexpressing M2 muscarinic receptor or α2-adrenergic receptor and heterotrimeric G proteins were prepared as indicated previously (Cladman and Chidiac, 2002). Baculovirus encoding the α2a-adrenergic receptor was generously provided by

Figure 4.1

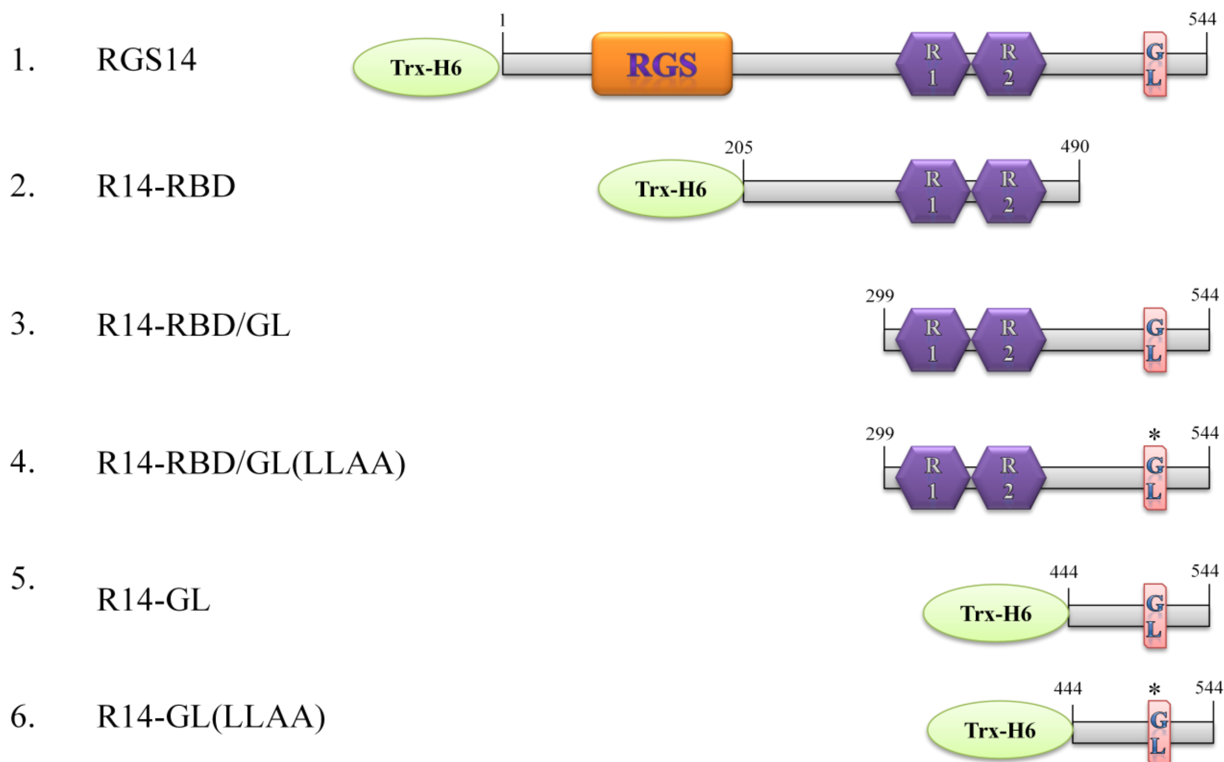


Fig. 4.1. Diagram of constructs used in this study. Tx- and H6-tagged full-length RGS14 (1) (TxH6-RGS14), truncated versions of the protein which contain the RB domains (2) (aa205-490, R14-RBD), RB domains with active (3) or inactive GoLoco domain (4) (aa299-544, R14-RBD/GL, R14-RBD/GL(LLAA)), or the wild type (5) or inactivated GoLoco domain (6) (aa444-544, R14-GL or R14-GL(LLAA)).

Dr Johnny Näsman (Åbo Akademi University, Turku, Finland), and other baculoviruses were as described previously (Cladman *et al.*, 2002;Mao *et al.*, 2004). Sf9 cell membranes (8 μg protein/tube) were assayed for agonist-stimulated GTP hydrolysis at 30ºC for 5 minutes in the absence or presence of the indicated purified proteins in reaction buffer (20 mM Hepes, pH 7.5, 1 mM EDTA, 1 mM DTT, 0.1 mM PMSF, 10 mM NaCl, 2 mM $MgCl_2$ (7.5 mM free Mg^{2+}) 1 μM GTP, 1 mM ATP, [γ-^{32}P] GTP (1×10^6 cpm per assay) and protease inhibitors) in a total reaction volume of 50 μL. The assay was stopped by adding ice-cold 5% (w/v) Norit in 0.05 M NaH_2PO_4. The reaction mixture was centrifuged and the level of $^{32}P_i$ in the resulting supernatant was determined by liquid-scintillation counting. The nonspecific GTPase activity was defined as that in the presence of either the M2 muscarinic receptor inverse agonist tropicamide (10 μM) or the α2-adrenergic receptor inverse agonist rauwolscine (10 μM), as appropriate, and these values were subtracted from the total counts per minute to yield agonist- and receptor-dependent GTP hydrolysis rates.

4.3.3 GTPγS BINDING ASSAY

Purified His_6-Gαi1 (100 nM) was incubated for 1 hour at 4°C in binding buffer (20 mM Hepes (pH 8.0), 1 mM EDTA (pH 8.0), 100 mM NaCl, 1 mM DTT, 2 mM $MgCl_2$, 0.1 mg/ml BSA, 0.1% Lubrol, PMSF and 1 μg/ml leupeptin, 10 μg/ml aprotinin) in the presence or absence of 1 μM RGS14 or its truncated mutants. Binding assays were initiated by adding 0.5 μM [^{35}S]-GTPγS (1.25×10^5 cpm/pmol). The incubation continued for 30 minutes at 30ºC. The assay was terminated by adding ice-cold stop buffer (20 mM Tris (pH 8.0), 10 mM $MgCl_2$, 100 mM NaCl, 0.1% Lubrol, 1 mM GTP and 0.1 mM

DTT). Quenched samples were filtered through nitrocellulose membranes (Millipore) followed by washing four times with 2 mL ice-cold wash buffer (20 mM Tris (pH 8.0), 100 mM NaCl, 10 mM $MgCl_2$). Radioactivity was measured using liquid-scintillation counting. The nonspecific binding was determined in the presence of 100 μM unlabeled GTPγS, and these values were subtracted to yield specific binding.

4.3.4 PROTEIN-PROTEIN INTERACTION ASSAY

Different mutants of RGS14 (500 nM) were incubated with equimolar amount (500 nM) of GST-tagged RGS4 or RGS5. The protein mixture was incubated on a rotating platform at 4ºC for 2 hours in binding buffer (50 mM Tris (pH 7.5), 0.6 mM EDTA, 150 mM NaCl, 1 mM DTT, 0.1% Triton-X 100, PMSF 2 μg/ml leupeptin, and 20 μg/ml aprotinin). Glutathione Sepharose 4B beads (20 μL bed volume) were then added into the protein mixture and incubated overnight. The protein mixture was washed three times with binding buffer and the pellets were resuspended in 2X Laemmli buffer. Eluted proteins were separated on a 12% SDS gel and transferred to a Polyvinylidene Fluoride Transfer (PVDF) membrane (Pall Corporation) for immunoblotting.

4.3.5 IMMUNOBLOTTING

Membranes were incubated with blocking buffer (Tris-Buffered Saline Tween-20 (TBST) with 5% skim milk) for 1 hour and then probed with anti-His or anti-GST antibody (1:1000) (Santa Cruz biotechnology) diluted in blocking buffer overnight on a rotating platform at 4ºC. Blots were subsequently washed 3 times with TBST and then

incubated with HRP-conjugated secondary antibody (1:2000) (Promega) diluted in TBST for 1 hour at room temperature. After another 3 washes with TBST, the blot was visualized by LumiGLO Reserve Chemiluminescence substrate (KPL, Inc) using a FluorChem 8000 imaging system.

4.4 RESULTS

4.4.1 RGS14 STIMULATES M2 MUSCARINIC RECEPTOR-ACTIVATED G PROTEIN GTPASE ACTIVITY.

A previous study from our labs showed that full-length RGS14 acts as a GAP on receptor-activated Gi/o proteins, however, for reasons that are unclear the isolated RGS domain of RGS14 exhibited little or no discernable GAP activity (Hepler *et al.*, 2005). These results suggested two possibilities: 1) The isolated RGS domain of RGS14 on its own is not sufficient to act as a GAP under such conditions, thus amino acid residues outside the RGS domain may act to enhance its GAP activity. 2) The observed GAP activity of full length RGS14 comes from domains other than the RGS domain. To test these possibilities, first, we examined the potential GAP activity of a truncated form of RGS14 lacking the RGS domain but containing the RBD and GoLoco domains (R14-RBD/GL, Construct 3) using a receptor-stimulated GTPase assay. As shown in *Figure 4.2*, full-length RGS14 enhanced the agonist-dependent, receptor-stimulated steady-state GTPase activity of all four Gi/o proteins (*Figure 4.2A*). In contrast, R14-RBD/GL displayed little or no effect on GTP hydrolysis even at micromolar concentrations (*Figure 4.2B*). These results argue against the possibility that RGS14 contains a second GAP domain.

4.4.2 R14-RBD/GL INCREASES RECEPTOR-DEPENDENT GAP ACTIVITY OF RGS PROTEINS.

We have previously shown that R14-RBD/GL potentiates steady-state receptor-promoted GAP activity of RGS4 on Gαi2 and Gαo, apparently by increasing the affinity of RGS4 for Gαi2 and Gαo (Hepler *et al.*, 2005). Here, we tested whether this activity was also

2004). We found that 1 μM R14-RBD/GL does indeed enhance the GAP activity of a submaximally activating concentration of RGS4 on all four Gi/o protein subtypes (*Figure 4.3*), leading to 70-160% increases under the conditions tested. These effects do not appear to be dependent on the M2 muscarinic receptor *per se*, as comparable effects were found with α2-adrenergic receptor-activated G proteins (*Figure 4.4A*). We also examined whether R14-RBD/GL could enhance the GAP activity of other RGS proteins. Indeed, R14-RBD/GL facilitated the GAP activity of each R4 subfamily RGS protein that we tested, including RGS4, RGS5 (*Figure 4.4B*), and RGS16 (data not shown). It is unlikely that the observed increases in GTPase activity could be attributable to the GDI activity of the GoLoco domain since (i) this would be expected to limit nucleotide exchange and thus inhibit/decrease the RGS GAP effects under steady-state conditions and (ii) the positive effect occurs with G protein subunits which are considered to be insensitive to the GDI activity of the protein. Still, it is conceivable that the GoLoco domain, through its ability to bind to Gα, could somehow facilitate RGS effects on the latter, and further experiments were designed to test this possibility.

4.4.3 THE ENHANCEMENT OF RGS PROTEIN GAP ACTIVITY BY RGS14 IS NOT DUE TO ITS GOLOCO MOTIF.

We next investigated which domain or region within the C-terminal half of RGS14 might be involved in the observed enhancement of RGS protein function. To do this, we used three other constructs, namely R14-RBD/GL(LLAA) (construct 4), which contains an altered GoLoco domain that has greatly reduced GDI activity (*Figure 4.8A*), and the shorter truncation mutants R14-GL and R14-GL(LLAA) (constructs 5 and 6), which lack

Fig. 4.2. Effect of RGS14 on M2 muscarinic receptor stimulated GTPase activity. Membranes derived from Sf9 cells co-expressing the M2 muscarinic acetylcholine receptor plus heterotrimeric Gαi1, Gαi2, Gαi3 or Gαo were assayed with the agonist carbachol (100 μM) either alone or in the presence of full-length RGS14 (A) or R14-RBD/GL (B) at the concentrations indicated. Nonspecific signal with each membrane was defined as that observed in the absence of RGS protein and in the presence of inverse agonist tropicamide (10 μM) and this was subtracted to yield the values indicated. Bars represent mean values ± S.E.M of three independent experiments.

Figure 4.2

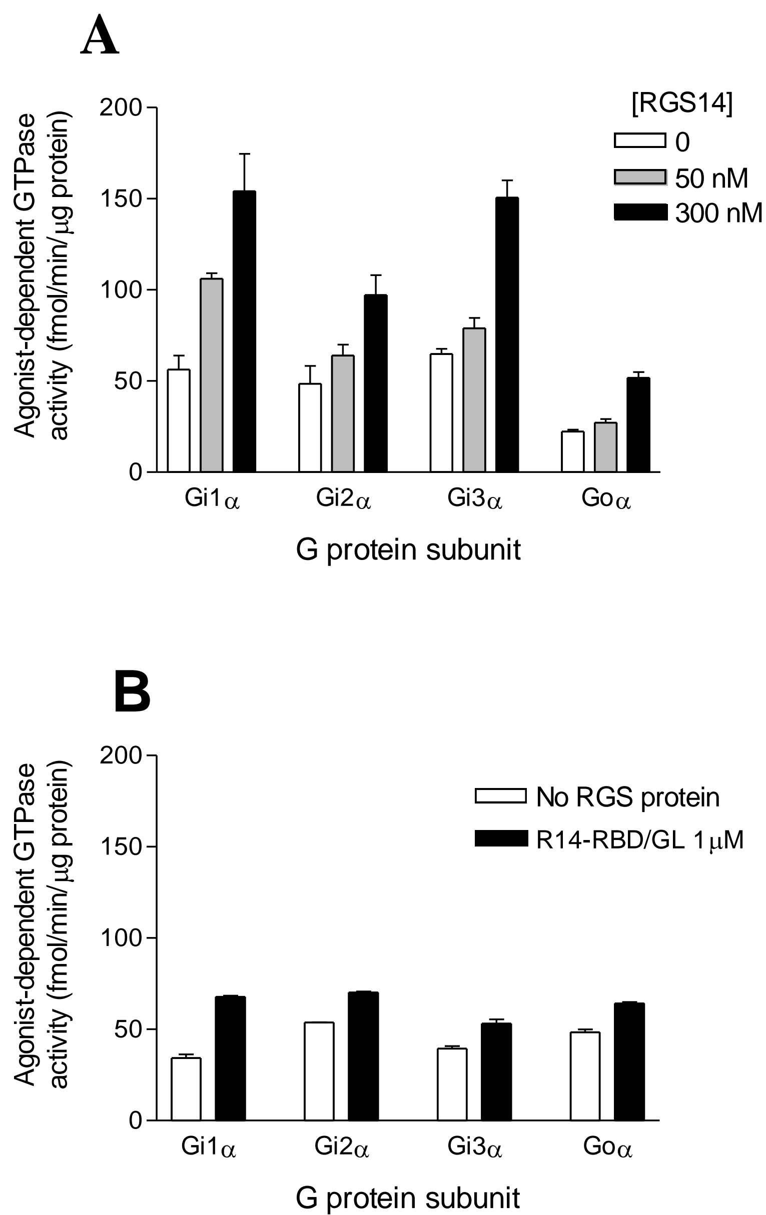


Fig. 4.3. Effect of R14-RBD/GL on RGS4 GAP activity. Membranes derived from Sf9 cells co-expressing the M2 muscarinic acetylcholine receptor plus heterotrimeric Gαi1, Gαi2, Gαi3 or Gαo were assayed with the agonist carbachol (100 μM) either alone or in the presence of either R14-RBD/GL, RGS4 or both R14-RBD/GL and RGS4 at the concentrations indicated. Nonspecific signal with each membrane was defined as that observed in the absence of RGS protein and in the presence of tropicamide (10 μM) and this was subtracted to yield the values indicated. Bars represent mean values ± S.E.M. of three independent experiments. ## $P<0.01$, *** $P<0.005$ compared to agonist alone (One way ANOVA with Tukey's Multiple Comparison Test).

Figure 4.3

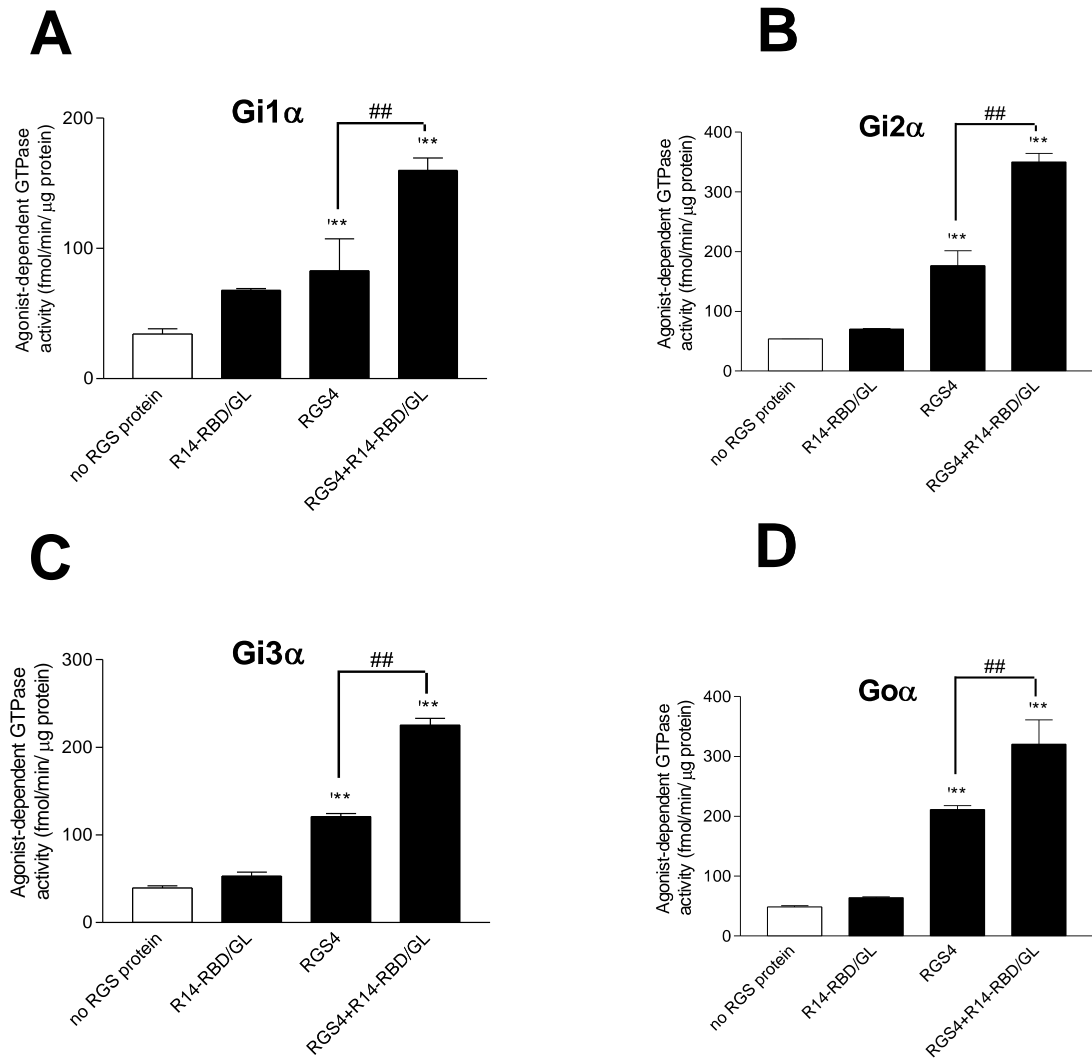


Figure 4.4

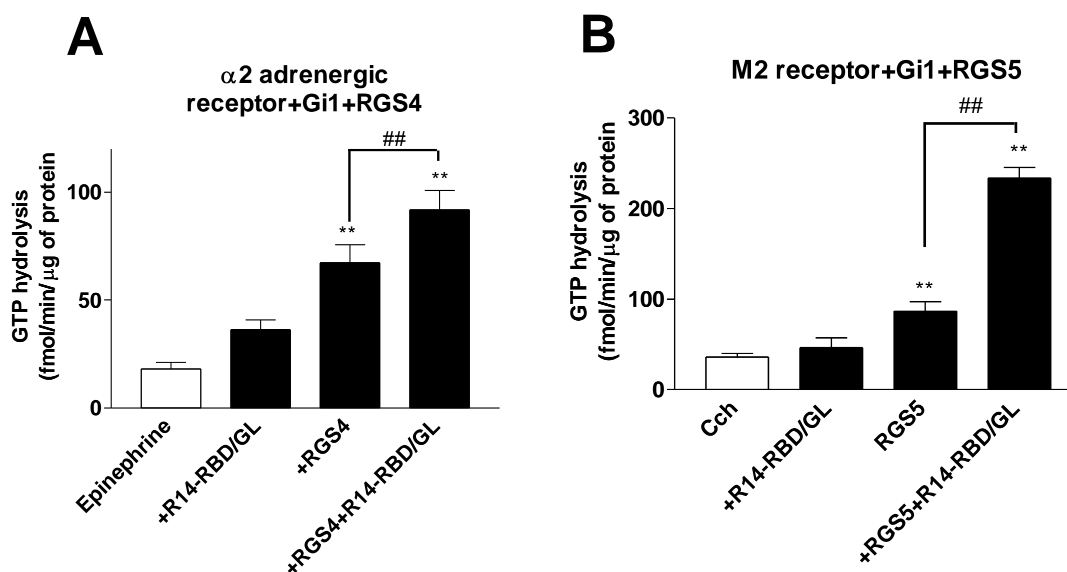


Fig. 4.4. Effect of R14-RBD/GL on steady-state GTPase activity. Membranes derived from Sf9 cells co-expressing the α2-adrenergic receptor plus heterotrimeric Gαi2 (A) or M2 muscarinic acetylcholine receptor plus heterotrimeric Gαi3 were assayed with the agonist epinephrine (alpha2-adrenergic receptor (10μM)), or carbachol (M2 muscarinic acetylcholine receptor (100 μM)) either alone or in the presence of RGS proteins as indicated. Nonspecific signal was defined as that observed in the absence of RGS protein and in the presence of rauwolscine (α2 adrenergic receptor (10μM)), or tropicamide (M2 muscarinic acetylcholine receptor (10 μM)) and this was subtracted to yield the values indicated. Data represent mean values ± S.E.M. of three independent experiments. ** $P<0.01$, compared to agonist alone, ## $P<0.01$, compared to RGS protein alone, (One way ANOVA with Tukey's Multiple Comparison Test).

both the RGS domain and RB domains, and respectively contain an active or an inactive GoLoco domain. All three of these constructs showed little or no effect on receptor-driven Gα GTPase activity in the absence of RGS proteins (*Figure 4.5A*). In the presence of RGS proteins, as shown in *Figure 4.5B*, mutation of the GoLoco motif results in at most a slight decrease in activity ($P > 0.05$), while removal of the region encompassing the RB domains has a more profound effect ($P<0.001$). These trends were confirmed in dose response experiments with increasing concentrations of the various RGS14 constructs. *Figure 4.5C* shows that R14-RBD/GL and R14-RBD/GL(LLAA) are able to enhance RGS protein GAP activity in a dose-dependent manner, whereas the GoLoco domain alone has little or no effect. Overall, these results clearly suggest that the GoLoco domain alone is not sufficient to increase GTP hydrolysis, whereas residues 300-444, where both of the RB domains are located, is primarily responsible for this RGS GAP enhancing activity.

4.4.4 THE RB DOMAIN REGION OF RGS14 CAN DIRECTLY INTERACT WITH RGS PROTEINS.

Our previous study suggested that the enhanced GAP effect of R14-RBD/GL may reflect an increase in RGS domain affinity for G proteins. Here we examined whether the RB domain can directly interact with RGS proteins. In pull-down experiments using purified components, R14-RBD/GL directly interacted with both RGS4 and RGS5, however, the GoLoco domain alone showed no appreciable binding to the RGS proteins (*Figure 4.6A*). This result reinforces the idea that residues outside the GoLoco motif are responsible for

Fig. 4.5. R14-RB domains enhance the GAP activity of RGS proteins on Gα proteins. Membranes derived from Sf9 cells co-expressing the M2 muscarinic acetylcholine receptor plus heterotrimeric Gαi3 were assayed with the agonist carbachol (100 μM) either alone or in the presence of different mutants of RGS14 (A), RGS4 or both RGS14 and RGS4 (B and C) at the concentrations indicated. Nonspecific signal with each membrane was defined as that observed in the absence of RGS protein and in the presence of the inverse agonist tropicamide (10 μM) and this was subtracted to yield the values indicated. Data represent mean values ± S.E.M. of three independent experiments. ## $P<0.01$, *** $P<0.005$ compared to agonist alone (One way ANOVA with Tukey's Multiple Comparison Test).

Figure 4.5

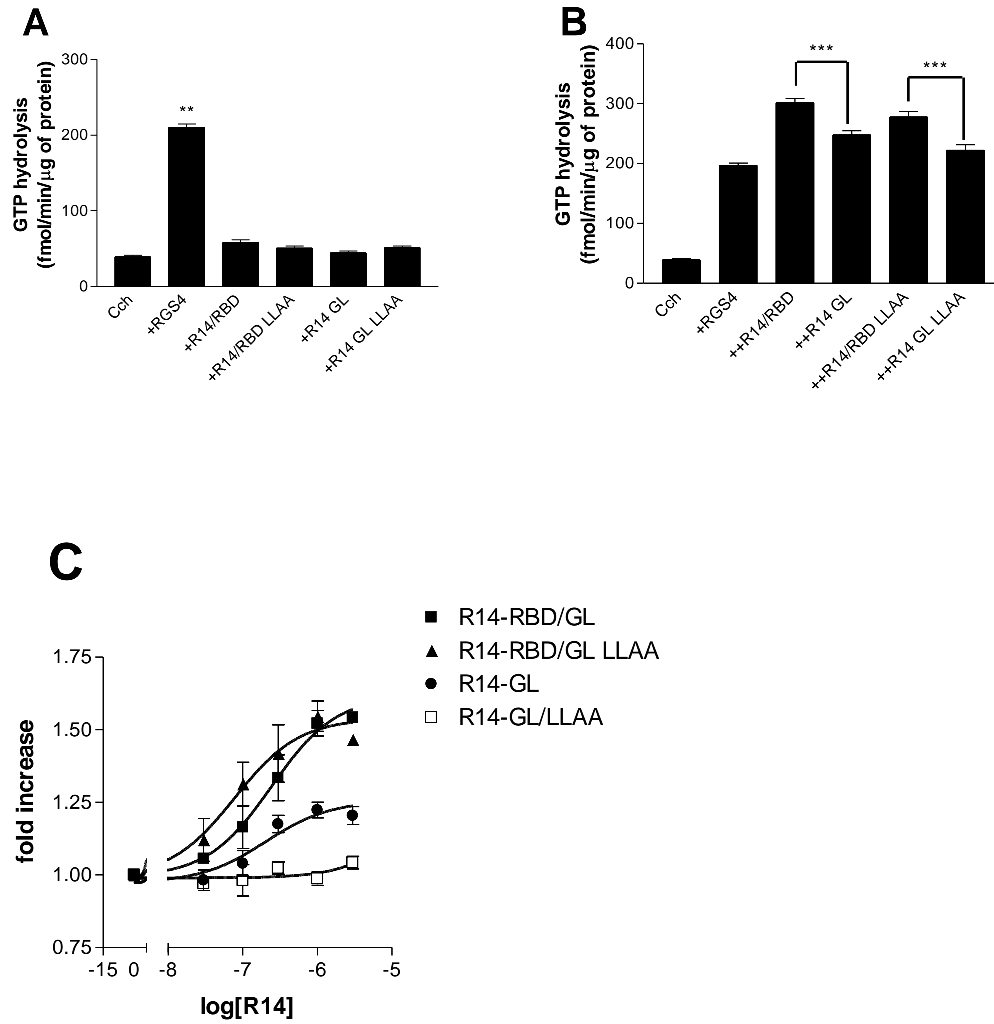
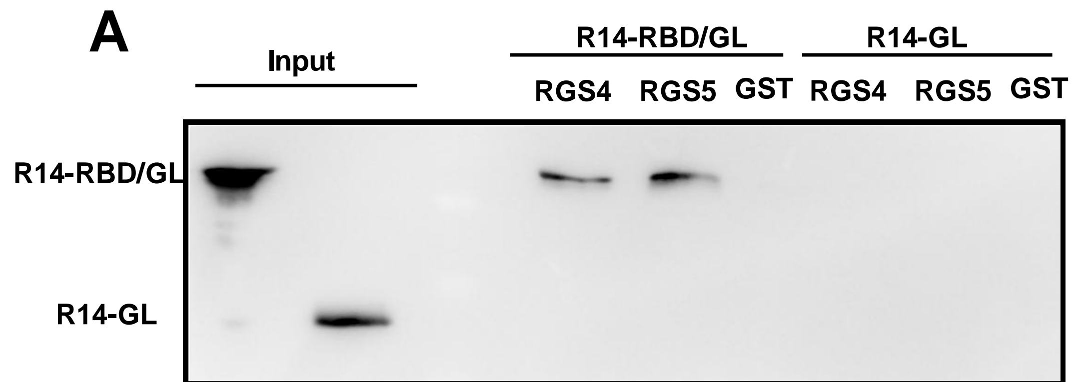
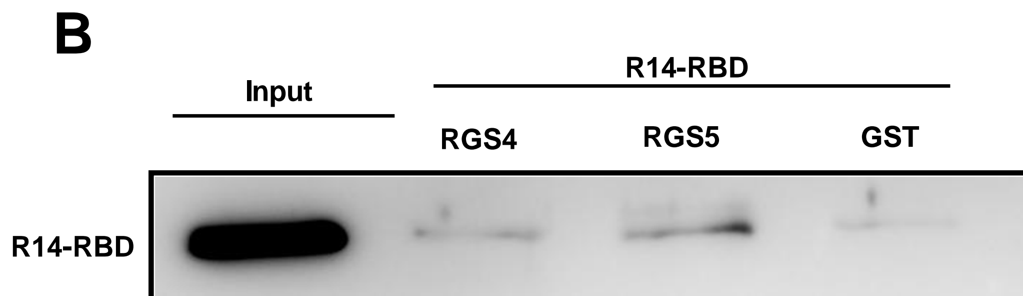


Fig. 4.6. Protein-protein interaction between R14-RB domains and RGS proteins. Purified His6-R14-RBD/GL, His6-R14-GL (A) or R14-RBD (B) was incubated with an equimolar amount of GST-tagged RGS4 or RGS5 for 2 hours at 4ºC. Glutathione Sepharose 4B beads were then added into the protein mixture and incubation continued overnight. The protein mixture was washed three times with binding buffer and the beads were separated by SDS-page and transferred to a PVDF membrane for immunoblotting. Data are representative of three independent experiments.

Figure 4.6





pull-down: GST

immunoblot: anti-His

the effect of R14-RBD/GL on RGS domain GAP activity, and further suggests that this activity may due to a direct interaction between the RB domains and the RGS protein.

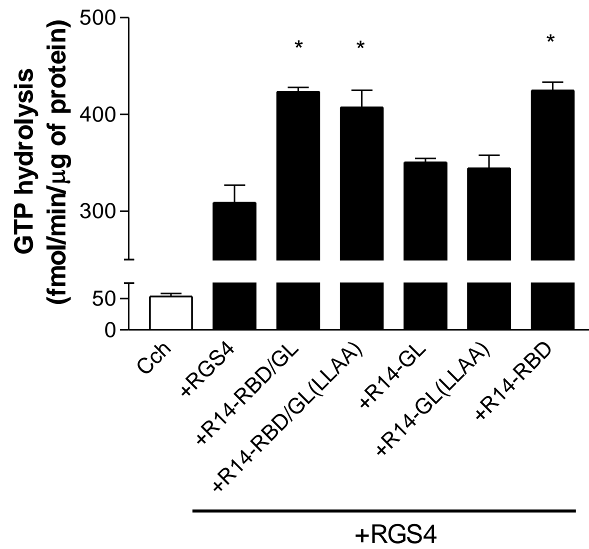
We next generated a construct lacking both the RGS domain and the GoLoco domain (R14-RBD, construct 2), and examined its ability to interact with RGS proteins. As shown in *Figure 4.6B***,** this domain is sufficient to interact with RGS proteins. No binding was observed between R14-RBD and the GST control protein. Since the RGS domain of RGS14 is homologous to those of RGS4 and RGS5 (43% identical, and 19% similar), this result also suggests that it is possible that interdomain interactions within RGS14 may be important for RGS14 GAP activity.

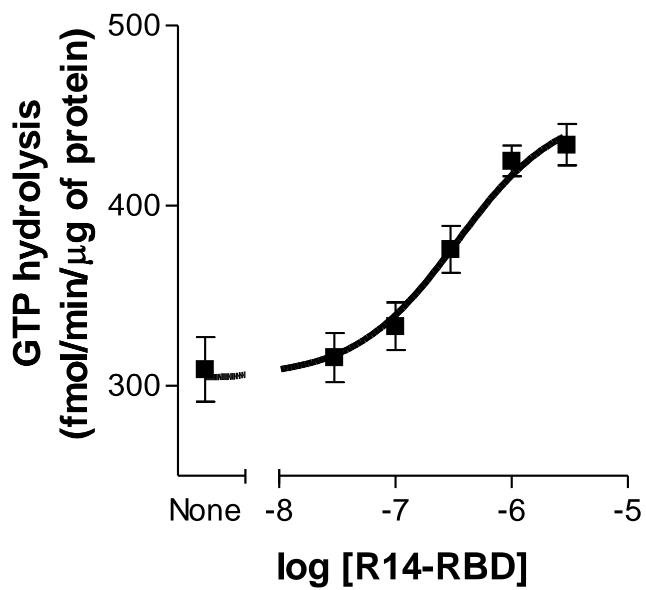
4.4.5 THE RB DOMAINS OF RGS14 ARE SUFFICIENT TO ENHANCE RGS PROTEIN GAP ACTIVITY.

Based on the ability of the RBD region to bind to RGS proteins, we further tested its ability to enhance RGS protein GAP activity. As shown in *Figure 4.7A*, R14-RBD can increase RGS protein GAP activities to a level similar to R14-RBD/GL and R14-RBD/GL (LLAA). On the other hand, the GoLoco domain alone has no significant effect. *Figure 4.7B* suggests that the RB domain alone is able to enhance the GAP activity of RGS4 in a dose-dependent manner.

Fig. 4.7. Effect of isolated RGS14 RB domains on RGS4 GAP activity. Steady-state GTPase assays were preformed in the presence of agonist carbachol (100 μM) either alone or in the presence of RGS4 (300nM) with or without various RGS14 constructs (A) or at increasing concentrations of R14-RBD in the presence of RGS4 (300nM) (B). Nonspecific signal with each membrane was defined as that observed in the absence of RGS protein and in the presence of tropicamide (10 μM) and this was subtracted to yield the values indicated. Data represent mean values ± S.E.M. of at least three independent experiments. * $P<0.5$ compared to agonist alone (One way ANOVA with Tukey's Multiple Comparison Test).

Figure 4.7



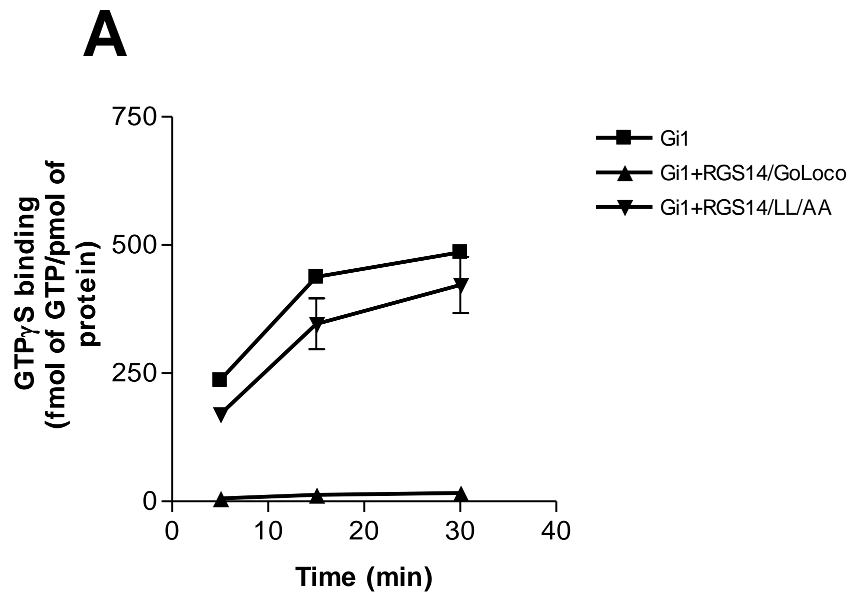


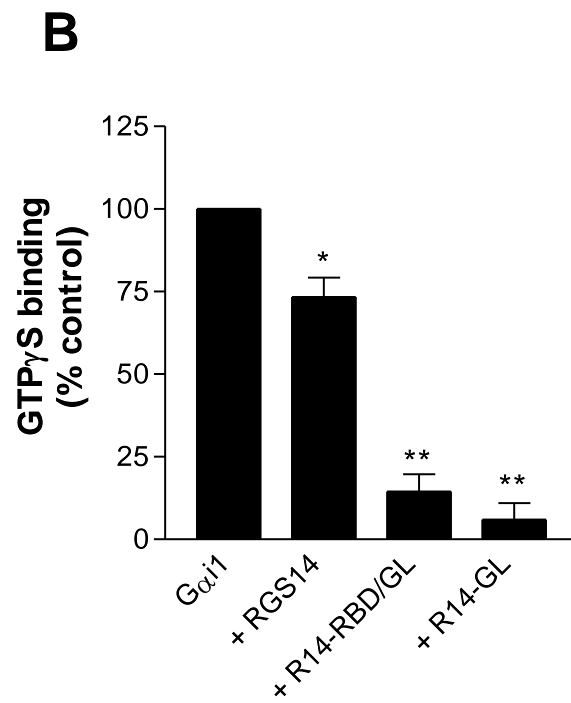
4.4.6 Removal of the RGS domain enhances RGS14 GDI activity.

If the RB domains act as an anchor to enhance interactions between RGS proteins and G proteins, then it follows that the RGS domain in turn may alter the activities of other RGS14 functional domains. We used a single-turnover GTPγS binding assay to address this issue. We compared the GDI activity of full-length RGS14 and the truncated mutant forms of RGS14 which lack the RGS domain or both RGS and RB domains (Constructs 1, 2, and 4). Full-length RGS14 exhibited significantly lower GDI activity compared to R14-RBD/GL (*Figure 4.8B*), while the additional removal of the RBD region resulted in little or no further change (*Figure 4.8B*). This result suggests that the RGS domain may interfere with the productive association between the GoLoco motif and G protein.

Fig. 4.8. Effect of the RGS domain on RGS14 GDI activity. Purified His6-Gαi1 was incubated for 1 hour at 4°C in binding buffer in the presence or absence of RGS14 or its mutants. Binding assays were initiated by adding [^{35}S]-GTPγS. The incubation continued for 30 minutes at 30ºC. The assay was terminated by adding ice-cold stop buffer. Samples were filtered through nitrocellulose membranes (Millipore) followed by washing four times with 2mL ice-cold wash buffer. Radioactivity was measured using liquid-scintillation counting. The nonspecific binding was determined in the presence of 100 μM unlabeled GTPγS, and these values were subtracted to yield specific binding. Data represent mean values ± S.E.M. of at least three independent experiments. * $P<0.5$, ** $P<0.01$ compared to agonist alone (One way ANOVA with Tukey's Multiple Comparison Test).

Figure 4.8





4.5 DISCUSSION

Our study reveals novel biochemical properties of RGS14 and suggests that at least some of its domains have multiple functions. Most notably, the C-terminal half of RGS14, which contains the RB and GL domains, promotes the GAP activity of several different RGS proteins, and this RGS-enhancing effect maps to the region in and around the RB domains (amino acids 300-444). The increase in GAP activity appears to reflect an RB domain-mediated increase in the affinity between the RGS protein and its target G protein (Hepler *et al.*, 2005), and the phenomenon is evident with multiple types of Gi/o proteins, RGS proteins, and GPCRs. In contrast to this positive modulatory effect on GAP activity, it appears that the RGS14 RGS domain itself may interfere with the GDI activity of the RGS14 GoLoco motif, indeed, the truncated forms of RGS14 lacking the RGS domain were found to have increased effects on Gαi/Gαo nucleotide exchange compared to full length RGS14.

The apparent ability of the RGS domain to impede GoLoco GDI activity, as well as the complex effects of Gαi1 on the ability of RGS14 to modulate Ras/Raf signalling reported by Shu and co-workers (Shu *et al.*, 2010), imply that interdomain interactions within RGS14 dictate its ability to engage its various functions. The present results similarly seem to point to an additional interdomain interaction wherein amino acid residues in and around the RB domains facilitate the GAP activity of the RGS domain in full length RGS14. Such a mechanism would be consistent with observations showing 1) the GAP-enhancing effects of R14-RBD and R14-RBD/GL on other RGS proteins, 2) the binding

of purified R14-RBD/GL and R14-RBD to RGS4 and RGS5, 3) that full length RGS14 is approximately 10 fold more potent as a GAP compared to the isolated RGS14 RGS domain in single turnover GTP hydrolysis assays with Gαi1 and Gαo (Hollinger *et al.*, 2001), and 4) that unlike full length RGS14 (*Figure 4.2A*), the isolated RGS domain has limited GAP activity in membrane-based assays of receptor-driven G protein activity (Hepler *et al.*, 2005).

The GAP-enhancing function of the greater RB region of RGS14 potentially could manifest itself *in vivo* not only as an interdomain effect, but also between RGS14 and other RGS proteins. It is not clear to what extent this could occur with the full length protein, as it is conceivable that the RGS domain of RGS14 might sterically interfere with the binding of other RGS proteins to the "RGS-enhancing" domain in cells. However, this would not be an issue if the RGS domain were absent, for example due to partial proteolysis of the full length protein or alternative splicing of RGS14 mRNA.

Presently available data suggest that RGS14 in humans can exist as four or more different splice variants (UniProtKB/Swiss-Prot entry: O43566; reference: http://www.expasy.org/cgi-bin/get-all-varsplic.pl?O43566). Studies to date have examined the full length form (isoform 1; UniProtKB/Swiss-Prot O43566-7; GenBank EAW85012.1) and a short variant (isoform 2; UniProtKB/Swiss-Prot O43566-4; GenBank AAM12650.1) containing part of the RGS domain and part of the first small G protein binding domain whose function remains unclear (Martin-McCaffrey *et al.*,

2004a;Cho *et al.*, 2005;Martin-McCaffrey *et al.*, 2005). In addition, there are two uncharacterized intermediate-length variants that contain the full RB and GoLoco domains and surrounding sequence but from which the RGS domain is either mostly missing (isoform 3; UniProtKB/Swiss-Prot O43566-5; GenBank: AAY26402.1) or completely absent (isoform 4; UniProtKB/Swiss-Prot O43566-6; GenBank: BAC85600.1). Knowledge about RGS14 mRNA splicing is less extensive in other species, however variants similar to human isoform 3 have been tentatively identified in mouse (GenBank: BAB22436.1) and chimpanzee (NCBI Reference Sequence: XP_001141818.1), and additionally a chimpanzee homologue of isoform 4 has been described (NCBI Reference Sequence: XP_001141745.1).

The existence of similar variants across species suggests that isoforms of RGS14 lacking the RGS domain may function in a way distinct from the full length protein. Notably, isoforms 3 and 4 are structurally similar to R14-RBD/GL, and thus the present results may shed light on the functions of these uncharacterized protein species. Based on our findings, we hypothesize that either or both of these naturally occurring forms of RGS14 may a) promote the GAP activity of subfamily B/R4 RGS proteins at GPCR-activated Gi/o proteins and b) have increased GoLoco GDI activity relative to full length RGS14.

Previous studies have examined the functions of the various domains of RGS14 piecemeal using both biochemical and cell-based approaches, and it may be of interest to reconsider some of these findings in light of the present results. For example, Traver and

colleagues investigated the effect of full length and truncated forms of RGS14 on M2 muscarinic receptor-mediated ERK activation. Their results showed that both the GoLoco and RGS domains are required for RGS14 to maximally inhibit carbachol-stimulated ERK activation (Traver *et al.*, 2004). One interpretation of this is that the GoLoco domain can prevent the G protein from being activated by the receptor, while the RGS domain promotes rapid signal termination, thus the GoLoco and RGS domains work individually to inhibit ERK phosphorylation (Traver *et al.*, 2004). Based on the current results, an alternative explanation is that the enhancement of the RGS14 GAP activity by its RB domains may increase the inhibitory effect of the RGS14 RGS domain on receptor-stimulated G protein signalling, and thus contribute to the greater inhibitory effect of the full length protein relative to the isolated RGS domain. Intriguingly, a truncation mutant containing both RBD and GoLoco domains produced a greater inhibitory effect than a shorter construct lacking the RBD (Traver *et al.*, 2004), and we interpret this to mean that the larger of these two modified RGS14 proteins may have promoted the GAP activity of other endogenous RGS proteins present in the cell.

Intramolecular binding has been demonstrated in other multidomain RGS proteins, and the present results suggest the possibility that RGS12, which like RGS14 contains both a GoLoco motif and a tandem RB domains along with its RGS domain (with comparable domain positioning) may also contain a GAP-enhancer function. Indeed, Snow *et al.* previously hypothesised that regions outside the RGS domain of RGS12 may mediate its GAP activity and/or receptor selectivity. They used different experimental approaches

and showed that the RGS12 N terminal domain can selectively bind to the alternative 3' exon form of RGS12 (Snow *et al.*, 1998;Snow *et al.*, 2002). However, the functional consequence of this interaction on RGS12 GAP activity was not reported.

Regarding the function of the RGS14 GoLoco domain, previous studies have shown that its GDI activity can be potentiated by phosphorylation at the threonine residue adjacent to the N-terminus of the GoLoco motif (Hollinger *et al.*, 2003), which could potentially reflect a phosphorylation-dependent change in tertiary structure. Here we used single-turnover GTPγS binding assays to compare the GDI activity of full-length RGS14 and its truncated mutants lacking the RGS domain or both RGS domain and RB domains. Our results suggest that the RGS domain limits RGS14 GDI activity (*Figure 4.8B*). It is possible that the interaction between the RB domains and the RGS domain interferes with the interaction between the GoLoco domain and G protein, thus limiting GDI activity. In our membrane-based assay, the GoLoco domain of RGS14 alone had little or no effect on steady-state GTP hydrolysis either in the absence or the presence of RGS proteins, and moreover the presence of the GoLoco domain did not appear to diminish steady-state RGS GAP activity, implying that little or no GDI effect occurred. In contrast, in solution-based steady-state GTPase assays in which nucleotide exchange is driven by Ric-8A, full length RGS14 produced a decrease rather than an increase in GTP turnover (Vellano *et al.*, 2011), implying that the GDI effect takes precedence under such conditions.

Overall, the results of our labs and others indicate that each of the multiple functions of RGS14 may manifest itself or remain quiescent under a given set of conditions depending upon intramolecular interactions, post-translational modifications, interactions with other proteins, and cellular context. The present findings advance our understanding of RGS14 function, and also raise a number of interesting questions about RGS14 and its splice variants. Future studies will aim to further elucidate how intramolecular interactions within RGS14 are altered by the binding of various partners such as heterotrimeric and small G proteins, and how this impacts on the ability of RGS14 to function as a signal integrator between these two types of guanine nucleotide-dependent signalling pathways.

4.6 References

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Chapter 5

GENERAL DISCUSSION

5 GENERAL DISCUSSION

5.1 SUMMARY OF RESEARCH PROJECTS

My studies have focused on two GoLoco motif containing proteins, G18 and RGS14. The overall objective of this research was to elucidate the mechanisms by which G18 and RGS14 regulate G protein activity. The **specific aims** of my work were:

1. **To characterize the effects of G18 on Gi/o protein activities and the respective contributions of its amino terminal domain and carboxyl terminal GoLoco motifs.**
2. **To characterize the effects of G18 on RGS protein GAP activity.**
3. **To characterize the effect of the GoLoco and small G protein binding domains of RGS14 on RGS protein GAP activities.**

The proposed studies reveal key aspects of how the different G protein binding domains within G18 and RGS14 work in combination to selectively regulate G protein mediated GPCR signaling. The study of how G protein activation and deactivation steps are regulated by multi-G protein binding domain containing proteins not only helps us further understand the basic aspects of G protein function, but also gives us a better idea of how G proteins can regulate cell signaling under normal and pathological conditions.

In Chapter 2, we demonstrate that the N-terminus of G18 exhibits a novel G protein regulatory activity. We have identified the N-terminal region of G18 as a novel G protein interacting domain, which is sufficient to bind with relatively high affinity to the active/transition state of both Gαi1 and Gαo. Thus, besides three GoLoco motifs, G18

contains a fourth G protein binding domain. When examining the biochemical activities of this domain, we found that it promotes nucleotide exchange on Gαi1, but inhibits net nucleotide turnover on Gαo without having effects on GTP hydrolysis. Thus, the N-terminal region of G18 may potentially play a role in differentiating signals between different G protein subtypes. Since G18 contains multiple G protein binding domains that exhibit distinct regulatory activity, we also examined the overall effect of G18 on G protein activity under steady-state condition. Interestingly, in solution based assays, full-length G18 acts as a GEF on Gαi1, suggesting that the N-terminal domain is dominant under this experimental condition. However, in membrane based, agonist/receptor-stimulated GTPase assays, full-length G18 inhibits RGS protein promoted GTP hydrolysis in a dose dependent manner, and apparently acts as a GDI. Thus, the effect of G18 on G protein activity may depend on its cellular localization. That is, free intracellular Gαi1 could be a substrate for G18 GEF activity, whereas the GoLoco motifs would appear to inhibit receptor-dependent Gαi1 activation at the plasma membrane.

In Chapter 3, we looked at the potential interaction and cross-talk between RGS proteins and G18 in regulating G protein activity. We observed endogenous expression of G18 in primary aortic smooth muscle cells both at the mRNA and protein levels. Both endogenous and overexpressed G18 exhibited a cytosolic expression pattern, whereas co-expression of Gαi recruits G18 to plasma membrane (*Figure 5.1*). We also identified a direct interaction between G18 and RGS5, another G protein regulator that is selectively expressed in the vascular system (Manzur and Ganss, 2009). The interaction between G18 and RGS5 leads to an increase in the GAP activity of RGS5, but has little or no

Figure 5.1

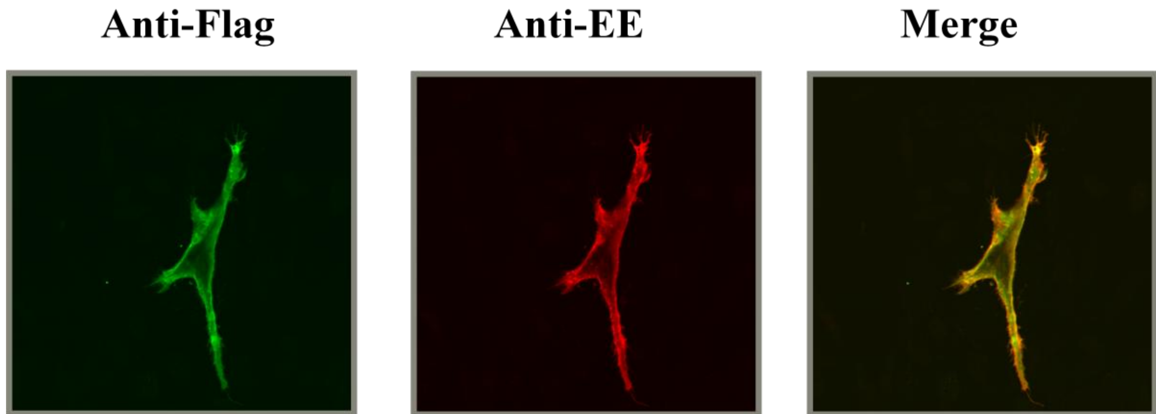


Fig. 5.1. Co-localization between G18 and Gi1. CHO cells were seeded in 10 cm plates and transiently transfected with plasmid encoding Flag-G18 ± EE-Gi. 48 hours after transfection, cells were fixed and subjected to immunofluorescence study (B). Blots or fixed cells were probed with anti-Flag antibody (1:500), anti-EE antibody (1:500) AlexaFluor 488 goat-anti rabbit or AlexaFluor 594 goat-anti mouse secondary antibodies (Invitrogen). Data are representative of three independent experiments.

effect on either G18 GDI or GEF activities. The underlying mechanism of this enhancement in RGS5 GAP activity by G18 is unclear, but our observations are consistent with a mechanism wherein active G protein, can form a three protein complex with RGS5 and G18 which results in increased RGS5 GAP activity, GDP-bound Gα protein in contrast competes with RGS5 for G18 binding.

In Chapter 4, we looked at the molecular mechanisms of how different molecular domains of RGS14 contribute to its effects on G protein activity. We identified a region within RGS14 (amino acid residues 300-444) where the tandem Ras bindng domains are located is sufficient to enhance the GAP activities of the RGS domains. It appears to do this by directly interacting with the RGS proteins and increasing the binding affinity between RGS proteins and G proteins. Since the RGS domain of RGS14 exhibits a relatively weak effect on steady-state GTP hydrolysis, we also examined the ability of this GAP enhancing region to restore the GAP activity of the R14-RGS domain. Interestingly, a construct that encodes the RB domain and the amino acid residues between the RGS domain and Ras binding domain (amino acids 205-490) is able to modestly enhance GAP activity of the RGS14-RGS domain. This suggests that the amino acid residues between the RGS domain and the Ras binding domain may also contribute to RGS14 GAP activity. In addition, the interaction between RGS domain and RB domain not only contains a GAP enhancing activity, it also seems to interfere with the predictive binding between GoLoco domain of RGS14 and G protein and thus inhibit GDI activity.

5.2 EFFECTS OF GOLOCO PROTEINS ON RECEPTOR-STIMULATED G PROTEIN SIGNALING

The effects of GoLoco proteins on nucleotide exchange on Gα proteins have been well studied. However, little is known about their effects on receptor-G protein coupling or receptor-mediated G protein activation. It has been observed that the high affinity binding of agonist to 5-HT1 receptor coupled to Gi is inhibited up to 70% by the addition of an AGS3-GoLoco peptide. This inhibition would appear to be primarily due to the interaction between the GoLoco motif and the G protein, since an inactive mutation of this peptide which does not bind to G protein has little or no effect on receptor-G protein coupling (Peterson *et al.*, 2000). Thus, it has been suggested that the binding between the GoLoco motif and Gα protein may not simply be replacing Gβγ subunits. The binding of Gβγ subunits which seems to be able to maintain the receptor's high affinity for agonists, but the GoLoco motif on the other hand may stabilize the receptor under a conformation that has low affinity for agonists (Nanoff and Freissmuth, 1997). Similarly, in a separate study using BRET, agonists binding greatly decreased the BRET signal obtained between α2 adrenergic receptor and AGS3 in the presence of Gα proteins (Oner *et al.*, 2010a). These two studies reinforce the possibility that GoLoco-G protein interactions and agonist activation of the receptor might be mutually inhibitory. Supporting this idea, the BRET signal obtained from AGS3 and G proteins coupling is decreased about 30-40% upon receptor activation. This decrease is Gβγ independent, since both basal and receptor-mediate BRET signal between AGS3 and Gαi1 were unchanged by co-expression of the carboxyl terminus of GRK2, which binds Gβγ, and apparently activation of the receptor can lead to a physical dissociation of AGS3 and Gα.

Chapter 2 and Chapter 4 of this thesis examined the abilities of two GoLoco proteins, G18 and RGS14, to inhibit receptor–stimulated G protein activation respectively. Interestingly, the two proteins exhibit different effects on receptor-driven G protein activity in our membrane based steady-state GTPase assays. G18 exhibits inhibitory activity through either its GoLoco motifs or N-terminal domain whereas RGS14 serves as a GAP via its RGS domain with no evidence of a GDI activity under these experimental conditions. Since both G18 and RGS14 contain more than one G protein binding domain, their net activities toward G proteins may depends on which domains are dominant in a particular circumstance, which may also vary in the presence and absence of the receptor.

In solution based steady-state GTPase assays, full-length RGS14 acts as a GDI both in the presence and absence of a receptor-independent GEF, Ric-8 (Vellano *et al.*, 2011), whereas RGS14 increased receptor-stimulated GTP hydrolysis via its RGS domain, reflecting its GAP activity (Chapter 4). One possible explanation for this difference is that even though Ric-8 is a GEF, the rate of nucleotide exchange in the presence of Ric-8 is still not fast enough to reveal the GAP activity of the RGS domain (i.e. nucleotide exchange may still be rate limiting). Another interpretation is that in the presence of the receptor or Gβγ subunits, which interfere with the binding between GoLoco motif and G proteins, perhaps the GoLoco cannot function as a GDI. In single turnover GTPase assays, both full-length protein and isolated RGS domain of RGS14 exhibit GAP activities indicating that the RGS domain is functioning. However, the EC_{50} of full-length RGS14 is 10 times lower than the isolated RGS domain of RGS14, suggesting that

isolated R14-RGS has lower affinity and/or is a relatively weak GAP, and this may also account for the loss of GAP activity in solution based stead-state GTPase assays. Thus, amino acid residues outside the RGS box may act as an adaptor to facilitate RGS14 GAP activity (Hollinger *et al.*, 2001). Indeed, in Chapter 4, we identified that the tandem Ras binding domains region of RGS14 contains a novel GAP enhancing activity, which is sufficient to increase not only R14-RGS but also other RGS proteins GAP activities.

The effect of G18 on G protein signaling is multifaceted and complex. In solution-based steady-state GTPase assays using purified Gαi1, the GEF activity of its N-terminal domain appears to predominate over the GDI activity of the GoLoco motifs, thus full length G18 increases nucleotide turnover. However, in the presence of the receptor, which also acts as a GEF upon ligand binding, the net effect of G18 on Gi1 appeared to be predominately mediated via its GoLoco motifs (Chapter 2, (Zhao *et al.*, 2010)). Coupling between G18 and Gαi in the presence or absence of receptor was also examined in living cells. Even though we observed direct interaction between the N-terminus of G18 and activated Gαi/o proteins *in vitro*, little or no BRET signal was detected between this region and G protein in cells (Oner *et al.*, 2010b). The difference may be due to the C-terminal location of the R-luc tag in the BRET study, which was optimized for detecting GoLoco-G protein binding. Also, the G proteins might be in their inactive GDP-bound state rather than active state in the BRET assays, and thus the affinity for the N-terminal domain of G18 may be relatively low. Interestingly, a modest but detectable BRET signal was observed when using constitutively active Gαi1 (Gαi1Q204L) and G18 (Oner *et al.*, 2010b), suggesting that G18 may couple to active G protein. Our results

suggest the possibility that such as interaction may occur through a different region other than the GoLoco motifs, specifically, the proline-rich N-terminus which binds to activated Gαi1. Similar to AGS3, G18-Gαi1 and receptor can form a complex, which suggests that G18 may be able to regulate receptor-mediated signaling in cells (Oner *et al.*, 2010b). However, this hypothesis needs to be examined experimentally.

The effect of G18 on Gαo is dominated by its N-terminal domain both in solution and in membrane based assays. Thus, even though the GoLoco motif is Gαo insensitive, G18 may still serve as a regulator for Gαo signaling (Kimple *et al.*, 2004;Cao *et al.*, 2004;Zhao *et al.*, 2010). It will be interesting to see if G18 can regulate Gαo activity in a cellular context, and whether nucleotide concentration may affect G18's effect on Go signaling.

5.3 Regulation of RGS GAP activity by adapter domains

Apart from the RGS14 RGS domain, many RGS proteins exhibit relatively weak GAP activity and require molecular adapters to target them to G proteins, For example, RGS9-1 is specifically expressed in retina and regulates transducin-mediated cell signals (Anderson *et al.*, 2009). The Gαt effector PDE-γ can increase the affinity between RGS9-1 and Gαt by over 20 fold (Skiba *et al.*, 2000). Interestingly, a splice variant of RGS9-1, RGS9-2, contains a C-terminal domain that shares sequence homology to PDE-γ, and this C-terminus of RGS9-2 serves a similar role as PDE-γ and promotes RGS9-2 GAP activity. In addition, it also has the ability to increase RGS9-1 GAP activity to a similar level as PDE-γ (Martemyanov *et al.*, 2003). There are ~100 amino acids between the RGS domain and the two Ras binding

domains of RGS14, but the biological function of this region remains unknown. In chapter 4, we examined if the “GAP enhancing domain” is able to restore the GAP activity of R14-RGS domain. Our current data, as well as unpublished observations suggest that this region maybe required as an adaptor domain for the RB domain to target the R14-RGS domain. Interestingly, it seems to be not required for the former to target other RGS proteins. Moreover, sequence BLAST results indicate that this region shares homology with the C-terminal domain of RGS9-2 (Identities = 20/52 (39%), Positives = 27/52 (52%)). The alignment of these three potential adapter domains exhibits ~27% identical or similar residues. Most of the similar residues are hydrophobic amino acids or positively charged residues that are important for protein folding, indicating that these proteins may share a similar secondary structure. Several studies imply that both N-terminal domain and the C-terminal domain of PDE-γ contribute to stabilization of the RGS9-Gα complex (Slep *et al.*, 2001;Guo and Ruoho, 2011). Taken together, the amino acid residues between the RGS domain and the Ras binding domains of RGS14 may act as an anchor domain which is necessary for RGS14 GAP activity.

Although the crystal structure of full length RGS14 has not been solved, the structures of the RGS domain, the second Ras binding domains and GoLoco motif either alone or coupled with binding partners have been studied by NMR and crystallography, respectively (Kimple *et al.*, 2002;Soundararajan *et al.*, 2008). However, the structure of the region between the RGS domain and the Ras binding domains still remains unknown.

The fact that the R14-Ras binding region is able to enhance the GAP activity of multiple RGS proteins suggests a common regulatory mechanism wherein it increases affinity between RGS protein and G protein, thus enhancing the GAP activities. G18 also exhibits RGS enhancing activity but this activity is limited to RGS5, as it was not observed with other RGS proteins that have been tested (Chapter 3). Thus, although both G18 and RGS14 are able to enhance RGS protein GAP activities, the domains of these two GoLoco proteins that mediate these RGS-enhancing effects appear to be dissimilar.

RGS4 and RGS5 share a high degree of sequence similarity in their RGS domains (56% identical and 82% positive). The fact that G18 increases RGS5 GAP activity but not that of RGS4 suggests that amino acid residues outside the RGS domain may be important in regulating RGS5 GAP activity. Indeed, unlike RGS4, the short N-terminus of RGS5 seems to have an inhibitory effect on RGS5 GAP activity (Zhou *et al.*, 2001). Thus, the binding of RGS5 to G18 may cause a conformational change in RGS5 that favors GAP activity. Another possibility is that as discussed in Chapter 3, since G18 may also interact with GTP-bound Gα proteins, the three proteins may form a complex where GTP hydrolysis is more rapid than with RGS5-Gα-GTP.

5.4 ALTERNATIVE SPLICING IN MULTI-G PROTEIN BINDING DOMAIN CONTAINING PROTEINS

Many signaling proteins are found to have alternative splice variants. Insertion or deletion of complete protein domains is one of the most common mRNA splice mechanisms (Kriventseva *et al.*, 2003). Using this mechanism, different variants may exist that distinct cellular localizations, functions and molecular activities. On the other

hand, comparing sequence differences between splice isoforms of a single gene may also provide important information regarding functional domain prediction (Wheelan *et al.*, 2000).

A database search indicates that there is a short isoform of G18 (GenBank ID: AAB47489.1) which contains the N-terminal 14 amino acids as well as residues from 62 to 160, where the three tandem GoLoco motifs are located (*Figure 5.2*). The human G18 gene consists of 4 exons separated by three introns. The short isoform of G18 is missing the second exon plus the first quarter of the third exon. Interestingly, the amino acid sequence corresponding to this missing region may be sufficient to account for the novel GEF domain of G18 (*Figure 5.3*). These results suggest that a single gene may encode different protein isoforms that contain distinct biochemical and cellular functions. RGS14 presents as a more complicated situation. As discussed in Chapter 4, there are 4 potential alternate isoforms, none of which encode a functional RGS domain, which suggests that these forms may all serve a cellular role other than as a GAP, consistent with our observation of the ability of the RB domains to enhance GAP activity of most R4 family proteins. The shorter forms of RGS14 thus may act as molecular adapters to facilitate the activities of other RGS proteins. RGS12 is another relatively large RGS protein that shares a high level of sequence homology with RGS14. It also contains an RGS domain, and two tandem Ras binding domains followed by a GoLoco domain. Splice variant isoforms have also been found in RGS12, most of which involve on the N-

Figure 5.2

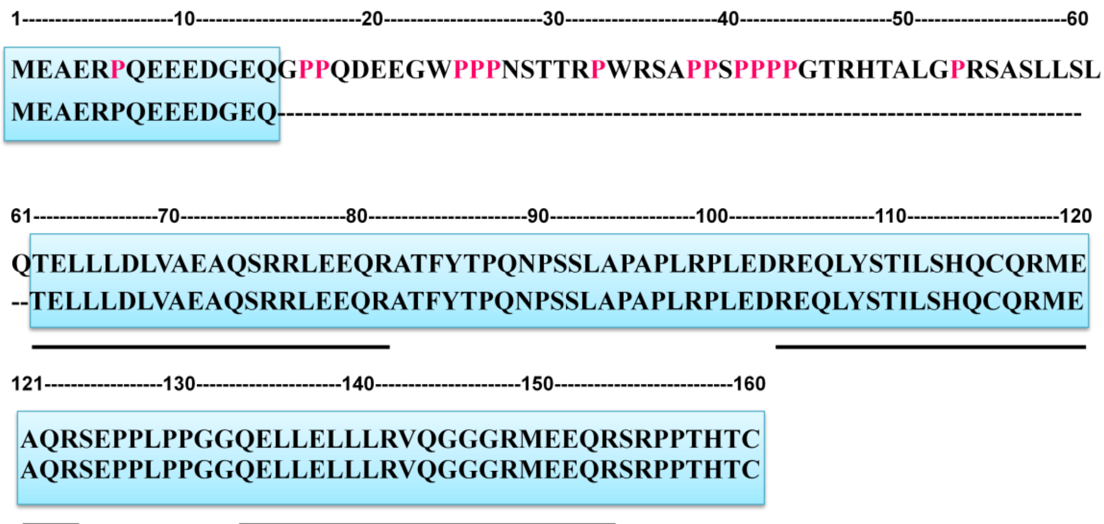


Fig. 5.2. Amino acid sequence of G18 and potential splice variant. Prolines in the N-terminus of G18 are labeled in red and three GoLoco motifs are underlined.

Figure 5.3

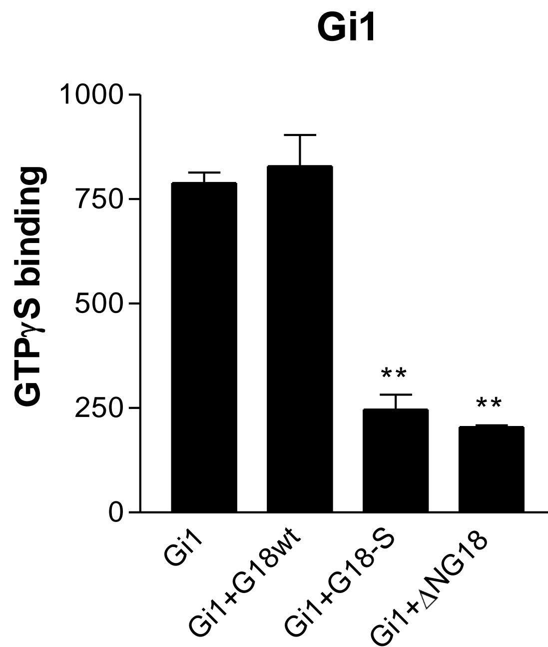


Fig. 5.3. The effect of G18-short (G18-s) on nucleotide exchange. Purified His6-Gαi1 (100 nM) was preincubated with G18 and its mutants (1 μM) at 4ºC. Binding assays were initiated by adding 0.5 μM [^{35}S] GTPγS (1.25×10^5 cpm/pmol) at 30ºC. The binding of GTPγS to Gα proteins was measured after 30 min of incubation. Nonspecific binding was estimated in the presence of excess unlabeled GTPγS, and these values were subtracted from the results. The data are presented as the mean ± S.E.M. of 3 independent experiments performed in duplicate. ** $P<0.01$, compared to G protein alone (One way ANOVA with Tukey's Multiple Comparison Test).

terminal PDZ domain and the C-terminus of the protein (Snow *et al.*, 1998;Chatterjee and Fisher, 2000). It is possible that RGS12 may also contain splice variants that exhibit activity similar to the Ras binding domains of RGS14. However, further experiments are required to test this hypothesis.

5.5 CONTRIBUTIONS OF THIS THESIS TO THE FIELD OF G PROTEIN REGULATION RESEARCH

5.5.1 PHYSIOLOGICAL RELEVANCE OF G18 ON REGULATING RECEPTOR-DEPENDENT AND -INDEPENDENT G PROTEIN SIGNALING

Most studies focus on the biochemical activities of G18, little is known regarding how G18 is functioning in a cellular context. Data from Chapter 2 and Chapter 3 of the current thesis along with the work of other groups have highlighted the potential regulatory role of G18 on Gi/o signaling (Kimple *et al.*, 2004;Cao *et al.*, 2004). Alteration in Gi and Go signaling has been suggested to play important mechanistic roles under different pathological conditions. In addition, we have shown that G18 activity and expression patterns may also vary depending on the cellular environment. Thus understanding the cellular function of G18 may provide insight regarding how G protein signaling is regulated, and further help us to identify novel druggable targets for future therapeutical use.

G18 was found to be primarily expressed in the cardiovascular system and immune systems (Cao *et al.*, 2004;Zhao *et al.*, 2010). The important role of GPCR mediated signals in the cardiovascular system has been clearly demonstrated. Altered Gi/o

mediated cell signaling has been linked to many pathological conditions, such as hypertension, hypertrophy and finally can lead to heart failure (Sato and Ishikawa, 2010). In general, Gi signaling serves a protective role in the cardiovascular system. An increase in Gi expression in human heart failure can be viewed as an adaptational response of the heart (El-Armouche *et al.*, 2003). For example, an increase in myocardial Gαi level occurs as one of the earliest events in animal models of hypertention (Anand-Srivastava, 1996). Expression of Gi was increased up to about 40% in the aorta of 6 week old spontaneously hypertensive rats (SHR) rats compared to normotensive rats (WKY), whereas Gs expression was unchanged (Anand-Srivastava, 1992). In addition, temporal inactivation of Gi/o delayed hypertension development in SHR (Li and Anand-Srivastava, 2002). Interestingly, the expression of G18 is also elevated in aortic smooth muscle cells isolated from SHR rats compared to WKY rats (*Figure.5.4*). Thus, the inhibitory effect of G18 on Gi activation could conceivably have a protective role under hypertensive condition.

Chapter 3 of this thesis discussed the combined effects of G18 and RGS5 on G protein activation and provided direct evidence of the positive effect of G18 on RGS5 GAP activity. Gene expression studies revealed that the activity and expression pattern of RGS5 *in vivo* is dynamically regulated, suggesting its role in the regulation of adaptive processes and vascular remodeling (Manzur *et al.*, 2009). Besides regulating normal vascular function such as blood pressure, RGS5 was also shown to be an important regulator of tumor vessel angiogenesis (Berger *et al.*, 2005). The mRNA level of RGS5

Figure 5.4

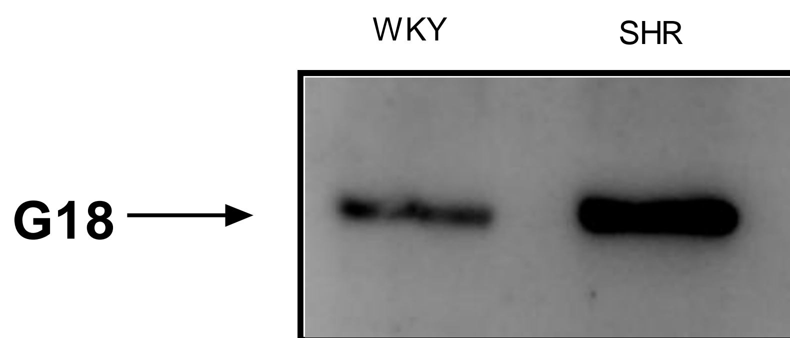


Fig. 5.4. Expression of G18 in WKY and SHR cells. Aortic smooth muscle cells isolated from normotensive rat (WKY) or spontaneous hypertensive rat (SHR) were lysed and cell lysate were subjected to SDS-page and immunoblotting using anti-G18 antibody (1:1000).

was found to be unregulated up to 5 fold in pancreatic islet tumors compared to normal pancreas. In situ hybridization analyses showed that RGS5 was expressed in cells that are closely associated with tumor blood vessels, and it also temporally and quantitatively coincided with tumor-induced angiogenic activity (Berger *et al.*, 2005). Furthermore, strong RGS5 expression was not only found in tumor angiogenesis, but also during the wound healing process, suggesting that RGS5 may also play a role in vascular cell migration (Lovschall *et al.*, 2007). The molecular mechanisms of how RGS5 regulates pericyte maturation and angiogenesis still remains to be investigated. Consistent with the expression pattern of RGS5, in a subcellular localization study of G18, we found that beside its cytosolic localization, G18 also localized at the lamellipodia of the smooth muscle cell (*Figure.5.5*). Lamellipodia are a characteristic feature at the front, leading edge, of motile cells, thus this localization of G18 may reflect its potential role in wound healing and cell migration. Thus, G18 may also play a role in RGS5 mediated regulation of cell migration via direct interaction and enhancement of RGS5 GAP activity. Moreover, G18 itself may also alter vascular remodeling through an RGS5 independent pathway.

G protein mediated cell signaling has also been suggested to regulate multiple aspects of the immune response. So far, the only G proteins that have been found interact with G18 are the Gαi/o subfamily proteins. In lymphocytes, Gαi2 and Gαi3 expression significantly exceeds the average expression present in a panel of cell types and tissues, whereas Gαi1 expression is relatively low (Kehrl, 2004). Numerous studies implicate GPCRs that signal through Gi in the regulation of lymphocyte function including T-cell

differentiation and cytokine production. The most striking functional role of Gi is its regulation of lymphocyte migration (Bargatze and Butcher, 1993). As discussed above, RGS5 acts as a key gene in abnormal vascular tumor morphology. Loss of RGS5 leads to pericyte maturation, and vascular normalization. In addition, $RGS5^{-/-}$ tumors also exhibit an enhanced influx of immune effector cells and markedly prolong the survival of tumor-bearing mice (Hamzah *et al.*, 2008). Thus, since G18 has a profound effect on regulating both Gi proteins activity and RGS5 function, it may inhibit Gαi triggered integrin activation, firm adhesion of lymphocytes to high endothelial venules and transmigration. At the same time, it may also contribute to RGS5 dependent alteration of immune cell influx (Zhou *et al.*, 2001).

Besides their effects on receptor dependent G protein signaling, many GoLoco proteins have been found to be involved in receptor independent G protein signaling such as asymmetric and symmetric cell division (see introduction). Among these proteins, G18 may be of particular interest, since it contains a GEF domain in addition to the three GoLoco motifs. Furthermore, the GEF activity of G18 is predominant in the absence of the receptor, suggesting this domain may indeed activate G proteins when they are not coupled to the receptor (Chapter 2). Most studies have indicated that the role of G protein signaling in cell division is independent of the receptor. Thus receptor independent GEFs are critical for nucleotide exchange on Gα subunits as well as subunit rearrangement, and Gβγ signaling. The only GEF that has been suggested to play such a role is Ric-8 (Afshar *et al.*, 2004;Hess *et al.*, 2004). A recent report demonstrates that the GoLoco-G protein complex is required for the proper function of Ric-8 which is

necessary for mitotic spindle orientation (Woodard *et al.*, 2010). To complete the G protein cycle, RGS proteins have also been suggested to be involved in the process (Hess *et al.*, 2004). Compared to the Ric-8/GoLoco-G protein complex/RGS pathway, G18 holds an advantage in that the GEF domain and GoLoco motifs are located within a single protein. This certainly makes the whole process simpler and easier where the C-terminus GoLoco-Gα complex may serve as a substrate for the N-terminal domain to act as a GEF. Furthermore, the direct coupling between G18 and RGS5 in cells also provides a mechanism to turn off the pathway, thus, RGS5 may serve a termination role corresponding to the function of RGS7 in *C. elegans*.

5.5.2 Physiological relevance of RGS14 on regulation of RGS protein GAP activity

Chapter 4 of this thesis uncovered a previously unidentified GAP enhancing activity of the Ras binding region of RGS14. Thus the RB domains of RGS14 and possibly those of RGS12 may act as integrators of heterotrimeric G protein signaling and monomeric G protein Ras/Raf signaling pathways. It has been suggested that the binding of Gαi1 and Raf to RGS14 tends to be mutually inhibitory (Shu *et al.*, 2010). Functionally, co-expression of Gαi1 reversed RGS14 inhibition of PDGF signaling (Shu *et al.*, 2010). Our data suggests that the RGS domain and the RB domains of RGS14 may undergo an intramolecular interaction and that this interaction may increase the affinity between the RGS domain and G protein and further promotes GAP activity of the RGS domain. At the same time, removal of the RGS domain leads to an enhancement in RGS14 GDI activity. Taken together, it is possible that RGS-RBD-Gα can form a complex and facilitates GTP hydrolysis, and after terminating the heterotrimeric G protein activation,

the RGS domain dissociates from Gα, which in turn releases the RB domain from the complex and allows it to interact with small G proteins and activate the Ras/Raf mediated MAP kinase pathway.

The RB domains of RGS14 also participate in determining the subcellular localization of RGS14. The nuclear localization of RGS14 depends on its RGS and RB domains, whereas the RB domains are sufficient for RGS14 localization to centrosomes (Shu *et al.*, 2007). However, the functional consequence of RB domain-centrosome colocalization still remains unknown. Co-expression of inactive GDP-bound Gα protein but not active GTP-bound Gα protein recruits RGS14 to the plasma membrane, suggesting that even though the GDI activity of the GoLoco motif is inhibited by the RGS-RBD interaction (Chapter 4), it may be important for targeting RGS14 to the cell membrane.

5.6 Future Directions

The novel findings of this thesis provide some interesting and exciting information regarding how GoLoco motif containing proteins regulate receptor-stimulated G protein activation, as well as how various domains within a single protein work together and modulate each other's activity. This thesis also raises some important questions that may be addressed in the future, and will be important to further enhance our knowledge of regulation of cell signaling by GoLoco motif containing proteins.

5.6.1 *FURTHER CHARACTERIZING THE INTERACTION BETWEEN N-TERMINUS OF G18 AND G PROTEINS*

Chapter 2 of this thesis identified a novel interaction between the proline rich N-terminus of G18 and Gαi1/o. However, this interaction may not be limited within the Gαi/o subfamily of G proteins. The data from earlier reports suggested that there is no appreciable binding between the C-terminus GoLoco motifs to other G proteins such as Gαs, and Gαq, (Kimple *et al.*, 2004;Cao *et al.*, 2004). However, the interaction between the N-terminal domain or full-length G18 and these G proteins remain unknown.

The amino acid residues important for the binding between the N-terminal domain of G18 and the G protein still remain to be elucidated. As mention in the discussion of Chapter 2, the relatively high proportion of proline residues of this region is of particular interest. The unique side chain structure and its effects on overall protein conformation may contribute to the observed interaction. In addition, the overall amino acid composition of the N-terminal region of G18 shares a common sequence feature of unfolded proteins and suggests that it may be highly flexible in solution. Thus, this region may be able to adopt different conformations for binding to different partners.

Another interesting observation is that the interaction between the N-terminal domain of G18 and Gαi or Gαo proteins results in different functional consequences. The reason for these seemingly opposite effects remains unclear. However, studies on another receptor independent GEF, Ric-8, suggest that it preferentially interacts with the nucleotide-free state of G proteins, thus it may stabilize G proteins under their nucleotide free state and

prevent nucleotide association. Interestingly, in a recent paper, the Tall group has examined the apparent GEF activity of Ric-8B on Gs (Chan *et al.*, 2011). However, due to the high affinity between Ric-8 and nucleotide free G protein, this activity is only observed under high concentration of GTP. It is currently unknown if G18 shares a similar mechanism, and ongoing experiments are trying to address this issue by looking at the nucleotide exchange process in more detail. We are currently examining the effect of G18 on GDP dissociation from the G protein. Another potential interesting experiment will be to perform the nucleotide titration assays and to see if the different effects of G18 on Gi and Go are due to changing in the affinity between G protein and GTP after binding to G18.

5.6.2 CELLULAR FUNCTION OF G18 AND ITS SPLICE VARIANTS

Chapter 2 of this thesis suggests that even though via different mechanisms, G18 can inhibit receptor-stimulated activation of both Gi and Go proteins. This observation indicates that it may reverse the inhibitory role of Gi signaling on AC activity and cAMP production. Looking directly looking at cAMP production upon overexpression of different G protein binding domains of G18 may provide some insight information of how G18 regulates G protein function inside cells and which G protein binding domain is responsible for its effect. Another interesting project would be to look at the expression patterns, regulation and activities of the short splice variants of G18. The distinct domain composition suggests that their biochemical as well as physiological functions may be different. Consistent with this hypothesis, GTPγS binding assays have shown that unlike the full-length protein which exhibits little or no effect on nucleotide exchange on Gαi1

due to opposite effects of its GEF and GDI activities, whereas the short isoform of G18 exhibit a profound GDI activity (*Figure 5.3*).

Besides different G protein binding domains, other binding partners and post-translational modifications may also regulate the cellular function of G18. For example, little or no GDI activity is observed when G18 is coupled to RGS5 in a receptor stimulated steady-state GTPase assay, suggesting that when coupling to RGS5, G18 may rather enhance RGS5 GAP activity. Thus its cellular function may vary under different circumstances. Another potential mechanism that may regulate G18 function is phosphorylation. It has been suggested that the fragment of G18 in the major histocompatibility complex immunoprecipitates was actually phosphorylated at Ser-59, which is just upstream of the first GoLoco motif (Cao *et al.*, 2004). Phosphorylation of RGS14 at a corresponding site dramatically increased its GDI activity (Hollinger *et al.*, 2003). This suggests that phosphorylation may influence the interaction between G18 and G protein and provide a regulatory mechanism for signal input or subcellualr location.

Finally, it would be interesting to look at the potential role of G18 in cell division. Similar to other GoLoco motif containing proteins, in smooth muscle cell, the subcellular localization of endogenous G18 involves its association with microtubules both during interphase and during mitosis (unpublished observation, *Figure 5.5, 5.6*). Thus, G18 may play a role in regulating microtubule dynamics and spindle pole organization.

Overexpression and siRNA approaches may be used to elucidate the effects of G18 on microtubule assembly and spindle pole orientation.

5.6.3 Characterizing RGS14 GAP adapter domain and enhancing domain

Chapter 4 of this thesis provides more detailed information regarding the GAP enhancing activity of the RGS14 Ras binding region on different RGS proteins. However, the mechanism underlying this is still unclear. The specific binding site on both the RGS protein and the RB domains still remain to be solved. It is currently unknown whether the binding of the RGS proteins and Rap/Raf kinase to the RB domains share a similar binding site, or whether the binding of small G protein binding is able to inhibit the GAP enhancing activity of the RB domains. It has been suggested that small G protein binding has no effect on the GAP or GDI activities of full-length RGS14 (Mittal and Linder, 2006), however, RB domains are not directly involved in those activities. Future experiments might be directed at examining the effect of RGS14-RB domains on GAP activity upon the binding of small G proteins.

5.7 General conclusion

The major components of GPCR-mediated signal transduction are transmembrane receptors, second messengers and effectors. The amplitude and duration of the signal output may also be regulated by different accessory proteins. Chapter 2, Chapter 3 and Chapter 4 of the current thesis focus on two accessory proteins of G protein signaling G18 and RGS14. In addition, these two proteins both contain more than one G protein binding domains that are able to modulate G protein activity via different mechanisms.

Figure 5.5

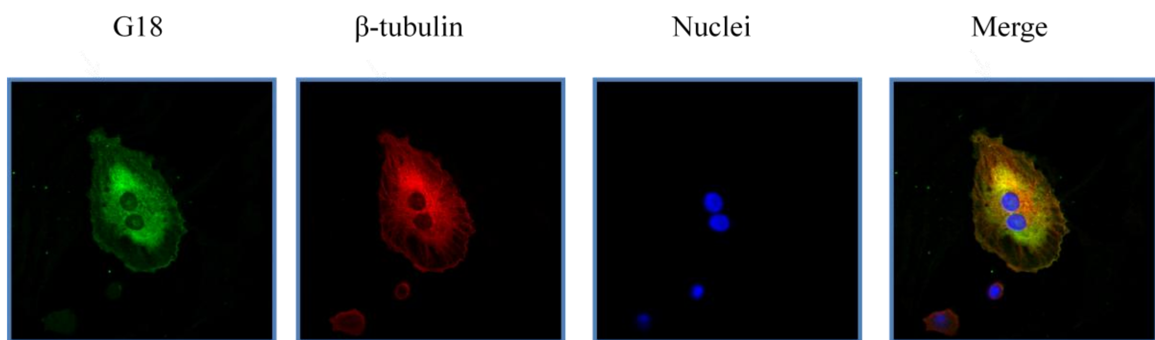
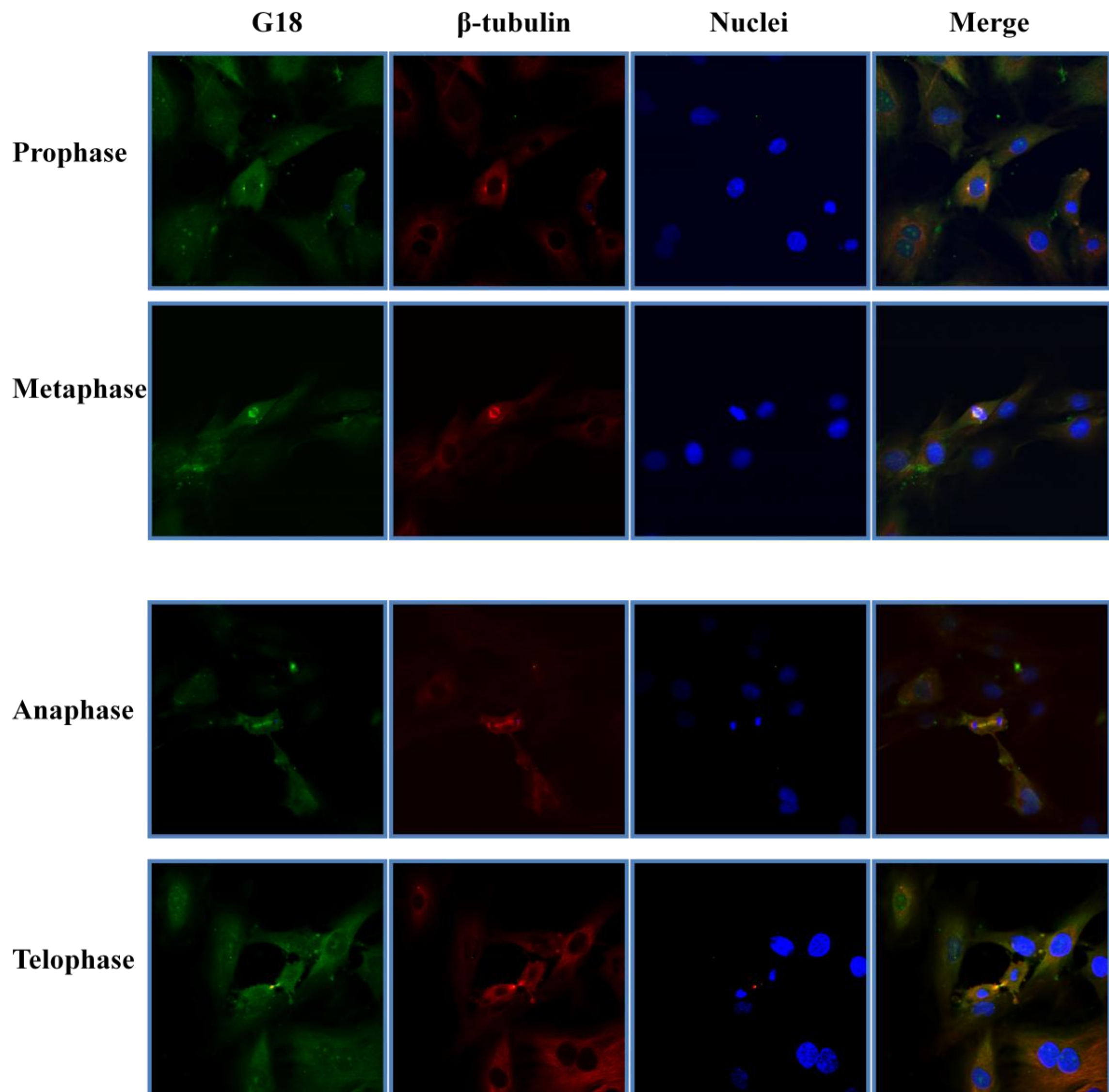


Fig. 5.5. Co-localization between G18 and β-tubulin during interphase. Primary aortic smooth muscle cells were fixed and subjected to immunoflurescence study. fixed cells were probed with anti-G18 antibody (1:500), anti β-tubulin antibody (1:500) and AlexaFluor 488 goat-anti rabbit or AlexaFluor 594 goat-anti mouse secondary antibodies (Invitrogen). DAPI was used to stain the nuclei.

Fig. 5.6. Co-localization between G18 and β-tubulin during Mitosis. Primary aortic smooth muscle cells were serum starved for 24 hours, and 20 hours after serum replacement, cells were fixed and subjected to immunoflurescence study. fixed cells were probed with anti-G18 antibody (1:500) and anti β-tubulin antibody (1:500) and AlexaFluor 488 goat-anti rabbit or AlexaFluor 594 goat-anti mouse secondary antibodies (Invitrogen).

Figure 5.6



We have discovered a novel G protein interacting domain located N-terminal to the GoLoco motifs of G18, and this domain has the ability to alter nucleotide exchange on Gα proteins. We have also identified a novel interaction between G18 and RGS5 which enhances RGS5 GAP activity but has a seemingly negligible effect on G18 function. Finally, we characterize an intramolecular interaction between the RGS domain and the small G protein binding domain of RGS14 and how this interaction affects RGS14 GAP and GDI activities. Thus, our general conclusion is: GoLoco motif containing proteins G18 and RGS14 are the organizers of G protein signaling that also modulate RGS function. Although the physiological function and regulation of G18 and RGS14 still remain to be elucidated, data presented in this thesis provide some insight information regarding how these two proteins regulate G protein activation and deactivation cycle and their own activity from both molecular and biochemical perspectives. This information will assist us to better interpret the mechanisms of the cellular function of these two proteins. Furthermore, it will also help us to understand how G protein signaling is regulated by different accessory proteins.

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CURRICULUM VITAE

Peishen Zhao

EDUCATION

- **Doctor of Philosophy**
  Department of Physiology and Pharmacology
  University of Western Ontario, Canada
  Major: Pharmacology
  Supervisor: Dr. Peter Chidiac

- **Bachelor of Science 2006** Pharmacology, Shenyang Pharmaceutical University, Shenyang, Liaoning, China
  Department of Pharmaceutical Science
  Shenyang Pharmaceutical University, China
  Major: Pharmacology

TRAINING AND MENTORING EXPERIENCE:

- Teaching Assistant for "Pharmacology 3580y – Pharmacology lab course"
  The Course is designed for third year Science student, major in pharmacology. The purpose of this lab course is to illustrate some of the key concepts presented in the lecture courses, as well as to introduce you to some common approaches to research in pharmacology.

  My role is to coordinate with instructors, prepare material for the student lab, helping student with calculations, interpret experimental results, marking lab report, quiz and answer questions.

  As a graduate student of Dr. Peter Chidiac who is one of the instructors, I also assistant Dr. Chidiac develop specific lab module (Drug Actions on Intracellular Signaling Mechanisms and Gene Transcription). This include: establishing stable cell line, design experiments and write experimental protocols.

- Student mentor for "Pharmacology 4980 – Honors research project"
  - Jeff Giles Sep. 2007-Apr. 2008
    Project: The effect of the GoLoco domain of RGS14 on RGS protein GAP activity
  - Kyle Salvador Sep.2008-Apr.2009
    Project: The effect of the RBD domains of RGS14 on RGS protein GAP activity

- Graduate Student Supervision
  - William Xue, MSc student, University of Western Ontario, Sep. 2009 –
    Project: Molecular determinants for eIF2Bε interaction with RGS2

- Judging undergraduate posters for departmental research day 2009, 2010, 2011

RESEARCH EXPERIENCE:

University of Western Ontario, Canada Sep. 2006- present

PhD student, Department of Physiology and Pharmacology
Research Mentor: **Dr Peter Chidiac**

- **Projects:** To investigate the role of multi- G protein regulatory domain containing protein on cell signaling
- **Responsibilities:**
  - Responsible for design and management of multiple projects. Maintain daily lab running including ordering, equipment maintenance, radioactive protocols and animal inventory.
  - Investigate biochemical and cellular role of multi-G protein regulatory domain containing protein such as G18 (AGS4) and RGS14.
  - Help lab mates with their projects, provide necessary technique and skill support.
- **Techniques:** Culture of primary cells (mouse hepatocytes, rat cardiomyocytes); gene and protein expression (real-time RT-PCR (Sybr Green and Taq-man methods), western blotting); in vitro signaling assays (cAMP accumulation, inositol phosphates accumulation, thymidine incorporation, white adipose tissue lipolysis, GTPase activity assay); whole animal physiology (indirect calorimetry, glucose tolerance, insulin tolerance); basic molecular techniques (plasmid amplification and purification, protein purification, RNA isolation)

Institute of material Medica, Chinese Academy of Medical Sciences and Peking Union Medical College Jan. 2006 – Jun. 2006

4th year thesis project, Department of Pharmacology
Principal Investigator: **Dr. Zhufang Shen**

- **Project**: The effect of saturated fatty acid (SFA) and unsaturated fatty acid on the blood free fatty acid level in Chinese Hamster
- **Responsibilities:**
  - Investigate the different effect of different concentration of SFA and UFA on Chinese Hamster plasmid lipid level.
  - Determine the differences in the distribution, and clearance of SFA/UFA in wild type and MSG induced type 1 diabetes mice.
- **Techniques:** Basic animal handling, I.P. injection, intragastric administration, mouse tail vein injection, blood collection, plasmid lipid measurement.

Shenyang Pharmaceutical University
Jan. 2005-Dec.2005

Research Assistant, Department of Pharmacology
Principal Investigator: **Dr. Yuyang Zhang**

- **Discipline: Clinic Pharmacology**

SCHOLARSHIPS:

Graduate School:
Sep.2006- present University of Western Ontario
-Western Graduate Research Stipends (WGRS) – Pharmacology

Undergraduate:
Sep.2005-Jul. 2006 University Scholarship (First Prize)
Sep.2004-Jul.2005 University Scholarship (Second Prize)
Sep.2003-Jul.2004 University Scholarship (Third Prize)

Awards:
2010 SSMD the Graduate Incentive Funds
2010-2011 Graduate Thesis Research Fund (GTRF) Awards
2010-2011 Chinese Government award for Outstanding Self-financed Students Abroad

PROFESSIONAL SOCIETY MEMBERSHIP:

Canadian Society of Pharmacology and Therapeutics, 2008 –

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Zhao P, Nguyen CH, Chidiac P. **The proline-rich N-terminal domain of G18 exhibits a novel G protein regulatory function**. J Biol Chem. 2010 Mar 19;285(12):9008-17. Epub 2010 Jan 22.

Nguyen CH, Ming H, **Zhao P**, Hugendubler L, Gros R, Kimball SR, Chidiac P. **Translational control by RGS2**. J Cell Biol. 2009 Sep 7;186(5):755-65.

Nunn C*, **Zhao P***, Zou MX, Guglielmo C, Summers K, Chidiac P. **Body fat regulation in RGS2-deficient mice**. *Paper in press (accepted manuscript, cellular signaling) (*These authors contributed equally to this work)*

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Nguyen CH, **Zhao P**, Shu FJ, Hepler JR, Chidiac P. **Dexras1 inhibits RGS14 GAP activity**. *Manuscript in submission.*

In Preparation

Zhao P, Chidiac P. **Regulation of RGS5 GAP activity by GoLoco motif containing protein G18**.

Nguyen C, **Zhao P**, Sobiesiak A, Chidiac P **Rgs2 is a component of the cellular stress response**.

Zhao P, Chidiac P **Role of G18 in cell division.**

Published poster abstracts

Zhao P, Nguyen C, Nunn C, Chidiac P (2008) **The effect of the GoLoco motif-containing protein G18 on regulation of G protein signaling.**

Zhao P, Nguyen CH, Chidiac P (2008). **Effects of the GoLoco motif containing protein G18 on RGS protein GAP activity.**

CONFERENCE ATTENDED AND POSTER PRESENTED:

2007 GPCR retreat Sep. 27th to 29th, 2007. London ON,
Poster presented: The effect of the GoLoco motif-containing protein G18 on regulation of G protein signalling"

3rd RGS Protein Colloquium Apr. 4th to 5th, 2008. San Diego CA.
Poster presented: Effects of the GoLoco motif-containing protein G18 on G protein signalling reveal a novel regulatory function."

2008 GPCR retreat Oct. 16th to 18th, 2008. Chateau Bromon Bromont Qubec
Poster presented: The regulatory effect of the N-terminus of G18/AGS4 on G protein signalling."

2009 GPCR retreat Oct. 15th to 18th, 2009. University of Rochester
Poster presented: The effect of G18 and RGS5 GAP activity"

2010 GPCR retreat, Oct. 21st to 23rd, 2010. University of Toronto
Poster presented: The effect of RBD domain on RGS14 GAP and GDI activity"

2011 Experimental Biology, Apr. 9th to 13th, 2011. Washington, DC
Poster presented: Regulation of RGS5 GAP activity by the GoLoco motif containing protein G18/AGS4

4th RGS Protein Colloquium, Apr. 13th to 14th, 2011. Washington, DC
Poster presented: RGS14 regulates GAP activity via its C-terminal RB/GoLoco domains

SPECIAL SKILLS:

Techniques and skills:

Molecular techniques: Molecular Cloning, mutagenesis,
Cell biology: basic cell culture, transit transfection, stable transfection, ELISA,

Virology: Adenovirus amplification, Baculovirus amplification, cell infection, virus titre determination

DNA RNA and protein techniques: DNA purification, RNA purification, protein purification (affinity purification,), Reverease Transcriptase PCR, real-time PCR, SDS page, western blot, protein-protein interaction assay (pull-down, Co-immunopercipitation), FPLC, antibody design

Animal care: basic animal care and handling, injection,mice body weight measurment, body temperature measurment, transgeneic mice genotyping, mice blood glucose measurment, aortic ring organ bath

Primary cell isolateion: mouse embryonic fibroblast (MEF), neonatal rat cardiomyocyte, mice adipocyte, mice hepatocyte

Biochemical assays: kinetic assays, cAMP accumulation; [35S]GTPγS binding; inositol phosphates accumulation, GTPase assay

Other: confocal microscopy,