Regulation of the Gαi – G-protein Regulatory (GPR) Module and the Biological Function of GPR-containing Proteins in Chemokine Signal Processing of Primary Leukocytes

by

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Abstract

WILLIAM GENE ROBICHAUX III. Regulation of the Gαi – G-protein Regulatory (GPR) Module and the Biological Function of GPR-containing Proteins in Chemokine Signal Processing of Primary Leukocytes. (Under the direction of Joe B. Blumer)

Activators of G-protein Signaling (AGS) proteins modulate G-protein signaling in diverse and unexpected ways and have functional roles in numerous physiological systems. The functions of AGS proteins in the immune system, where Gαi-coupled chemokine receptors are predominantly involved in dynamic signaling events, are poorly understood. The Group II AGS proteins AGS3 and AGS4 express multiple G-protein regulatory (GPR) motifs, each of which dock GDP-bound Gαi/o/t subunits and effectively compete with Gβγ for binding. This unique ability positions these proteins to modulate downstream signaling of Gαi and Gβγ, thus promoting signal diversity from seven-transmembrane receptors (7TMR). However, regulatory mechanisms and functional roles for the Gαi2–GPR module in leukocytes are poorly understood. Using a bioluminescence resonance energy transfer (BRET) platform, we demonstrated chemokine regulation of Gαi2–GPR modules that were receptor-proximal. Generation of fusion proteins with Gαi physically tethered to the 7TMR revealed that regulation of Gαi2–GPR was independent of endogenous G-protein cycling subsequent to receptor activation, suggesting that Gαi-GPR couples to 7TMRs analogous to Gαβγ heterotrimer. Additional modes of regulation for AGS4 were also investigated including identification of alternative binding proteins ARID1b and eEF1d,

suggesting potential modulatory functions for AGS4 in transcription and protein translation, and phosphorylation of AGS4-Y108 by JAK2 and Src, which regulates the Gαi-AGS4 interaction. Furthermore, regulation of Gαi2–GPR by chemokine receptors and expression of AGS3 and AGS4 in immune cells and tissues suggested functional roles of these proteins in the immune system. Investigating chemoattractant signal processing in primary leukocytes from wild-type, AGS3-null and AGS4-null mice demonstrated 25-40% decreased migration with corresponding reduction in ERK1/2 activation of null-animals. The importance of the Gαi–GPR interaction in chemokine signaling provides a novel platform for development of pathway targeted small molecules, identified in preliminary screening for modulators of the Gαi–GPR interaction. These studies have broad implications for G-protein signal processing and Gαi-GPR complexes in immune function.

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List of Abbreviations

7TMR – seven transmembrane spanning receptor (see also GPCR)

AGS – activator of G-protein signaling

AR – adrenergic receptor

ARID1b/2 – AT-rich interacting domain-containing proteins 1b/2

ATP adenosine triphosphate

BMDC – bone marrow derived dendritic cells

cAMP – cyclic adenosine monophosphate

CCL – chemokine (C-C motif) ligand

CCR – C-C chemokine receptor

CCT – chaperonin containing TCP-1

CD3 – cluster of differentiation 3

CD4 – cluster of differentiation 4

CD8 – cluster of differentiation 8

Cdc42 – cell division control protein 42

CNS – central nervous system

CXCL – chemokine (C-X-C motif) ligand

CXCR – C-X-C chemokine receptor

DAG – diacylglycerol

DPBS – Dulbecco's Phosphate Buffeted Saline (containing no Ca++ or Mg++)

EDTA – ethylenediaminetetraacetic acid

eEF1d/2 – eukaryotic elongation factors 1d/2

eEF2K – eukaryotic elongation factor 2 kinase

ERK1/2 – extracellular signal-regulated kinases 1/2

FITC – fluorescein isothiocyanate

fMLP – N-Formyl-methionyl-leucyl-phenylalanine

GAP – GTPase-accelerating protein

GDI – guanine nucleotide dissociation inhibitor

GDP – guanosine diphosphate

GEF – guanine nucleotide exchange factor

GIRK channel – G-protein coupled inwardly-rectifying potassium channel

GIV/Girdin – Gα interacting vesicle-associated protein

GLL – Gγ-like domain

GM-CSF – granulocyte macrophage colony-stimulating factor

GPCR – G-protein coupled receptor (see also 7TMR)

GPR motif – G-protein regulatory domain (also GoLoco motif)

Gpsm – G-protein signaling modulator

GRK – G-protein regulatory kinase

GST – glutathione S-transferase

GTP – guanosine-5'-triphosphate

ICAM1 – intracellular adhesion molecule 1

iDC – immature dendritic cell

IFN-γ – interferon gamma

IgG – immunoglobulin G

IgM – immunoglobulin M

IL-1 – interleukin 1

IL-2 – interleukin 2

IL-6 – interleukin 6

IP – intraperitoneal

IP3 – inositol 1,4,5-triphosphate

JAK2 – Janus kinase 2

JAM-A – junctional adhesion molecule-A

LFA-1 – lymphocyte function-associated antigen 1

LKB1 – liver kinase B1

LPS – lipopolysaccharide

mDC – mature dendritic cell

mInsc – mammalian inscuteable

PCP2 – Purkinje cell protein 2

PE – phycoerythrin

PECAM – platelet endothelial cell-adhesion molecule

PhLP – phosphoducin-like protein

PI3K – phosphoinositide 3 kinase

Pins – partner of inscuteable

PIP2 – phosphatidylinositol 4,5-bisphosphate

PKC – protein kinase C

PLCβ – phospholipase Cβ

PMA – phorbol 12-myristate 13-acetate

PSGL-1 – P-selectin glycoprotein ligand-1

PTX – Pertussis toxin

RGS – regulators of g-protein signaling

Ric8A/B – resistance to inhibitors of cholinesterase 8 A/B

Rluc – Renilla luciferase

SDF-1 – stromal cell-derived factor-1

SDS-PAGE – sodium dodecyl sulfate polyacrylamide gel electrophoresis

STAT – signal transducer and activator of transcription

SWI/SNF – SWItch/Sucrose NonFermentable complex

TM – transmembrane domain

TNF-α – tumor necrosis factor alpha

TPR – tetratricopeptide repeat

TSLP – thymic stromal lymphopoietic protein

VCAM1 – vascular cell-adhesion molecule 1

VLA-4 – very late antigen 4

WT –wild-type

YFP – yellow fluorescent protein

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Chapter 1

Literature Review

Introduction to Canonical G-protein Signal Transduction

Signaling through G-proteins is a fundamental transduction pathway for cells. G-protein coupled receptors (GPCRs) are the most commonly used mechanism for cells to process extracellular stimuli into appropriate signal output. Signifying the importance and diversity of G-protein signaling, 3-4% of the entire human genome is dedicated to the genes of GPCRs, thus representing the single largest gene superfamily (Katritch et al 2013). Such a variety of receptors allows for G-proteins to be integrated into signaling pathways from countless stimuli, and as a consequence, perturbations to this core signaling system are linked to the development of cardiovascular, metabolic, neurodegenerative, cancer and immunological diseases. The practically universal use of G-proteins by cells has made the development of therapeutics to this pathway quite attractive, leading to greater than 30% of known pharmaceuticals targeting GPCRs or downstream effectors of these signaling pathways (Overington et al 2006, Rask-Andersen et al 2011). However, although this pathway is well exploited for drug development, the manner in which cells are able to achieve appropriate signal specificity, efficiency, and strength and maintain flexibility and adaptability in the face of dynamic alterations of external stimuli is still largely unknown.

Although involved in a vast array of signaling pathways, receptors from the GPCR superfamily all contain general structural similarities including an

extracellular amino-terminus, seven transmembrane-spanning (7TM) alpha helical domains, and an intracellular carboxy-terminus forming three extracellular and three intracellular loops as seen in solved crystal structures of rhodopsin, M2 muscarinic acetylcholine, β2 adrenergic, and CXCR4 receptors. (Haga et al 2012, Palczewski et al 2000, Pierce et al 2002, Rasmussen et al 2007, Wu et al 2010). Even with these common structural features, the superfamily is further categorized into five subclasses: rhodopsin and adrenergic receptors, secretin receptors, glutamate receptors, adhesion receptors and frizzled/taste receptors (Fredriksson et al 2003). The primary conduit of signal transduction for this receptor superfamily are heterotrimeric G-proteins, although G-protein independent mechanisms are also reported, including β-arrestin-mediated signaling cascades and Janus kinase 2 (JAK2) activation of Stat1 and Stat3 (Azzi et al 2003, Godeny et al 2007, Lefkowitz & Shenoy 2005, Luttrell & Lefkowitz 2002).

Initially, GPCRs were viewed as simple “on-off” switches for activation of G-protein heterotrimers; however, recent studies demonstrate the dynamic conformations these receptors can adopt. In the most basic context, extracellular ligand binding triggers conformational changes in the transmembrane domains and intracellular loops, thus stabilizing active, thermodynamically-favorable conformations which have increased affinity for heterotrimeric G-protein coupling and subsequent activation (Azzi et al 2003, Bockenhauer et al 2011, Kahsai et al 2011, Malik et al 2013, Park 2012, Vilardaga et al 2005, Yao et al 2009). Of the conformational changes occurring in the receptor, three notable clusters of

highly-conserved residues in the transmembrane domains (TM), termed micro-switches, are hypothesized to be of utmost importance for complete receptor activation (Katritch et al 2013, Nygaard et al 2009). These residues are pivotal points in the receptor that adopt completely altered conformations after receptor activation thus mediating larger conformational changes throughout the remaining receptor complex, especially TMVI and TMVII. Although these are not the sole residues that undergo conformational changes, these three micro-switch motifs are commonly thought to contribute to complete receptor activation (Katritch et al 2013, Nygaard et al 2009).

One highly conserved motif throughout GPCRs is the CWxP motif. This motif is strategically located near the ligand binding pocket in TMVI and is in close proximity to the proline kink found in TMVI (Crocker et al 2006, Ruprecht et al 2004, Schwartz et al 2006, Shi et al 2002). Activation of the receptor results in a conformational change in position of the Trp residue of the CWxP motif resulting in a rotation of the Trp side chain toward TMV where the residue can partake in aromatic interactions with a highly conserved Phe/Tyr residue thus stabilizing the active conformation (Shi et al 2002). This rotation is suggested to result in an outward bending of the TMVI domain of the receptor allowing G-proteins to access the cytoplasmic binding pocket (Crocker et al 2006, Ruprecht et al 2004, Schwartz et al 2006, Shi et al 2002). Conformational rotation of the Trp in the CWxP motif has not been as dramatic as predicted in crystal structures recently published, while the subsequent two micro-switches conformational changes were readily visualized in X-ray crystal structures of GPCRs

(Rasmussen et al 2011, Scheerer et al 2008). The NPxxY motif is the second of these highly conserved motifs suggested to be intricately involved in receptor activation. Located in TMVII, the Asn residue of this motif has been observed to interact with TMVI either through additional polar residues in TMVI or through a hydrogen binding network involving a water molecule in receptor inactive state(s) (Govaerts et al 2001, Okada et al 2002). Additionally, in rhodopsin the Tyr residue of this motif associates with helix VIII (typically Phe) through aromatic interactions, while this residue exists in a upward tilted conformation in other GPCRs where it is involved in a hydrogen bonding network with water (Barak et al 1995, He et al 2001, Li et al 2004). Activation of the receptor results in bending of the TMVII causing a shift in the Asn residue of the NPxxY motif to face the middle of TMII, thus alleviating the hydrogen bond lockdown of TMVI, allowing TMVI to swing open (Barak et al 1995, Govaerts et al 2001, Nygaard et al 2009). In addition, the bending of TMVII also orients the Tyr residue of the NPxxY motif to obstruct TMVI from withdrawing from the outward movement and thereby leaving the cytoplasmic pocket open for G-protein binding (Barak et al 1995, Govaerts et al 2001, Nygaard et al 2009, Park et al 2008a). A third highly conserved micro-switch region located in the cytoplasmic end of TMIII of most GPCRs is known as the DRY motif (Asp/Glu-Arg-Tyr residues). Association of the Arg/Glu of the DRY motif with TM-VI results in what has been deemed an “ionic lock” resulting in TM-VI occupying the cytoplasmic pocket and sterically hindering G-protein coupling (Rasmussen et al 1999, Xie & Chowdhury 2013, Yao et al 2006b). The importance of the Arg/Glu residue of the DRY micro-switch

was demonstrated through site-directed mutagenesis studies resulting in increased constitutive activity which suggested this residue assisted in stabilizing inactive conformations of the receptor (Alewijnse et al 2000, Arnis et al 1994, Ballesteros et al 2001, Malik et al 2013, Rasmussen et al 1999, Zhu et al 1994). Although constitutive activity of the receptor is the most common observation obtained when mutating this residue, there are reports of no change to receptor activity, but rather to receptor folding (Chung et al 2002, Lu et al 1997). Overall, the conformational changes induced by these micro-switches in receptor tertiary structures are required to transfer external signals to the intracellular environment for further signal processing. Determination of the crystal structures of Gα and Gβγ subunits assisted in the development of hypotheses on the ability of GPCRs to propagate cellular signals through the G-protein heterotrimer.

Initial crystals for Gα subunits were obtained in the 90's and revealed structural characteristics that lead to hypotheses of how the Gα subunit functioned and coupled to GPCRs. Crystallography revealed that Gα subunits primarily consist of two α-helical domains that bind nucleotide and one of which harbors an intrinsic GTPase domain (Ras-like domain) illustrated in previous biochemical studies (Lambright et al 1994, Lambright et al 1996, Mixon et al 1995, Sunahara et al 1997). The nucleotide binding "pocket" is positioned between the Ras-like domain and helical domain and is bordered by flexible regions known as switch domains. Aside from hydrolyzing guanosine nucleotides, the Ras-like domain is also a suitable binding surface for interacting-proteins such as GPCRs, Gβγ dimer, effectors, and other signal modulators such

as regulators of G-protein signaling (RGS) proteins (Tesmer et al 1997a, Tesmer et al 1997b). Post-translational lipid modifications of the amino terminus of Gα subunits by myristolylation and palmitoylation also suggest the amino terminus to be important for anchoring the Gα subunit to the plasma membrane for signaling (Degtyarev et al 1994, Preininger et al 2003). Coupling of Gα subunits with GPCRs is thought to depend on the associations of the C-terminus, α4 – β6 loop, and N-terminus of the Gα subunits with the cytoplasmic "pocket" of a GPCR in an active conformation (Bae et al 1999, Cai et al 2001, Hamm et al 1988, Ho & Wong 2000). The Gα C-terminus was found to contact TM6 or intracellular loop 3 (ICL3) in the cytoplasmic "pocket" of the GPCR, where it can be modulated by the receptor upon activation (Cai et al 2001, Hu et al 2010, Rasmussen et al 2011, Scheerer et al 2008). The solved structure for the β2 adrenergic receptor (AR) – GαsGβγ complex in an active receptor conformation and nucleotide-free, intermediate Gα bound state supported many interactions and crystal structures previously determined for active GPCRs mentioned previously (Rasmussen et al 2011). Of note, the α3 – β5 loop of Gα subunits previously postulated to interact with receptors was not observed in the solved β2AR – GαsGβγ complex; however, this may imply different conformational states of the receptor which may bind Gα subunits in altered orientations or different Gα subunits preferentially (Grishina & Berlot 2000).

There are sixteen Gα subunit isoforms in mammals (Downes & Gautam 1999, Wettschureck & Offermanns 2005). These Gα subunits are typically grouped into four distinctive classes based on effector regulation: Gαs, Gαi/o,

Gαq/11 and Gα12/13 (Downes & Gautam 1999, Wettschureck & Offermanns 2005). Activation of adenylyl cyclase by the $G\alpha_s$ subunit is known to catalyze the conversion of adenosine triphosphate (ATP) to cyclic adenosine monophosphate (cAMP). On the other hand, Gαi subunits confer an inhibitory effect on adenylyl cyclases, suppressing cAMP production. Therefore, $G\alpha_s$ and Gαi/o regulation of adenylyl cyclase enzymes acts to counter one another's effects on cAMP levels within the cell (Chen-Goodspeed et al 2005, Chen et al 1997, Dessauer et al 1998, Taussig et al 1994). Active $G\alpha_{GTP}$ subunits have also been implicated in the activation of tyrosine kinase signaling cascades through direct association with the catalytic domain of c-Src resulting in increased activity of this kinase (Corre et al 1999, Ma et al 2000). Further support of crosstalk between Gα subunits and receptor tyrosine kinase downstream signaling was illustrated in mice expressing constitutively active Gαi2, where this mutation results in activation of c-Src, MAPKs, and STATs (Edamatsu et al 1998, Ram et al 2000). Interestingly, the $G\alpha_{GDP}$ subunit is also a substrate for phosphorylation by c-Src which is able to modulate coupling of the Gα subunit to 7TMRs (Hausdorff et al 1992). $G\alpha_{q/11}$ subunits increase intracellular calcium through activation of phospholipase Cβ (PLCβ) (Blank et al 1991). The final Gα group, $G\alpha_{12/13,}$ activates Rho-GTPases mediating actin cytoskeletal remodeling and subsequent cellular migration (Sugimoto et al 2003). Of the four groups of Gα subunits, the Gαi/o class is the most highly expressed, thus one working hypothesis is that the relative pool of Gβγ released subsequent to GαiGβγ heterotrimer activation is higher than for other Gα classes, which would explain why many of the

responses from Gαi-coupled GPCRs are predominantly mediated by Gβγ (Blumer & Tall 2012).

In comparison to Gα subunits, there are five Gβ subtypes and twelve Gγ subtypes (Downes & Gautam 1999, Wettschureck & Offermanns 2005). The Gβ subunit adopts a structure resembling a seven-bladed propeller consisting of WD40 repeats and interestingly contains no catalytic domain (Sondek et al 1996, Wall et al 1995). Gγ subunits are relatively small proteins (~8-10 kDa) consisting of two alpha helices joined by a single loop (Sondek et al 1996, Wall et al 1995). Gβ and Gγ subunits are bound through interaction of the Gγ N-terminal helix with the N-terminal alpha helical domain of the Gβ subunits, while the C-terminal helix of Gγ subunit is found to associate with propellers five and six of the Gβ subunit (Sondek et al 1996, Wall et al 1995). The Gβγ dimer is quite stable once assembled; however, proper folding and assembly of this complex requires the chaperone function of chaperonin containing TCP-1 (CCT) to assist with Gβ subunit folding with subsequent release of the properly folded subunit by the co-chaperone phosphoducin-like protein (PhLP) to allow association with the Gγ subunit (Lukov et al 2006, Lukov et al 2005, McLaughlin et al 2002, Wells et al 2006). Further post-translational lipid modifications of the Gγ subunits C-terminus by farnesyl or geranylgeranyl group addition illustrates the importance of Gγ subunits in membrane targeting of Gβγ dimers (Takida & Wedegaertner 2003). One exception to the rule of Gβγ dimer formation involves $G\beta_5$ subunits, which shares the least homology amongst the Gβ isoforms and interacts with and is stabilized by RGS proteins containing a Gγ-like (GLL) domain in the absence of

Gγ to prevent the degradation of GLL containing proteins (Chen et al 2003, Snow et al 1998, Witherow et al 2000).

The absence of a catalytic domain illustrated that Gβγ subunits mediate effects by protein-protein interactions on surface domains often referred to as Gβγ "hot spots" (Lin & Smrcka 2011, Lodowski et al 2003, Panchenko et al 1998, Scott et al 2001). Although each effector binds Gβ using unique residues, the general area in which many of these effectors associate with the Gβ subunit is directly associated with the Gα subunit in the inactive heterotrimer complex thus preventing Gβγ effector activation until the heterotrimer dissociates/rearranges upon activation (Davis et al 2005). Perhaps contrary to expectations, crystallography of the β2 adrenergic receptor (AR) – GαsGβγ complex in the nucleotide-free transition state demonstrated no direct associations of the Gβγ dimer with the GPCR suggesting that the GPCR does not act on the Gβγ directly in this conformation; however, these results do not exclude the potential importance of Gβγ subunits to initial coupling of heterotrimeric G-proteins to the 7TMR (Rasmussen et al 2011, Wu et al 2000).

G-protein heterotrimers are the major conduit for signal transfer from receptor to effector. Upon ligand binding the GPCR undergoes conformational changes as described previously, resulting guanosine nucleotide exchange factor (GEF) activity of the receptor, which induces the exchange of guanosine diphosphate (GDP) for guanosine triphosphate (GTP) bound to the Gα subunit (Figure 1.1 [1]), (Chung et al 2011, Malik et al 2013, Rasenick et al 1994, Rasmussen et al 2011). Agonist-bound receptor-mediated rearrangement of the

Gα subunit results in a large conformational shift of the helical domain that dramatically opens the nucleotide binding pocket to allow GDP release and exchange for GTP as had long been hypothesized (Chung et al 2011, Noel et al 1993, Rasmussen et al 2011, Van Eps et al 2011, Westfield et al 2011, Yao & Grant 2013). Changes in switch regions of the Gα subunit result in dissociation or rearrangement of the $G\alpha_{GTP}$ subunit and Gβγ dimer of the G-protein heterotrimer exposing the effector binding surface on Gβγ, allowing the two G-protein entities to interact with and regulate distinct downstream signaling pathways (Chung et al 2011, Davis et al 2005, Van Eps et al 2011). Intrinsic GTPase activity of the Gα subunit induces hydrolysis of the terminal phosphate of the GTP molecule serving as a time dependent 'off' switch for Gα subunits and its effectors (Figure 1.1 [2]), (Graziano et al 1989, Kleuss et al 1994, Linder et al 1990). This process is relatively slow, but is accelerated by association with accessory proteins known as regulators of G-protein signaling (RGS) proteins (Berman et al 1996b, Doupnik et al 1997, Watson et al 1996). Conversion of $G\alpha_{GTP}$ to $G\alpha_{GDP}$ terminates the effector signaling of the Gα subunit. Furthermore, with the Gα subunit returning to a basal inactive state, the Gα subunit is free to re-associate with the Gβγ dimer and effectively terminate the Gβγ effectors as well (Figure 1.1 [3]). The intact G-protein heterotrimer is then able to couple once again with the receptor for additional signal transmissions.

Figure 1.1

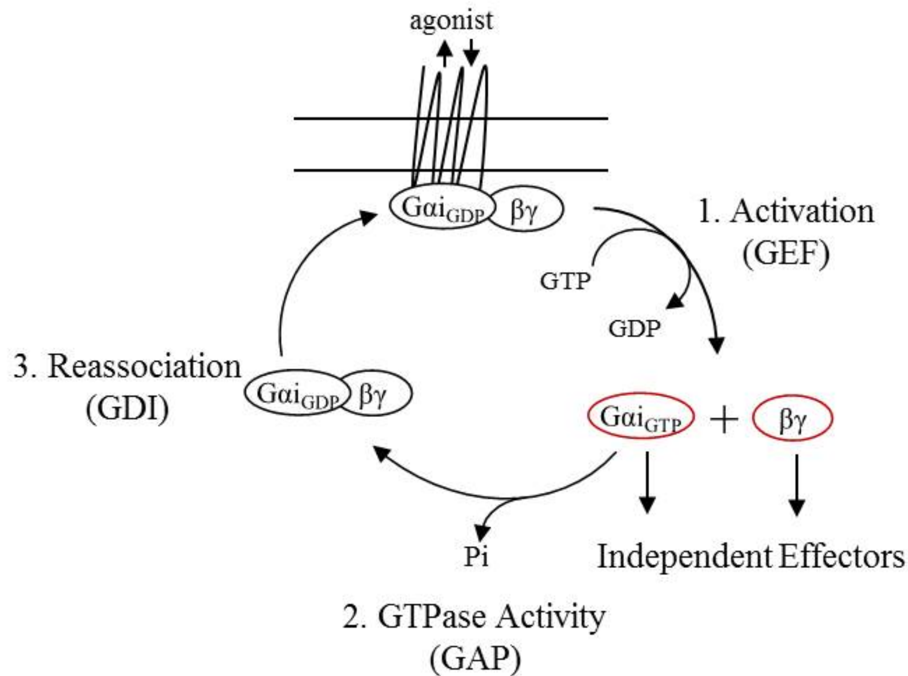

Figure 1.1. The G-protein activation-deactivation cycle

(1) After engaging the receptor, G-proteins are activated in response to agonist stimulation and subsequent conformational changes that occur within the receptor to catalyze nucleotide exchange of the Gαi subunit. This promotes subunit dissociation to reveal effector binding motifs for activation of independent downstream signaling cascades for Gαi and Gβγ. Activation has also been demonstrated by guanine nucleotide exchange factors (GEF) proteins in the absence of receptor activation.

(2) Intrinsic GTPase activity of the Gαi subunit hydrolyzes GTP back to GDP to turn off Gαi signaling. This process is relatively slow, but is often catalyzed by

GTPase accelerating (GAP) proteins such as regulators of G-protein signaling (RGS) proteins.

(3) The resulting GDP-bound Gαi subunit is free to reassociate with Gβγ to effectively inhibit further Gβγ effector signaling and then return to the receptor for further signal transmission. The inactive Gαi is also a target of guanine nucleotide dissociation inhibitors (GDI) proteins that bind to the Gαi and can exclude Gβγ from reassociating.

Physiological responses initiated by G-protein signaling are determined by a diverse group of proteins that act as secondary messengers or have a direct action on cellular responses through interactions with Gα or Gβγ (Kristiansen 2004). This diverse group includes enzymes such as adenylyl cyclases and phospholipases, ion channels, adhesion proteins, tubulin, as well as a large number of novel G-protein effectors (Hewavitharana & Wedegaertner 2012, Woehler & Ponimaskin 2009). Gα subunits are commonly associated with activation/inhibition of adenylyl cyclase enzymes. Generation of elevated cAMP levels by $G\alpha_s$ sequentially activates second messengers such as protein kinase A (PKA), while Gαi balances this activation by demonstrating an opposing effect on adenylyl cyclase to inhibit the signaling (Wan & Huang 1998). A second major signaling pathway of G-protein activation involves the release of intracellular calcium resulting from activation of phospholipase-Cβ (PLCβ) isoforms by Gβγ and also Gαq/11 (Blank et al 1991, Boyer et al 1992, Park et al 1993, Smrcka & Sternweis 1993). Upon activation, PLCβ hydrolyzes phosphatidylinositol 4,5-bisphosphate (PIP2) cleaving the phospholipid into diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP3) (Park et al 1993, Smrcka & Sternweis 1993). DAG further propagates signaling via activation of protein kinase C (PKC), while IP3 travels to the endoplasmic reticulum where it engages IP3 receptors and stimulates release of intracellular calcium stores (Berridge 1989, Bishop & Bell 1988). Gβγ also activates multiple other signaling cascades including but not limited to phosphoinositide 3 kinase (PI3K), G-protein coupled inwardly-rectifying

potassium channels (GIRK channels), specific adenylyl cyclase isoforms and MAPK cascades (Lin & Smrcka 2011).

Termination of signal processing at the receptor has also been observed to be an important aspect of G-protein signaling. Following receptor activation, GPCRs undergo an endocytosis process to sequester and desensitize the receptor signaling by removing receptor from the cell surface and consequently, the agonist. Activated protein kinases such as protein kinases A and C as well as G-protein regulatory kinases (GRKs) phosphorylate the carboxyl terminus and three intercellular loops of the GPCRs (Benovic et al 1989, Nobles et al 2011). Additionally, phosphorylation of the receptor enhances the recruitment of arrestins from the cytoplasm to the receptor's intracellular domain (Lohse et al 1992). The recruitment of arrestin is essential to serves as an adaptor protein between the receptor and clathrin protein heavy chain (Goodman et al 1996). Arrestins are also responsible for binding to β2 adaptin subunits, resulting in the organization of the AP-2 complex and targeting of the complex to clathrin coated pits (Laporte et al 2000, Laporte et al 1999). The receptor-arrestin-AP2 complex then undergoes clathrin-mediated endocytosis where the clathrin coat disassembles leaving an endosome containing the GPCR for receptor resensitization (Pippig et al 1995).

Although classical G-protein signaling is well documented in numerous biochemical studies, the malleability of G-protein signaling to variations in signaling inputs still remains elusive to investigators. One possible explanation for this flexibility is the presence of proteins that are secondary to the core

signaling unit, which could potentially modulate the signal transfer from receptor to G-protein or G-protein to effector referred to as accessory proteins.

Modulation of Canonical Signal Transduction by Accessory Proteins

In light of the common features of activation shared by nearly all GPCR receptor and heterotrimers, one of the key questions in biology is the nature of GPCR signal diversity. One possible explanation is the existence of additional proteins outside of the core signaling triad (receptor, G-protein, effector) that are able to act on and modify signal transfer from receptor to G-protein or G-protein to effector resulting in signal diversity from a commonly shared mechanism.

Looking simplistically at the G-protein activation cycle, there are three critical points that are prime targets for potential regulation (Figure 1.1). The first is transfer of signal from an agonist-bound receptor or other protein with guanine nucleotide exchange factor (GEF) activity, to stimulate nucleotide exchange on Gα. Subsequently, Gα and Gβγ subunits dissociate/rearrange to activate effectors, thus propagating the signal. Secondly, intrinsic GTPase activity of Gα results in hydrolysis of the gamma phosphate of GTP that returns the Gα subunit to the inactive state. Finally, $G\alpha_{GDP}$ and Gβγ reassociate to reform an intact heterotrimer, which is available to couple to the receptor in order to reinitiate the cycle.

Accessory proteins, which are proteins distinct from the core signaling triad of receptor, G-protein and effector, modulate signal transfer at each of these three points to regulate signal strength, efficiency, duration, location and providing mechanisms for signal flexibility to adapt to a changing environment. One such scenario is interference of receptor – G-protein coupling or proteins

possessing GEF activity *in lieu* of the receptor (Ahn et al 2003, Attramadal et al 1992, Chan et al 2011, Cismowski et al 2000, Cismowski et al 1999, Collins et al 1990, Ferguson et al 1996, Garcia-Marcos et al 2009, Klattenhoff et al 2003, Lohse et al 1992, Tall et al 2003, Wilden et al 1986). A second instance of modulation involves accelerating the intrinsic GTPase activity of the Gα subunit, resulting in enhanced signal termination, increasing the turnover rate of Gα and reassociation with Gβγ (Berman et al 1996b, Hunt et al 1996). Lastly, accessory proteins could bind Gα subunits subsequent to GTP hydrolysis to decrease the rate of heterotrimer reassociation (Blumer & Lanier 2003, Cismowski et al 1999, De Vries et al 2000, Natochin et al 2000, Takesono et al 1999). Taken together, one can begin to envision how accessory proteins may influence signal adaptation of a commonly shared GPCR activation mechanism, thus providing additional texture and functional diversity to G-protein signaling systems. Furthermore, disruption or alteration of either the core signaling triad or the accessory proteins that modulate it may underlie pathophysiological or disease states. An additional line of thought suggests that aside from acting as modulators of the canonical heterotrimeric signaling event, accessory proteins may also serve as independent signaling modules separate and distinct from GPCRs (Oner et al 2010a, Oner et al 2010b).

A well-studied regulatory mechanism used by accessory proteins to modulate transfer of signal from receptor to G-protein is by effectively uncoupling the G-protein from the receptor. A family of serine/threonine kinases referred to as G-protein regulatory kinases (GRKs) are responsible for many

phosphorylation events on receptors subsequent to agonist-mediated activation and contribute to receptor desensitization (Benovic et al 1989, Premont et al 1996, Rockman et al 1996). Of the seven GRKs expressed in mammalian tissues, GRK2, 3, 5, and 6 are ubiquitously expressed and employed by cells for receptor desensitization. The other GRK variants have more restricted expression profiles in visual tissues and testes (Premont et al 1996). Phosphorylation of the receptor in turn enhances the recruitment of arrestins, most commonly arrestin 2 (β-arrestin 1) or arrestin 3 (β-arrestin 2), to the receptor (Lohse et al 1992). At the receptor, arrestin proteins unfold to reveal a polar core that interacts with the phosphorylated C-terminus residues of the receptor (Shilton et al 2002). This displaces the C-terminus of the arrestin protein, which then adopts an active conformation that is accessible for N- and C-terminal interactions with intracellular loops of the receptor (Shilton et al 2002). Arrestin association with the receptor intercellular loops sterically precludes G-proteins from coupling to the receptor and targets the receptor for internalization (Ahn et al 2003, Attramadal et al 1992, Ferguson et al 1996). In the past, investigators predicted that this event would mark the ending of G-protein signaling until the receptor was able to return to the surface to signal further; however, recent studies have suggested that although G-protein coupled receptors are trafficked from the membrane and away from ligands, the receptors actually continue signaling and contributing to overall cAMP levels through coupling G-protein heterotrimers while present on the endosome membrane (Calebiro et al 2009, Ferrandon et al 2009, Irannejad et al 2013). Additionally,

arrestin proteins in complex with 7TMRs can act as an alternative signaling complex for activation of ERK1/2 through G-protein independent, arrestin-mediated mechanism (Gesty-Palmer et al 2006).

Another mechanism utilized by accessory proteins to regulate receptor to G-protein signal processing is by exhibiting GEF activity in the absence of receptor thereby presenting as non-receptor GEFs. One example of an accessory protein that expresses this ability, although not the sole function, is the resistance to inhibitors of cholinesterase 8 (Ric8) proteins (Chan et al 2011, Miller et al 2000, Tall & Gilman 2004, Tall et al 2003). Ric8 was initially found in *C. elegans* and demonstrated positive regulation of neurotransmitter release through Gα signaling events (Miller et al 2000, Reynolds et al 2005, Schade et al 2005). Two variants of Ric8 were found in mammals and demonstrated divergent binding of Gα subunits (Klattenhoff et al 2003, Tall et al 2003). Ric8a was found to bind to GDP-bound Gαi/o, Gα12/13, and Gαq/11 subunits, while Ric-8B demonstrated binding to Gαs/olf subunits in the absence of Gβγ (Chan et al 2011, Klattenhoff et al 2003, Tall et al 2003, Von Dannecker et al 2005). *In vitro*, Ric8A and Ric8B both demonstrate stabilization of a nucleotide free transition state, via the switch II and C-terminus contact sites of Gαs/i, to catalyze nucleotide exchange, similar to that seen by activated GPCR on heterotrimeric G-proteins (Chan et al 2011, Kataria et al 2013, Nagai et al 2010, Rasmussen et al 2011, Tall et al 2003, Thomas et al 2008). Upon Gα subunit nucleotide exchange, GTP-bound Gα no longer acts as a substrate for Ric8 and the complex dissociates (Chan et al 2011, Tall et al 2003). A multifunctional role for

Ric8A emerged through studies involving pertussis toxin treatment to block the GEF activity, which incidentally revealed a secondary chaperone-like function of Ric8 where in the presence of Ric8A, Gα subunit expression was markedly increased (Oner et al 2013b). This report along with other supportive studies suggests a multifunctional role for Ric8 through regulation of Gα subunit folding during biosynthesis (Chan et al 2013, Gabay et al 2011, Oner et al 2013b). Ric8 proteins are functionally characterized to regulate positioning of the mitotic spindle, asymmetric division, and acting as molecular chaperones for Gα subunits (Afshar et al 2004, Chan et al 2011, David et al 2005, Gabay et al 2011, Oner et al 2013b, Wang et al 2005, Woodard et al 2010). Thus, through direct regulation of receptor or activation of G-protein signaling in the absence of receptor, accessory proteins present many unexpected roles in influencing G-protein signal processing.

The existence of proteins that modulate G-protein activation suggests the existence of proteins that may facilitate the opposing side of the G-protein cycle, i.e. signal termination. A large family of accessory proteins that fills this niche is known as the regulators of G-protein signaling (RGS) protein family. Initially found in *S. cerevisiae*, this family of proteins is comprised of more than thirty human members containing RGS domains (Berman et al 1996b, Chan & Otte 1982, De Vries et al 1995, Siderovski et al 1996). RGS proteins increase speed of Gα subunit deactivation by accelerating the GTPase activity of these proteins, classifying them as GTPase-accelerating proteins (GAPs) (Berman et al 1996b, Faurobert & Hurley 1997, Hunt et al 1996). Investigation of the crystal structure

for RGS4 bound to $G\alpha i1_{GDP}$-AlF_4^- suggested that the mechanism of action for RGS proteins was to stabilize the switch domains of Gα subunits, rather than contributing additional residues to the active site on Gα subunits, (Berman et al 1996a, Tesmer et al 1997a). These studies provide substantial evidence for roles of accessory proteins in the deactivation of the G-protein activation cycle.

The initiation and deactivation of G-protein signaling are extensively studied and yet still present unexpected mechanisms of regulation that augment the canonical signaling paradigm as is the case for the final family of accessory proteins. Discovered over a decade ago using a yeast-based functional screen for activators of heterotrimeric G-proteins independent of cell surface receptors, the Activators of G-protein signaling (AGS) proteins remain an important focus of study in the investigation of G-protein signal modulation (Cao et al 2004, Cismowski et al 1999, Sato et al 2011b, Takesono et al 1999).The AGS proteins were further subdivided into four specific groups dependent on functional aspects of each protein and Gαi subunit binding (Blumer et al 2005, Blumer et al 2007, Sato et al 2006a, Sato et al 2011b).

Group I AGS proteins are classified as non-receptor GEFs and include proteins such as AGS1, Ric8a, Ric8b and Gα interacting vesicle-associating protein (GIV/Girdin) (although the latter three may be considered Group I AGS proteins, they were not actually identified in the original yeast-based functional screen as described previously),(Chan et al 2011, Cismowski et al 2000, Cismowski et al 1999, Garcia-Marcos et al 2009, Klattenhoff et al 2003, Tall et al 2003). AGS1 is related to the Ras small-GTPase superfamily and was found to

interact with Gαi/o to facilitate nucleotide exchange to regulate ERK1/2 and adenylyl cyclase activation (Cismowski et al 2000, Cismowski et al 1999, Graham et al 2002, Graham et al 2004, Nguyen & Watts 2005, Takesono et al 2002). Functionally, AGS1 is involved in regulation of hormone secretion, circadian rhythm, and is anti-proliferative (Cheng et al 2004, Lellis-Santos et al 2012, McGrath et al 2012, Vaidyanathan et al 2004). GIV/Girdin is another Group I AGS protein determined to have GEF activity on Gα subunits (Garcia-Marcos et al 2009). Knockout of GIV/Girdin in a mouse model demonstrated defects in angiogenesis, neurogenesis, and cell migration (Enomoto et al 2005, Kitamura et al 2008, Wang et al 2011). This protein has also been implicated in cell autophagy where GIV acts on Gα subunits complexed with Group II AGS proteins at autophagic vesicles (Garcia-Marcos et al 2011, Garcia-Marcos et al 2009). Overall, Group I AGS proteins have unveiled many unexpected inputs into G-protein signaling linked to numerous physiological responses.

Group II AGS proteins are functionally classified as GDIs and have seven family members that all share a common ~20 amino acid motif, the G-protein regulatory (GPR) motif (Figure 1.2) (Takesono et al 1999). Four members express multiple (2-4) of these GPR motifs and include AGS3 (4 GPRs), LGN (4 GPRs), AGS4 (3 GPRs), PCP2 (2 GPRs), while the other three members, RGS12, Rap1Gap, and RGS14, express a single GPR motif (Figure 1.2), (Cao et al 2004, Jordan et al 1999, Kimple et al 2001, Luo & Denker 1999, Mochizuki et al 1996, Takesono et al 1999). The GPR motif (also referred to as the GoLoco motif) binds and stabilizes inactive, GDP-bound Gαi/o/t subunits, inhibit GTPγS

binding to Gαi/o/t subunits, and competes with Gβγ subunits for binding Gαi/o subunits (Bernard et al 2001, Cao et al 2004, Kimple et al 2001, Natochin et al 2001, Peterson et al 2000, Peterson et al 2002, Siderovski et al 1999, Takesono et al 1999). Interestingly, members of this family containing a single GPR motif also contain a secondary GAP domain to accelerate GTP hydrolysis of Gα subunits suggesting possible multifunctional roles of these proteins in Gα subunit cycling mechanisms yet to be identified (Brown et al 2015, Vellano et al 2013, Zhao et al 2013). Additionally, the reported ability for GIV/Girdin and Ric8A to act on Gαi-GPR complexes provides an additional mode of signaling cross-talk between Group I and Group II proteins (Garcia-Marcos et al 2011, Oner et al 2013b). Previous studies have linked GPR proteins to several disease pathologies including drug addiction and craving, learning and memory, ischemia reperfusion injury, polycystic kidney disease, blood pressure control, energy expenditure and metabolism, and rheumatoid arthritis and inflammatory pathways (Blumer et al 2008, Bowers et al 2004, Branham-O'Connor et al 2014, Conley & Watts 2013, Giguere et al 2013, Kwon et al 2012, Lee et al 2010, Nadella et al 2010, Regner et al 2011, Yao et al 2005). Group II AGS proteins, being the focus of this thesis, will be discussed in more depth in the following section.

Figure 1.2

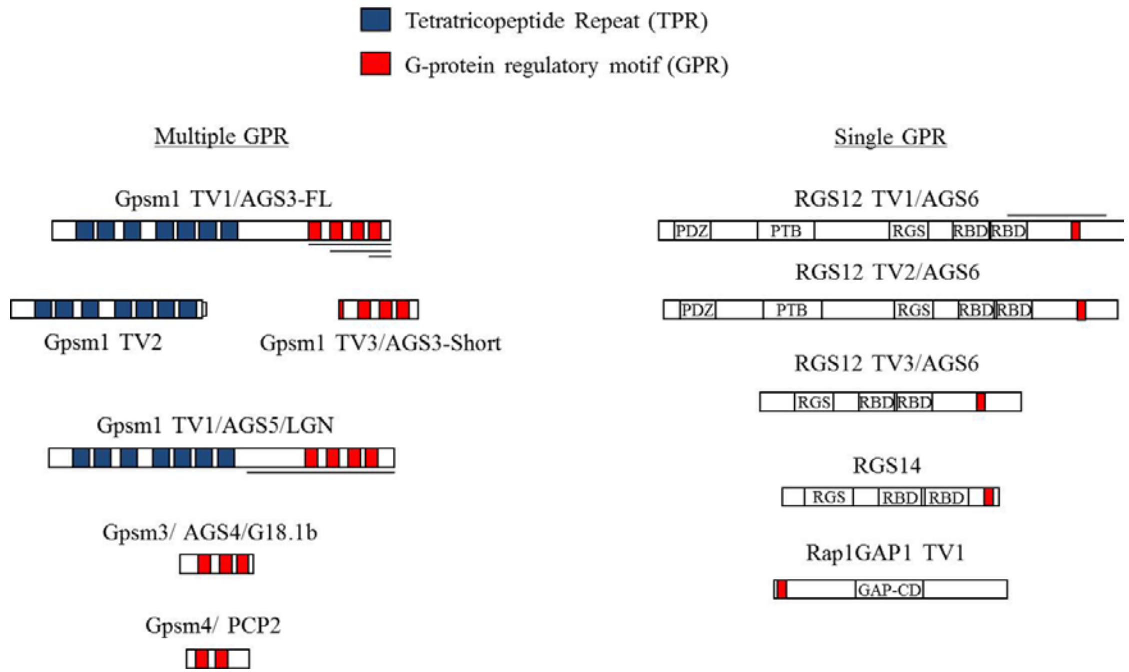

Figure 1.2. Group II AGS proteins

Left, Depicted representations of Group II AGS proteins exhibiting more than one G-protein regulatory (GPR, red) motifs. Tetratricopeptide repeats (TPR, blue) domains are also labeled where applicable. *Right,* Representative domain organization for Group II AGS proteins exhibiting a single GPR motif (red).

Unlike the Group I and Group II AGS proteins, Group III AGS proteins demonstrate binding to Gβγ (Cismowski et al 1999, Sato et al 2006b, Sato et al 2009, Yuan et al 2007). Overall, these proteins function to activate Gβγ signaling either by dissociation of the heterotrimeric complex through binding shared 'hot spot' domains with the Gα subunit or adopting an adaptor role between Gβγ, in associated heterotrimer with Gα subunit, and Gβγ effectors to promote effector activation without heterotrimer dissociation (Sato et al 2006b, Sato et al 2009, Yuan et al 2007). One member of the Group III AGS proteins, AGS8, is involved in hypoxia-induced apoptosis of cardiomyocytes and exhibited the ability to activate PLCβ2 in the absence of subunit dissociation (Sato et al 2006b, Sato et al 2014, Sato et al 2009, Yuan et al 2007). In addition, AGS2 (Tctex-1), which was initially described as a light chain component for dynein, demonstrated roles in neurogenesis and neurite outgrowth and recently phosphorylation of AGS2 was linked to cilia resorption prior to cell cycle entry into S-phase (Gauthier-Fisher et al 2009, King et al 1996, Li et al 2011, Sachdev et al 2007, Yeh et al 2013). Subsequent investigation determined activation of the insulin-growth factor 1 receptor (IGF-1R) and stabilization of GDP-bound Gα subunits by AGS3 induce activation of AGS2 by released Gβγ which is required for cilia resorption and progression of the cell cycle (Yeh et al 2013). This study provides yet another mode of connectivity between multiple AGS proteins working synergistically to modulate signal transduction in the cell. However, more studies of the Group III AGS proteins roles in G-protein signaling are needed to completely elucidate the function of this group.

Diverging from the other groups of AGS proteins, the more recently identified Group IV AGS proteins (AGS11-13) are transcription factors and revealed non-receptor mediated activation of G-protein signaling utilizing $G\alpha_{16}$ and, to a lesser extent, $G\alpha_s$ in yeast functional screens (Sato et al 2011b). Although functional roles of these proteins have yet to be identified, Group IV AGS proteins are expressed in the hypertrophic mouse heart, where nuclear localization of $G\alpha_{16}$ was reported in the presence of AGS11 resulting in a substantial increase to claudin 14 mRNA expression (Sato et al 2011b). This unique group of AGS proteins is an interesting addition to the family of AGS proteins, but further structural and functional studies of these proteins are needed to determine if there exist additional roles for these proteins in tissues other than the heart.

In summary, accessory proteins have demonstrated the ability to modulate signal transfer of the core signaling triad at every step of the G-protein activation/deactivation cycle. Interestingly, a family of proteins identified in a yeast based functional screen has revealed a functionally diverse group of proteins that effectively influence signal processing and integration in various ways. Further analysis of specific members of this group of proteins will reveal novel mechanisms of G-protein signaling with broad implications on G-protein signal processing.

Roles of Group II AGS Proteins in G-protein Signal Processing

As mentioned, the discovery of AGS proteins through yeast-based functional screens for proteins that activated G-protein signaling through Gαi, but not $G\alpha_s$ or $G\alpha_{16}$ lead to the classification of seven mammalian Group II AGS proteins (Cao et al 2004, Cismowski et al 1999, Takesono et al 1999). These proteins were found to harbor at least one GPR motif that competes with Gβγ for binding Gαi/o/t subunits in the GDP-bound state, thus stabilizing the inactive conformation and resulting in the inhibition of nucleotide exchange (Bernard et al 2001, Cao et al 2004, Kimple et al 2001, Natochin et al 2001, Peterson et al 2000, Peterson et al 2002, Siderovski et al 1999, Takesono et al 1999). Interestingly, the number of GPR containing genes has expanded throughout evolution. There exists a single GPR protein in *D. melanogaster* that has been linked to cell polarity and asymmetric cell division known as Partner of Inscuteable (Pins), while *C. elegans* contains three GPR motif-containing proteins, GPR1/GPR2, which are 99% identical and functionally redundant and F32A6.4/AGS3, which plays a role in feeding behavior (Bergstralh et al 2013, Colombo et al 2003, Gotta et al 2003, Hofler & Koelle 2011, Nipper et al 2007, Schaefer et al 2001, Schaefer et al 2000, Srinivasan et al 2003, Yu et al 2000). GPR1/2 are involved in asymmetric cell division, but were also observed to have a role in a $G\alpha_o$ food-seeking behavioral mechanism (Colombo et al 2003, Gotta et al 2003, Hofler & Koelle 2011, Srinivasan et al 2003). In mammals there are seven genes encoding proteins with GPR motifs suggesting the need for a larger

repertoire of these modulatory proteins as signaling events became more complex.

Of particular interest, within Group II AGS proteins are members expressing more than one GPR motif, which confer the capability to bind an equivalent number of Gα subunits simultaneously (Figure 1.2), (Adhikari & Sprang 2003, Bernard et al 2001, Oner et al 2010a, Oner et al 2010b). Selectivity of GPR motifs for Gα subunits is typically observed as Gαi1-3 > Gαt > Gαo, but individual motifs of GPR containing proteins have demonstrated preferences for certain Gαi/o/t subunits (Cao et al 2004, McCudden et al 2005b, Mittal & Linder 2004, Peterson et al 2000, Willard et al 2006). Thus, the Group II AGS proteins containing multiple GPR motifs may potentially “seed” multiple Gα subunits to 7TM receptors and/or receptor-independent GEFs (Blumer & Lanier 2014, Blumer et al 2007, Sato et al 2006a).

Of the four Group II AGS proteins with multiple GPR motifs, the most extensively studied is AGS3 (Takesono et al 1999). This protein expresses seven amino-terminal tetratricopeptide repeat (TPR) domains followed by four carboxy-terminal GPR motifs (Figure 1.2), (Bernard et al 2001, De Vries et al 2000, Takesono et al 1999). These structural domains allow for the possibility of four Gαi/o subunits to be bound to AGS3 at any given time, while the TPR domains have demonstrated importance in mediating protein-protein interactions for subcellular targeting of AGS3 and function as intramolecular modulators for GPR binding of Gα subunits (Adhikari & Sprang 2003, An et al 2008, Bernard et al 2001, Blumer et al 2003, Oner et al 2010a, Oner et al 2013c, Pan et al 2013,

Vural et al 2010). The enriched expression of AGS3 in the brain and heart (AGS3-short), as well as expression in smooth vascular tissue and leukocyte populations was valuable in the determination of function for AGS3 in tissues of the body (Blumer et al 2008, Branham-O'Connor et al 2014, Pizzinat et al 2001).

Absence of AGS3 was found to further exacerbate dysregulation of spindle orientation as seen with disruption of Gβγ signaling during neurogenesis, thus implicating AGS3 in asymmetric cell division similar to its ortholog Pins (Sanada & Tsai 2005). To further support a role for AGS3 in asymmetric cell division, the TPR domain of AGS3 interacts with liver kinase B1 (LKB1), an ortholog of PAR-4 protein in *C. elegans,* which is also involved in asymmetric cell division (Blumer et al 2003, Watts et al 2000). LKB1 phosphorylated the GPR domains of AGS3 as demonstrated by substitution of a phospho-mimetic aspartic acid residue within the GPR motif to impede association with Gα subunits (Blumer et al 2003). Further investigation into the role of AGS3 in the central nervous system revealed a functional role for AGS3 in drug-seeking behavior (Bowers et al 2008, Bowers et al 2004, Fan et al 2009, Yao et al 2005, Yao et al 2006a). AGS3 is upregulated in the prefrontal cortex of mice after withdrawal from repeated cocaine administration, and silencing of AGS3 expression by antisense oligonucleotides injected into the nucleus accumbens effectively prevented drug-seeking behavior in mice withdrawn from heroin, ethanol, and cocaine (Bowers et al 2008, Bowers et al 2004, Yao et al 2005). The role of AGS3 in kidney disease has also recently been of keen interest to the field. Under normal conditions, AGS3 levels in the kidney are quite low, but upon

injury, the expression of AGS3 in this tissue drastically increases (Kwon et al 2012, Nadella et al 2010, Regner et al 2011). Impaired renal tubule recovery from ischemia reperfusion injury and increased rate of cyst progression in polycystic kidney disease models were observed upon loss of AGS3 expression, again mimicking the loss of Gβγ signaling in these same models (Kwon et al 2012, Nadella et al 2010, Regner et al 2011).

An additional binding partner of the TPR domain of AGS3, mammalian Inscuteable (mInsc), has demonstrated multifaceted functions when bound to AGS3 proteins that further expand the roles of AGS3 in mammalian cells (Kamakura et al 2013, Vural et al 2010). Ectopic and endogenous AGS3 associates with pre-aggresomal structures in the cytoplasm that can be readily diffused by increasing levels of Gα subunits, but binding of mInsc reverts AGS3 back into the punctate structures (Vural et al 2010). Recently, binding of AGS3 to mInsc at the leading edge of neutrophils also targeted of the Par3-Par6-atypical protein kinase C (aPKC) complex to induce directional migration in these cells (Kamakura et al 2013). Another ortholog of Pins and member of Group II AGS proteins, LGN, which is over 60% identical to AGS3, has a similar domain organization to AGS3 and has important regulatory roles in asymmetric cell division, cytokinesis, and cell polarity (Blumer et al 2002, Blumer et al 2006, Du & Macara 2004, Du et al 2001, Du et al 2002, Fuja et al 2004, Fukukawa et al 2010, Kaushik et al 2003, Lechler & Fuchs 2005, Zheng et al 2013). LGN-Gαi complexes are known to localize to spindle poles, centrosomes and the midbody during cell division through association of the LGN-TPR domain with the nuclear

mitotic protein NuMA (Blumer et al 2002, Blumer et al 2006, Du & Macara 2004, Du et al 2001, Du et al 2002, Fuja et al 2004, Kaushik et al 2003). The binding of microtubules by NuMA is disrupted upon association with LGN-Gαi complexes (Du et al 2002). However, the Gαi-LGN complex bound to NuMA was observed to also be substrate for the non-receptor GEF Ric8a, resulting in nucleotide exchange of the Gαi subunit (Tall & Gilman 2005). Upon Gαi activation, NuMA is released from LGN allowing NuMA to return to modulating microtubule dynamics (Tall & Gilman 2005). Silencing expression of NuMA was also shown to disrupt LGN localization to spindle poles and improper segregation of chromosomes (Du & Macara 2004). Additionally, LGN interacts with mInsc and aPKC-Par6 complexes further implicating this protein in mitotic spindle orientation and polarity in cells (Izaki et al 2006, Lechler & Fuchs 2005, Yasumi et al 2005). Further studies demonstrate the involvement of LGN in regulation of the basal activity of G-protein regulated ion channels such as the Gβγ-dependent GIRK channel (Wiser et al 2006).

Taken together, these studies indicate a diverse array of functions for AGS3 and the related protein LGN and have demonstrated roles for AGS3 in many pathologies such as drug addiction and neuronal plasticity, ischemia reperfusion injury and polycystic kidney disease, blood pressure control, energy expenditure and metabolism, autophagy, membrane protein trafficking, and directional migratory response (Blumer et al 2008, Bowers et al 2004, Conley & Watts 2013, Garcia-Marcos et al 2011, Kamakura et al 2013, Kwon et al 2012,

Nadella et al 2010, Pattingre et al 2003, Regner et al 2011, Vural et al 2010, Yao et al 2005).

One of the less investigated member of the Group II AGS proteins, AGS4, harbors three separate GPR motifs for binding $G\alpha i_{GDP}$ (Cao et al 2004). Unlike the previously described multi-GPR proteins AGS3 and LGN, AGS4 is absent of any obvious regulatory domains (Figure 1.2). Rather, the amino terminus of AGS4 contains a 56 amino acid proline-rich domain, which is reported to have potential guanine nucleotide exchange properties (Zhao et al 2010). In one report the amino terminus of AGS4 was also found to interact with Gβ subunits, although this is in direct contrast to previous biochemical studies demonstrating that AGS4 does not associate with Gβγ subunits (Cao et al 2004, Giguere et al 2012c). Another key feature of AGS4 is restricted expression to cells of hematopoietic lineage, and thus a role for AGS4 in the immune system is of expanding interest to the field (Cao et al 2004, Giguere et al 2013, Giguere et al 2014, Zhao et al 2010). Investigations into the role of AGS4 in a model of acute inflammatory arthritis demonstrated that AGS4 expression was required for the increase in number of pro-inflammatory monocytes, and loss of AGS4 reduces the onset of inflammation-mediated arthritis (Giguere et al 2013).

The final member of Group II AGS proteins with multiple GPR motifs is PCP2. Somewhat similar to AGS4, PCP2 expresses two GPR motifs with no defined regulatory protein domains (Zhang et al 2002). PCP2 demonstrates restricted expression in the cerebellum and retinal bipolar neurons where it interacts with Gαi/o subunits through the two GPR motifs (Dhingra et al 2008,

Redd et al 2002). Early investigations into PCP2 deficient mice illustrated no altered behavior or cerebellar anatomy (Mohn et al 1997, Vassileva et al 1997); however, a recent study investigating mice with inactivated PCP2 has reported sex-dependent differences in anxiety and fear conditioning suggesting an active role for PCP2 in the cerebellum responses (Walton et al 2012).

The ability of the Group II AGS proteins to stabilize the inactive Gαi subunit suggests possible mechanisms by which multiple GPR motif-containing proteins may modulate G-protein signaling. One possible scenario is that GPR proteins compete for binding Gα subunits in complex with Gβγ to initiate subunit dissociation of the heterotrimer in the absence of receptor or presence of unknown signal to prompt this competition, thus promoting or prolonging Gβγ signaling and inhibiting Gα signaling (Figure 1.3A), (Bernard et al 2001, Ghosh et al 2003, Schaefer et al 2001). One could also envision a scenario where GPR motif-containing proteins bind Gα subunits subsequent to hydrolysis of GTP preventing reassociation with Gβγ subunits, either during basal G-protein cycling events or post receptor-mediated activation of the G-protein heterotrimer (Figure 1.3B), (Blumer et al 2005, Blumer & Lanier 2003, Blumer & Lanier 2014, Blumer et al 2012, Blumer et al 2007, Cismowski et al 1999, Kinoshita-Kawada et al 2004, Sato et al 2006a, Takesono et al 1999, Webb et al 2005, Yao et al 2005, Yao et al 2006a). Again, the end result of the second scenario would be enhanced or prolonged Gβγ-regulated effector signaling while inhibiting signaling though Gα subunits. These two situations would implicate AGS3/LGN/AGS4 in the regulation of subunit interactions to decrease receptor – G-protein coupling in

a manner to suppress signaling through Gα effectors while facilitating Gβγ signaling.

A third scenario also suggests that Gαi – GPR complexes form novel signaling modules distinct from the canonical Gαβγ heterotrimer (Figure 1.3C). In this scenario, GPR proteins could act in a manner analogous to Gβγ by serving as substrates for receptor or non-receptor GEFs, resulting in nucleotide exchange and subsequent dissociation of the $G\alpha_{GTP}$ from the GPR protein. In support of such a scenario, GIV/Girdin and Ric8A have demonstrated GEF activity on Gαi-GPR complexes (Garcia-Marcos et al 2011, Oner et al 2013b, Tall et al 2003, Thomas et al 2008, Vellano et al 2011a, Vellano et al 2011b, Woodard et al 2010). Additionally, experiments investigating regulation of the Gαi-GPR complex have demonstrated close proximity of the GPCRs to AGS3/AGS4 proteins bound to Gαi1, which is regulated by agonist activation of the GPCRs in live cells (Oner et al 2010a, Oner et al 2010b). The Gαi-GPR complex may also act as a noncanonical signaling entity as was recently described by the formation of the Gαi-GPR complex acting as a scaffold for recruitment of mInsc-Par3-Par6-aPKC polarity complex required for chemokine-directed migration of neutrophils (Kamakura et al 2013). It is also interesting to envision AGS3/LGN/AGS4 forming scaffolds of multiple Gα subunits simultaneously to generate larger signaling complexes to increase signal efficiency whether through noncanonical signaling pathways or by cycling Gα subunits back to the receptor (Adhikari & Sprang 2003, Bernard et al 2001, Blumer & Lanier 2014).

The discovery of Group II AGS proteins with multiple GPR domains has revealed many unexpected regulatory mechanisms for G-protein signaling systems. Further investigation of the Group II AGS proteins have unveiled a surprisingly large number of roles these proteins participate in, both in canonical G-protein signaling through GPCRs as well as new and exciting alternative signaling mechanisms. While the interactions of the GPR motif with the Gαi subunit have been extensively studied biochemically, the cellular regulation of these proteins in the aspect of G-protein signaling require further exploration (Adhikari & Sprang 2003, Bernard et al 2001, Peterson et al 2000, Peterson et al 2002). Although beginning to surface, in order to fully appreciate the roles of these multi-GPR proteins in the immune system further investigations are required (Branham-O'Connor et al 2014, Giguere et al 2013, Kamakura et al 2013).

Figure 1.3

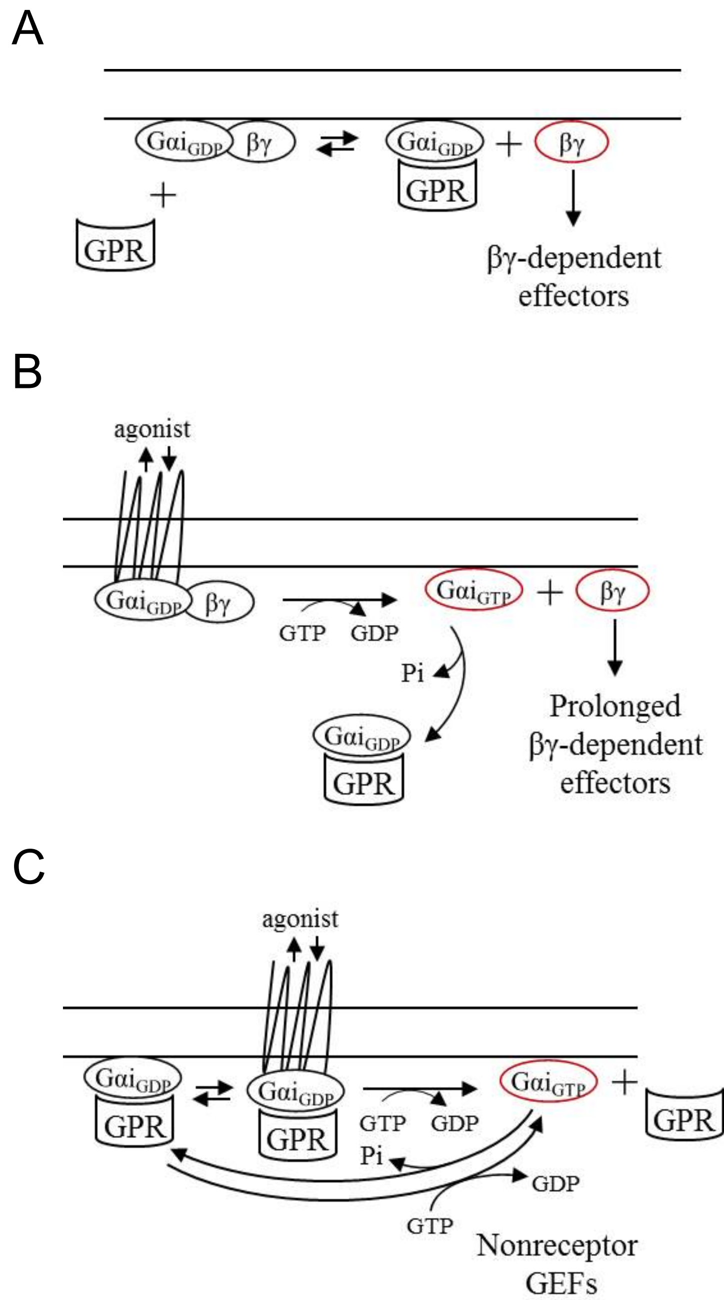

Figure 1.3. Working hypotheses for the action of GPR motif-containing proteins in modulation of Gαi signal processing

(A) Scenario depicting competitive binding of GPR-proteins and Gβγ for inactive Gαi resulting in subsequent heterotrimer dissociation and promoting Gβγ activation in the absence of receptor or presence of an unknown signal mediator.

(B) Scenario illustrating receptor activation of Gαiβγ, followed by sequestration of Gαi by GPR-containing proteins subsequent to GTP hydrolysis, but prior to reassociation of Gαiβγ heterotrimer, thus prolonging Gβγ effector signaling.

(C) Scenario displaying the coupling of novel signaling complexes, Gαi–GPR, to a 7TMR to initiate nucleotide exchange independent of Gαiβγ heterotrimer. In this scenario, the Gαi–GPR would signal analogous to the Gαiβγ heterotrimer, being substrate for 7TMRs as well as non-receptor GEFs as depicted in the illustration. Upon nucleotide exchange the Gαi–GPR would dissociate and following GTP hydrolysis the Gαi subunit would be available for reassociation with GPR proteins or Gβγ subunits.

Chemokine-Stimulated Activation of G-proteins and Potential Role for GPR-containing proteins in Signal Processing

Cells of the immune system have to adapt to a complex array of signals to coordinate the movement and redistribution of leukocytes to maintain proper host immunosurveillance. To accomplish this, the immune system employs chemokine receptors in the targeting of leukocytes to secondary sites of development or inflammation (Beider et al 2003, Forster et al 1999, Henderson et al 2003, Lu et al 1998, Wright et al 2002). Directed leukocyte migration either to secondary lymphoid organs or to sites of inflammation is accomplished through a series of events leading to the extravasation of the leukocyte into the tissue as first observed by electron microscopy nearly forty years ago (Anderson & Anderson 1976). Infiltration of pathogens into host tissue results in activation of sentinel resident leukocytes that secrete pro-inflammatory cytokines, which facilitate endothelial cells to increase surface expression of adhesion molecules including E-selection and P-selectin to recruit circulating lymphocytes (Alon et al 1994, Bosse & Vestweber 1994, Jung & Ley 1999, Jutila et al 1994, Labow et al 1994, Mayadas et al 1993). Parallel expression of adhesion molecules such as L-selectin and P-selectin glycoprotein ligand 1 (PSGL-1) on the surface of circulating leukocytes mediates transient interactions of the adhesion molecules resulting in a characteristic, tethered “rolling” and decreased velocity of circulating leukocytes (Bosse & Vestweber 1994, Ley et al 1993, Xia et al 2002, Yang et al 1999). The slowing of leukocytes allows for increased exposure to chemokines presented on the luminal surface by the endothelial cells, thus

activating leukocyte integrins lymphocyte function –associated antigen 1 (LFA-1) and very late antigen 4 (VLA-4) to engage stronger adhesion forces with immunoglobulin family members intracellular adhesion molecule 1 (ICAM1) and vascular cell-adhesion molecule 1 (VCAM1), respectively, and promoting cell immobilization (Alon et al 1995, DiVietro et al 2007, Dustin et al 1986, Makgoba et al 1988, Rothlein et al 1986, Yang et al 2005). Once the mobility of the cell is arrested, extravasation of the cell through the endothelium can occur. This activity requires the use of a variety of cell-adhesion molecules, including platelet endothelial cell-adhesion molecule (PECAM) and junctional adhesion molecule-A (JAM-A), inevitably transferring the leukocyte from the blood, through the endothelium, and into the inflamed tissue region (Corada et al 2005, Khandoga et al 2005, Schenkel et al 2004a, Schenkel et al 2004b, Thompson et al 2001). Following proper extravasation into the inflamed tissue, leukocytes will continue following the chemotactic gradient to infiltrate the primary site of inflammation. The highly orchestrated transendothelial cell migration is required for efficient immune system development and function through redistribution of leukocytes, while perturbations of chemokine signaling in this process lead to immunological pathologies (Hornquist et al 1997, Jin et al 2008, Moreno et al 2015, Ohman et al 2002, Rudolph et al 1995).

Chemokine receptors are Gαi-coupled GPCRs and divided into four groups dependent on the chemokine ligand to which the receptor binds including CCRs, CXCRs, CX3CRs, XCRs (Arai & Charo 1996, Haribabu et al 1997, Murphy et al 2000, Polakis et al 1988). Although the primary role of this receptor

family is to regulate directed cell movement, functional overlap with the processes of cytokine and superoxide production are also documented after chemokine treatment (Hirsch et al 2000, Li et al 2000, Sasaki et al 2000). Chemokine-induced receptor activation leads to nucleotide exchange of GDP for GTP on the Gαi subunit leading to dissociation of the heterotrimer. Gβγ is then free to associate and activate downstream effectors essential for cell migration, PI3Kγ and PLCβ2/3 (Arai et al 1997, Bach et al 2007, Goldman et al 1985, Hannigan et al 2002, Hirsch et al 2000, Li et al 2000, Neptune & Bourne 1997, Neptune et al 1999, Rickert et al 2000, Sotsios et al 1999). PI3Kγ activation results in the generation of PIP_3 and occurs largely at the leading edge of migrating leukocytes where it is involved in the maintenance of polarity and migratory signaling in these cells (Hannigan et al 2002, Rickert et al 2000, Servant et al 2000, Sotsios et al 1999, Wang et al 2014). Absence of PI3Kγ in leukocytes results in ineffective T-cell development and activation, as well as defects in migration and oxidative burst in neutrophils (Hirsch et al 2000, Li et al 2000, Sasaki et al 2000). A second key Gβγ effector in leukocytes is PLCβ2/3, which hydrolyzes PIP_2 into DAG and IP_3 leading to the activation PKC and release of calcium from intracellular stores, respectively (Berridge 1989, Bishop & Bell 1988, Goldman et al 1985, Park et al 1993, Smrcka & Sternweis 1993). To further support a role for PLCβ2/3 in the immune system, PLCβ2/3-deficient neutrophils demonstrated defects in superoxide production and protein kinase regulation, but , interestingly, not chemotaxis (Li et al 2000). In contrast, T-cells deficient in PLCβ2/3 exhibited decreased chemotaxis, mirroring results obtained

with intracellular calcium chelating agents (Bach et al 2007). Additionally, a recent report that used a small molecule to trigger heterotrimer dissociation in the absence of nucleotide exchange indicated that Gβγ signaling was required for maximal chemotaxis in neutrophils; however, treatment with this small molecule combined with pertussis toxin demonstrated further decreases in chemotaxis, revealing a lesser, but observable Gαi component to chemokine-induced migration of neutrophils (Surve et al 2014). Nevertheless, many of the downstream responses mediated by chemokine receptors have long been correlated to Gβγ effects from activation of G-protein heterotrimers (Arai et al 1997, Goldman et al 1985, Neptune & Bourne 1997, Neptune et al 1999, Spangrude et al 1985).

Numerous studies on the role of Gαi in lymphocyte biology, aided by the use of pertussis toxin to inhibit Gαi/o heterotrimer signaling and the development of Gαi2 and Gαi3 knockout mice, revealed that perturbations of G-protein signaling in lymphocytes negatively affect lymphocyte development and chemokine-induced signaling (Bargatze & Butcher 1993, Chaffin & Perlmutter 1991, Cyster & Goodnow 1995, Dalwadi et al 2003, Han et al 2005, Huang et al 2003, Hwang et al 2010, Hwang et al 2007, Jin et al 2008, Pero et al 2007, Rudolph et al 1995, Skokowa et al 2005, Spangrude et al 1985, Thompson et al 2007, Warnock et al 1998, Wiege et al 2013, Wiege et al 2012, Zarbock et al 2007). Initially demonstrating the importance of Gαi-dependent signaling in migration and homing to secondary lymphoid organs, lymphocytes treated with pertussis toxin to inhibit Gαiβγ signaling events revealed substantial defects in

lymphocyte and neutrophil migration, improper egress of mature T cells from the thymus, as well as defective homing and chemokine-induced arrest prior to transmigration into peripheral lymphatics (Chaffin & Perlmutter 1991, Cyster & Goodnow 1995, Spangrude et al 1985, Warnock et al 1998, Zarbock et al 2007). In addition to impaired chemokine-directed migration, Gαi2 null lymphocytes also exhibited reduced migration velocity compared to wild-type lymphocytes (Han et al 2005, Hwang et al 2007).

In the intact animal, Gαi2 deficiency was linked to enhanced expression of IFN-γ, IL-1, IL-6, and TNF-α accumulating into a pro-inflammatory Th1 $CD4^+$ response with development of severe immunological pathologies such as fatal cases of ulcerative colitis and adenocarcinoma (Gotlind et al 2011, He et al 2000, Hornquist et al 1997, Ohman et al 2002, Pena et al 2009, Rudolph et al 1995). The induction of cytokine expression in Gαi2-null macrophages, splenocytes, and lymphoid dendritic cell populations was also observed (He et al 2000, Huang et al 2003, Pena et al 2009). Interestingly in the absence of Gαi2, the proliferative response of T effector lymphocytes was demonstrated to be less susceptible to suppression by regulatory T lymphocytes resulting from increased secretion of pro-inflammatory cytokines including IL-6, which is known to be antagonistic to regulatory T lymphocyte activity (Gotlind et al 2011). The potential functional redundancy of Gαi2 versus Gαi3, in chemokine signaling events is of considerable interest and addressed with the use of knockout models for each of the Gαi genes. Gαi2 is required for T-cell migration via activation of CXCR3, while loss of Gαi3 resulted in increased migration via the CXCR3 receptor

suggesting a competitive or exclusionary nature between the two Gαi subunits (Thompson et al 2007). In B-cells, similar defects in chemotaxis to CXCL12, CXCL13, and CCL19 were indeed observed in Gαi2 null cells, while, again, the removal of Gαi3 appeared to amplify B-cell migration to these chemotaxis-inducing molecules (Hwang et al 2010). Splenic architecture and B-cell development were also impacted by the absence of Gαi2 (Dalwadi et al 2003, Hwang et al 2010). The absence of Gαi2 was also protective against graft-versus-host disease (Jin et al 2008). In contrast, Gαi3-deficient T-cells adoptively transferred exacerbated the disease compared with wild-type counterparts (Jin et al 2008). Corroborating these results, Gαi2 null mice also have decreased chemotaxis to CXCL10 and CXCL11, while Gαi3 null mice demonstrated higher levels of response to the same chemokines (Jin et al 2008). Additional support of the notion that Gαi2 is responsible for proper migration and trafficking of immune cells was reported for CCL2 and C5a-induced macrophage stimulation, while Gαi2 in endothelial cells also was shown to be required for proper transmigration of neutrophils (Pero et al 2007, Wiege et al 2013, Wiege et al 2012). Although not used predominantly in cell migration, Gαi3 is capable of substituting for Gαi2 during C5a-induced activation of macrophages and bacterial-induced cytokine production (Fan et al 2007, Wiege et al 2013). Functional defects in cytokine production, migration, and trafficking revealed by the loss of Gαi offers insight into the contribution of G-protein signaling in the autoimmune pathologies caused by the absence of the Gαi proteins.

The ability of leukocytes to process, prioritize and respond appropriately to multiple signals remains a key question in immunology. The modulation of G-protein signaling by accessory proteins in chemokine directed migration is of particular interest. As mentioned above, depletion of Gαi2 resulted in decreased migration of leukocytes to chemotactic stimuli (Hwang et al 2010, Hwang et al 2007, Jin et al 2008, Pero et al 2007, Thompson et al 2007, Wiege et al 2013, Wiege et al 2012). The modulation of Gαi signaling by RGS1 was initially described in B-cells where loss of RGS1 resulted in impaired desensitization of chemokine signaling leading to increased migration and abnormal lymphocyte trafficking deficiency (Han et al 2005, Hwang et al 2010, Moratz et al 2004b). Subsequent studies using an RGS-insensitive Gαi2 knock-in mouse model reported impaired migration of neutrophils from bone marrow and impaired pathogen clearance linked to defective desensitization and unregulated chemokine signaling, as well as enhanced basal calcium levels and irregular B-cell distribution and migration (Cho et al 2012, Hwang et al 2015). Additionally, roles for AGS3 and AGS4 in cell chemotaxis were recently described (Branham-O'Connor et al 2014, Giguere et al 2013, Kamakura et al 2013). AGS3 demonstrated upregulated expression upon leukocyte activation, whose importance was illustrated by depletion of AGS3 in dendritic cells, T lymphocytes and B lymphocytes exhibiting defective chemokine-mediated chemotaxis, calcium mobilization, and phosphorylation of Erk and Akt (Branham-O'Connor et al 2014). The loss of AGS4 also demonstrated decreased chemotaxis and instilled a protective phenotype from arthritis in these mice (Giguere et al 2013).

Interestingly, AGS3 was found to form a complex with Gαi and mInsc to target the Par3-Par6-aPKC complex to the leading edge, divulging an unexpected mechanism for AGS3 induced polarity and migration in neutrophils (Kamakura et al 2013). Migration of macrophages and tumor cells was also determined to require the GEF activity of GIV/Girdin on Gαi3 to induce maximal chemotaxis (Ghosh et al 2008). These studies suggest the importance of accessory proteins in modulation of the activation state of the G-protein alpha subunit, and thus possible promotion of prolonged Gβγ signaling, in chemokine receptor signaling in immune cells.

The roles of Gαi subunits in cell migration continue to be debated in the literature (Kamakura et al 2013, Lehmann et al 2008, Neptune & Bourne 1997, Neptune et al 1999, Rudolph et al 1995, Surve et al 2014). Migration is generally considered a Gβγ-driven process, relying on the activation of numerous second messengers that contribute to cellular movement as mentioned above. The molecular function of the active $G\alpha i_{GTP}$ released upon chemokine receptor activation of the heterotrimer is less understood. Although not completely defined, elevated levels of cAMP are implicated in inhibition of neutrophil chemotaxis supporting the action of active Gαi subunits to potentiate migration by inhibiting adenylyl cyclase in immune cells (Harvath et al 1991). More recent reports further demonstrate chemotaxis requires Gαi coupled receptors and even non-canonical Gαi signaling complexes for proper migration of cells (Kamakura et al 2013, Neptune et al 1999). Moreover, there is growing evidence for roles of accessory proteins that associate with Gαi in having modulatory functions in the

migratory response of immune cells (Branham-O'Connor et al 2014, Cho et al 2012, Giguere et al 2013, Han et al 2005, Hwang et al 2010, Hwang et al 2015, Kamakura et al 2013, Moratz et al 2004b). Investigations into the function of accessory proteins in the context of chemokine receptor signaling have only begun to scratch the surface in terms of the importance of these proteins in innate and adaptive immunity.

Specific Aims

Herein, we developed three specific aims to further the knowledge of the dynamic and regulated Gαi – GPR module in 7TMR signaling and the requirement of two representative Group II AGS proteins, AGS3 and AGS4, in maximal chemokine signal integration of primary leukocytes. *Specific aims are as follows:*

1) Determine if the Gαi – GPR complex is regulated by 7TM receptors and in a manner analogous to canonical Gαβγ heterotrimer.

2) Define alternative modes of regulation for the Gαi – AGS4 interaction through potential interacting proteins identification and phosphorylation of key residues that influence complex formation.

3) Demonstrate that the Gαi – GPR module is required for maximal 7TM chemokine receptor signal processing.

Rationale for specific aim one is based on previous studies that demonstrate 7TMRs regulating the association of Gαi – GPR module in a receptor proximal manner. To explore the possibility that 7TMR directly couple the Gαi – GPR complex, we generated a fusion protein containing a 7TMR tethered to Gαi for targeting the Gαi – GPR module to the receptor microdomain and to observe the effect of receptor activation on the interaction between Gαi and GPR proteins through bioluminescence resonance energy transfer (BRET) techniques. Use of pertussis toxin-insensitive fusion proteins and sequestration of endogenous G-proteins demonstrated that the observed regulation is not due

to endogenous G-protein subunit cycling after receptor activation of canonical Gαiβγ heterotrimer. Additionally, previous studies depicting the importance of Gαi2 subunits in leukocyte signaling and expression of GPR proteins in immune tissues prompted the hypothesis that chemokine receptors regulate the Gαi2 – GPR complex to modulate Gαi signaling. Thus, using the chemokine receptor CXCR4 labeled with a fluorescent acceptor protein, we were able to determine if chemokine receptors could elicit regulation of the Gαi2 – GPR module.

For specific aim two, we initially hypothesized that AGS4, which lacks well-defined protein interaction motifs, binds alternative proteins to modulate its subcellular localization and/or biological function. As an initial approach to test our hypothesis we generated a cell line that expressed AGS4 fused to a tandem affinity purification tag to isolate interacting proteins followed with mass spectrometry identification. We then determined the consequence of AGS4 phosphorylation on the interaction with Gαi and potential kinases responsible for this phosphorylation through an *in vitro* kinase assay and BRET techniques. An initial screen for small molecule modulators of the Gαi – GPR complex identified compounds that may serve as a platform for development of reagents and/or targeted therapeutics towards the Gαi – GPR module.

Aim three arose from the hypothesis that AGS3 and AGS4 are involved in chemokine signal integration of leukocytes. Defects in chemokine-induced signal processing (e.g. directed migration and activation of ERK1/2) observed in AGS3-null and AGS4-null mice indicated functional roles for AGS3 and AGS4 in chemokine signal processing. Furthermore, an *in vivo* model of peritonitis

revealed a biological role of AGS4 in inflammation-induced neutrophil migration. Taken together, these aims are focused to demonstrate the Gαi – GPR interaction is a dynamic and regulated event in cells of the immune system where it is required for maximal 7TMR-mediated responses.

Significance

There are growing reports implicating G-proteins as regulators of numerous physiological signaling cascades. The increasing for G-proteins in various systems continues to divulge unexplored regulatory mechanisms to encompass the vast signaling repertoire required for proper signal integration, while retaining flexibility to adapt to dynamic extracellular signals. The discovery and subsequent characterization of AGS proteins lead to a conceptual advancement in terms of G-protein signal adaptation. AGS proteins were found to fit into various biological niches of the G-protein activation cycle (GEFs, GDIs, and effectors), allowing for adaptation from classical heterotrimeric signaling. Thus, the regulation by such a diverse group of signal modulators is of high importance when one considers the immense number of signaling cascades that AGS proteins potentially affect and the pathologies that present through disruption of these regulatory systems only beginning to be described.

Of particular interest are Group II AGS proteins that express multiple GPR motifs capable of binding 2-4 GDP-bound Gαi/o/t simultaneously that could assemble unique signaling modulators to alter G-protein activation. Two representative members of this group, AGS3 and AGS4, have been found in immunological tissues; however, the biological significance of their expression is not well elucidated. Interestingly, a major receptor class utilized by hematopoietic cells is the chemokine receptor class, which happens to be comprised of Gαi-coupled 7TMRs. Aside from a prospective role of these proteins in inflammatory pathologies, signaling through chemokine receptors is also involved in the

migration of malignancies of hematopoietic origin that “hijack” this receptor signaling system to home to secondary sites of metastasis such as bone and lymph nodes (Cunningham et al 2010, Singh et al 2010). Thus, AGS3 and AGS4 may potentially modulate chemokine signal processing in these pathologies. Moreover, considering that one major limitation to targeting G-proteins in cells has been their ubiquitous expression in all tissues contributing to side effects, the role of AGS3 and AGS4 in chemokine signaling may provide a novel platform for developing therapeutics by virtue of the tissue specific distribution of these proteins.

Group II AGS proteins like AGS3 and AGS4 provide insight into novel modes of signal input and regulation of heterotrimeric G-protein signaling and provide a platform for discovering mechanisms underlying signal strength, specificity, and integration of G-protein mediated cellular responses. Furthermore, they provide unexpected targets for development of therapeutics for diseases which result from altered heterotrimeric G-protein signaling. This dissertation addresses key questions in the field with respect to G-protein signal processing and will advance novel concepts for the role of accessory proteins in immune cell responses and function.

Chapter 2

Multifaceted Regulation of the Gαi – GPR complex through Coupling of a Seven Transmembrane Span Receptor and Alternative Binding Proteins

***Note: This chapter contains a portion of the manuscript:**

Direct Coupling of a Seven Transmembrane Span Receptor to a $G\alpha_i$ – GPR Complex

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Submitted for publication

Introduction

The discovery of Activators of G-protein Signaling (AGS) proteins, originally identified in a yeast-based functional screen for mammalian cDNAs that activated G-protein signaling in the absence of a receptor, revealed both unexpected regulatory mechanisms for G-protein signaling systems and expanded functional roles for the G-protein subunits (Cao et al 2004, Cismowski et al 1999, Sato et al 2006b, Sato et al 2011b, Takesono et al 1999). Group I AGS proteins encompass non-receptor guanine nucleotide exchange factors whereas Group II AGS proteins, all of which contain a G-protein regulatory (GPR) motif, engage Gαi/o/t as guanine nucleotide dissociation inhibitors. Group III AGS proteins appear to engage Gβγ, whereas Group IV AGS proteins, which were just recently identified, interact with Gα16 (Sato et al 2011a).

AGS3 and AGS4 are representative members of two distinct subgroups of AGS proteins. AGS3 has four GPR motifs downstream of a series of tetratricopeptide repeat domains (TPR) involved in protein interactions and intramolecular regulatory events, whereas AGS4 is a smaller protein with three GPR motifs without any clearly defined protein interaction domains upstream of the GPR motifs. As the GPR motif stabilizes the GDP-bound conformation of Gα free of Gβγ, regulation of this interaction could effectively alter G protein signal processing and may be subject to regulation by a multitude of signal modulators

including 7TMRs, alternative interacting proteins, or post-translational modifications.

Interestingly, AGS3 expression is upregulated upon lymphocyte activation and AGS4 has a restricted expression profile to cells of hematopoietic origins (Chapter 3), (Branham-O'Connor et al 2014, Cao et al 2004, Giguere et al 2013, Zhao et al 2010). Additionally, migrating leukocytes, as well as malignant hematopoietic cells, signal through chemokine receptors primarily by coupling to Gαi2, the predominant isoform of Gαi in these tissues (Arai et al 1997, Neptune & Bourne 1997, Wright et al 2002). However, initial studies investigating the regulation of AGS3, AGS4, and RGS14 by 7TMRs were demonstrated for Gαi1 by $\alpha_{2A/D}$-adrenergic receptor ($\alpha_{2A/D}$-AR) (Oner et al 2010a, Oner et al 2010b, Vellano et al 2013). Therefore, regulation of GPR protein complexes in the context of the immune system has not been investigated. As an approach to address this question, we utilized a BRET platform with GPR proteins AGS3 and AGS4 fused to *Renilla* luciferase and Gai2 fused to YFP between the aB-aC loops in the helical domain as previously described (Gales et al 2005, Gales et al 2006, Gibson & Gilman 2006) together with the chemokine receptor CXCR4.

We hypothesized that Gαi2 – GPR complexes are regulated by chemokine receptors to modulate G-protein signaling. Two initial questions about the regulation of the Gαi2 – GPR complex by G-protein coupled chemokine receptors then arise: (1) Is the Gαi – GPR complex modulated specifically by chemokine receptor activation and, if so, (2) is the Gαi – GPR complex situated in the adjacent proximity of the receptor to be directly regulated in a manner similar to

that observed with the G-protein Gαβγ heterotrimer (Lambright et al 1996, Rasmussen et al 2011, Wall et al 1995). Alternatively, the regulation of GαGPR may be secondary to canonical 7TM receptor coupling to Gαβγ subsequent to G-protein subunit flux within the microenvironment of a signaling complex. It was also recently postulated that Groups I-III AGS proteins may actually represent a signaling triad that parallels that of the well characterized 7TM receptor – Gαβγ – effector system (Blumer & Lanier 2014).

As part of a broader approach to explore these concepts, we examined the 7TM receptor -mediated regulation of the Gαi-GPR complex when Gαi was actually tethered to the 7TM receptor itself. Thus the Gαi-GPR interaction would be highly localized and could also be monitored independent of endogenous Gαβγ as the tethered G-protein could be rendered pertussis toxin insensitive by a single point mutation. The results of these studies suggest direct coupling of a 7TM receptor to the GαGPR complex, which has broad implications for G-protein signal processing.

In addition to potential direct coupling to the receptor, the Gα-GPR cassette may also be regulated by alternative binding partners. Such regulation is observed for some GPR proteins such as AGS3, LGN, and RGS14 by virtue of their interaction with alternative binding partners via defined protein-protein interaction motifs (An et al 2008, Blumer et al 2003, Blumer et al 2002, Du & Macara 2004, Du et al 2001, Pizzinat et al 2001, Shu et al 2007). Interestingly, AGS4, aside from its three GPR motifs, does not contain any obvious protein interaction domains; however, the amino-terminus of AGS4 contains a poly-

proline rich region that may subserve a regulatory function for AGS4 (Cao et al 2004, Takesono et al 1999). As an initial approach to examine these potential modes of regulation for AGS4, we utilized a tandem affinity purification system and subsequent mass spectrometry analysis to identify potential AGS4 binding partners.

Furthermore, post-translational modifications within or proximal to GPR motifs may differentially regulate the Gαi-GPR interaction (Adhikari & Sprang 2003, Blumer et al 2003, Hollinger et al 2003, Kimple et al 2004). Phosphorylation has been linked to regulation of subcellular localization and modulation of Gαi binding of AGS3 and LGN (Blumer et al 2003, Groves et al 2010, Johnston et al 2009). In contrast, phosphorylation of RGS14 appeared to enhance $G\alpha i_{GDP}$ interaction (Hollinger et al 2003). These studies suggest that phosphorylation may play a key modulatory role in regulating GPR-containing proteins; however, the functional consequences of phosphorylation on another Group II AGS protein, AGS4, are incompletely characterized (Giguere et al 2012b, Rush et al 2005, Zarling et al 2000, Zhong et al 2012). Additionally, the kinases responsible for the phosphorylation of AGS4 are unknown. AGS4 contains only two tyrosine residues, Y85 and Y108; interestingly, in a recent study to identify the phosphoproteomic changes induced by the cytokine thymic stromal lymphopoietin (TSLP) in a lymphocyte cell line, AGS4 was identified as being phosphorylated on Y108 (Zhong et al 2012). TSLP plays critical roles in shaping and regulating immune responses and is a critical mediator of allergic inflammation and hypersensitivity disorders, in particular asthma and other atopic

diseases (He et al 2008, Liu et al 2007, Redhu et al 2013, Roan et al 2012, Wilson et al 2013, Ziegler 2012, Ziegler et al 2013). TSLP also promotes maturation of dendritic cells and the induction of inflammatory-driven, type 2 helper T-cell (Th2)-mediated immune responses, which underlies its involvement in allergic and hypersensitivity reactions. TSLP induces the phosphorylation and activation of several tyrosine kinases, including JAK2 and Src (Zhong et al 2012). Interestingly, AGS4-Y108 is a consensus phosphorylation residue for both JAK2 and Src. Therefore, as an initial approach to determine the role of AGS4 phosphorylation, we generated aspartic acid mutations to serine and tyrosine residues to investigate modulation of Gαi binding to these residues upon phosphorylation. Additionally, *in vitro* kinase assays were used to determine if JAK2 and Src are able to effectively phosphorylate AGS4.

Potential regulation of AGS4 by TSLP signaling further suggests a role for Group II AGS proteins in immune cell signal processing. Indeed, loss of AGS4 conferred decrease in proinflammatory signaling and disease progression in a model of rheumatoid arthritis (Giguere et al 2013). Additionally, AGS3 was identified in a complex with Gαi and mInsc targeting the Par3-Par6-aPKC polarity complex to the leading edge of neutrophils and was required for directed migration in these cells (Kamakura et al 2013). We also recently demonstrated that AGS3 is required for proper chemokine signal processing and migration of B and T lymphocytes as well as bone marrow derived dendritic cells (Branham-O'Connor et al 2014). These collective studies as well as those indicating a role for GPR proteins in drug addiction and neuronal plasticity, ischemia reperfusion

injury and polycystic kidney disease, blood pressure control, energy expenditure and metabolism, autophagy, membrane protein trafficking, and directional migratory response suggest that the Gαi – GPR complex may be a prime target for therapeutic intervention (Blumer et al 2008, Bowers et al 2008, Bowers et al 2004, Branham-O'Connor et al 2014, Cao et al 2004, Giguere et al 2013, Kwon et al 2012, Nadella et al 2010, Regner et al 2011, Yao et al 2005, Yao et al 2006a, Zhao et al 2010). Thus, using a BRET-based drug screening platform, we identified seven potential inhibitors of the Gαi – GPR interaction to be further developed as biochemical tools for further investigation of the Gαi – GPR interaction and/or a potential therapeutic.

Experimental Procedures

Materials and reagents:

Polyethylenimine (PEI) (25 kDa molecular mass, linear form), was obtained from Polysciences, Inc (Warrington, PA) and Lipofectamine 2000 used for stable transfections was purchased from Life Technologies (Grand Island, NY). Benzyl-coelenterazine was obtained from NanoLight Technology (Pinetop, AZ). UK14304, rauwolscine HCl, AMD3100, pertussis toxin, and β-actin antiserum (A5441) were purchased from Sigma-Aldrich (St. Louis, MO) and recombinant human CXCL12/SDF-1α was purchased from BioAbChem (Laden, SC). Gray 96-well Optiplates were obtained from Perkin Elmer (Waltham, MA). GFP antiserum was obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). $G\alpha i_{1/2}$ and $G\alpha i_3$ antiserum was kindly provided by Dr. Thomas Gettys (Pennington Biomedical Research Center, Baton Rouge, LA). AGS4 antibody (AP5725c) and GRK2 antibody (ab50633) were obtained from Abgent (San Diego, CA) and Abcam (Cambridge, MA), respectively. Protease inhibitor mixture tablets (Complete Mini) were obtained from Roche Applied Science (Indianapolis, IN). Strepavidin resin and gallein were purchased from G Biosciences (St. Louis, MO) and Tocris Biosciences (Minneapolis, MN), respectively. Small molecules used in the screening for potential inhibitors were obtained through the ChemBridge DIVERSet library of compounds housed at MUSC Hollings Cancer Center Drug Discovery and Screening core facility.

Plasmids:

AGS3, AGS3-short (AGS3-sh), AGS4 and AGS4-short (AGS4-sh) fused at the carboxyl terminus to *Renilla* luciferase (Rluc) as well as mutations to each GPR motif in these constructs (AGS3-Q/A and AGS4-Q/A), and $\alpha_{2A/D}$-AR constructs were generated as previously described (Oner et al 2010a, Oner et al 2013a, Oner et al 2010b). Gαi1-yellow fluorescent protein (Gαi1YFP) and Gαi2-yellow fluorescent protein (Gαi2YFP) were generated by Dr. Scott Gibson (Gibson & Gilman 2006) and kindly provided by Dr. Greg Tall (University of Rochester, Rochester, NY) and Dr. Nathan Dascal (Tel Aviv University, Tel Aviv, Israel), respectively. YFP was inserted within the αB-αC loops in the helical domain of Gαi as described (Gibson & Gilman 2006, Oner et al 2010a, Oner et al 2010b). CXCR4 constructs were kindly provided by Dr. Michel Bouvier (Universite de Montreal) and pcDNA3::GRK2-CT, which encodes amino acids Tyr^{466} – Leu^{689} in the carboxyl terminus of GRK2, was kindly provided by Dr. Jeffrey Benovic (Thomas Jefferson University, Philadelphia, PA). All other reagents and materials were obtained as described elsewhere (Oner et al 2010a, Oner et al 2013a, Oner et al 2010b).

Site-directed Mutagenesis and fusion protein plasmid construction:

The α_{2A}AR – Gαi2YFP fusion protein was generated by polymerase chain reaction (PCR) using the rat $\alpha_{2A/D}$AR as template and primer sets containing specific sites for restriction enzyme digest as follows: XhoI, α_{2A}AR forward primer 5'-AAA CTC GAG GCC GCC ACC ATG GGC TCC CTG CAG CCG GAC-3'; EcoRI, α_{2A}AR reverse primer 5'-CAT GAA TTC CTG CAA GCT TCC TCC TCC

TCC GGA CAC GAT CCG CTT-3’. The reverse primer also encodes a SGGGS linker between α_{2AD}AR and Gαi2YFP. Digestion of pcDNA3::Gαi2YFP or pcDNA3::Gαi2YFP^{C352I} constructs at upstream XhoI/EcoRI sites followed by ligation with the digested receptor-linker resulted in in-frame construction of the α_{2A}AR – Gαi2YFP fusion proteins. Cysteine 352 (C352) in Gαi2, which is the site of ADP-ribosylation by pertussis toxin (PTX), was converted to isoleucine to render the protein PTX insensitive by site-directed mutagenesis using the pcDNA3::Gαi2YFP construct with the following primer set: Gαi2YFP^{C352I} forward primer 5’-AAC AAC CTG AAG GAC ATT GGC CTC TTC TGA-3’; Gαi2YFP^{C352I}reverse primer 5’-TCA GAA GAG GCC AAT GTC CTT CAG GTT GTT-3’.

Cell Culture, Transfection, Immunoblotting, BRET, Plasmid expression:

BRET measurements and immunoblotting were performed as previously described (Oner et al 2010a, Oner et al 2013a, Oner et al 2010b). Plasmid transfection of HEK293 cells with PEI was conducted as previously described (Oner et al 2010a, Oner et al 2013a, Oner et al 2010b, Oner et al 2013b). Experiments measuring BRET between AGS3-Rluc or AGS4-Rluc and Gαi2YFP and $\alpha_{2A/D}$AR or CXCR4 had HEK293 cells transfected with 10 ng phRLuc$_{N3}$::AGS3 or 2 ng phRLuc$_{N3}$::AGS4, respectively, 500 ng pcDNA3::Gαi2YFP, and 500 ng pcDNA3::RG20, or pcDNA3::CXCR4. In experiments measuring BRET between AGS3-Rluc or AGS4-Rluc and $\alpha_{2A/D}$AR-Venus or CXCR4-Venus in the presence or absence of Gαi2, HEK293 cells were transfected with 10 ng phRLuc$_{N3}$::AGS3 or 2 ng phRLuc$_{N3}$::AGS4, respectively,

750 ng pcDNA3::$\alpha_{2A/D}$AR-Venus or pIRESpuro3::CXCR4, and 750 ng pcDNA3::Gαi2 where indicated, HEK293 cells were transfected with 10 ng phRLuc$_{N3}$::AGS3 or 2 ng phRLuc$_{N3}$::AGS4, respectively and 500 ng pcDNA3::$\alpha_{2A/D}$AR-Gαi2YFP or pcDNA3::$\alpha_{2A/D}$AR-Gαi2YFP^{C352I} in experiments measuring BRET between AGS3-Rluc or AGS4-Rluc and $\alpha_{2A/D}$AR-Gαi2YFP or $\alpha_{2A/D}$AR-Gαi2YFP^{C352I}. Experiments to measure BRET between AGS3-sh-Rluc or AGS4-sh-Rluc and $\alpha_{2A/D}$AR-Gαi2YFP or Gαi2YFP were conducted in HEK293 cells transfected with 2 ng phRLuc$_{N3}$::AGS3-sh or 2 ng phRLuc$_{N3}$::AGS4-sh, respectively and 750 ng pcDNA3::$\alpha_{2A/D}$AR-Gαi2YFP or Gαi2YFP. Based upon a series of preliminary experiments we optimized the system to generate levels of $\alpha_{2A/D}$AR-Gαi2YFP and $\alpha_{2A/D}$AR-Gαi2YFP^{C352I} that bracketed the levels of endogenous Gαi2 as determined by immunoblotting. For BRET saturation experiments, AGS3–Rluc and AGS4-Rluc were expressed as above with increasing amounts (0 – 1000 ng) of pcDNA3::$\alpha_{2A/D}$AR-Gαi2YFP or pcDNA3::$\alpha_{2A/D}$AR-Gαi2YFP^{C352I}. Forty-eight hours after cell transfection, cells were dispensed in triplicate at 1 x 10^5 cells/well in gray 96-well Optiplates (Perkin Elmer (Waltham, MA). Fluorescence and luminescence signals were measured using a TriStar LB 941 plate reader (Berthold Technologies) with MikroWin 2000 software. Cells were incubated with the α_2-AR agonist (UK14304 – 10 μM) or vehicle in Tyrode's solution (140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 0.37 mM NaH_2PO_4, 24 mM $NaHCO_3$, 10 mM (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), pH 7.4 and 0.1% glucose (w/v)) for 5 minutes prior to addition of coelenterazine H. Coelenterazine H (Nanolight

Technology, 5 μM final concentration) was added to each well and luminescence measured after two minutes (donor: 480 $\pm$ 20 nm; acceptor: 530 $\pm$ 20 nm) with the TriStar LB 941 plate reader. Gαi2YFP or $\alpha_{2A/D}$AR-Gαi2YFP fusion protein expression was monitored by measuring YFP fluorescence (excitation 485 nm, emission 535 nm). AGS3- and AGS4-Rluc expression was monitored by measuring the intensity of the luminescence signal. BRET signals were determined by calculating the ratio of the light intensity emitted by the YFP divided by the light intensity emitted by Rluc. Net BRET values were determined by first calculating the 530 ± 20:480 ± 20 nm ratio and then subtracting the background BRET signal determined from cells transfected with the donor plasmids $phRLuc_{N3}$::AGS3 or $phRLuc_{N3}$::AGS4 alone. Cell lysates and immunoblotting were performed as previously described using 10-13% sodium dodecyl sulfate polyacrylamide gel electrophoresis (Oner et al 2010a, Oner et al 2010b). Where indicated, cells were incubated with α_2-AR agonist UK14304 (10uM), α_2-AR antagonist rauwolscine (100 μM), CXCR4 agonist CXCL12 (100 ng/mL), CXCR4 antagonist AMD3100 (1 μg/mL) for the times indicated and/or pretreated with pertussis toxin (100 ng/ml) 18 hours prior to BRET measurements.

Stable cell line generation:

Both NTAP AGS4 and Gαi1YFP stably expressing cells were generated in HEK293 cells using Lipofectamine 2000 (Invitrogen). HEK293 cells were seeded at ~70% confluency for transfection in 6-well dishes. Lipofectamine 2000 working solution was made by adding Lipofectamine 2000 (10 μl) to 250 μl Dulbecco's

Modified Eagles Medium (DMEM, HyClone™) supplemented with 5% fetal bovine serum (FBS). DNA working solutions were made by adding NTAP-taggged protein, NTAP empty vector, or Gαi1YFP (4 µg total DNA) to 250 µl of DMEM + 5% FBS. The two working solutions were combined, vortexed for 3 sec and incubated at room temperature for 20 min, after which the DNA:lipid complexes were added to each well of HEK293 cells. After 24 hours, the transfection media was removed, cells were dispersed in a 10cm dish in 10 ml of DMEM + 5% FBS supplemented with 2 µg/ml puromycin or 800 µg/ml G418, to select for NTAP AGS4 and Gαi1YFP expressing cells, respectively. Forty-eight hours post-transfection, cells were transferred into 15cm dishes with 20 ml fresh media containing selection antibiotics. After 7-10 days, single colonies were selected and isolated using cloning cylinders and separated into 10cm dishes with appropriate selection media. Immunoblotting and fluorescence measurements validated expression of the appropriate plasmid.

Tandem Affinity Purification (TAP):

Purification of TAP-tagged AGS4 required $1x10^8$ cells of NTAP AGS4 stably expressing HEKs (NTAP-only cells used as negative controls). Cells were harvested using serum free DMEM, centrifuged at 500 x g for 5 min, and resuspended in 300 µl streptavidin binding buffer (50mM TRIS-HCl pH 8, 150mM NaCl, 10% glycerol (v/v), 1% NP-40 lysis buffer, phosphotase inhibitors) to be lysed for 20 min on ice. After this incubation the lysates were centrifuged at 20,000 x g, 4°C to isolate the protein in each sample. During this time, 500 µl streptavidin resin per condition (GBiosciences) was equilibrated in streptavidin

binding buffer as a 50% slurry. After centrifugation, 30 μl (10%) of the protein lysates was taken as an input control. The remaining lysate was added to the equilibrated 500 μl streptavidin resin for at least 2 hours, 4°C with constant rotation. After incubation, a 25 μl aliquot was taken for later analysis and the resin was then pelleted by centrifugation on a picrofuge for 5-10 s. Cleared lysate was removed and stored for later analysis. Resin bound to the TAP proteins was washed three times with 500 μl streptavidin binding buffer followed by subsequent burst centrifugations 5-10 s to pellet the resin, while cleared washes were removed for later analysis. Samples were resuspended in 150 μl streptavidin binding buffer and TAP tagged proteins were then eluted by 100 μl 5x Laemmli sample buffer, followed by boiling of the resin for five minutes. The supernatant ~250 μl was transferred to a fresh microcentrifuge tube, where samples were subjected to SDS-PAGE, and transferred to polyvinylidene difluoride (PVDF) membranes. Membranes were then blocked with 50% Odyssey Buffer [LI-COR Biosciences] and 50% Tris-buffered saline + 0.01% Tween (TBST) or 5% blotto for 30 min at room temperature, immunoblotted for AGS4 (1:250 dilution) antibody, $G\alpha i_{1/2}$ antiserum (1:10,000 dilution), or $G\alpha i_3$ antiserum (1:10,000 dilution), followed three 10 min washes in TBST. Membranes were then exposed to 1:5,000 or 1:20,000 dilutions of horseradish peroxidase-conjugated goat anti-rabbit IgG, respectively, followed by three 30 min washes with TBST and subsequent exposure with ECL. Additionally, SDS-PAGE was run with subsequent Coomassie blue staining to visualize bands from the elution that

were excised and sent to the MUSC Mass Spectrometry/Proteomics Core facility for identification of additional purified proteins.

Excised gel plugs were washed with 50 mM ammonium bicarbonate for 10 minutes. Next, the plugs were de-stained using 25mM ammonium bicarbonate in 50% acetonitrile for 15 minutes, repeated twice. The plugs were dehydrated with 100% acetonitrile for 15 minutes, and then dried in a speedvac. Each gel plug was covered with Proteomics Grade Trypsin (Sigma) and incubated at 37C overnight.

The supernatant was collected in a clean dry eppendorf tube. Peptides were further extracted with 1 wash of 25mM ammonium bicarbonate for 20 minutes and three washes of 5% formic acid, 50% acetonitrile for 20 minutes each. The supernatant was collected and pooled after each wash then dried down in a speedvac to ~2 uL. Prior to LC/MS analysis the samples were reconstituted with 10 ul of 2% acetonitrile and 0.2% formic acid.

Enzymatically digested samples were analyzed via liquid chromatography (LC)-electrospray ionization (ESI) -tandem mass spectrometry (MS/MS) on a linear ion trap mass spectrometer (LTQ, Thermo Finnigan) coupled to a Dionex 3000 nano LC system. A 25cm 75micron C-18 reversed phase LC column (packed in house, with Waters ODS C18) was utilized with a 120 minute gradient from 2% acetonitrile, 0.2% formic acid to 60% acetonitrile, 0.2% formic acid. Data Dependent Analysis was utilized on the LTQ to perform MS/MS on the 10 most intense ions in each MS spectra with a minimum ion count of 1000.

Dynamic Exclusion was set to exclude ions from MSMS selection for 3 minutes after being selected 2 times in a 30 second window.

The MS/MS data was searched against a human database using Sequest via Bioworks 3.0 SP1 (Thermo). Variable modifications of methionine oxidation were considered. Protein identifications must have an Xcorr vs charge state > 1.5, 2.0, 2.5 for +1, +2, and +3 ions, with at least 2 unique peptides matching the protein, and a good match for at least 4 consecutive y or b ion series from the MS/MS spectra.

^{32}P autorad in vitro kinase assay:

Purified GST-AGS4-short (Leu^{57}-Cys^{160}) was generated as described previously (Cao et al 2004). GST-AGS4-short-Y108F was generated by site-directed mutagenesis and purified according to Cao et al. Five μg purified GST, GST-AGS4-short and GST-AGS4-short-Y108F was incubated with 1 μg purified active JAK2 (Leu^{808}-Gly^{1132}) and Src kinases in kinase buffer (50 mM HEPES pH 7.5, 10 mM $MgCl_2$, 3 μM Na_3VO_4, 1 mM EGTA, 1 mM DTT, 10 uM ATP and 10 μCi γ-32P-ATP) for 30min at 25C. Samples were subjected to SDS-PAGE and autoradiography.

Small Molecule Screening:

Gαi1YFP stably expressing HEK293 cells were generated by Lipofectamine 2000 (Invitrogen) transfection and G418 antibiotic selection as described in the above section *Stable cell line generation*. Expression levels of Gαi1-YFP were determined to be 291,729 ± 77,278 RFU. The 50,080 compound DIVERSet library from ChemBridge, (MUSC Drug Discovery Core) was used. HEK293 cells

stably expressing Gαi1YFP were transfected with 2 ng $phRluc_{N3}$::AGS4 using PEI for forty-eight hours prior to drug treatment. Cells were dispensed at 1 x 10^5 cells/well in gray 96-well Optiplates (Perkin Elmer (Waltham, MA). Prior to reading fluorescence or luminescence, compounds were administered to each well (10 compounds per well or individual in secondary screen) at a final concentration of 1 μM for 1 hour at 37°C, 5% CO_2. Total fluorescence (ex. 485 nm; em. 535 nm) was measured to monitor Gαi1YFP expression using the TriStar LB 941 plate reader (Berthold Technologies). Coelenterazine H (Nanolight Technology, 5 μM final concentration) was added to each well and luminescence measured after two minutes (donor: 480 $\pm$ 20 nm; acceptor: 530 $\pm$ 20 nm) with the TriStar LB 941 plate reader with MikroWin 2000 software. BRET signals were determined by calculating the ratio of the light intensity emitted by the YFP divided by the light intensity emitted by Rluc. Net BRET was calculated as previously described using individual wells transfected with AGS4-Rluc alone to subtract background signals for each set of compounds.

Data Analysis:

Statistical significance for differences involving a single intervention was determined by one-way analysis of variance (ANOVA) followed with a post hoc Tukey's test using GraphPad Prism version 4.03 (GraphPad Software, San Diego).

Results and Discussion

Agonist-sensitive regulation of the Gαi2–GPR module by chemokine receptors

Two Group II AGS proteins containing multiple GPR motifs, AGS3 and AGS4, were used as model proteins to identify regulatory mechanisms for Gα – GPR interactions. AGS3 and AGS4 differ in the number of GPR motifs expressed and the domain composition of the amino terminus of each protein. AGS3 contains a series of TPR domains involved in protein interactions and intramolecular regulatory events, while AGS4 is absent in these protein binding domains and only expresses a short proline-rich segment upstream of the GPR motifs. Although initial observations depict regulation of Gαi1 with AGS3 and AGS4 by $\alpha_{2A/D}$-AR, the regulation of these proteins with Gαi2, the predominant isoform in the immune system has yet to be determined (Branham-O'Connor et al 2014, Cao et al 2004, Giguere et al 2013, Oner et al 2010a, Oner et al 2010b). Thus, as an initial approach to address the hypothesis that Gαi2 – GPR complex is regulated by chemokine receptors, BRET measurements were taken from HEK293 cells expressing AGS3-Luc or AGS4-Luc, Gαi2-YFP and the chemokine receptor CXCR4 (Figure 2.1A). The previously investigated receptor, $\alpha_{2A/D}$-AR was used as a positive control (Figure 2.1A) (Oner et al 2010a, Oner et al 2010b). Indeed, Gαi2 - GPR complexes were regulated by agonists for both CXCR4 and a2-AR, reflected as a decrease in BRET signal upon receptor activation, suggesting either dissociation or rearrangement of the Gαi2 – GPR signaling complex (Figure 2.1B and 2.1C). This decrease was effectively blocked by appropriate antagonist or pertussis toxin (PTX), which prevents Gαi coupling

to receptor by ADP ribosylating a cysteine residue four residues from the carboxyl terminus of Gαi/o subunits (Figure 2.1B and C). Additionally, mutation of a critical glutamine residue in each of the GPR motifs from glutamine to alanine (Q/A) completed blocked GPR association with Gαi2 subunits in the BRET system similar to results previously ascertained for Gαi1 (Figure 2.1B and C) (Oner et al 2010a, Oner et al 2010b, Peterson et al 2002).

Figure 2.1

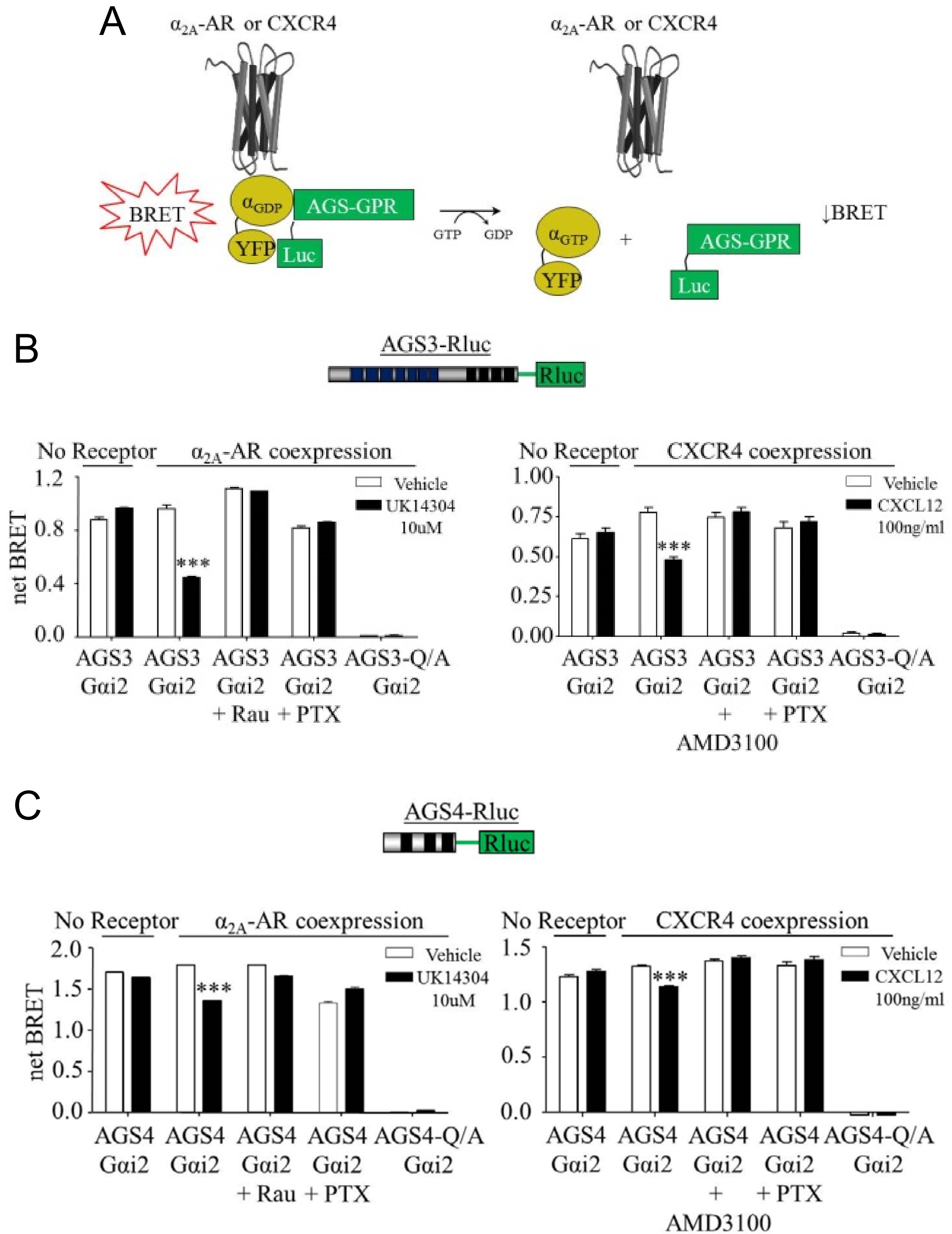


Figure 2.1. Regulation of Gαi2 – GPR interaction by Gαi-coupled receptor activation

(A) Schematic of bioluminescence resonance energy transfer (BRET) system along with representation of hypothesized agonist-induced regulation of Gαi2YFP – GPR-Rluc BRET association by receptor activation.

(B) Net BRET signals were obtained from HEK cells transfected with 10 ng $phRLuc_{N3}$::AGS3 or 10 ng $phRLuc_{N3}$::AGS3-Q/A-Rluc along with 500 ng pcDNA3::Gαi2-YFP. Cells were also transfected in the presence or absence of 500 ng pcDNA3::$\alpha_{2A/D}$-AR (*left panel*) or pcDNA3::CXCR4 receptor (*right panel*). Vehicle (Tyrode's solution), $\alpha_{2A/D}$-AR agonist UK14304 (10 μM), or CXCL12 (100ng/mL) were added to cells as indicated followed by fluorescence and luminescence readings as described in "Experimental Procedures." The $\alpha_{2A/D}$-AR antagonist rauwolscine (10 μM) and CXCR4 antagonist AMD3100 (1 μg/mL) were added 10 minutes prior to agonist stimulation as indicated. Cells were treated with pertussis toxin (PTX, 100 ng/mL) 18 hours prior to receptor stimulation where indicated.

(C) Net BRET signals were obtained from HEK cells transfected with 2 ng $phRLuc_{N3}$::AGS4 or 2 ng $phRLuc_{N3}$::AGS4-Q/A-Rluc along with 500 ng pcDNA3::Gαi2-YFP. Cells were also transfected in the presence or absence of 500 ng pcDNA3::$\alpha_{2A/D}$-AR (*left panel*) or pcDNA3::CXCR4 receptor (*right panel*). Vehicle (Tyrode's solution), $\alpha_{2A/D}$-AR agonist UK14304 (10 μM), or CXCL12 (100ng/mL) were added to cells as indicated followed by fluorescence and luminescence readings as described in "Experimental Procedures." The $\alpha_{2A/D}$-AR

antagonist rauwolscine (10 μM) and CXCR4 antagonist AMD3100 (1 μg/mL) were added 10 minutes prior to agonist stimulation as indicated. Cells were treated with pertussis toxin (PTX, 100 ng/mL) 18 hours prior to receptor stimulation where indicated.

All data are expressed as means ± SEM from at least 3 independent experiments with triplicate determinations (N=9). Notations of *** signify p-values < 0.0001 as compared with vehicle stimulated control group based on Tukey's post hoc test following ANOVA.

Association of chemokine receptor with GPR-containing proteins is Gαi dependent and disrupted subsequent to receptor activation

As an initial approach to test the hypothesis that the Gαi2 – GPR complex was in close enough proximity to 7TMRs to facilitate the observed regulation (Figure 2.1B and C), the BRET system was adapted by converting the receptor to the acceptor (Venus) while the donor (Rluc) was retained on AGS3 or AGS4 to observe the consequence of receptor activation on the association between GPR-containing proteins and receptors (Figure 2.2A). The association was indeed a Gαi2-mediated event subject to regulation by receptor activation, similar to that observed previously for Gαi1 (Figure 2.2B and C) (Oner et al 2010a, Oner et al 2010b). Agonist regulation of the Gαi-GPR complex was blocked by treatment of the appropriate antagonist or PTX, and substitution of GPR-Q/A mutations completely eliminated BRET signals between the receptor and GPR proteins (Figure 2.2B and C). These results suggest the existence of a ternary complex of GPR-Gαi2-GPCR, which is regulated by agonist stimulation and analogous to canonical Gαβγ – GPCR coupling. Additionally, regulation of these complexes by receptor activation was observed to be PTX sensitive, whereas Gαi-GPR interaction in the absence of agonist is unaffected by PTX (Figure 2.1B and C and Figure 2.2B and C) (Oner et al 2010a, Oner et al 2010b).

Figure 2.2

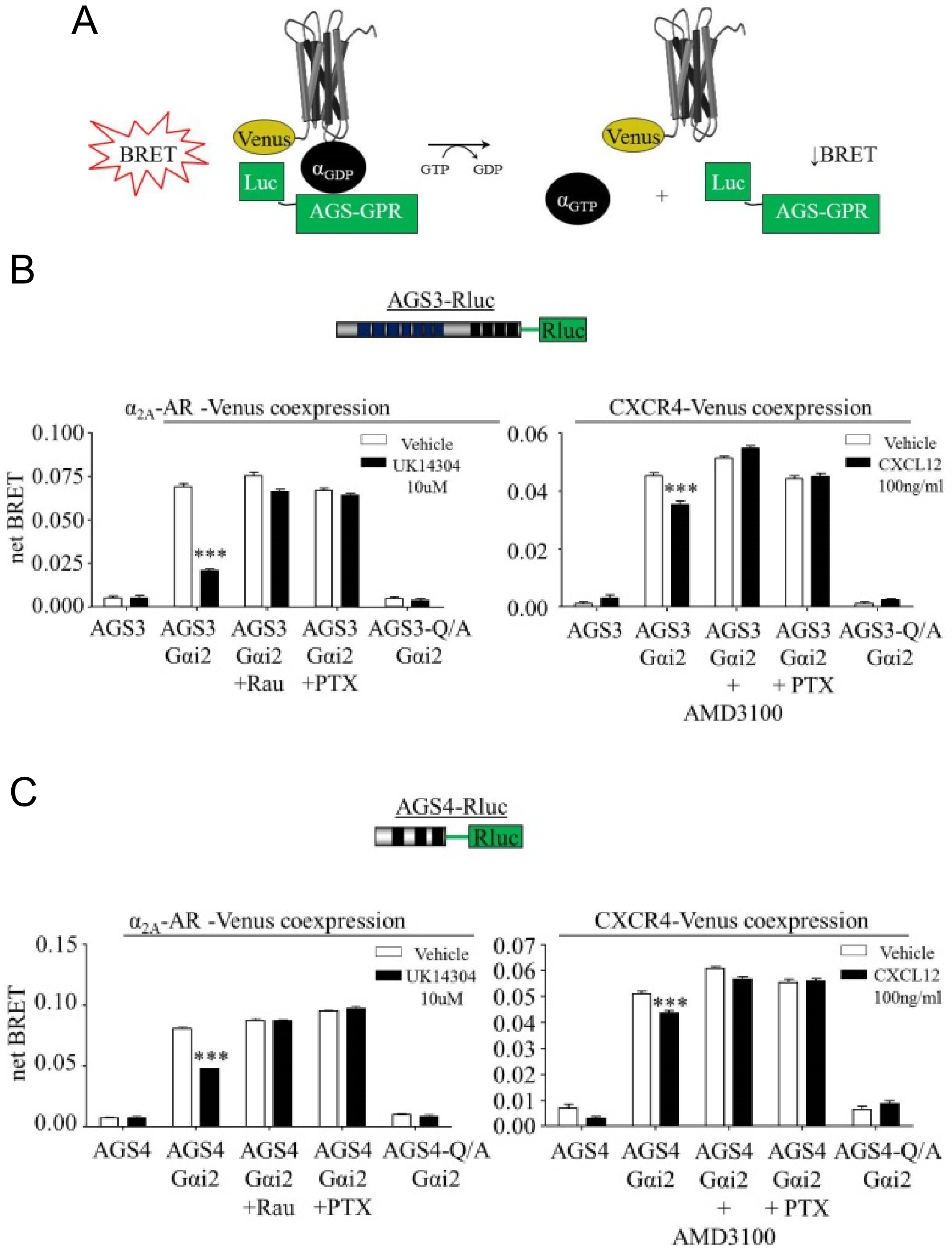


Figure 2.2. Regulation of Gαi2-dependent proximity of GPR protein to receptor by Gαi-coupled receptor activation

(A) Schematic representing BRET system to measure effect of receptor activation on proximity of GPR-Rluc protein to the receptor-venus.

(B) HEK cells were transfected with 10 ng $phRLuc_{N3}$::AGS3 or 10 ng $phRLuc_{N3}$::AGS3-Q/A along with 750 ng pcDNA3::a_{2A}-AR-Venus or pIRESpuro3::CXCR4-Venus in the presence or absence of 750 ng pcDNA3::Gαi2 as indicated. Vehicle (Tyrode's solution), $\alpha_{2A/D}$-AR agonist UK14304 (10 μM), or CXCL12 (100ng/mL) were added to cells as indicated followed by fluorescence and luminescence readings as described in "Experimental Procedures." The $\alpha_{2A/D}$-AR antagonist rauwolscine (10 μM) and CXCR4 antagonist AMD3100 (1 μg/mL) were added 10 minutes prior to agonist stimulation as indicated. Cells were treated with pertussis toxin (PTX, 100 ng/mL) 18 hours prior to receptor stimulation where indicated.

(C) HEK cells were transfected with 2 ng $phRLuc_{N3}$::AGS4 or 2 ng $phRLuc_{N3}$::AGS4-Q/A along with 750 ng pcDNA3::a_{2A}-AR-Venus or pIRESpuro3::CXCR4-Venus in the presence or absence of 750 ng pcDNA3::Gαi2 as indicated. Vehicle (Tyrode's solution), $\alpha_{2A/D}$-AR agonist UK14304 (10 μM), or CXCL12 (100ng/mL) were added to cells as indicated followed by fluorescence and luminescence readings as described in "Experimental Procedures." The $\alpha_{2A/D}$-AR antagonist rauwolscine (10 μM) and CXCR4 antagonist AMD3100 (1 μg/mL) were added 10 minutes prior to agonist

stimulation as indicated. Cells were treated with pertussis toxin (PTX, 100 ng/mL) 18 hours prior to receptor stimulation where indicated.

All data are expressed as means ± SEM from at least 3 independent experiments with triplicate determinations (N=9). Notations of *** signify p-values < 0.0001 as compared with vehicle stimulated control group based on Tukey's post hoc test following ANOVA.

Regulation of Gαi2 – GPR module by physical tethering and subsequent activation of 7TMRs

Specific regulation of the Gαi2 – GPR complex and its proximity to the receptor suggests that the complex may directly couple to the 7TMR in a manner analogous to heterotrimeric G-proteins. As an initial approach to address the hypothesis regarding direct receptor coupling to Gαi2GPR, we generated a fusion protein in which Gαi2YFP was tethered to the carboxyl terminus of the $\alpha_{2A/D}$AR via a flexible glycine linker (Bahia et al 1998, Bertin et al 1994, Burt et al 1998, Seifert et al 1999, Wise et al 1997) Similar results were obtained for untethered Gαi2YFP and $\alpha_{2A/D}$-AR constructs. We also generated a variant of the $\alpha_{2A/D}$AR-Gαi2YFP fusion protein that was PTX-insensitive ($\alpha_{2A/D}$AR-Gαi2YFP^{C352I}). We then examined the ability of GPR proteins to interact with the tethered Gαi2. Interestingly, both AGS3 and AGS4 interacted with the tethered WT and PTX-insensitive Gαi2 as indicated by the robust basal levels of BRET (Figure 2.3B and E). Expression and functionality of $\alpha_{2A/D}$AR-Gαi2YFP and $\alpha_{2A/D}$AR-Gαi2YFP^{C352I} were confirmed by immunoblotting (Figure 2.3C) and agonist-induced phosphorylation of ERK1/2 (Figure 2.3D). $\alpha_{2A/D}$AR-Gαi2YFP:AGS3-Rluc BRET and $\alpha_{2A/D}$AR-Gαi2YFP:AGS4-Rluc BRET were not observed with the GPR-insensitive Gαi^{N149I} mutant or with AGS3 or AGS4 that were rendered incapable of binding Gαi by mutation of a conserved glutamate residue in each of the GPR motifs, thus demonstrating the specificity of the interaction (Figure 2.5A, B, and C) (Oner et al 2010a, Oner et al 2010b, Peterson et al 2002, Sato et al 2004, Willard et al 2008).

Incubation of cells with the $\alpha_{2A/D}$AR agonist UK14304 reduced the $\alpha_{2A/D}$AR-Gαi2YFP:AGS3-Rluc BRET by ~40% (Figure 2.3D, left panel). Significant agonist-induced reductions in $\alpha_{2A/D}$AR-Gαi2YFP:AGS4-Rluc BRET were also observed, although not to the same magnitude as that observed for AGS3-Rluc (Figure 2.3D, right panel). Both the basal $\alpha_{2A/D}$AR-Gαi2YFP:AGS3-Rluc BRET and the magnitude of the agonist-induced decrease in BRET observed for AGS3-Rluc or AGS4-Rluc with tethered Gαi2YFP were similar to that observed with untethered Gαi2YFP. Similar results were obtained for untethered Gαi2YFP and $\alpha_{2A/D}$-AR constructs. Thus, these data indicate that a 7TM agonist is regulating a GαGPR complex that is directly anchored to the receptor.

A similar distinction between AGS3 and AGS4 with respect to the magnitude of agonist-induced changes in BRET was also observed with untethered Gαi1YFP (Oner et al 2010a, Oner et al 2010b). It is not clear if the differences in the magnitude of the agonist-induced changes in GαiYFP:AGS3-Rluc versus GαiYFP:AGS4-Rluc BRET reflect different coupling efficiencies, stoichiometric considerations and/or the relative spatial positioning of the acceptor and donor for AGS3 versus AGS4. To investigate if the amino terminal domains of AGS3 or AGS4 were responsible for this inconsistency with agonist response, we used AGS3-short (lacking all TPR domains and one GPR domain) and AGS4-short (proline-rich amino terminal deleted) tagged with Rluc in the context of the $\alpha_{2A/D}$-AR fusion protein BRET platform. Truncation of the amino terminal domains of AGS3 or AGS4 did not alter the agonist-induced reduction in

BRET signal for either construct as compared to the full-length counterparts (Figure 2.5C). These data suggest the difference in agonist regulation of these proteins does not involve the amino terminal domains, but rather there lies an intrinsic difference between the GPR domains and/or residues between these domains of AGS3 and AGS4 (Figure 2.5C). These differences may be reminiscent of results depicting that the 3rd GPR domain of AGS3 requiring flanking residues to attain proper GDI activity (Adhikari & Sprang 2003). In addition, the receptor-mediated regulation of GαiAGS3 and GαiAGS4 also differ in that agonist-mediated regulation of the GαiAGS3 complex results in translocation of AGS3, but not AGS4, to the Golgi apparatus (Oner et al 2013c).

Figure 2.3

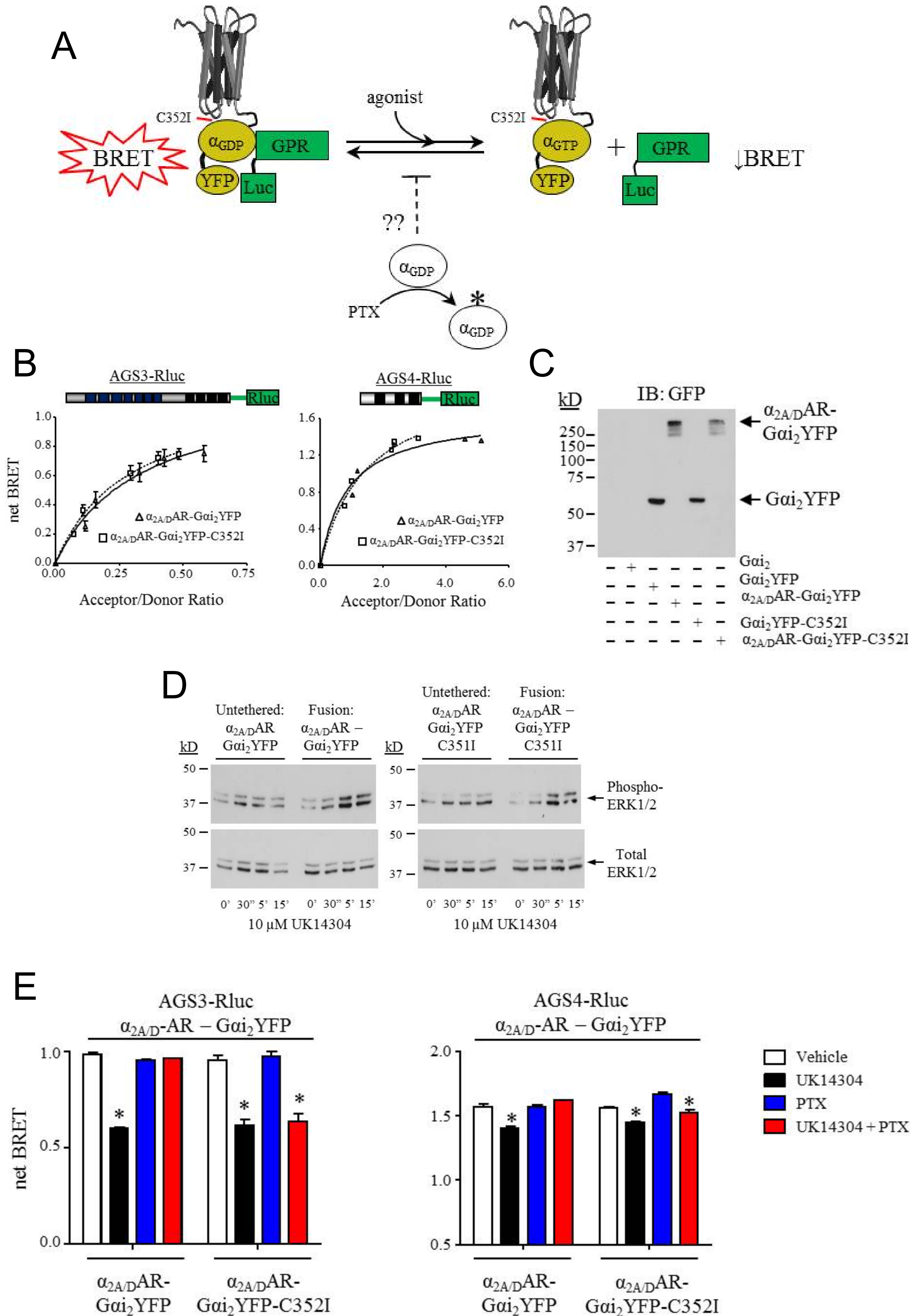


Figure 2.3. Agonist-induced regulation of an $\alpha_{2A/D}$AR-Gαi2 fusion protein complexed with the GPR proteins, AGS3 and AGS4

(A) Schematic representing approach taken to determine the influence of endogenous, untagged Gαi on GPR-Rluc – $\alpha_{2A/D}$-AR-Gαi2YFP BRET. Mutation of Cys352 to Ile (C352I) renders Gαi2YFP insensitive to pertussis toxin and was used to prevent flux by endogenous Gαi subunits, which may be released from endogenous Gαβγ heterotrimer coupling to the $\alpha_{2A/D}$-AR-Gαi2YFP fusion protein. Agonist bound to the receptor is denoted by an asterisk (*).

(B) HEK293 cells expressing a fixed amount of AGS3-Rluc (*left*) or AGS4-Rluc (*right*) and increasing amounts (0, 100, 200, 500, 750, and 1000ng) of $\alpha_{2A/D}$AR-Gαi2YFP (squares) or $\alpha_{2A/D}$AR-Gαi2YFP^{C352I} (triangles) were processed for BRET measurements as described in "Experimental Procedures."

(C) Lysates (50 µg) from control HEK293 cells or HEK293 cells expressing Gαi2, Gαi2YFP, $\alpha_{2A/D}$AR-Gαi2YFP or $\alpha_{2A/D}$AR-Gαi2YFP^{C352I} (750 ng each plasmid) were subjected to sodium dodecyl sulfide – polyacrylamide gel electrophoresis (SDS-PAGE), transferred to a PVDF membrane and immunoblotted with GFP antiserum.

(D) Lysates (50 µg) from HEK293 cells expressing untethered $\alpha_{2A/D}$AR (500 ng) and Gαi2YFP or Gαi2YFP^{C352I} (750 ng each plasmid) or fusion proteins $\alpha_{2A/D}$AR-Gαi2YFP or $\alpha_{2A/D}$AR-Gαi2YFP^{C352I} (750 ng each plasmid). These lysates were subjected to SDS-PAGE, transferred to PVDF membranes and immunoblotted with total ERK1/2 and phospho-ERK1/2 (Y402) antibodies as indicated.

(E) HEK293 cells expressing AGS3-Rluc (*left panel*) or AGS4-Rluc (*right panel*) and $\alpha_{2A/D}$AR-Gαi2YFP or $\alpha_{2A/D}$AR-Gαi2YFP^{C352I} were incubated in the absence or presence of pertussis toxin (100 ng/mL) for 18 hours as described in "Experimental Procedures." Cells were then washed and incubated with vehicle (Tyrode's solution) or $\alpha_{2A/D}$AR agonist UK14304 (10 μM) for five minutes followed by fluorescence and luminescence readings to obtain net BRET signals as described in "Experimental Procedures." (D, *Left panel*) AGS3-Rluc relative luminescence units (RLU): AGS3-Rluc + $\alpha_{2A/D}$AR-Gαi2YFP – 335,234 ± 9,929; AGS3-Rluc + $\alpha_{2A/D}$AR-Gαi2YFP + PTX – 327,626 ± 15,110; AGS3-Rluc + $\alpha_{2A/D}$AR-Gαi2YFP^{C352I} – 385,996 ± 22,073; AGS3-Rluc + $\alpha_{2A/D}$AR-Gαi2YFP^{C352I} + PTX – 373,388 ± 17,790. Relative fluorescence units (RFU): $\alpha_{2A/D}$AR-Gαi2YFP – 111,523 ± 3,246; $\alpha_{2A/D}$AR-Gαi2YFP + PTX – 112,991 ± 2,545; $\alpha_{2A/D}$AR-Gαi2YFP^{C352I} – 110,420 ± 2,416; $\alpha_{2A/D}$AR-Gαi2YFP^{C352I} + PTX – 112,565 ± 3,072. *(B, right panel*) AGS4-Rluc RLU: AGS4-Rluc + $\alpha_{2A/D}$AR-Gαi2YFP – 87,143 ± 6,516; AGS4-Rluc + $\alpha_{2A/D}$AR-Gαi2YFP + PTX – 71,193 ± 5,723; AGS4-Rluc + $\alpha_{2A/D}$AR-Gαi2YFP^{C352I} – 148,939 ± 7,362; AGS4-Rluc + $\alpha_{2A/D}$AR-Gαi2YFP^{C352I} + PTX – 133,482 ± 11,038. RFU: $\alpha_{2A/D}$AR-Gαi2YFP – 106,882 ± 5,325; $\alpha_{2A/D}$AR-Gαi2YFP + PTX – 109,976 ± 5,497; $\alpha_{2A/D}$AR-Gαi2YFP^{C352I} – 142,380 ± 2,980; $\alpha_{2A/D}$AR-Gαi2YFP^{C352I} + PTX – 166,057 ± 8,005.

All BRET data are expressed as means ± S.E. from at least 3 independent experiments with triplicate determinations (N=9) and immunoblots are representative of three independent experiments. *, $p < 0.05$ compared with vehicle treated control group based on Tukey's post hoc test following ANOVA.

Direct 7TMR regulation of the Gαi2 – GPR module occur independent of endogenous G-protein cycling

Regulation of the $\alpha_{2A/D}$AR-Gαi2YFP:GPR-Rluc complex by agonist may reflect the ability of the Gαi2GPR cassette to directly sense agonist-induced conformational changes in the receptor (Figure 2.3A) as is the case for 7TM receptor coupling to Gαβγ heterotrimer. Alternatively, the agonist-induced reduction of $\alpha_{2A/D}$AR-Gαi2YFP:GPR-Rluc BRET may reflect displacement of AGS3- or AGS4-Rluc from the 7TM receptor-Gαi2YFP fusion protein by Gβγ or Gα subsequent to receptor coupling to either endogenous Gαβγ heterotrimer (Burt et al 1998) or the $\alpha_{2A/D}$AR-Gαi2YFP fusion protein where endogenous Gβγ is bound to the tethered Gαi2YFP.

To address these questions, we conducted two sets of experiments. First, we studied the effect of agonist on $\alpha_{2A/D}$AR-Gαi2YFP:GPR-Rluc BRET after rendering the tethered Gα subunit PTX insensitive by mutation of the cysteine that is actually ADP ribosylated by pertussis toxin (Figure 2.3A). Such an approach would allow us to eliminate receptor coupling to endogenous Gαβγ, but retain the coupling integrity of the $\alpha_{2A/D}$AR-Gαi2YFP^{C352I} fusion protein (Bahia et al 1998). Thus, we have an experimental platform that provides a highly localized readout of receptor-mediated regulation of Gαi2GPR.

The agonist-induced regulation of $\alpha_{2A/D}$AR-Gαi2YFP:AGS3-Rluc or $\alpha_{2A/D}$AR-Gαi2YFP:AGS4-Rluc BRET observed with untethered or tethered Gα was completely blocked by incubation of cells with PTX (Figure 2.3D). However, the agonist-induced regulation of untethered or tethered Gαi^{C352I} was not altered

by PTX pretreatment, which blocked receptor coupling to endogenous Gαi/oβγ (Figure 2.3D). These data indicate that the agonist-induced regulation of $\alpha_{2A/D}$AR-GαiYFP:AGS3-Rluc or $\alpha_{2A/D}$AR-GαiYFP:AGS4-Rluc BRET is spatially localized and not likely due to exchange of endogenous Gαi/o for GαYFP bound to the GPR protein or to the displacement of GαYFP bound to the GPR protein by Gβγ subsequent to receptor-mediated coupling to Gαβγ heterotrimer.

In addition to interacting with the GPR proteins AGS3 and AGS4, the $\alpha_{2A/D}$AR-Gαi2YFP fusion protein may also interact with endogenous Gβγ. Agonist induced activation of the $\alpha_{2A/D}$AR-Gαi2YFP:Gβγ complex may "release" Gβγ, which could potentially displace AGS3 or AGS4 from the $\alpha_{2A/D}$AR-Gαi2YFP fusion protein reducing $\alpha_{2A/D}$AR-Gαi2YFP:GPR-Rluc BRET. To address this issue, we used the carboxyl terminus of G-protein coupled receptor kinase 2 (GRK2-CT) to scavenge any Gβγ that may be "released" by agonist-induced activation of $\alpha_{2A/D}$AR-Gαi2YFP:Gβγ (Figure 2.4A). GRK-CT expression was confirmed by immunoblotting (Figure 2.4B and C lower panels). Expression of GRK2-CT did not alter the agonist induced regulation of the BRET observed with AGS3-Rluc or AGS4-Rluc and the untethered[2] or tethered Gαi2YFP (Figure 2.4B and C upper panels). Under similar experimental conditions with untethered Gαi2YFP, expression of Gβγ reduces basal Gαi2YFP:GPR-Rluc BRET (Oner et al 2010a, Oner et al 2010b) and this effect of Gβγ was reversed by GRK2-CT providing an internal control that indicates effective Gβγ scavenging (Figure 2.4B and C lower panels). The lack of effect of GRK2-CT on agonist-induced regulation of the interaction of GPR proteins with the tethered GαiYFP is

consistent with previous observations using untethered GαiYFP (Oner et al 2010a). Furthermore, the Gβγ inhibitor gallein also did not alter the basal or agonist-regulated BRET between AGS3-Rluc or AGS4-Rluc and either Gαi2YFP or the $\alpha_{2A/D}$AR–Gαi2YFP fusion protein (Figure 2.4 D). These data suggest that the agonist-induced regulation of the interaction of Gαi with GPR proteins does not involve subunit flux subsequent to receptor coupling to Gαβγ.

The ability for AGS3 and AGS4 to bind multiple Gαi subunits simultaneously suggests the intriguing possibility for these proteins to assist in scaffolding of larger signaling complexes to further increase the efficiency of signal transduction (Blumer & Lanier 2014). To determine if multiple Gαi subunits tethered to the $\alpha_{2A/D}$AR were effectively bound by AGS3 and AGS4 we again utilized our BRET fusion protein platform. Using subsequent Q/A mutations in the GPR domains of AGS3 and AGS4 rendering the motifs unable to bind Gαi subunits, the level of basal BRET was reduced stepwise with each additional GPR mutation (Figure 2.5A and B). Furthermore, agonist induced reduction of the BRET signal was maintained even in the presence of a single functioning GPR motif (Figure 2.5A & B). These data suggest that all GPR motifs of AGS3 and AGS4 are able to functionally bind tethered Gαi subunits and contribute to the overall BRET signal.

Figure 2.4

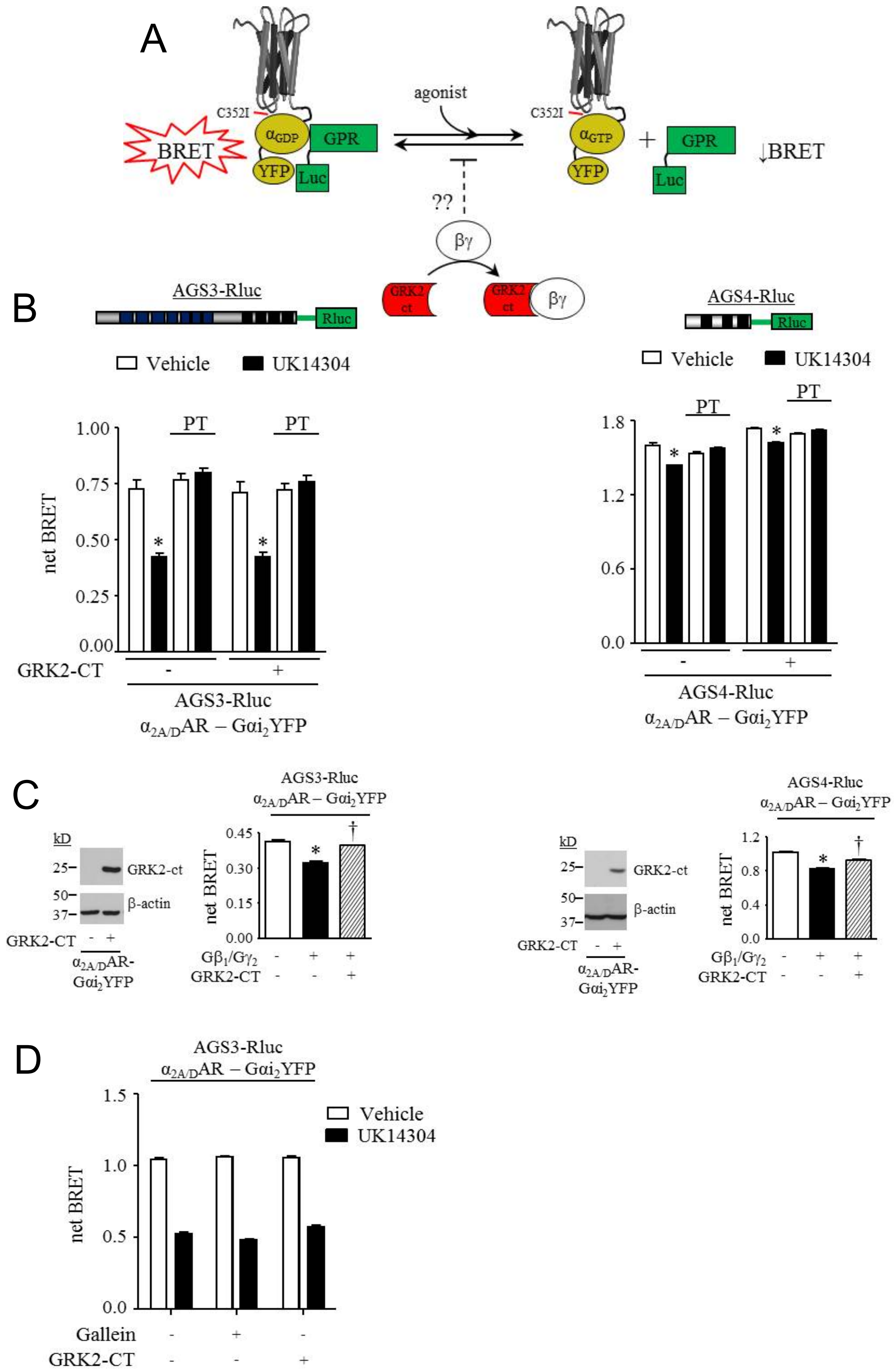


Figure 2.4. Influence of a Gβγ scavenger on the agonist-induced regulation of GαiGPR where Gαi is tethered to the receptor

(A) Schematic representing approach taken to determine the effect of endogenous Gβγ subunits, which may engage the $\alpha_{2A/D}$-AR-Gα_i2YFP fusion protein resulting in altered BRET signals. GRK2-CT was expressed as a "sink" for free Gβγ released subsequent to receptor activation. Agonist bound to the receptor is denoted by an asterisk (*).

(B) Net BRET values obtained from HEK293 cells expressing AGS3-Rluc (*left panel*) or AGS4-Rluc (*right panel*) and $\alpha_{2A/D}$AR-Gαi2YFP as described in "Experimental Procedures." Where indicated, cells also expressed GRK2-CT. Cells were incubated with vehicle (Tyrode's solution) or UK14304 (10 μM) for 5 minutes. For experiments involving PTX, cells were incubated with PTX (100 ng/mL) for 18 hours prior to agonist exposure. (*B, left panel*): RLU: AGS3-Rluc – 195,791 ± 15,175; AGS3-Rluc + PTX – 178,887 ± 24,596; AGS3-Rluc + GRK2-CT – 218,392 ± 12,663; AGS3-Rluc + GRK2-CT + PTX – 220,238 ± 19,824. RFU: $\alpha_{2A/D}$AR-Gαi2YFP – 110,414 ± 2,294; $\alpha_{2A/D}$AR-Gαi2YFP + PTX – 104,532 ± 2,263; $\alpha_{2A/D}$AR-Gαi2YFP + GRK2-CT -106,967 ± 2,562; $\alpha_{2A/D}$AR-Gαi2YFP + GRK2-CT + PTX – 116,045 ± 3,266. (*B, right panel*) RLU: AGS4-Rluc – 147,140 ± 7,740; AGS4-Rluc + PTX – 150,290 ± 8,165; AGS4-Rluc + GRK2-CT – 155,576 ± 8,972; AGS4-Rluc + GRK2-CT + PTX – 147,944 ± 10,565. RFU: $\alpha_{2A/D}$AR-Gαi2YFP – 109,090 ± 2,942; $\alpha_{2A/D}$AR-Gαi2YFP + PTX – 112,983 ± 3,019; $\alpha_{2A/D}$AR-Gαi2YFP + GRK2-CT – 124,288 ± 2,273; $\alpha_{2A/D}$AR-Gαi2YFP +

GRK2-CT + PTX – 112,371 ± 2,189. *, $p < 0.05$ compared with vehicle treated control group.

(C) Lysates (50 µg) from a representative experiment as described in *B* were subjected to SDS-PAGE and immunoblotting with GRK2 and β-actin antisera as indicated (*far left panel*). HEK293 cells expressing AGS3-Rluc (10 ng plasmid) and $\alpha_{2A/D}$AR-Gαi2YFP (250 ng plasmid) in the absence and presence of $G\beta_1$, $G\gamma_2$ and/or GRK2-CT (500 ng each plasmid) as indicated were subjected to BRET measurements as described in "Experimental Procedures" (*left panel*). Lysates (50 µg) from a representative experiment as described in the upper panel of *B* were subjected to SDS-PAGE and immunoblotting with GRK2 and β-actin antisera as indicated (*right panel*). HEK293 cells expressing AGS4-Rluc (2 ng plasmid) and $\alpha_{2A/D}$AR-Gαi2YFP (250 ng plasmid) in the absence and presence of $G\beta_1$, $G\gamma_2$ and/or GRK2-CT (500 ng each plasmid) as indicated for 48h were subjected to BRET measurements as described in "Experimental Procedures" (*far right panel*).

(D) Net BRET signals were obtained from HEK cells transfected with AGS3-Rluc (10 ng plasmid) and α_{2A}-AR-Gαi2YFP (750 ng plasmid). BRET signals were measured as described in "Experimental Procedures." Cells were also transfected with GRK2-CT (500 ng plasmid) as indicated or incubated with a pharmacological Gβγ inhibitor, gallein (10µM), for 30 minutes prior to receptor stimulation, followed by two subsequent washes with Tyrode's solution to remove any remaining fluorescent gallein from the solution. Vehicle (Tyrode's solution) or α_{2A}-AR agonist UK14304 (10 µM) were added to cells as indicated followed by

fluorescence and luminescence readings as described in “Experimental Procedures.”

All BRET data are expressed as means ± S.E. from at least 3 independent experiments with triplicate determinations (N=9) and immunoblots are a representative image of three independent experiments. *, $p < 0.001$ compared with control group. †, $p < 0.001$ compared with $G\beta_1\gamma_2$-expressing group based on Tukey’s post hoc test following ANOVA.

Figure 2.5

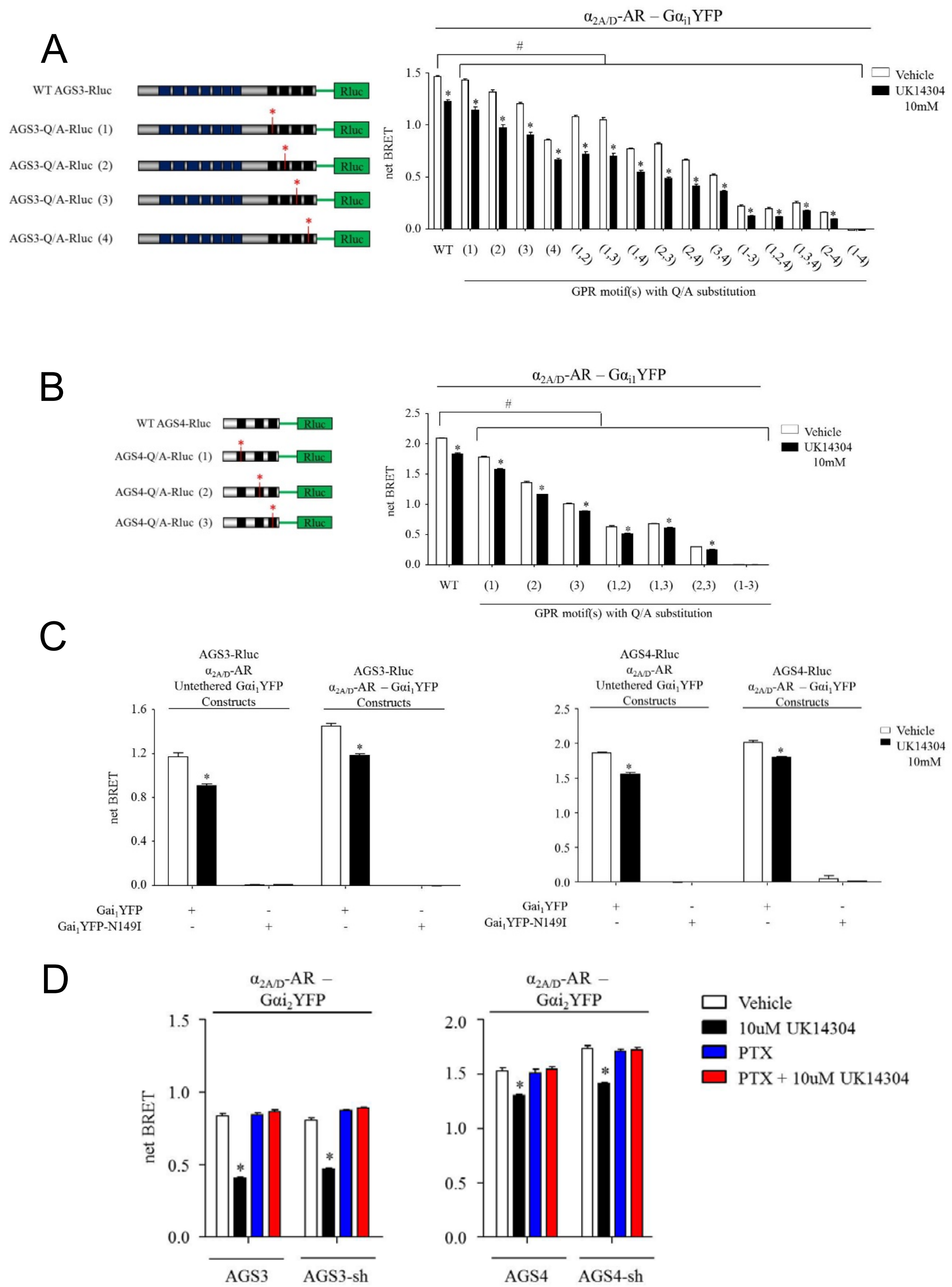


Figure 2.5. Single mutations in the GPR motifs progressively reduce association of GPR proteins with Gαi, while the amino terminus of AGS3 and AGS4 does not effect this regulation

(A) Schematic representing the $phRLuc_{N3}$::AGS3 constructs used in the following experiment that contain Q/A point mutations in each subsequent or a combination of the four GPR motifs (AGS3-Q/A) inhibiting the binding of Gαi to the mutated domains (*left panel)*. Net BRET measurements from HEKs transfected with 750 ng pcDNA3:: α_{2A}-AR-Gαi1YFP and 10 ng $phRLuc_{N3}$::AGS3 or 10 ng of $phRLuc_{N3}$::AGS3 constructs containing Q/A point mutations in each subsequent or a combination of the four GPR motifs (AGS3-Q/A). BRET signals were measured as described in "Experimental Procedures." Vehicle (Tyrode's solution) or α_{2A}-AR agonist UK14304 (10 µM) were added to cells as indicated followed by fluorescence and luminescence readings as described in "Experimental Procedures" (*right panel).*

(B) Schematic representing the $phRLuc_{N3}$::AGS4 constructs used in the following experiment that contain Q/A point mutations in each subsequent or a combination of the three GPR motifs (AGS4-Q/A) inhibiting the binding of Gαi to the mutated domains (*left panel)*. Net BRET measurements from HEKs transfected with 750 ng pcDNA3:: α_{2A}-AR-Gαi1YFP and 2 ng $phRLuc_{N3}$::AGS4 or 2 ng of $phRLuc_{N3}$::AGS4 constructs containing Q/A point mutations in each subsequent or a combination of the three GPR motifs (AGS4-Q/A). BRET signals were measured as described in "Experimental Procedures." Vehicle (Tyrode's solution) or α_{2A}-AR agonist UK14304 (10 µM) were added to cells as indicated

followed by fluorescence and luminescence readings as described in "Experimental Procedures" (*right panel).*

(C) Net BRET measurements from HEKs transfected with untethered Gαi1YFP or Gαi1YFP^{N149I} (750 ng each plasmid) and α_{2A}-AR (500 ng) or fusion proteins α_{2A}-AR-Gαi1YFP or α_{2A}-AR-Gαi1YFP^{N149I} (750 ng each plasmid) in the presence of AGS3-Rluc (10 ng, *left panel*) or AGS4-Rluc (2 ng, *right panel*). BRET signals were measured as described in "Experimental Procedures." Vehicle (Tyrode's solution) or α_{2A}-AR agonist UK14304 (10 μM) were added to cells as indicated followed by fluorescence and luminescence readings as described in "Experimental Procedures."

(D) Net BRET measurements from HEKs transfected with 750 ng pcDNA3:: α_{2A}-AR-Gαi2YFP and 10 ng phRLuc$_{N3}$::AGS3, 2 ng phRLuc$_{N3}$::AGS3-short (AGS3-sh) (*left panel)*, 2ng phRLuc$_{N3}$::AGS4 or 2 ng phRLuc$_{N3}$::AGS4-short (AGS4-sh) (*right panel)*. BRET signals were measured as described in "Experimental Procedures." Vehicle (Tyrode's solution) or α_{2A}-AR agonist UK14304 (10 μM) were added to cells as indicated followed by fluorescence and luminescence readings as described in "Experimental Procedures" Cells were treated with pertussis toxin (PTX, 100 ng/mL) 18 hours prior to receptor stimulation where indicated.

All data are expressed as means ± SEM from at least 3 independent experiments with triplicate determinations (N=9). Notations of * signify p-values <0.001 compared to vehicle stimulated controls, and # signifies p-value <0.01 compared

to vehicle treated wild-type AGS constructs, respectively, based on Tukey's post hoc test following ANOVA.

Our data suggest that a 7TM receptor couples directly to a GαiGPR complex, ostensibly promoting exchange of GDP for GTP in a manner that may be similar to 7TM receptor engagement of Gαβγ heterotrimer. Agonist-mediated activation of a 7TM receptor coupled to GαiGPR apparently results in reversible dissociation of the GPR protein from Gαi (Oner et al 2010a, Oner et al 2010b). Upon termination of agonist-induced activation, the GPR protein then re-associates with $G\alpha i_{GDP}$, representing a cycle that is conceptually analogous to the Gαβγ activation – deactivation cycle (Oner et al 2010a, Oner et al 2013a, Oner et al 2010b, Oner et al 2013c). There are several interesting conceptual thoughts that emanate from this work. As the regulation of both the GαiGPR complex and the Gαiβγ heterotrimer is PTX sensitive (Figures 2.1, 2.2, 2.3) (Oner et al 2010a, Oner et al 2010b), this raises the intriguing possibility that functional effects associated with PTX may be mediated in part by 7TM regulation of Gαi-GPR complexes. Secondly, as Group II AGS proteins may complex with multiple Gα subunits simultaneously (Figure 2.5) (Adhikari & Sprang 2003, Bernard et al 2001, Jia et al 2012, Kimple et al 2004), AGS3 and AGS4 may scaffold receptors and Gα subunits within a larger signaling complex (Blumer & Lanier 2014, Jahangeer & Rodbell 1993). Finally, of particular interest, the coupling of a receptor to the GαGPR complex or the Gαβγ heterotrimer may represent differentially regulated pathways preferred by particular hormones, neurotransmitters and small molecules.

Identification of novel interacting proteins for AGS4

AGS4 was selected to define potential interacting partners due to the lack of distinct protein interaction domains preceding the three GPR motifs as are present in other Group II AGS proteins (Cao et al 2004, Takesono et al 1999). To address the hypothesis that AGS4 binding partners regulate its subcellular location or function, cells stably expressing an amino-terminal tandem affinity purification (NTAP) tag linked to AGS4 (NTAP AGS4) were generated and demonstrated effective pulldown of known binding partners of AGS4, Gαi1/2 and Gαi3, thus validating my approach (Figure 2.6A & B). Three bands that specifically bound to NTAP-AGS4 were observed subsequent to resolving the eluate by gel electrophoresis and staining with Coomassie blue (Figure 2.6C). Trypsin digestion and mass spectrometry analysis of these bands demonstrated the presence of three protein classes including eukaryotic elongation factor 1d (eEF1d), AT-rich interacting domain 1b (ARID1b), and 14-3-3 isoforms (Figure 2.6C). Subsequent to my obtaining these results, a report implicated AGS4 binding to 14-3-3 isoforms that affected the subcellular localization of AGS4 (Giguere et al 2012b). Additionally, previous yeast-two hybrid screens using AGS4 as bait identified two proteins, eEF2 and ARID2 (mKIAA1557) (Cao 2005). Interestingly, these two proteins are closely related to eEF1d and ARID1b, respectively, which were identified in my NTAP-AGS4/mass spectrometry approach (Figure 2.6). Using GST tagged eEF2 and GST tagged ARID2, AGS4 was precipitated validating the association of AGS4 with these two binding partners (Cao 2005). An interesting study demonstrated that eEF2 kinase, one of

the kinase that phosphorylates eEF2 protein, binds directly and is activated by a GPCR (Park et al 2008b). Phosphorylation of eEF2 results in inhibition of protein synthesis, and it is interesting to speculate that this may be a form of regulation of protein synthesis by GPCRs. Although the site of interaction between AGS4 and eEF2 does not contain the residue that is phosphorylated by eEF2K or the nucleotide binding domain, the interaction site does coincide with the site on eEF2 known to be ADP-ribosylated and inactivated by diphtheria toxin (Cao 2005, Van Ness et al 1980). Therefore, AGS4 may act as an inhibitory molecule to eEF2 protein and result in decreased protein synthesis. The binding of ARID family members with AGS4 was quite unexpected. The ARID proteins identified are rather large proteins involved in the SWI/SNF chromatin remodeling complex of cells (Mohrmann et al 2004, Wang et al 2004). One possibility is that AGS4 binds ARID proteins in a manner to exclude them from the nucleus, resulting in altered transcription within the cell. These potential regulatory roles of AGS4 in protein synthesis and gene transcription were unanticipated, but require further studies to delineate the true nature and biological functions of these interactions.

Figure 2.6

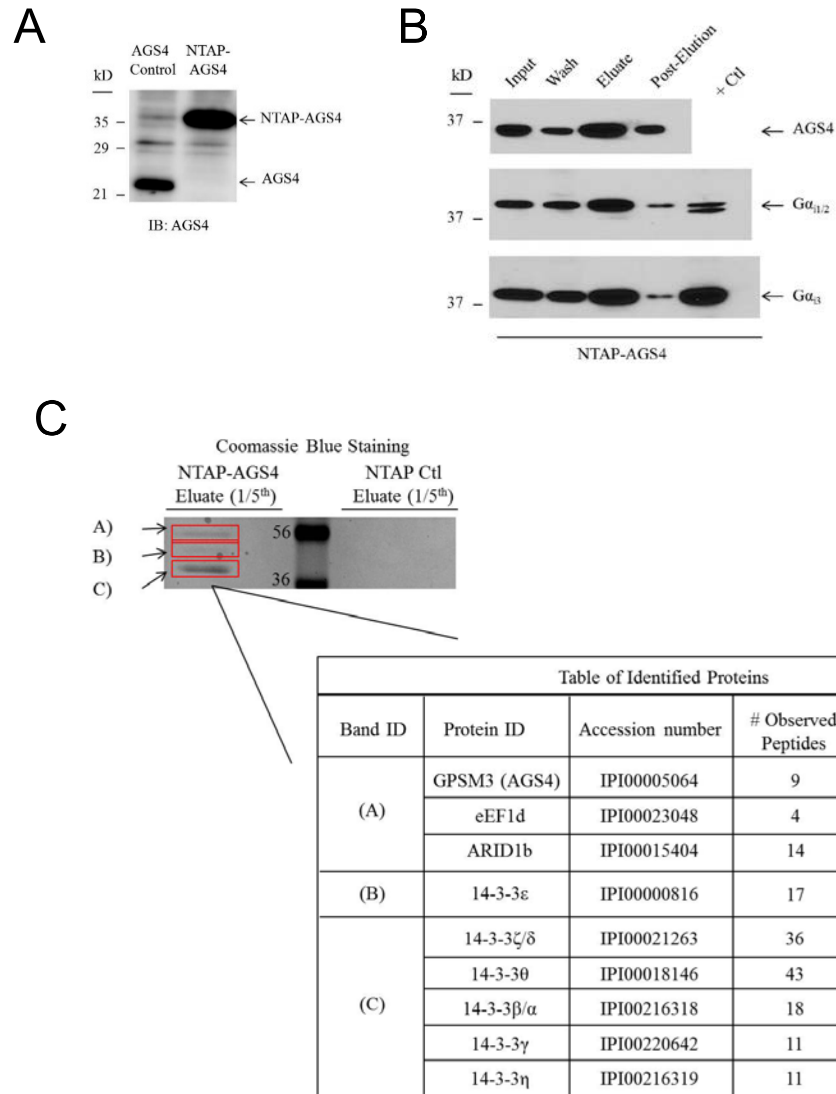


Table of Identified Proteins

Band ID	Protein ID	Accession number	# Observed Peptides	Percent Coverage
(A)	GPSM3 (AGS4)	IPI00005064	9	45.00
	eEF1d	IPI00023048	4	4.27
	ARID1b	IPI00015404	14	2.64
(B)	14-3-3ε	IPI00000816	17	29.02
(C)	14-3-3ζ/δ	IPI00021263	36	53.88
	14-3-3θ	IPI00018146	43	36.33
	14-3-3β/α	IPI00216318	18	36.18
	14-3-3γ	IPI00220642	11	25.91
	14-3-3η	IPI00216319	11	17.07

Figure 2.6. Identification of AGS4 interacting proteins by tandem affinity purification (TAP)

(A) Depicted is a representative immunoblot of lysates (50 μg) from HEK cells stably expressing pglue NTAP::AGS4 or transiently transfected with 1.5 μg pcDNA3::AGS4 using AGS4-specific antisera as described in "Experimental Procedures."

(B) Depicted is a representative immunoblot of lysates from NTAP-AGS4 stably expressing HEK cells processed through the tandem affinity purification (TAP) system. Samples are loaded fractionally (Input 1/200th, Wash 1/40th, Eluate 1/40th, Pose-Elution 1/20th) and control HEK cells transfected with 750 ng pcDNA3::Gαi2 or 750 ng pcDNA3::Gαi3 are loaded are loaded at 50 μg per lane. Immunobloting was conducted as described in "Experimental Procedures" with AGS4-specific antisera, $G\alpha_{i1/2}$ antisera, and $G\alpha_{i3}$ antisera.

(C) TAP eluates were subjected to polyacrylamide gel electrophoresis followed by coomassie blue staining as described in "Experimental Procedures." Bands were excised & sent to the MUSC mass spectrometry facility for protein identification. Illustrated is a tabular representation of the mass spectrometry results for each band.

Depicted immunoblots are a representative image of at least three independent experiments.

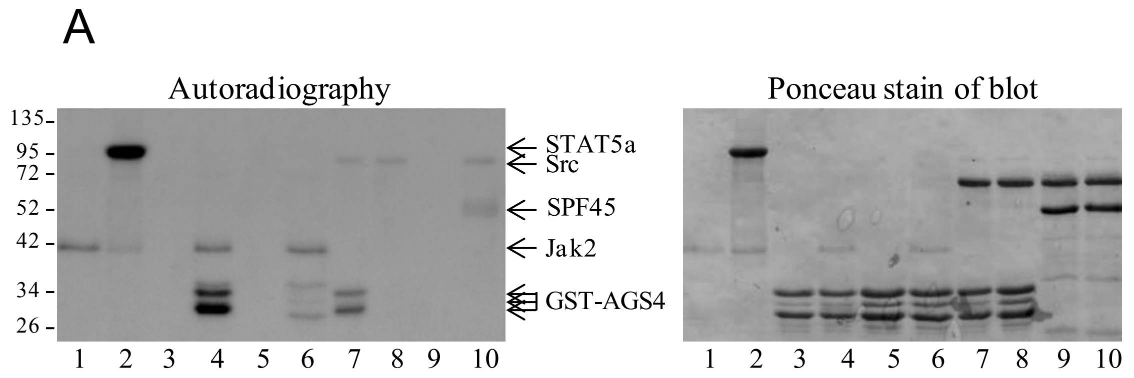
Decreased Gαi interaction with AGS4 upon phosphorylation of single tyrosine by JAK2 and Src kinases

Phosphorylation is another form of regulation seen in proteins containing GPR motifs (Blumer et al 2003, Groves et al 2010, Hollinger et al 2003, Johnston et al 2009). The importance of post-translational modification of the linker region between TPR and GPR motifs of Pins (ortholog of LGN and AGS3 in mammals) was illustrated through phosphorylation by Aurora kinase-A , which was required to assemble the Discs large (Dlg) / Khc-73 polarity complex to properly orient the mitotic spindle in *Drosophila* cells (Johnston et al 2009). Additionally, the phospho-mutant of AGS3 with all GPR serine and threonine amino acids mutated to alanine showed defective ability of these proteins to induce macroautophagy (Groves et al 2010). Previous studies also demonstrated phosphorylation of the GPR motif in AGS3 inhibits GDI activity, while phosphorylation just upstream of the RGS14 GPR motif demonstrated increased GDI activity as measured by decreased GTPγS binding of Gαi subunits (Blumer et al 2003, Hollinger et al 2003). Indeed, AGS4 is a known phospho-protein, with multiple residues demonstrated to be phosphorylated (Giguere et al 2012b, Rush et al 2005, van Bodegom et al 2012, Zarling et al 2000, Zhong et al 2012). As an initial platform to determine the functional consequence of AGS4 phosphorylation, we generated Rluc tagged AGS4 constructs with serine and tyrosine residues known to be phosphorylated and mutated them to aspartic acid to mimic phosphorylation. Using our BRET system, mutation of tyrosine 108 to aspartic acid (Y108) resulted in a dramatic decrease in G-protein binding compared to

wild-type AGS4 (Figure 2.7B). Interestingly, AGS4-Y108 is phosphorylated in response to thymic stromal lymphopoietic protein (TSLP), and this residue is a consensus phosphorylation site for tyrosine kinases Janus kinase 2 (JAK2) and Src, which are activated by TSLP (van Bodegom et al 2012, Zhong et al 2012). In addition, Janus kinase 2 (JAK2) and Src kinase are reported to be activated either by $G\alpha i_{GTP}$ or directly by some GPCRs (Corre et al 1999, Ma et al 2000, Marrero et al 1995, Vila-Coro et al 1999). As an initial approach to determine the phosphorylation of AGS4 by JAK2 and Src, we used an *in vitro* kinase assay with purified, recombinant active JAK2 or Src and GST-AGS4, GST-AGS4-Y108F, or GST as substrates. Interestingly, both JAK2 and Src were observed to phosphorylate wildtype AGS4 but not AGS4-Y108F (Figure 2.7A). Taken together, these data suggest that the activation of JAK2 and Src results in phosphorylation of AGS4-Y108 resulting in a decrease of the Gαi – GPR interaction. The regulatory function mediated by JAK2 and Src could signify a positive feedback loop of the kinases in response to activation by Gαi and or GPCRs counteracting the GDI activity of AGS4 and possibly other GPR proteins (Corre et al 1999, Ma et al 2000, Marrero et al 1995, Vila-Coro et al 1999). Positive regulation of kinase activity may result in prolonged activation of JAK2 and Src causing enhanced activation of downstream targets for the kinases including signal transducer and activator of transcription 1 and 3 (STAT1 & STAT3), thereby promoting gene transcription and cellular growth (Cao et al 1996, Darnell et al 1994).

Figure 2.7

A



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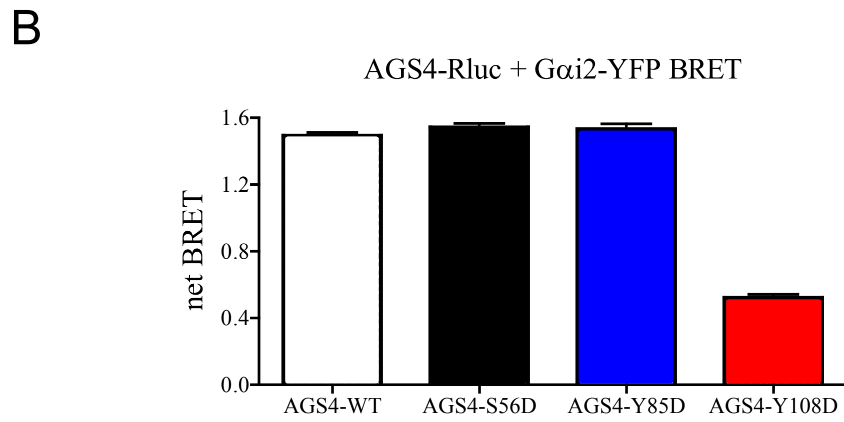


Figure 2.7. AGS4 is phosphorylated on Tyr108 by JAK2 and Src *in vitro*, which profoundly inhibits the AGS4 – Gαi interaction

(A) (*Left panel*) *In vitro* kinase assay with purified active JAK2 (Leu^{808}-Gly^{1132}) and Src kinases and recombinant purified GST-AGS4 and GST-AGS4-Y108F for 30min at 25C. Legend: 1) JAK2 only; 2) JAK2 + STAT5a positive control; 3) GST-AGS4 only (no kinase control); 4) GST-AGS4 + JAK2; 5) GST-AGS4-Y108F only (no kinase); 6) GST-AGS4-Y108F + JAK2; 7) GST-AGS4 + Src; 8) GST-AGS4-Y108F + Src; 9) His-SPF45 + Src no ATP control; 10) His-SPF45 + Src positive control. (*Right panel)* Ponceau S staining of the same nitrocellulose membrane used for the autoradiograph from the left panel. *In vitro* kinase assay performed with Dr. Scott Eblen

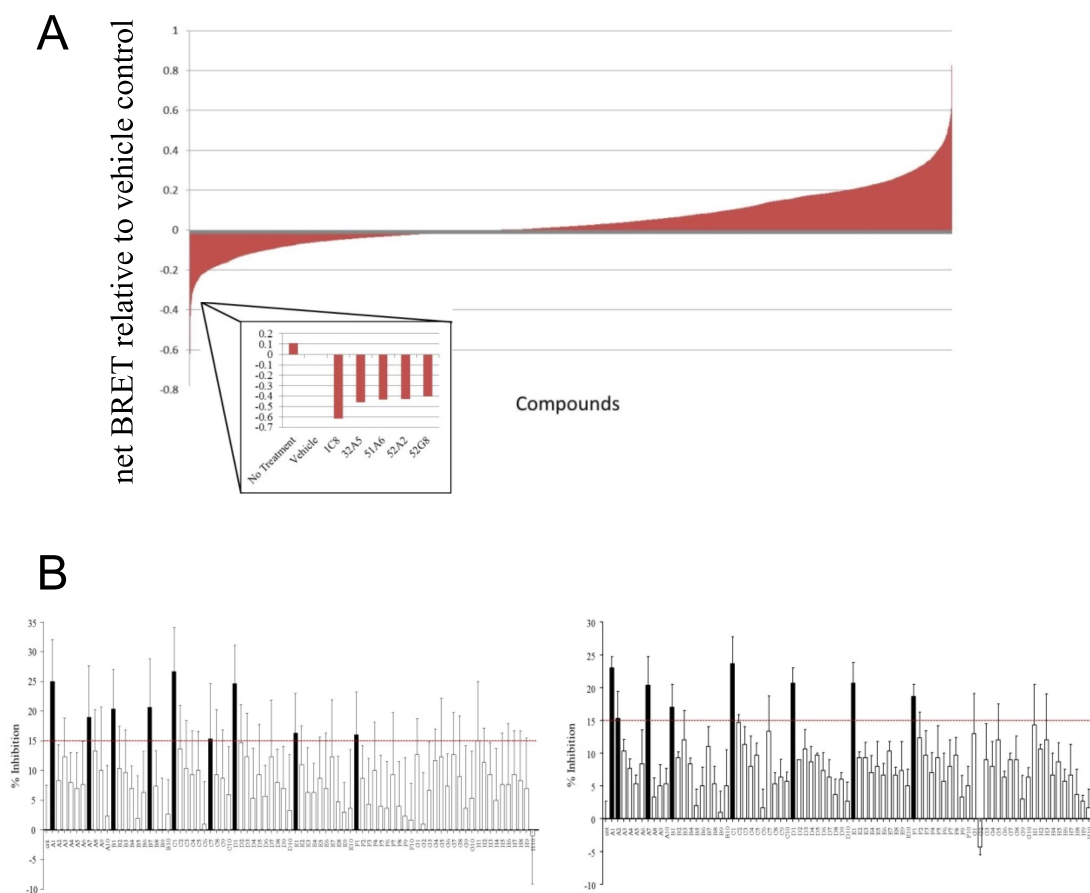
(B) HEK293 cells were transfected with 2ng AGS4-Rluc-WT, -S56D, -Y85D or –Y108D + 500ng Gαi2-YFP. Forty-eight hours after transfection, BRET measurements were taken as described in Experimental Procedures. Whereas Asp-mutations of S56 or Y85 had no effect, the placement of a negatively charged residue mimicking phosphorylation on Y108 reduced the AGS4 – Gαi interaction by nearly 70%.

BRET screening platform for small molecule modulators of the Gαi–GPR interaction

The Gαi – GPR interaction appears to be a dynamic and regulated event in signaling pathways of immune cells where perturbation of this complex results in immunological pathologies; furthermore, deficiency in AGS4 recently demonstrated an immunoprotective phenotype inhibiting the progression of arthritis in mice (Branham-O'Connor et al 2014, Cao et al 2004, Giguere et al 2013, Kamakura et al 2013, Zhao et al 2010). Therefore, using our AGS4-Rluc – Gαi1-YFP BRET system as a platform, we screened the 50,080 ChemBridge DIVERSet compound library for potential modulators of the Gαi – AGS4 interaction. Initial results from wells containing pools of ten compounds identified compounds in eight wells with the highest inhibitory properties (Figure2.8A). These 80 compounds were then individually assessed for inhibition of Gαi interaction with AGS3 and AGS4 by BRET to demonstrate specificity of these compounds for the Gαi – GPR module. From this pool, seven molecules were found to retain inhibitory action against the Gαi interaction with either AGS3 or AGS4 (Figure 2.8B). The subsequent development of these compounds will be an asset to the field in the investigation of the Gαi – GPR interaction in other members of the Group II AGS proteins either as a reagent or possibly in the development of therapeutics for pathologies linked to GPR proteins, e.g. drug

addiction and craving, ischemia reperfusion injury, polycystic kidney disease, blood pressure control, energy expenditure and metabolism, and rheumatoid arthritis and inflammatory pathways (Blumer et al 2008, Bowers et al 2004, Branham-O'Connor et al 2014, Conley & Watts 2013, Giguere et al 2013, Kwon et al 2012, Lee et al 2010, Nadella et al 2010, Regner et al 2011, Yao et al 2005).

Figure 2.8



C

$G\alpha_i$ – GPR Inhibitory Compounds		
Well ID	Chemical ID	Structure
A1	2,2,4-trimethyl-1,2-dihydro-6-quinolinyl 4-(acetylamino)benzenesulfonate	
A7	4-(4-methoxy-3-methylbenzyl) morpholine	
B1	N-(3-fluorophenyl)-2-{[1-(4-methoxyphenyl)-2,5-dioxo-3-pyrrolidinyl]thio}acetamide	
C1	1-[(5-bromo-2-thienyl)sulfonyl] piperidine	
D1	N-(4-methoxyphenyl)-N'-(3-nitrophenyl)thiourea	
E1	2-[5-(benzylthio)-1,3,4-oxadiazol-2-yl] pyridine	
F1	sodium 4-(2-methyl-1H-indol-3-yl)-4-oxobutanoate	

$G\alpha_i$ – AGS3 Specific Inhibitory Compounds		
Well ID	Chemical ID	Structure
B7	[3-(1-naphthyloxy) propyl]hydrazine	
C7	N-(6-butyl-1,3-benzothiazol-2-yl)nicotinamide	

$G\alpha_i$ – AGS4 Specific Inhibitory Compounds		
Well ID	Chemical ID	Structure
A2	N'-benzylidene-4-tert-butylbenzohydrazide	

Figure 2.8. Identification of Gαi – GPR small molecule inhibitors

(A) Net BRET measurements from HEK cells stably expressing pcDNA3::Gαi1YFP transfected with 2 ng $phRLuc_{N3}$::AGS4. Cells were treated with 50,080 compounds (distributed along the x-axis) in 10 compound pools at 1μM for 1hr at 37°C, 5% CO_2 prior to fluorescence and luminescence readings as described in "Experimental Procedures." Data are represented as the Gαi – AGS4 net BRET from drug-treated cells ***minus*** the net BRET measurement for vehicle only (DMSO) control (mean net BRET value 0.81 ± 0.25). Data are expressed as means ± SEM with triplicate determinations.

(B) Net BRET measurements from HEK cells transfected with 750 ng pcDNA3::Gαi1YFP and 10 ng $phRLuc_{N3}$::AGS3 (*left panel)* or 2 ng $phRLuc_{N3}$::AGS4 (*right panel)*. Cells were treated with 80 compounds (identified in from the initial screening in pooled formulation to have the most pronounced inhibition) at 10 μM for 1hr at 37°C, 5% CO_2 prior to fluorescence and luminescence readings as described in "Experimental Procedures." Data are expressed as means ± SEM with triplicate determinations and are represented in percent inhibition compared to DMSO control; red dotted line depicts 15% inhibition of BRET signal.

(C) Chemical names and structures of the compounds identified from the ChemBridge DIVERSet library to have the largest inhibition of the Gαi – GPR interaction (*left*) or inhibitory action on Gαi – AGS3 or Gαi – AGS4 interactions specifically (*right*) utilizing http://www.hit2lead.com for identification.

Chapter 3

Defective Chemokine Signal Processing in Leukocytes Lacking Activator of G Protein Signaling 3 (AGS3) and Activator of G-protein Signaling (AGS4)

***Note: This chapter contains a portion of the paper:**

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Defective Chemokine Signal Integration in Leukocytes Lacking Activator of G Protein Signaling 3 (AGS3).

Branham-O'Connor M, Robichaux III WG, Zhang XK, Cho H, Kehrl JH, Lanier SM, and Blumer JB

Introduction

Integration of signals emanating from chemokine receptors represents one of the most commonly used mechanisms for leukocyte distribution and recruitment to lymphoid organs and the periphery. Such signal integration involves not only the regulated expression of individual chemokine receptors and the stoichiometries of the core signaling triad of receptor, heterotrimeric G-protein and effectors but also cell-type-specific accessory proteins which modulate signals across this core signaling system. Such accessory proteins bestow upon leukocytes and other cells the ability to tightly control signaling pathways to maximize signal efficiency, strength, and duration while at the same time providing flexibility to quickly adapt to changes in environmental stimuli (Cho & Kehrl 2009, Sato et al 2006a).

Perturbations of heterotrimeric G-protein signal input or duration result in defective leukocyte development, trafficking, motility and overall chemokine responsiveness (Cho et al 2012, Han et al 2005, Hwang et al 2007, Pero et al 2007, Rudolph et al 1995, Skokowa et al 2005, Zarbock et al 2007). In addition, accessory proteins at the GPCR – G-protein interface also play key roles in regulating leukocyte function by modulating G-protein activity and responsiveness to chemokines. Many regulators of G-protein signaling (RGS) proteins are expressed in leukocytes (Cho & Kehrl 2009, Moratz et al 2004a) and play important functions in regulating chemokine responsiveness. For example,

RGS1 plays an important role in modulating lymphocyte motility and trafficking (Han et al 2005). RGS1$^{-/-}$ lymphocytes move more rapidly in response to chemokines, suggesting that modulating the duration of Gαi activation in response to chemokines plays an important role in leukocyte activation and trafficking (Han et al 2005). Additional mechanisms for modulation of Gαi activity are also likely important for spatio-temporal regulation of leukocyte responsiveness and for integration of signals from multiple chemokines at any given time.

Another group of accessory proteins, the Activators of G-protein Signaling (AGS) proteins, were identified in a yeast-based functional screen for receptor-independent activators of heterotrimeric G-proteins (Cismowski et al 1999, Takesono et al 1999) and can be broadly categorized into three groups based on their input into the G-protein activation/deactivation cycle (Blumer et al 2007). Group II AGS proteins are characterized by the presence of up to four G-protein regulatory (GPR) motifs (also referred to as LGN or GoLoco motifs (Ponting 1999, Siderovski et al 1999)) which bind free Gαi/o/t subunits in the GDP-bound conformation and act as guanine nucleotide dissociation inhibitors (GDI) (Blumer et al 2012, McCudden et al 2005a). GPR motif proteins thus provide a novel mode of signal input to heterotrimeric G-proteins that may operate distinct from the super-family of G-protein coupled receptors (GPCR) and may also function as binding partners for Gαi subunits independent of heterotrimer formation. These thoughts have broad implications for signal processing and provide a

mechanism for unexpected functions of G-proteins as signal transducers within the cell.

A member of the Group II AGS proteins, AGS3 (gene name – G-protein signaling modulator-1 (Gpsm1)) contains seven tetratricopeptide repeats (TPR) which are involved in protein-protein interactions and four G-protein regulatory (GPR)/GoLoco motifs, allowing AGS3 to simultaneously bind up to four $G\alpha i_{GDP}$ subunits free of Gβγ. Previous data suggest functional roles for AGS3 in such diverse processes as neuronal plasticity and addiction, autophagy, membrane protein trafficking, polycystic kidney disease, cardiovascular regulation and metabolism (Blumer et al 2008, Bowers et al 2008, Bowers et al 2004, Fan et al 2009, Groves et al 2007, Kwon et al 2012, Nadella et al 2010, Pattingre et al 2003, Regner et al 2011, Vural et al 2010, Yao et al 2005). As part of an expanded approach to more fully understand the *in vivo* role of AGS3 in G-protein signal processing, we previously reported the generation of a conditional AGS3 null mouse strain ($Gpsm1^{-/-}$), which is a valuable model to dissect physiological functions of AGS3 (Blumer et al 2008, Kwon et al 2012, Regner et al 2011).

A second, less investigated, member of the Group II AGS proteins is AGS4 (gene name – G protein signaling modulator-3 (Gpsm3)). Unlike AGS3, AGS4 does not possess well-defined protein interaction domains and contains three GPR motifs for binding $G\alpha i_{GDP}$ free of Gβγ (Cao et al 2004). Although lacking defined protein interaction domains, the amino terminus of AGS4 is reported to confer GEF activity on Gαi/o (Zhao et al 2010), while another study

suggested that this segment could bind Gβ subunits (Giguere et al 2012c); this is in contrast to earlier biochemical analysis illustrating that AGS4 competes for Gαi binding with Gβγ (Cao et al 2004, Giguere et al 2012c, Oner et al 2010b, Zhao et al 2010). Although not extensively investigated, one fundamental characteristic of AGS4 is a restricted expression profile to immunological tissues (Cao et al 2004, Giguere et al 2013, Giguere et al 2014, Zhao et al 2010). Loss of AGS4 inhibited progression of arthritis induction (Giguere et al 2013). Additionally, cytokine signaling through thymic stromal lymphopoietic protein (TSLP) was found to phosphorylate AGS4, suggesting a possible regulatory mechanism for AGS4 in this immunological signaling cascade (van Bodegom et al 2012, Zhong et al 2012). Moreover, AGS4 mRNA expression is observed to be similar or even more pronounced in additional immunological cell lineages including dendritic cells, T leukocytes, and neutrophils (www.immgen.org and www.biogps.org) and was demonstrated to be decreased after anti-inflammatory treatment (Schmidt et al 2012). In this study, we used an AGS4 null (Gpsm3$^{-/-}$) mouse model as an initial approach to understand the functional role of AGS4 in chemokine-induced signal processing in leukocytes.

Our goal in this study was to define the functional roles of AGS3 and AGS4 in leukocytes, beginning with their roles in chemokine receptor signal processing. Our data suggest that AGS3 and AGS4 play key roles in the integration of signals from the receptor to the chemotactic machinery, including ERK1/2 phosphorylation and leukocyte motility. Neutrophils, which demonstrate the highest level of AGS4 expression, require AGS4 for maximal response to

chemoattractants, and an *in vivo* model of inflammation demonstrated the importance of AGS4 in migration of neutrophils to sites of acute inflammation. These data indicate key roles for GPR proteins AGS3 and AGS4 in the integration of chemokine receptor signaling and expand the functional repertoire of accessory proteins in the immune system.

Experimental Procedures

Materials and reagents:

Pertussis toxin, β-actin antibody (A5441), lipopolysaccharides (LPS) from *E. coli* 0111:B4 (L4391), N-Formyl-Met-Leu-Phe (fMLP) (F3506), AMD3100 (A5602), and Thioglycollate Broth (USP Alternative, 70157) were purchased from Sigma-Aldrich (St. Louis, MO). Recombinant mouse GM-CSF, CXCL12 and CCL19 were obtained from BioAbChem Inc. (Ladson, SC). AGS3-antisera generated by immunization of rabbits with a GST-AGS3 fusion protein encoding the GPR domain (Ala^{461}–Ser^{650}) of AGS3 was kindly provided by Dr. Dzwokai Ma (University of California, Santa Barbara, CA, described in (Groves et al 2010)). AGS4 antibody (AP5725c), anti-phospho-ERK (Tyr^{402}), and total ERK were purchased from Abgent (San Diego, CA), Santa Cruz Biotechnology (Dallas, TX) and Abcam (Cambridge, MA), respectively. Protease inhibitor cocktail tablets (Complete Mini) were obtained from Roche Applied Science. ACK Lysing Buffer (0.15 M NH_4Cl/ 0.01M $KHCO_3$/10 μM EDTA, 10-548E) was obtained from Lonza (Basel, Switzerland) and Percoll™ (17-089-02) from GE Healthcare Life Sciences (Pittsburgh, PA). Dynabeads® Untouched™ Mouse T Cells kit (11413D) was purchased from Invitrogen Life Technologies (Grand Island, NY). Corning HTS Transwell®-96 well plates (09-761-83) as well as other materials and media for cell culture were obtained from Fisher Scientific (Waltham, MA). Conjugated antibodies FITC-CD11b (557396), isotype FITC-Rat IgG_{2a},κ (553929), PE-Ly-6G (551461), and isotype PE-Rat IgG_{2b},κ (553989) were

purchased from BD Biosciences (San Jose, CA).Other materials were obtained as described elsewhere (Oner et al 2010a, Oner et al 2010b).

Mouse models:

Gpsm1$^{-/-}$ mice used in this study were generated as previously described (Blumer et al 2008). Wild-type and Gpsm1$^{-/-}$ female littermates at 6-12 weeks of age from Gpsm1$^{+/-}$ intercrosses were used. Gpsm1$^{+/-}$ breeding pairs were generated from backcrosses onto C57BL/6J mice for more than 12 generations. Gpsm3$^{-/-}$ mice generated in the C57BL/6 background were obtained through the Knockout Mouse Project (KOMP) consortium. Wild-type and Gpsm3$^{-/-}$ littermates at 6-12 weeks of age from Gpsm3$^{+/-}$ intercrosses were used. Genotyping of these mice was performed using a three-primer polymerase chain reaction (PCR) using the following primer set: mgAGS4 16651 forward primer 5'-TGA CGG GTG GAC ACA GGA GAC TTG GGA AAG-3'; Common 3' forward (universal RAF5 forward) 5'-CAC ACC TCC CCC TGA ACC TGA AA-3'; CSD-Gpsm3-SR1 5'-CAG GGA AAG TGG GTG GTA AAT ACA G-3'. Tissues and lysates were prepared and processed for immunoblotting as described (Blumer et al 2002) .

Complete Blood Count Analysis:

Cardiac puncture was administered to euthanized WT or Gpsm3$^{-/-}$ mice using a 1 ml syringe fitted with a 21G needle to harvest fresh blood from the left ventricle slowly to prevent cardiac collapse of the heart and subsequently collected in BD Microtainer tubes containing EDTA. Samples were maintained at constant temperature and humidity throughout processing and analysis. Complete blood cell counts (CBCs) were performed using a HemaVet 950 (Drew Scientific,

Dallas, TX) instrument to measure leukocyte, erythrocyte, and thrombocyte levels in each sample. Machine calibration and performance were verified each day that samples were analyzed using MULTI-TROL standard solution (Dog, Drew Scientific, Dallas, TX). All samples were run within 2 hr of initial collection.

Primary cells:

Dendritic cells – Bone marrow was isolated from WT, Gpsm1$^{-/-}$ or Gpsm3$^{-/-}$ mouse femurs and tibiae using a 25G syringe to flush the bone marrow out with 10 mL of DPBS (PBS, Ca^{++} and Mg^{++} free). Isolated bone marrow was then filtered through a 40-μm nylon cell strainer, centrifuged at 4°C 500 x g and decanted. Red blood cells were lysed with 5 mL of ice-cold ACK lysing buffer (0.17 M NH_4Cl/0.17 M Tris) for 5 min at room temperature, followed by an additional spin at 4°C 500 x g to pellet the harvested bone-marrow cells. Isolated cells were then resuspended in 10 mL DC I media (RPMI-1640 supplemented with 10% FBS, 100 U/mL penicillin, 100 mg/mL streptomycin, and 20 ng/ml rmGM-CSF), and plated 4-5 x 10^5 cells/mL in a 10 cm tissue culture dish. On day four, 10 mL fresh DC I media was added to each dish. On day eight, non-adherent and loosely adherent cells were harvested, centrifuged 4°C 500 x g, decanted and re-seeded in 10 mL fresh DC II media (RPMI-1640 supplemented with 10% FBS, 100 U/mL penicillin, 100 mg/mL streptomycin, and 10 ng/ml rmGM-CSF) to generate immature dendritic cells (iDC). Day nine cells were treated with or without 200 ng/mL LPS for the indicated times or for 24 hours to generate mature dendritic cells (mDC).

B and T lymphocytes – Spleens of WT, Gpsm1$^{-/-}$ or Gpsm3$^{-/-}$ mice were gently crushed between frosted glass slides in 10 mL serum-free RPMI. Spleen homogenate was centrifuged at 4°C 500 x g and decanted. Red blood cells were lysed with 10 mL of ice-cold ACK lysing buffer for 5 min at room temperature, followed by an additional spin at 4°C 500 x g to pellet the splenocytes. Splenocytes were then washed once and resuspended in DPBS supplemented with 0.1% BSA and 2 mM EDTA at 5 x 10^7 cells/mL or 1 x 10^8 cells/ml for subsequent B or T cell isolation, respectively. Cell isolation was performed according to Invitrogen Dynabeads protocol for untouched B cell isolation or negative T cell isolation.

Neutrophils – Bone marrow was isolated from WT or Gpsm3$^{-/-}$ mouse femurs and tibiae using a 25G syringe to flush the bone marrow out with 10 mL of DPBS. Isolated bone marrow was then filtered through a 40-μm nylon cell strainer, centrifuged at 4°C 500 x g and decanted. Pelleted cells were resuspended in 2 mL DPBS followed by subsequent careful layering on top a 3-layer Percoll™ density gradient. The density gradient was generated by diluting 100% Percoll™ in DPBS to required densities represented by 78%, 64%, 52% Percoll dilutions. Stacking of the different layers was conducted as follows, 3 mL 78% Percoll, 2 mL 64% Percoll, and 2 mL 52% Percoll followed by subsequent 2 mL of sample. After centrifugation, 1500 x g for 40 min at 4°C, the 78/64% Percoll interface was carefully isolated and added to 9 mL of DPBS to disrupt the remaining gradient. Isolated cells were then centrifuged 4°C, 1500 x g for 5 min, decanted, and subjected to 1 mL of ice-cold ACK lysis buffer for 5 min at room temperature to

remove any remaining red blood cells. Cells were then resuspended in 1-2 mL phenol red-free RPMI supplemented with 0.1% BSA and 2mM EDTA.

Immunoblotting:

Single-cell suspensions from spleen and thymus were prepared by crushing freshly dissected tissues between frosted glass slides in 10 mL DPBS. After centrifugation 4°C 500 x g 5min, samples were decanted and red blood cells lysed with 10 mL ice-cold ACK lysis buffer for 5 min at room temperature followed a second round of centrifugation at 4°C 500 x g for 5 min. ACK lysis buffer was then decanted and pellets were resuspended in 100-300 μL 1% NP40 lysis buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 5 mM EDTA, 1% Nonidet P-40) with protease inhibitors. Samples were incubated on ice for 20 min followed by centrifugation at 10,000 x g for 30 min at 4°C. Dendritic cells were harvested using cell scrappers and neutrophils samples were collected after Percoll density centrifugation to be processed in the 1% NP-40 lysis buffer with protease inhibitors as described above. Protein concentration was determined by a Pierce BCA protein assay. Protein samples were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE, 10-13.5%), then separated proteins were transferred to polyvinylidene difluoride (PVDF) membranes for immunoblotting as described (Blumer et al 2002). Immunoblotting with AGS4 antibodies (Abgent, San Diego, CA) was conducted as follows: Membranes were then blocked with 50% Odyssey Buffer [LI-COR Biosciences] and 50% Tris-buffered saline + 0.01% Tween (TBST) for 30 min at room temperature, incubated with AGS4 antibody (1:250 dilution) overnight 4°C, followed by three

10 min washes in TBST. Membranes were then exposed to 1:5,000 dilution of horseradish peroxidase-conjugated goat anti-rabbit IgG 30 min room temperature, followed by three 30 min washes with TBST and subsequent exposure with ECL. Cell pellets processed for phosphorylated proteins were lysed in 1% NP40 buffer with protease inhibitors and additional phosphatase inhibitors (50 mM NaF, 5 mM sodium pyrophosphate, 40 mM β-glycerophosphate and 200 μM Na_3VO_4) on ice for 20 min followed by centrifugation at 10,000 x g for 30 min at 4°C. Samples were subjected to SDS-PAGE, proteins transferred to PVDF membranes and immunoblotted for anti-phospho-Erk (Y402) (Santa Cruz Biotechnology, Dallas, TX, USA), or total Erk (Abcam, Cambridge, MA, USA) antibodies. Densitometric quantification of the immunoblotted bands was performed using ImageJ densitometry software (Version 1.49i, National Institutes of Health, Bethesda, MD). Selected bands were quantified based on their relative intensities and normalized to total Erk.

Chemotaxis:

Corning Transwell 24-well inserts (6.5 mm diameter, 5.0 μm pore size) or 96-well inserts (5.0 μm pore size) were used for all chemotaxis assays. For dendritic cell chemotaxis, 235 μL of serum-free RPMI with or without CXCL12 (10-500 ng/mL) or CCL19 (250 ng/mL) was added to each lower chamber and 75 μL of approximately 3 x 10^6 cells/ml were loaded in at least triplicate determinations into the upper chambers. Where indicated, dendritic cells were pre-incubated with 100 ng/mL of pertussis toxin for 18 hr at 37°C prior to measuring chemotaxis. For lymphocytes, 235 μL of serum-free RPMI supplemented with

0.1% BSA and 2mM EDTA with or without CXCL12 (50-300 ng/ml) or CCL19 (50-300 ng/ml) was added to each lower chamber, and 75 μL of approximately 1 x 10^7 lymphocytes/mL were added in at least triplicate determination into the upper chambers. In the case of neutrophils, 235 μL of serum-free RPMI supplemented with 0.1% BSA and 2mM EDTA with or without fMLP (0.1-5.0 μM) was added to each lower chamber, and 75 μL of approximately 5 x 10^6 cells/ml were added in at least triplicate determinations into the upper chambers. Chemotaxis chambers were incubated at 37°C, 5% CO_2 for 20 hrs for dendritic cells, 5 hrs for lymphocytes, and 3 hrs for neutrophils. The upper chamber was removed and cells migrating to the bottom chamber as well as cells retained in the upper chamber were counted by flow cytometry. The percentage of cells migrated was calculated relative to the input, where the number of cells migrating to the bottom chamber in the absence of chemokine was subtracted.

Thioglycollate-induced intraperitoneal inflammation:

WT and Gpsm3$^{-/-}$ mice received intraperitoneal injections using an insulin syringe (28G) to deliver 1 mL of DPBS or 4% thioglycollate (in DPBS, sterilized, and aged for a minimum of 2 wks). After 2 hrs, intraperitoneal (IP) cavity lavage was carried out through injection of 10 mL cold DPBS and thorough subsequent agitation of the cavity. Blood (350 – 600 μL) was also collected by cardiac puncture and bone marrow was collected from femurs as described above in *Primary Cells* section. Isolated cells were centrifuged 4°C 500 x g 5 min, excess supernatant decanted, and red blood cells were lysed using 1 mL ice-cold ACK lysis buffer (5 min incubation followed by subsequent 500 x g centrifugation for 5

min). Blood samples required a minimum of two ACK lysis steps to remove all of the red blood cells. Isolated IP lavage cells were then resuspended in 50 μL of PBS with 1% BSA and 0.1% NaN_3 (PBS-BSA), isolated cells from the blood were resuspended in 200 μL of PBS-BSA, and isolated bone marrow cells were resuspended in 1 mL of PBS-BSA. Each sample then had 50 μL subjected to incubation with conjugated antibodies for analysis by flow cytometry as described below.

Flow cytometry and cell sorting:

Single-cell suspensions from spleen and thymus were prepared from female mice (Gpsm1$^{+/+}$ and Gpsm1$^{-/-}$ littermates) at 6 weeks of age by crushing freshly dissected tissues between frosted glass slides in PBS. After lysing red blood cells with 10 mL ACK lysis buffer (0.17 M NH_4Cl/0.17 M Tris), cells were counted and washed with PBS with 1% BSA and 0.1% NaN_3 (PBS-BSA). A total of 10^6 cells were first incubated with anti-FcγIII (CD16/CD32) for 30 min at 4°C to block Fc receptors, then cells were incubated with primary FITC or PE-conjugated Abs in PBS-BSA for 30 min at 4°C (BD Pharmingen, San Diego, CA). Cells were washed twice in PBS-BSA, resuspended in 500 μl of PBS-BSA, and analyzed on a flow cytometer (BD Pharmingen). Additionally, single-cell suspensions of WT and Gpsm3$^{-/-}$ neutrophils collected from Percoll density centrifugation or from thioglycollate-induced inflammation experiments were prepared as described above. Pellets cells were washed and resuspended in 50 μL PBS supplemented with 1% BSA and 0.1% NaN_3 (PBS-BSA). Cells were incubated with primary FITC-CD11b (0.3 μL, 0.15 μg) or PE-Ly-6G (2 μL, 0.4 μg) conjugated Abs or

isotype controls FITC-Rat IgG_{2a},κ (1 μL, 0.5 μg) or PE-Rat IgG_{2b},κ (3 μL,0.6 μg) in PBS-BSA for 30 min at 4°C (BD Pharmingen, San Diego, CA). Cells were washed thrice with 500 μL PBS-BSA with subsequent centrifugations 4°C 500 x g 5 min, and resuspended in 250-500 μl of PBS-BSA for analysis by flow cytometer (BD Pharmingen). Neutrophil populations were observed as being dual positive ($CD11b^+$, $Ly\text{-}6G^+$).

Data Analysis:

Statistical significance for differences involving a single intervention was determined by one-way analysis of variance (ANOVA) followed with a post hoc Tukey's test using GraphPad Prism version 4.03 (GraphPad Software, San Diego).

Results and Discussion

Accessory proteins for G-protein signaling systems have revealed surprising diversity in modes of heterotrimeric G-protein signal processing, including but not limited to the modulation of signal strength, duration, location, termination and the formation of signal transduction complexes (Refer to Chapter 2) (for review, see (Blumer et al 2012, Blumer et al 2007, McCudden et al 2005a, Sato et al 2006a)). In order to attain a proper response to dynamic chemotactic stimuli, leukocytes require highly specialized and spatially integrated G-protein signaling mechanisms (Cho & Kehrl 2009, Kehrl et al 2009). Moreover, increasing evidence indicates that proteins containing GPR motifs play key roles in dynamic biological signaling systems where signal integration is required for appropriate and efficient responsiveness of the system (Bowers et al 2008, Bowers et al 2004, Branham-O'Connor et al 2014, Fan et al 2009, Giguere et al 2012a, Kamakura et al 2013, Sanada & Tsai 2005, Yao et al 2005). As an initial approach to define the role of GPR proteins in such modes of signal integration, we studied the role of the GPR proteins AGS3 and AGS4 in chemotactic signal integration of immune cells.

Functional roles for AGS3 in numerous physiological signaling systems have been described, including drug addiction and neuronal plasticity, ischemia reperfusion injury and polycystic kidney disease, blood pressure control, energy expenditure and metabolism, autophagy and membrane protein trafficking; however, the functional role(s) of AGS3 in the immune system is not defined (Blumer et al 2008, Bowers et al 2004, Conley & Watts 2013, Fan et al 2009,

Garcia-Marcos et al 2011, Groves et al 2007, Kamakura et al 2013, Kwon et al 2012, Nadella et al 2010, Pattingre et al 2003, Regner et al 2011, Sanada & Tsai 2005, Vural et al 2010, Yao et al 2005). Additionally, biological roles of AGS4, particularly in the immune system where its expression predominates, are poorly understood (Cao et al 2004, Giguere et al 2013, Giguere et al 2014, Schmidt et al 2012). Thus, the data described here begin to explore potential functions for AGS3 and AGS4 in the regulation of chemokine-induced signaling and response to inflammation in various leukocyte populations where these GPR containing proteins are expressed. Indeed, there are relatively few reports of GPCR signal modulation by GPR-containing proteins (Conley & Watts 2013, Fan et al 2009, Kinoshita-Kawada et al 2004, Sato et al 2004, Webb et al 2005, Wiser et al 2006, Yao et al 2005), underscoring the significance of the current study which makes use of primary cells obtained from genetic null $Gpsm1^{-/-}$ and $Gpsm3^{-/-}$ mice.

Analysis of protein expression and leukocyte populations from AGS3/$Gpsm1^{-/-}$ and AGS4/$Gpsm3^{-/-}$ mice

To investigate potential functional roles for AGS3 and AGS4 in leukocytes, we utilized two recently generated mouse models, namely the AGS3/Gpsm1-null and AGS4/Gpsm3-null mouse models. The AGS3/Gpsm1-null model was generated by Blumer et al. and the AGS4/Gpsm3-null was obtained from the Knockout Mouse Project (KOMP) (Blumer et al 2008). Initial investigation of immune tissues of these mice compared to WT tissues determined abundant expression of AGS3 (M_r ~ 74,000) and AGS4 (M_r ~18,000) in WT tissues and

complete absence of these proteins in the null animals (Figure 3.1A, 3.1B). As predicted from the mRNA expression of AGS4, neutrophil expression of AGS4 was observed to be the most abundant of the collected tissues (Figure 3.1B). With a documented role for Gαi and GPR proteins in asymmetric cell division and thus a potential impact on cell fate and differentiation, we measured leukocyte populations in AGS3 and AGS4-null animals (Dalwadi et al 2003, Gonczy 2008, Huang et al 2003, Knoblich 2010, Rudolph et al 1995). While initial results from Gpsm1$^{-/-}$ mice indicated that differentiation of lymphocyte populations are unaffected by the loss of AGS3 (Branham-O'Connor et al 2014), mice deficient in AGS4 expression demonstrated a mild but significant neutropenia and lymphocytosis while other populations were unaltered (Figure 3.2). Altered levels of circulating populations of neutrophils and lymphocytes in Gpsm3$^{-/-}$ mice may indicate a role for AGS4 in either differentiation or proper leukocyte trafficking mechanisms of these cells in these mice (Figure 3.2, 3.4B, 3.4D, 3.4E). In addition to these findings, our initial results validate the loss of AGS3 and AGS4 expression in immune cells and tissues in the knockout model systems, thus proving their utility in the investigation of the functional roles these GPR proteins in the immune system.

Figure 3.1

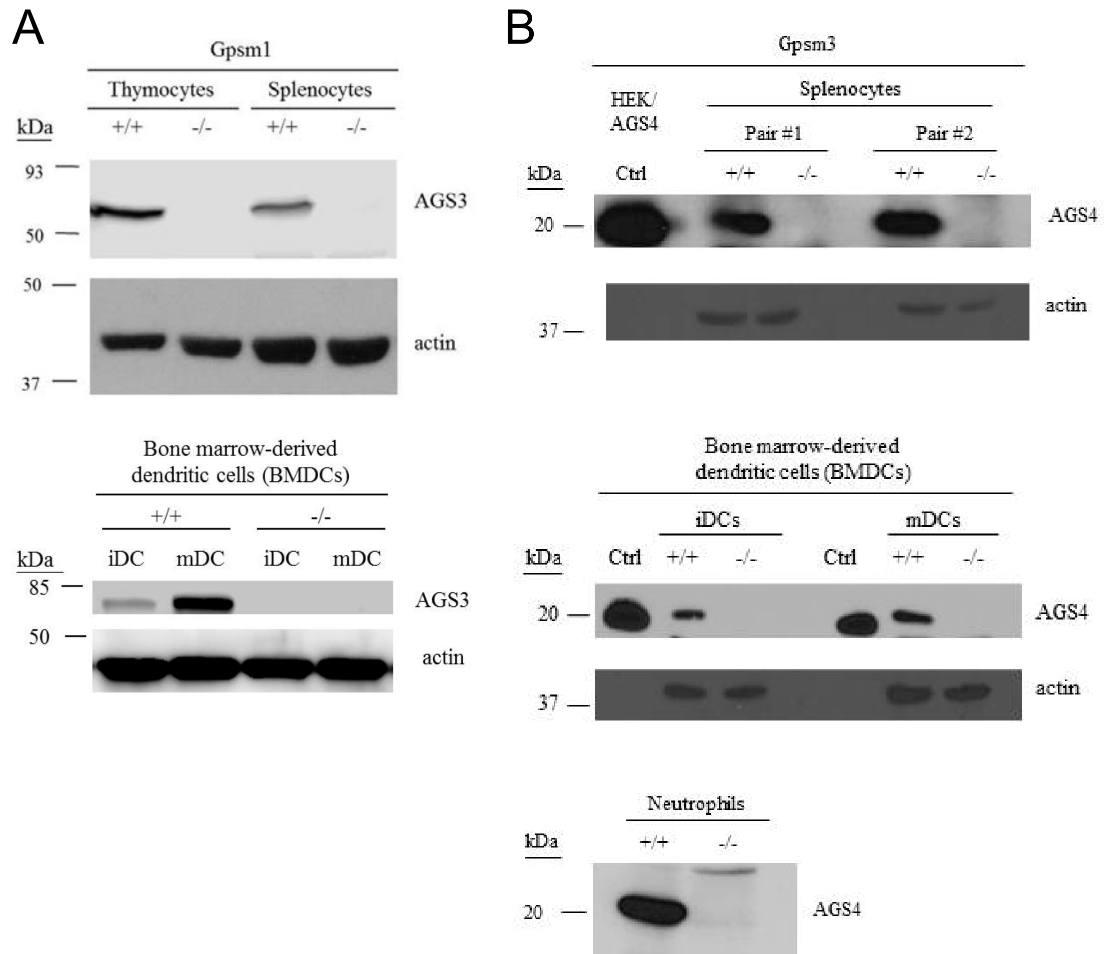


Figure 3.1. Expression of AGS3 and AGS4 in isolated primary leukocytes of WT, Gpsm1$^{-/-}$, Gpsm3$^{-/-}$

(A) Thymocytes and splenocytes (*top panel)* were isolated from WT vs Gpsm1$^{-/-}$ mice following red blood cell lysis and filtering to remove cell and tissue aggregates as described in Experimental Procedures. Harvested BMDCs from WT and Gpsm1$^{-/-}$ mice were also cultured to immature (iDC) and mature (mDC) dendritic cells (*lower panel)* as described in Experimental Procedures. Lysates were prepared with 1% NP-40 lysis buffer and subjected to SDS-PAGE (100 µg per lane) and immunoblotting with AGS3 and β-actin-specific antisera as described in Experimental Procedures. Immunoblots depicted are representative of at least 3 independent experiments.

(B) WT and Gpsm3$^{-/-}$ splenocytes (*top panel)* were isolated following red blood cell lysis and filtered to remove cell and tissue aggregates as described in Experimental Procedures. Harvested BMDCs from WT and Gpsm3$^{-/-}$ mice were cultured to immature (iDC) and mature (mDC) dendritic cells (*middle panel)* or used to isolate neutrophils (*lower panel)* by Percoll gradient centrifugation as described in Experimental Procedures. Lysates were prepared with 1% NP-40 lysis buffer and subjected immunoblotting (100 µg) with AGS4 and β-actin-specific antisera as described in Experimental Procedures. Immunoblots depicted are representative of at least 3 independent experiments.

Figure 3.2

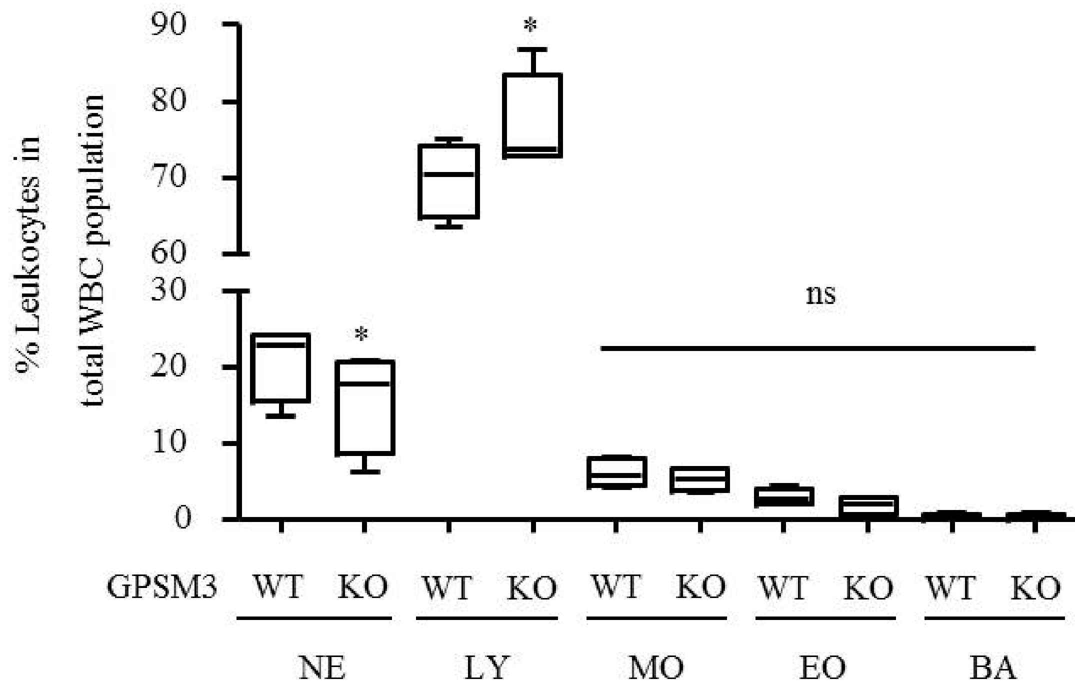


Figure 3.2. Altered circulating leukocyte populations are observed in Gpsm3$^{-/-}$ mice

Blood was collected from WT and Gpsm3$^{-/-}$ (KO) mice by cardiac puncture as described in Experimental Procedures. Complete blood cell counts (CBCs) were performed within 2 hr of blood collection using a HemaVet 950 instrument to measure leukocyte levels in each sample. The following populations were measured: neutrophils (NE), lymphocytes (LY), monocytes (MO), eosinophils (EO), basophils (BA). Percent of leukocyte populations in relation to total number of white blood cells was calculated and compared between WT and Gpsm3$^{-/-}$ littermate pairs. Data are representative of 5 independent experiments in box and whiskers plots depicting the median with quartiles 1 to 3 contributing to the surrounding box, while the maximum and minimum values are depicted by the whiskers. * denotes $p < 0.05$ based on Tukey's post hoc test following ANOVA.

Leukocyte stimulation enhances AGS3 and AGS4 protein levels

Activation of leukocytes often results in upregulation of proteins whose function is required for proper signal integration; thus, we postulated that AGS3 and AGS4 expression may be upregulated in response to stimulation of leukocytes. Activation of primary B lymphocytes with LPS or IgM for 12h demonstrated upregulation of AGS3 protein, but AGS4 protein expression was unaltered (Figure 3.3). Interestingly, stimulation of primary T lymphocytes with anti-CD3 and IL-2 illustrated enhanced expression of AGS4 over time, while AGS3 was significantly upregulated subsequent to CD3/IL-2 treatment followed by a moderate decrease in this expression after the time allotted (Figure 3.3). The differential expression of AGS3 and AGS4 during T-lymphocyte activation may represent unique, unexplored modulatory functions for each protein at different stages of this process. Additionally, bone marrow-derived dendritic cells (BMDCs) were observed to have increased expression of AGS3 in response to stimulation by LPS (Figure 3.1A), (Branham-O'Connor et al 2014). Moreover, these findings are in support of a recent report also depicting upregulation of AGS4 during monocyte differentiation from bone marrow by macrophage-colony stimulating factor over nine days, while decreased expression of AGS4 was observed in differentiation of macrophage-like cells using phorbol 12-myristate 13-acetate (PMA) over three days (Giguere et al 2013). Taken together, the upregulation of AGS3 and AGS4 in response to leukocyte activation further suggests a biologically significant role for these proteins in regulating immune responses to stimuli; furthermore, our results are consistent with regulated

expression of GPR proteins responding to extracellular cues (Bowers et al 2008, Bowers et al 2004, Cho et al 2000, Fan et al 2009, Giguere et al 2013, Giguere et al 2014, Kwon et al 2012, Nadella et al 2010, Regner et al 2011).

Figure 3.3

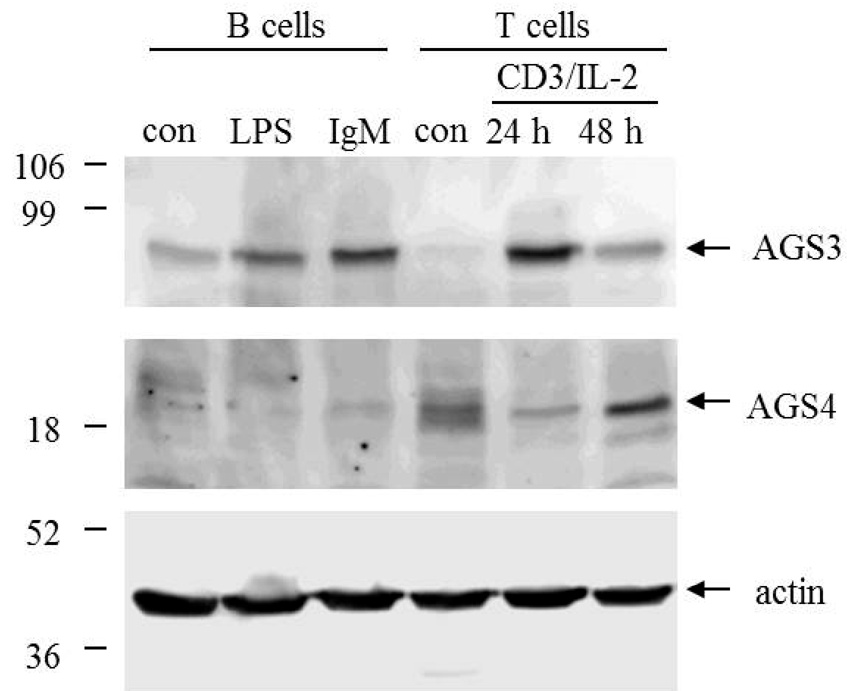


Figure 3.3. Differential upregulation of AGS3 and AGS4 protein expression in lymphocytes upon stimulation

Purified B- and T-lymphocytes from C57BL/6J mice were isolated as described in Experimental Procedures. Purified B cells were stimulated with 20 μg/mL anti-IgM F(ab')2 fragment or 1 mg/ml LPS for 12 h. Purified T cells were stimulated with 0.1 μg/ml CD3 and 20% interleukin (IL)-2 for 24 to 48 h. After treatment, cells were washed and lysed in SDS sample buffer and subjected to SDS-PAGE and immunoblotting with AGS3, AGS4 and actin-specific antisera as described in Experimental Procedures. Samples were kindly provided by Dr. John H. Kehrl, NIH-NIAID. Representative image from 3 independent experiments is depicted.

Defective chemotaxis in AGS3/Gpsm1$^{-/-}$ and AGS4/Gpsm3$^{-/-}$ leukocytes

Chemokine signaling is mediated by Gαiβγ heterotrimer activation, which is a critical component to this signaling cascade (e.g.(Delgado-Martin et al 2011)). Furthermore, the importance of Gαi subunits in chemokine signaling is commonly overshadowed by the effects imparted by the Gβγ subunit; however, previous reports have illustrated the importance of Gαi itself to the overall signal integration for chemokine receptors, as decreased chemokine-directed migration is observed in leukocytes isolated from Gαi-null animals (e.g. (Hwang et al 2007, Jin et al 2008). Thus, we investigated the functional consequence resulting from the absence of the Gαi binding proteins, AGS3 and AGS4, on chemokine-mediated signal integration in leukocytes. As an initial approach to address this question, chemotaxis of leukocytes isolated from WT, Gpsm1$^{-/-}$, and Gpsm3$^{-/-}$ mice were analyzed. Upregulation of AGS3 in activated B- and T-lymphocytes coincided with a nearly 50% reduction in chemotaxis towards either CXCL12/SDF-1 or CCL19 observed in isolated Gpsm1$^{-/-}$ B- and T-lymphocytes compared to WT lymphocytes (Figure 3.4A). Similarly, isolated T-lymphocytes, in which AGS4 expression is up-regulated after stimulation, from Gpsm3$^{-/-}$ mice demonstrated a ~ 25% decreases in chemokine-induced migration to CCL19, while chemotaxis to CXCL12 was reduced but not significantly (Figure 3.4B). The discrepancy in chemokine signal processing between these two Group II AGS proteins may signify unique regulatory functions for AGS3 and AGS4 in specific chemokine signaling pathways, further expanding the signaling diversity of these receptors.

Similar to the chemotactic defect observed in lymphocytes from Gpsm1-/- and Gpsm3-/- mice, bone marrow-derived dendritic cells (BMDCs) from AGS3-null and AGS4-null mice showed ~30% reduction and ~20% reduction, respectively, in migration towards the chemokine CXCL12 (Figure 3.4C and 3.4D). This effect was completely blocked by pertussis toxin pretreatment regardless of genotype (Figure 3.4D).

Significant levels of AGS4 expression in neutrophils prompted the investigation into chemokine-induced migration of neutrophils to fMLP (Figure 3.4E). Coinciding with the chemotactic reduction in lymphocytes and BMDCs, isolated neutrophils from Gpsm3$^{-/-}$ mice revealed > 30% reduction in fMLP-directed chemotaxis (Figure 3.4E).

Further analysis of the data indicated no significant difference in random migration between WT leukocytes and leukocytes lacking AGS3 and AGS4, suggesting that the chemotactic defect in Gpsm1$^{-/-}$ and Gpsm3$^{-/-}$ cells was primarily directional and not due to an overall decrease in the ability of the cells to migrate. Additionally, to rule out that loss of chemokine receptor expression in these cells was contributing to the reduction in cell migration, flow cytometry analysis revealed that chemokine receptor levels were unaltered in the absence of AGS3 or AGS4 indicating that the chemotactic defect in these leukocytes was not due to loss of chemokine receptor expression (Figure 3.5A and 3.5B).

Figure 3.4

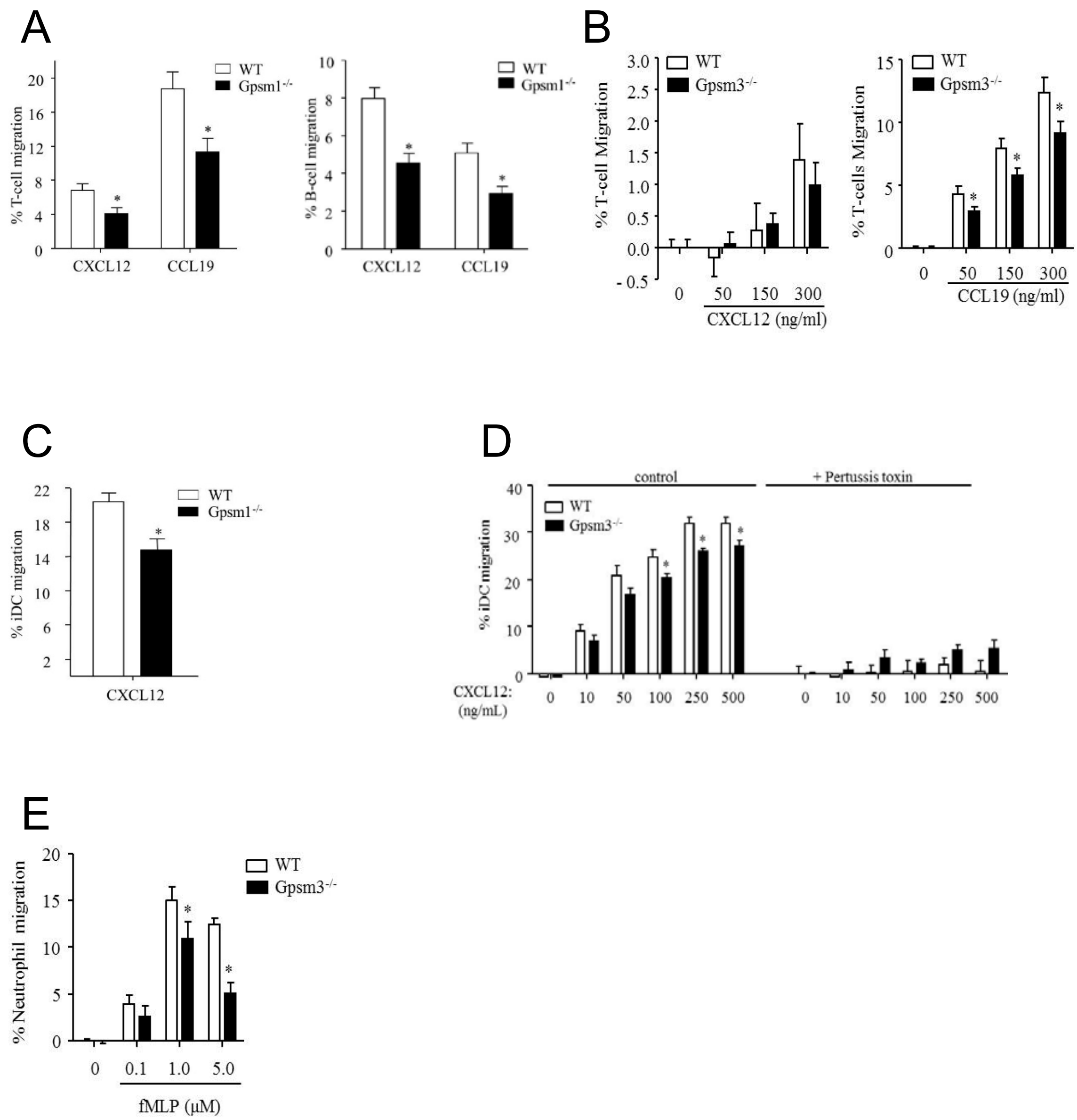


Figure 3.4. Chemotaxis of primary leukocytes from WT, Gpsm1$^{-/-}$, and Gpsm3$^{-/-}$ mice to chemokines CXCL12, CCL19, and fMLP

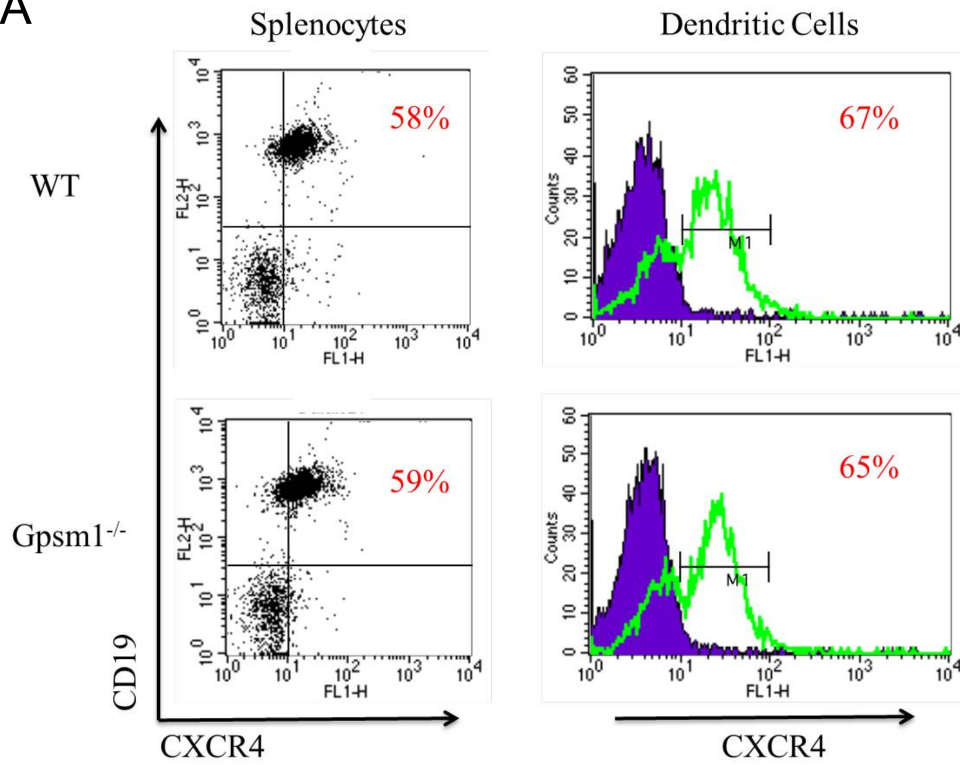
(A,B) T lymphocytes (*A, left panel and B)* and B lymphocytes (*A, right panel)* were separately isolated from freshly harvested splenocytes of WT, Gpsm1$^{-/-}$ (*A*) or Gpsm3$^{-/-}$ (*B*) mice. Cells were loaded in transwell migration chambers with the bottom chamber containing serum-free RPMI supplemented with 0.1% BSA and 2mM EDTA in the absence and presence of 300 ng/mL CXCL12 or CCL19 (*A*) or 50-300 ng/mL CXCL12 (*B, left panel*) or CCL19 (*B, right panel*). After 5h at 37°C, cells in the bottom chamber were counted, and the percentage of cells migrated was calculated relative to the input, where the number of cells migrating in the absence of chemokine was subtracted. Data are represented as the mean +/- S.E. of 3 independent experiments with at least triplicate determinations. *, $p < 0.01$ based on Tukey's post hoc test following ANOVA.

(C,D) Bone marrow cells were harvested from of WT, Gpsm1$^{-/-}$ (*C*) or Gpsm3$^{-/-}$ (*D*) mice and cultured as described in Experimental Procedures. After 8d, immature dendritic cells (iDCs) were cultured in the absence or presence of 100ng/ml Pertussis toxin for 18hr where indicated (*D)*. Dendritic cells were loaded in transwell migration chambers with the bottom chamber containing serum-free RPMI in the absence and presence of 250 ng/mL CXCL12 (*C)* or 0-500 ng/mL CXCL12 (*D)*. After 20hr at 37°C, cells in the bottom chamber were counted, and the percentage of cells migrated was calculated relative to the input, where the number of cells migrating in the absence of chemokine was subtracted. Data are represented as the mean ± S.E. of 3 independent

experiments with at least triplicate determinations. *, $p < 0.01$ based on Tukey's post hoc test following ANOVA.

(E) Neutrophils from WT and Gpsm3$^{-/-}$ mice were isolated from freshly harvested bone marrow of WT and Gpsm3$^{-/-}$ mice as described in Experimental Procedures. Isolated neutrophils were loaded in transwell migration chambers with the bottom chamber containing serum-free RPMI with 0.1% BSA and 2mM EDTA in the absence and presence of fMLP (0.1–5 μM). After 3h at 37°C, cells in the bottom chamber were counted, and the percentage of cells migrated was calculated relative to the input, where the number of cells migrating in the absence of chemokine was subtracted. Migration data are represented as the mean +/- S.E. of at minimum 4 independent experiments with at least triplicate determinations. *, $p < 0.01$ based on Tukey's post hoc test following ANOVA.

Figure 3.5



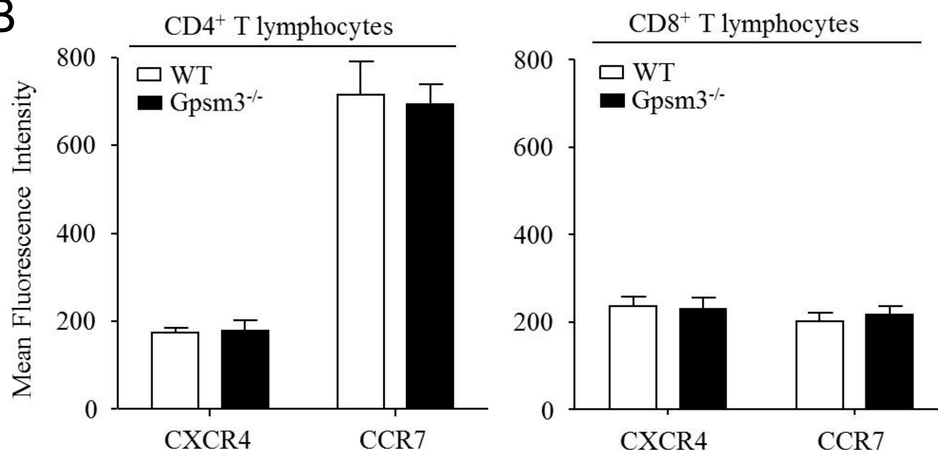


Figure 3.5. Receptor expression is unaltered in Gpsm1$^{-/-}$ and Gpsm3$^{-/-}$ leukocytes

(A) Splenocytes were isolated from WT and Gpsm1$^{-/-}$ mice following red blood cell lysis and filtering to remove cell and tissue aggregates as described in Experimental Procedures. WT and Gpsm1$^{-/-}$ total splenocytes (*left panel*) were stained with PE conjugated CD19 antibody and FITC conjugated CXCR4 antibody and BMDCs (*right panel*) were stained with FITC conjugated CXCR4 antibody with subsequent flow cytometric analysis. Results are representative of at least two independent experiments

(B) Splenocytes were isolated from WT and Gpsm1$^{-/-}$ mice following red blood cell lysis and filtering to remove cell and tissue aggregates as described in Experimental Procedures. CD4^{+} and CD8^{+} T lymphocytes were isolated and analyzed by flow cytometry through collaborative efforts with Dr. John H. Kehrl, NIH-NIAID. Results are representative of at least two independent experiments

Impaired chemokine-mediated signal processing in AGS3/Gpsm1$^{-/-}$ and AGS4/Gpsm3$^{-/-}$ leukocytes

Stimulation of chemokine receptors also activates downstream targets such as ERK1/2 kinases as part of the chemotactic process, predominately through Gβγ activation of PI3Kγ (Tilton et al 2000). Previous reports have demonstrated the importance of ERK1/2 activation subsequent to chemokine receptor stimulation in chemokine-mediated migration of leukocytes (Delgado-Martin et al 2011, Sagar et al 2012). Therefore, we hypothesized that the deficient chemotaxis observed with loss of AGS3 and AGS4 in leukocytes corresponded to deficient ERK1/2 phosphorylation. As an initial approach to explore this affect we isolated dendritic cells and splenocytes from WT, Gpsm1$^{-/-}$, and Gpsm3$^{-/-}$ bone marrow and spleens, respectively, and stimulated the leukocytes with CXCL12. Gpsm1$^{-/-}$ dendritic cells and splenocytes were unable to phosphorylate and sustain active ERK1/2 at similar levels to WT leukocytes (Figure 3.6A and 3.6C). Similarly, Gpsm3$^{-/-}$ leukocytes also demonstrated a reduction in chemokine-induced activation of ERK1/2 (Figure 3.6B and 3.6D). These results further demonstrate a role for AGS3 and AGS4 in chemokine-mediated signal processing for migrating leukocytes.

Figure 3.6

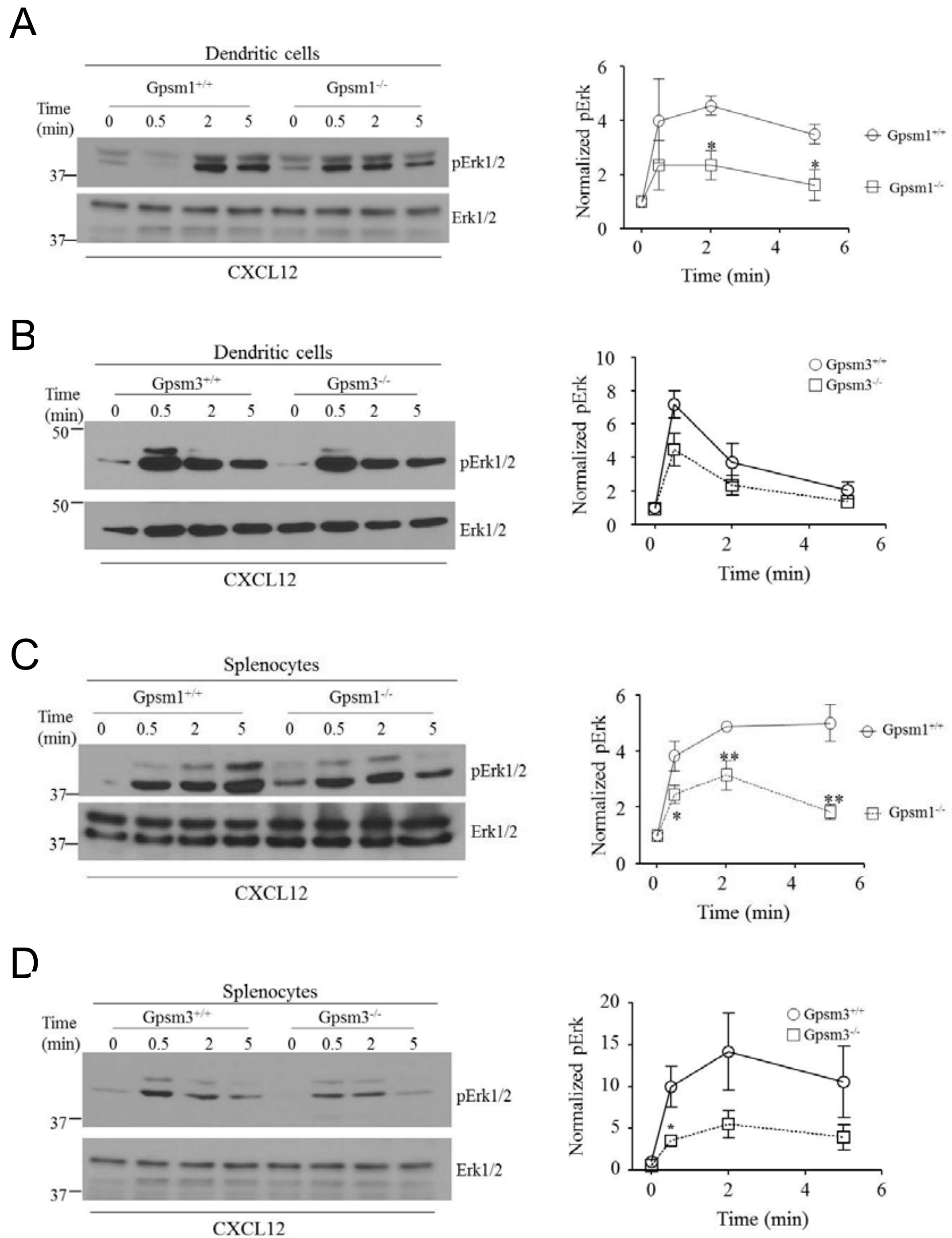


Figure 3.6. Gpsm1$^{-/-}$ and Gpsm3$^{-/-}$ dendritic cells and splenocytes exhibit reduced chemokine-stimulated phosphorylation of ERK1/2

(A,B) Single cell suspensions of WT, Gpsm1$^{-/-}$ (*A*), and Gpsm1$^{-/-}$ (*B*) cultured dendritic cells were treated in the absence or presence of CXCL12 (200 ng/mL) as described in Experimental Procedures. At the indicated times, cells were lysed in 1% NP40 lysis buffer containing protease and phosphatase inhibitors and lysates (50 µg per lane) were subjected to SDS-PAGE, transferred to PVDF and immunoblotted with anti-phospho-Erk (Y204) or total Erk-specific antibodies as described in Experimental Procedures. Representative immunoblots are shown in the left panels and densitometric analysis of at least 3 independent experiments normalized to total Erk levels and basal phosphorylated Erk at time point 0 (represented as means ± S.E.) are shown in the right panels. *, $p < 0.05$ based on Student's t-test analysis.

(C,D) Single cell suspensions of WT, Gpsm1$^{-/-}$ (*C*), and Gpsm1$^{-/-}$ (*D*) freshly isolated splenocytes were treated in the absence or presence of CXCL12 (200 ng/mL) as described in Experimental Procedures. At the indicated times, cells were lysed in 1% NP40 lysis buffer containing protease and phosphatase inhibitors and lysates (50 µg per lane) were subjected to SDS-PAGE, transferred to PVDF and immunoblotted with anti-phospho-Erk (Y204) or total Erk-specific antibodies. Representative immunoblots are shown in the left panels and densitometric analysis of at least 3 independent experiments normalized to total Erk levels and basal phosphorylated Erk at time point 0 (represented as means ±

S.E.) are shown in the right panels. *, $p < 0.05$; **, $p < 0.01$ based on Student's t-test analysis.

AGS4/Gpsm3$^{-/-}$ is required for maximal infiltration of neutrophils to sites of inflammation

The requirement for AGS4 in chemokine signal integration *ex vivo* prompted the question of how loss of AGS4 may affect chemokine signal processing in the intact animal. We hypothesized that AGS4 expression was required for neutrophils to efficiently migrate and to sites of inflammation *in vivo*. As an initial investigation into this biological role for AGS4, WT and Gpsm3$^{-/-}$ mice were injected with thioglycollate to induce peritonitis in the intraperitoneal cavity of these animals. WT mice demonstrated a significant accumulation of neutrophils at the initial site of inflammation (Figure 3.7A). This also coincided with an influx of neutrophils present in the blood (Figure 3.7B). In contrast, the level of neutrophils recruited to the IP cavity in Gpsm3$^{-/-}$ animals was drastically reduced by ~80% compared to WT littermates, while maintaining similar levels in the bone marrow during this period (Figure 3.7A and 3.7C). However, the presence of comparable induction of neutrophils in the blood similar to levels seen in WT mice (Figure 3.7B) suggests a possible deficiency in extravasation or a delayed response of neutrophils from the blood to the IP cavity in Gpsm3$^{-/-}$ mice after thioglycollate challenge. Interestingly, paracellular transendothelial migration of immune cells has been connected with signaling mechanisms involving Gαi2, as inhibition or loss of Gαi2 in immune cells and endothelial cells impairs neutrophil extravasation to sites of inflammation (Pero et al 2007, Warnock et al 1998, Wiege et al 2012, Zarbock et al 2007). Thus, aberrant transmigration of AGS4-null neutrophils from the blood to the IP cavity may

reflect simultaneous abnormal regulation of Gαi2 signaling in both endothelial cells and neutrophils, which together contribute to the observed defects in innate immunity.

Figure 3.7

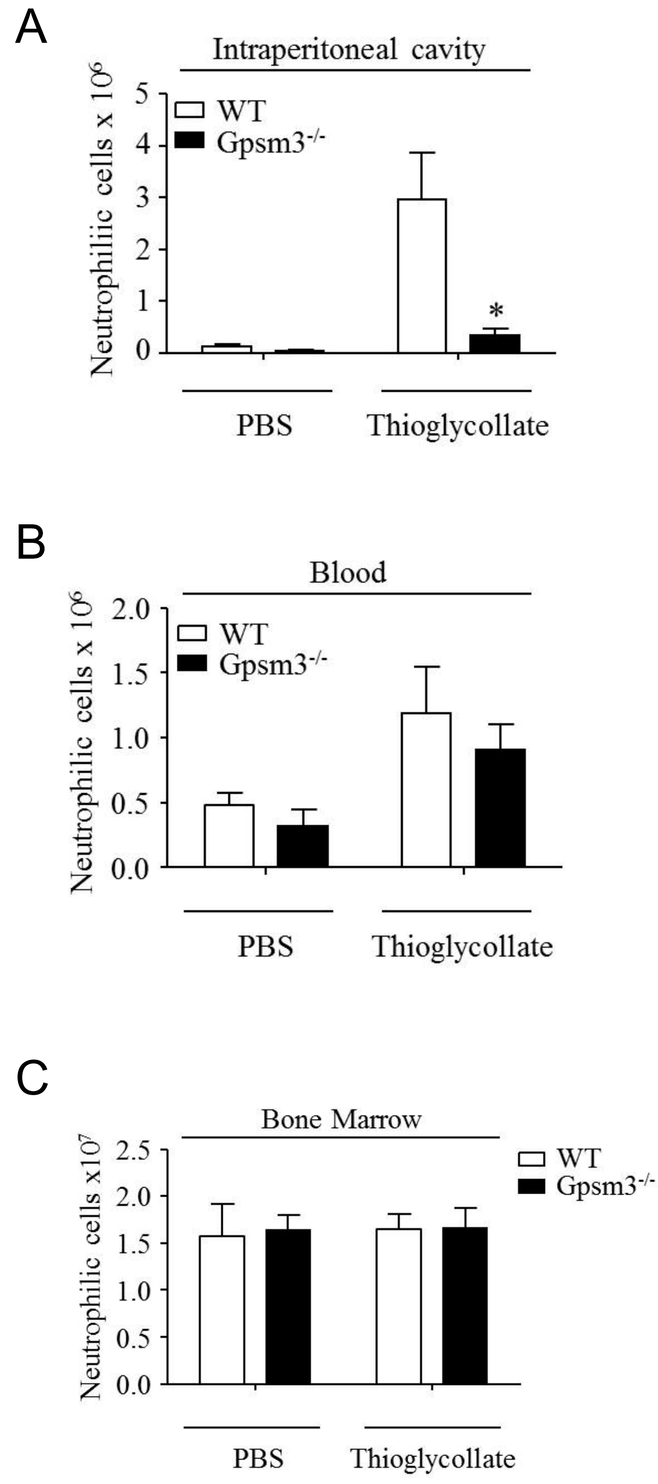


Figure 3.7. AGS4-null neutrophils demonstrate reduced migration in peritonitis model

WT and Gpsm3$^{-/-}$ mice received 1 mL intraperitoneal injections of sterile DPBS or 4% thioglycollate to induce localized inflammation as described in Experimental Procedures. Two hours post-injection, 10 ml of cold, sterile PBS was used to lavage the intraperitoneal cavity (A*)*. Blood was collected by cardiac puncture and femurs were processed to harvest bone marrow cells as described in Experimental Procedures (*B and C, respectively)*. Red blood cells were lysed from collected samples and cells were stained with FITC–CD11b and PE–Ly-6G for analysis of neutrophil levels in each tissue by flow cytometry. Neutrophil cell numbers were calculated using total events collected, applying flow rate and percentage of dual positive cells followed by dilutions carried out during processing of the cells. Data are represented as the mean +/- S.E. of 4 independent experiments. Notation of * signify p-values <0.001 based on Tukey's post hoc test following ANOVA.

In this report we demonstrate that chemokine-induced signal processing relies in part on AGS3 and AGS4, resulting in reduced chemokine-directed cell migration and ERK1/2 activation in the absence of either protein. An impaired neutrophil response to peritonitis further implicates GPR proteins in inflammatory signaling of leukocytes. Our results thereby expand the roles of GPR proteins, AGS3 and AGS4, to encompass chemokine signaling in leukocytes, where these proteins are predicted to modulate Gαi signal transduction of chemokine receptors. Various populations of AGS3-null and AGS4-null leukocytes demonstrate 25-40% reduction in chemotactic response, consistent with the idea that these are modulatory proteins that act to regulate the fundamental Gαi2 subunit signaling previously shown to be indispensable in chemokine stimulated systems (Cho et al 2012, Han et al 2005, Hwang et al 2007, Jin et al 2008, Pero et al 2007, Rudolph et al 1995, Skokowa et al 2005, Thompson et al 2007, Wiege et al 2012, Zarbock et al 2007). In light of these results, three hypotheses for the potential role of GPR containing proteins in modulation of chemokine receptor signal integration are as follows: 1) the 7TM chemokine receptor effectively couples the Gαi-GPR module to promote nucleotide exchange in a manner analogous to the Gαiβγ heterotrimer; 2) the distinct Gαi-GPR signaling module induces formation of a signaling complex to non-canonical signal mechanisms that direct chemokine-induced migration independent of Gβγ; and/or 3) GPR motifs of AGS3 and AGS4 act to sequester Gαi subunits and decrease the rate of heterotrimer reassociation thus prolonging or enhancing Gβγ signaling events that further facilitate chemokine-directed migration of leukocytes.

One hypothesis of the mode of action for GPR-proteins involves the chemokine receptor coupling to the Gαi – GPR complex as described in Chapter 2 (Oner et al 2010a, Oner et al 2010b) in a manner analogous to Gαiβγ heterotrimer to contribute to chemokine-stimulated downstream signaling. In heterologous systems, the Gαi – GPR module appears to directly engage with and is regulated by Gαi-coupled GPCRs (Oner et al 2010a, Oner et al 2010b) including CXCR4 (Chapter 2). Although the function of the Gαi-GPR signaling module is not fully elucidated for 7TMR signaling systems, reports of the importance of the Gαi subunit in chemokine-mediated signaling suggest that Gαi transduction may play a pivotal role in the systems. Gαi2 deficient immune cells demonstrate aberrant chemokine-directed migration and cytokine production (Huang et al 2003, Hwang et al 2007, Jin et al 2008, Thompson et al 2007, Wiege et al 2012). Additionally, maintaining low levels of intracellular cAMP was found to be essential to neutrophil chemotaxis (Harvath et al 1991). The active Gαi liberated by chemokine receptor activation can also activate the Src family of non-receptor tyrosine kinases leading to cascades which are implicated in cell migration and transmigration (Arefieva et al 2005, Fumagalli et al 2007, Ma et al 2000, Ptasznik et al 2002). Thus, the interaction of GPR containing proteins with Gαi subunits may in part participate in the modulation of chemokine activated Gαi-mediated signaling cascades.

The second hypothesis involves the Gαi – GPR complex formed after receptor activation but prior to heterotrimer reassociation, exhibiting a scaffolding function to recruit additional molecules to a non-canonical signaling complex.

This complex would then work in concert with Gβγ signaling events to promote efficient and appropriately directed cell motility. Indeed recent reports denote Gαi – AGS3 forming a large signaling complex with mammalian Inscuteable (mInsc) to recruit Par3-Par6-aPKC complex to leading edges of neutrophils and modulating directed migration (Kamakura et al 2013). These observations would suggest that $Gαi_{GDP}$ bound to GPR motif(s) would either function as an active signaling entity (Gonczy 2008) or serve as a platform for additional signal input as previously proposed (Blumer et al 2012).

As mentioned prior, signaling via chemokine receptors is generally understood to be primarily via Gβγ and its activation of downstream effectors (Arai et al 1997, Neptune & Bourne 1997, Neptune et al 1999, Peracino et al 1998, Spangrude et al 1985, Surve et al 2014). Gβγ-mediated stimulation of PI3Kγ (Stephens et al 1994, Stephens et al 1997), PLCγ (Wang et al 2000), ERK1/2 (Crespo et al 1994, Koch et al 1994) and exchange factors for small GTPases Rac and Cdc42 (Ueda et al 2008, Welch et al 2002) (reviewed in (Khan et al 2013)) as well as other scaffolding proteins (Sun et al 2011) appears to underlie the requirement of Gβγ for regulating chemoattractant-directed cell motility. Further supporting the critical role of Gβγ in chemokine signaling, recent reports demonstrate small molecule inhibitors and a small molecule activator of Gβγ are able to effectively respectively inhibit or promote chemotaxis of neutrophils and breast cancer cells (Kirui et al 2010, Lehmann et al 2008, Surve et al 2014). Although there is a solid foundation to support Gβγ as being an essential component of chemokine-mediated migration, even in the presence of

Gβγ small molecule inhibitors such as gallein, there still exists a degree of directed migration to chemokine suggesting that these cells may have additional signaling modules for promoting chemotaxis independent of Gβγ (Branham-O'Connor et al 2014, Lehmann et al 2008). Indeed, the use of a small molecule that activated Gβγ by heterotrimer dissociation recently demonstrated Gβγ signaling to be required for maximal neutrophil migration; but interestingly, in the co-treatment of both the small molecule and pertussis toxin, neutrophil chemotaxis was slightly reduced suggesting a minor, but observable contribution of Gαi to chemokine-induced migration of neutrophils (Surve et al 2014).

However within the context that Gβγ is primarily responsible for the majority of effects observed in chemokine signaling, AGS3 and AGS4 may influence interactions between Gαi and Gβγ subunits and thus impart a positive modulatory effect on cellular responses to chemokines. The competitive nature of the GPR motif with Gβγ for $G\alpha i_{GDP}$ binding (Bernard et al 2001, Ghosh et al 2003, Oner et al 2010a, Takesono et al 1999), suggests that GPR proteins may "grab" free $G\alpha i_{GDP}$ prior to heterotrimer reassociation to enhance or prolong Gβγ-regulated effector activation (Blumer & Lanier 2014, Blumer et al 2012, Blumer et al 2007). This hypothesis is supported by investigations in the broader context of GPR proteins influencing $G\alpha_i\beta\gamma$ subunit interactions (Kinoshita-Kawada et al 2004, Kwon et al 2012, Nadella et al 2010, Regner et al 2011, Sanada & Tsai 2005, Sato et al 2004, Takesono et al 1999, Webb et al 2005, Wiser et al 2006). Therefore, the observed defects in chemotaxis resulting from the absence of AGS3 and AGS4 may in part be attributed to increased rates of Gαiβγ

heterotrimer reassociation thereby decreasing periods of Gβγ activation, consequent phosphorylation of ERK1/2, and subsequent cell motility (Figure 3.4, 3.6 and 3.7).

Taken together, the data strongly support the hypothesis of GPR proteins contributing a modulatory function to chemokine-mediated Gβγ signaling. The data may also point to potential functional redundancy between AGS3 and AGS4 in the chemotactic migratory response. In this context multiple questions begin to develop. Are the actions of AGS3 or AGS4 independent of one another, or is the loss of one GPR-protein compensated by the capacity of the other? Do AGS3 and AGS4 have differential functions in subpopulations of immune cells and within certain periods of activation? Does absence of both GPR-containing proteins simultaneously lead to a potentiation of the defects observed in the absence of either protein alone? Generation of a dual knockout mouse for AGS3 and AGS4 would be a valuable asset in further exploring distinct functions of AGS3 and AGS4 and identifying mechanisms in which the GPR-proteins are interchangeable (see Chapter 4: Future Directions). Aside from the potential functional redundancy observed between AGS3 and AGS4 demonstrated by the above data, other GPR proteins are also expressed in immune tissues and cells, including LGN/Gpsm2 (Blumer et al 2002, Branham-O'Connor et al 2014, Oliaro et al 2010) and RGS14 (Cho et al 2000).Thus, it is possible that any of these proteins may be partially masking the effects of the loss of AGS3 or AGS4 in this process. Defining the roles of these GPR proteins in chemokine signal integration

may reveal additional functional capacity of the GPR motif in this context and is a focus of future efforts.

Chapter 4

Conclusions

Conclusions

The overall goal of this dissertation is to examine the regulation of GPR proteins, establish possible contributors of this regulation, and demonstrate a functional role for GPR proteins in chemokine signal processing. My experimental approach is as follows: 1) establish if the Gαi – GPR module is regulated by chemokine receptors; 2) determine if the regulation of the Gαi – GPR complex involves direct modulation by 7TMRs or flux of endogenous G-protein subunits; 3) identify alternative binding partners that may regulate function of AGS4; 4) a drug screen to identify potential small molecule modulators of the Gαi – GPR interaction; and 5) define functional roles for AGS3 and AGS4 in chemokine signal processing using null mice.

CXCR4 induces agonist-mediated regulation of the Gαi2 – GPR complex that is receptor proximal and Gαi2 dependent

Cells of hematopoietic origin have to constantly adapt to a dynamic extracellular environment. Many of these signals are transduced through the Gαi-coupled chemokine receptor family, which signals predominantly through the Gαi2βγ heterotrimer to direct leukocyte migration to secondary locales (Arai et al 1997, Neptune & Bourne 1997, Wright et al 2002). Interestingly, two GPR-

containing proteins, AGS3 and AGS4, which bind $G\alpha i_{GDP}$, are abundantly expressed in leukocytes (Chapter 3), (Branham-O'Connor et al 2014, Cao et al 2004, Giguere et al 2013, Zhao et al 2010). Additionally, previous reports indicated that the interaction between GPR-containing proteins (AGS3, AGS4, and RGS14) and Gαi1 can be regulated by 7TMRs (Oner et al 2010a, Oner et al 2010b, Vellano et al 2013). However, the regulation of the Gαi2 – GPR complex by chemokine receptors has not been investigated.

Using a BRET system to monitor the association between AGS3 or AGS4 with Gαi2 before and after CXCR4 receptor activation, we observed an agonist-dependent decrease in association between AGS3 or AGS4 with Gαi2 (Chapter 2, Figure 2.1). Similar to Gαiβγ heterotrimer, this regulation is effectively blocked by pre-treatment with pertussis toxin or addition of receptor antagonist. Further investigation into the regulation of the Gαi2 – GPR complex by chemokine receptors demonstrated that the interaction of AGS3 or AGS4 with the receptor was Gαi2-dependent and disrupted upon addition of agonist (Chapter 2, Figure 2.2). Taken together, these findings suggest of the formation of a ternary GPCR-Gαi2-GPR protein complex that is regulated by agonist-mediated activation of the CXCR4 receptor, which is analogous to agonist regulation of canonical Gαiβγ heterotrimer (Lambright et al 1996, Rasmussen et al 2011, Wall et al 1995).

Gαi – GPR complexes exhibit direct coupling to 7TMRs, where the observed regulation is independent of competitive displacement of tagged Gαi subunits by endogenous, untagged G-proteins

Although the previous data suggests the existence of a GPCR-Gαi2-GPR ternary complex, the regulation observed could also be attributed to a secondary event, namely the cycling of endogenous, untagged G-protein α and βγ subunits in the microenvironment of the signaling complex. This scenario could result from canonical receptor activation of Gαiβγ heterotrimer resulting in the displacement of tagged proteins with untagged endogenous G-protein subunits to diminish the signal (Burt et al 1998).

As an initial approach to delineate the regulation of the Gαi2 – GPR complex from an exchange of endogenous subunits with this signaling module, a fusion protein was generated between $\alpha_{2A/D}$-AR and Gαi2-YFP (Bahia et al 1998, Bertin et al 1994, Burt et al 1998, Seifert et al 1999, Wise et al 1997). This construct allowed for detection of Gαi2YFP– GPR-Rluc associations directly at a 7TM receptor in the plasma membrane distinct from alternative subcellular compartments. Additionally, site-directed mutagenesis of the cysteine residue in Gαi2 that is ribosylated by pertussis toxin (C352I) effectively blocks the inhibitory effect of pertussis toxin on the tagged proteins. Implementing these $\alpha_{2A/D}$-AR-Gαi2YFP fusion proteins, we demonstrated agonist regulation of the Gαi2 – GPR complex as previously observed for the unlinked proteins (Chapter 2, Figure 2.3E). A key observation was that treatment with pertussis toxin to inhibit endogenous Gαi coupling did not inhibit agonist-mediated regulation of the PTX-

insensitive fusion protein ($\alpha_{2A/D}$-AR-Gai2YFP) with either AGS3 or AGS4 (Chapter 2, Figure 2.3E). These data indicated that endogenous Gαi subunits were not involved in the observed agonist regulation of the Gαi2 – GPR module by 7TMRs. In addition, we also addressed the possibility that the agonist-mediated reduction in the Gαi2-GPR interaction may be due to competition with endogenous, untagged Gβγ subunits released subsequent to receptor activation. The expression of the Gβγ scavenger GRK2-CT or pharmacological inhibition of Gβγ by the small molecule inhibitor gallein had no effect on agonist-mediated regulation of the Gαi2 – GPR complex, indicating that endogenous, untagged Gβγ was not responsible for 7TM receptor regulation of the Gαi – GPR module (Chapter 2, Figure 2.4). Collectively, these data indicate that Gαi2 – GPR complexes may indeed couple to 7TMRs. Upon receptor activation, the Gαi – GPR module appears to sense conformational changes in the receptor analogous to canonical Gαiβγ heterotrimer, which disrupts Gαi2 association with the GPR motif presumably through nucleotide exchange and activation of the Gαi subunit. The implications of such a complex on the way we perceive GPCR signaling will be elaborated on in the following section.

Affinity purification identified ARID1b and eEF1d proteins directly binding AGS4

Many Group II AGS proteins are comprised of at least one GPR motif and additional protein interaction domains that are important for subcellular localization and/or function of these proteins (An et al 2008, Blumer et al 2003, Blumer et al 2002, Du & Macara 2004, Du et al 2001, Pizzinat et al 2001, Shu et

al 2007). However, AGS4 does not contain defined protein interaction domains aside from its three GPR motifs (Cao et al 2004, Takesono et al 1999). Thus, to define potential interacting partners, AGS4 was fused to an amino terminal tandem affinity purification (TAP) tag. Pulldown of these proteins and subsequent LC-MS/MS analysis revealed three previously unidentified AGS4 interacting proteins including 14-3-3, ARID1b, and eEF1d (Chapter 2, Figure 2.6C). One of the protein families, 14-3-3, was subsequently demonstrated to bind AGS4 and modulate subcellular location (Giguere et al 2012b). Furthermore, previous yeast-two hybrid screens revealed the association of AGS4 with ARID2 and eEF2, which are closely related to ARID1b and eEF1d, which we identified in the TAP purification screen (Cao 2005). GST-pulldown assays with both ARID2 and eEF2 demonstrated interaction with AGS4, thus validating the binding of AGS4 to these proteins (Cao 2005). Thus, while further experiments are required to delineate the functional role for these novel interactions, the direct binding of related proteins identified in two independent screens unexpectedly positions AGS4 to potentially be involved in modulation of protein translation and transcriptional regulation.

JAK2 and Src phosphorylate AGS4 on a tyrosine residue essential for maximal GPR capacity

The cytokine thymic stromal lymphopoietic protein (TSLP) was recently identified as initiating a signaling cascade that results in the phosphorylation of

AGS4 on tyrosine 108, a Janus kinase 2 (JAK2) and Src kinase consensus sequence (van Bodegom et al 2012, Zhong et al 2012). While phosphorylation of other GPR-containing proteins have demonstrated functional responses to this post-translational modification, the effect of phosphorylation on AGS4, remains largely unexplored (Blumer et al 2003, Groves et al 2010, Hollinger et al 2003, Johnston et al 2009, Rush et al 2005, Zarling et al 2000). In addition, AGS4 is the only GPR protein known to undergo tyrosine phosphorylation, which represents < 2% of total phosphorylation sites in cells (Hunter & Sefton 1980, Olsen et al 2006). Mutation of serine and tyrosine residues in AGS4 identified a critical tyrosine residue, Y108, which when replaced with a negatively charged aspartic acid residue to mimic phosphorylation, results in a dramatic reduction in Gαi binding (Chapter 2, Figure 2.7B). Furthermore, *in vitro* kinase assays revealed that the kinases JAK2 and Src effectively phosphorylate AGS4 on this critical Y108 residue (Chapter 2, Figure 2.7A). Regulation of AGS4 - Gαi interactions by phosphorylation reveals an additional, uninvestigated layer of regulation of AGS4 and further supports a connection between G-protein signaling to growth factor regulated kinases (Corre et al 1999, Della Rocca et al 1999, Luttrell et al 1997, Ma et al 2000, Marrero et al 1995, Maudsley et al 2000, Vila-Coro et al 1999).

Identification of small molecules that modulate Gαi – GPR interaction

Previous studies have linked the presence of GPR proteins to several disease pathologies including drug addiction and craving, learning and memory,

ischemia reperfusion injury, polycystic kidney disease, blood pressure control, energy expenditure and metabolism, and rheumatoid arthritis and inflammatory pathways (Blumer et al 2008, Bowers et al 2004, Branham-O'Connor et al 2014, Conley & Watts 2013, Giguere et al 2013, Kwon et al 2012, Lee et al 2010, Nadella et al 2010, Regner et al 2011, Yao et al 2005). Recently, studies have focused on the importance of GPR proteins in chemokine receptor signal processing where AGS3 was implicated in a non-canonical signaling complex directing migration of neutrophils and loss of AGS3 decreased chemotaxis and chemokine receptor processing of various leukocyte populations (Branham-O'Connor et al 2014, Kamakura et al 2013). Additionally, immunoprotective phenotypes for a model of rheumatoid arthritis were observed in the absence of AGS4, which has restricted expression to immunological tissues (Cao et al 2004, Giguere et al 2013, Zhao et al 2010). Thus, the Gαi – GPR complex may be a favorable target for development of therapeutics. A screening platform was developed to identify modulators of this interaction by observing alterations in the BRET signal between Gαi subunits and GPR proteins, using AGS4 as a model GPR protein. Primary and secondary screening identified seven potential hits that decreased the BRET signal between Gαi and AGS4 as well as AGS3 (Chapter 2, Figure 2.8). Further development of these compounds will be advantageous to future investigations of the Gαi – GPR interaction in other Group II AGS proteins as a reagent for blocking this association or development of novel therapeutics for pathologies linked to GPR proteins.

AGS3 exhibits a functional role in chemokine signal processing of leukocytes

Although the biological role of AGS3 in numerous physiological processes has been described, a functional role for AGS3 in the regulation of chemokine receptor signaling of hematopoietic cells was unknown (Blumer et al 2008, Bowers et al 2008, Bowers et al 2004, Fan et al 2009, Groves et al 2007, Kwon et al 2012, Nadella et al 2010, Pattingre et al 2003, Regner et al 2011, Sanada & Tsai 2005, Vural et al 2010, Yao et al 2005). Leukocytes incorporate dynamic signaling events through highly specialized, spatially integrated, G-protein signaling mechanisms to function properly (Cho & Kehrl 2009, Kehrl et al 2009). Similarly AGS3 is implicated in dynamic signaling processes of the central nervous system where adaptation of G-protein signaling is required for appropriate responses of the system (Bowers et al 2008, Bowers et al 2004, Fan et al 2009, Sanada & Tsai 2005, Yao et al 2005).

In recognition of these investigations, activation of primary AGS3-null leukocytes exhibited enhanced expression of AGS3 demonstrating that levels of AGS3 are influenced by changing extracellular environmental signals and suggesting a significant purpose for AGS3 in chemokine signal processing of these cells. Loss of AGS3 in leukocytes resulted in significantly decreased chemokine-directed migration that was not contributed to decreased receptor expression or decrease in chemokinesis as compared to WT leukocytes (Chapter 3, Figures 3.4A, 3.4C and 3.5A). Chemokine stimulated activation of ERK1/2, primarily contributed to Gβγ mediators, was also decreased demonstrating additional components of chemokine receptor processing are affected by loss of

AGS3 (Chapter 3, Figures 3.6A and 3.6C). These data suggest that a role for AGS3 in hematopoietic cells through positive modulation of Gβγ signaling events, whereas loss of this protein results in decreased periods of Gβγ activity.

A biological function for AGS4 in leukocyte chemokine signal processing was determined

Unlike AGS3, the biological function of AGS4 is less well understood. The expression of AGS4 is known to be restricted to tissues of immune origin, but only recently has AGS4 been found to be involved in pathways corroborating with its expression profile (Cao et al 2004, Giguere et al 2013, Giguere et al 2014, Schmidt et al 2012, van Bodegom et al 2012, Zhao et al 2010, Zhong et al 2012). Thus AGS4-null mice were used to define a functional role for AGS4 in primary leukocytes. Circulating populations of neutrophils and lymphocytes were altered in the absence of AGS4, possibly resulting from a defective trafficking mechanism (Chapter 3, Figure 3.2). Chemokine-directed migration of T lymphocytes, dendritic cells, and neutrophils was investigated and revealed a requirement for AGS4 in maximal chemotaxis in these cells (Chapter 3, Figures 3.4B, 3.4D, 3.4E). As seen with AGS3-null mice, levels of chemokine receptors and random migration were unaltered in the leukocytes from AGS4-null mice (Chapter 3, Figure 3.5B). Chemokine-mediated activation of ERK1/2, another mechanism in chemokine signal processing, was significantly reduced in the absence of AGS4. Furthermore, we demonstrated that the reduction in *ex vivo*

chemokine-directed migration of neutrophils translated to a deficiency of neutrophil recruitment to a site of induced-inflammation *in vivo*. The implications of defective chemokine signal processing upon loss of GPR-containing proteins will be addressed in the following section.

Perspective/Context in the Field

This dissertation expands on previous observations that suggested Gαi1 – GPR complexes were regulated by 7TMRs in a reversible manner (Oner et al 2010a, Oner et al 2010b, Vellano et al 2013). Developing this notion in the context of immunological systems, Gαi2 – GPR complexes were found to be regulated by agonist-dependent activation in close proximity to the chemokine receptor CXCR4. Further investigation into the direct coupling of this complex to the receptor demonstrated the observed regulation was proximal to the integral membrane protein $\alpha_{2A/D}$-AR and not resulting from subunit flux between endogenous and tagged proteins. My research further validated the direct coupling of 7TMRs to a GαiGPR complex, ostensibly promoting nucleotide exchange in a manner that may be similar to 7TM receptor engagement of Gαβγ heterotrimer. Several interesting conceptual thoughts emanate from this aspect of my research. Considering the simultaneous docking of up to 3-4 Gαi subunits on AGS4 and AGS3, my research provides further support to an interesting potential for these proteins to scaffold receptors and Gα subunits within a larger signaling complex (Adhikari & Sprang 2003, Bernard et al 2001, Blumer & Lanier 2014, Jahangeer & Rodbell 1993, Jia et al 2012, Kimple et al 2004). Also, previous reports and my research demonstrate the regulation of the GαiGPR complex is sensitive to pertussis toxin in a manner similar to that observed for canonical Gαiβγ heterotrimer, suggesting that functional effects imparted by pertussis toxin may in part be attributed to 7TMR regulation of GαiGPR complexes (Oner et al 2010a, Oner et al 2010b). Finally, differential regulation by

hormones, neurotransmitters, and small molecules may represent ligand bias of 7TMRs to couple GαGPR or canonical heterotrimer complexes thus modulating signal transmission. Therefore, characterization of the regulation of the novel GαGPR signaling complex positions 7TM receptors to potentially couple to one of three known (and possibly more) signaling complexes including Gαβγ, GαGPR and/or arrestin in a ligand-dependent, conformationally-selective manner to potentially generate alternate signaling pathways and allow flexibility of signal processing for these receptors (Azzi et al 2003, Barlic et al 2000, Lambright et al 1996, Lefkowitz & Shenoy 2005, Luttrell & Lefkowitz 2002, Rasmussen et al 2011, Wall et al 1995).

The regulation of subcellular distribution and function of GPR-containing proteins is typically attributed to the binding of interacting proteins or post-translational modifications (An et al 2008, Blumer et al 2003, Blumer et al 2002, Du & Macara 2004, Du et al 2001, Pizzinat et al 2001, Shu et al 2007). My research further builds upon these studies by identifying potential binding partners ARID1b, eEF1d and 14-3-3 proteins for AGS4, which, aside from Gαi, lacks defined interacting proteins. Interactions of AGS4 with these alternative binding proteins may reveal unexpected and potential modulatory functions for AGS4 in protein synthesis and transcriptional modulation. Potential binding of AGS4 to the site coinciding with diphtheria toxin inactivation of eEF2 suggests an interesting concept that binding of AGS4 to eEF2 may cause decreased protein synthesis (Van Ness et al 1980). The binding of ARID family members with AGS4 was quite unexpected, but this interaction may position AGS4 to bind

ARID proteins excluding them from the nucleus, thus potentially regulating transcription. These potential regulatory roles of AGS4 in protein synthesis and gene transcription were unanticipated, but require further studies to delineate the true nature of their biological functions. Additionally, tyrosine phosphorylation of AGS4 by the cytokine TSLP was recently described (van Bodegom et al 2012, Zhong et al 2012). Our data demonstrate that JAK2 and Src phosphorylate a critical tyrosine residue (Y108). Mutation of AGS4-Y108, which is positioned within the second GPR motif in AGS4, to Asp, reduced Gαi binding to AGS4 by nearly 70%, suggesting that Y108 phosphorylation may be a key (and novel) mechanism for cells to regulate the AGS4 – Gαi interaction. Interestingly, activation of JAK2 and Src is also linked to $G\alpha i_{GTP}$ and 7TMRs by multiple studies (Corre et al 1999, Luttrell et al 1997, Ma et al 2000, Marrero et al 1995, Vila-Coro et al 1999). The phosphorylation of AGS4 by JAK2 and Src may therefore represent a positive modulatory loop of the kinases. Reduction of AGS4 sequestration of Gαi subunits would enhance 7TMR activation of Gαi further prolonging activation of the kinases and downstream transcription factors including STAT1 and STAT3 (Cao et al 1996, Darnell et al 1994).

Reports have recently begun to implicate GPR proteins in immunological signaling pathways, which is supported by my findings of the significance of AGS3 and AGS4 expression in leukocytes (Branham-O'Connor et al 2014, Cao et al 2004, Cho et al 2000, Giguere et al 2013, Giguere et al 2014, Schmidt et al 2012, van Bodegom et al 2012, Zhao et al 2010, Zhong et al 2012). My research has defined signaling mechanisms that require AGS3 and AGS4 for maximal

signal integration including chemokine-induced chemotaxis and activation of ERK1/2. Interestingly, LGN expression (sharing structural domains and over 60% sequence homology with AGS3) is unaffected by AGS3 knockout and there appears to be no compensatory mechanism observed in these animals or animals that have both AGS3 and AGS4 absent (Figure 3.4, 3.6 and 4.4). These results suggest potentially divergent signaling pathways where chemokine signal processing may be more structured to AGS3 signaling while LGN is not a participant. However, the functions of AGS3 and AGS4 in chemokine signal processing that were observed could suggest a potential positive modulatory role for Gβγ signaling in this context. The restrictive expression of AGS4 to immunological tissues has led to recent investigations into its role as an inflammatory mediator in arthritis models (Giguere et al 2013, Schmidt et al 2012). This immunoprotective phenotype resulting from decreased proinflammatory signaling observed in the absence of AGS4 is supported by my research that defines the importance of AGS4 to chemokine signal processing including chemokine-directed migration of leukocytes to sites of inflammation. The use of *ex vivo* (primary cells) and *in vivo* animal models in my work has revealed novel functional roles for GPR proteins in integrating chemokine signals in the immune system. Furthermore, targeting of the GPR proteins may offer an alternative approach for development of therapeutics to pathologies that utilize chemokine receptor signaling (i.e. inflammatory diseases and certain cancers) (Branham-O'Connor et al 2014, Cunningham et al 2010, Giguere et al 2013, Koelink et al 2012, Singh et al 2010).

Moreover, investigation of the regulatory elements modulating the Gαi – GPR complex and identification of a biological role for AGS3 and AGS4 in chemokine signal processing suggests the Gαi – GPR module could provide a novel platform for the identification of pathway-targeted drugs. First steps into the initial exploration for potential modulators of the Gαi – GPR are described in this dissertation. Further development of these compounds will provide unique reagents to probe the functionality of the Gαi – GPR module and provide a starting platform for potential therapeutic manipulation. Therapeutics against the Gαi – GPR module may be advantageous in several disease pathologies connected with GPR containing proteins including drug addiction and craving, learning disability and memory, ischemia reperfusion injury, polycystic kidney disease, blood pressure control, energy expenditure and metabolism, and rheumatoid arthritis and inflammatory pathways (Blumer et al 2008, Bowers et al 2004, Branham-O'Connor et al 2014, Conley & Watts 2013, Giguere et al 2013, Kwon et al 2012, Lee et al 2010, Nadella et al 2010, Regner et al 2011, Yao et al 2005).

Emerging from the results demonstrated in this dissertation, three working hypotheses for the action of GPR proteins in leukocyte migratory response are supported (Figure 4.1). One hypothesis is that chemokine receptors directly couple to the Gαi – GPR complex sensing conformational changes in the receptor, analogous to Gαiβγ heterotrimer, resulting in guanine nucleotide exchange and subsequent release of the Gαi subunit to modulate chemotactic response (Figure 4.1). This hypothesis has support from previous reports

suggesting the existence of a Gαi – GPR signaling module and selective Gαi responses in chemokine signaling (Huang et al 2003, Hwang et al 2007, Kamakura et al 2013, Oner et al 2010a, Oner et al 2010b, Vellano et al 2011a, Wiege et al 2012). Thus, in this hypothesis, chemokine signaling would have a Gαi-mediated component in addition to the classically accepted Gβγ signaling mechanisms, where the Gαi – GPR module may be operational. A second possibility is that binding of Gαi by GPR proteins prior to reformation of the Gαiβγ heterotrimer could prolong or enhance Gβγ signaling through slowing heterotrimer reassociation and thereby facilitating directed migration (Figure 4.1). This hypothesis has support from previous studies that conclude chemokine receptor activation and subsequent directed migration occur predominantly through Gβγ mediated activation of PI3Kγ and PLCβ2/3 and the downstream mediators (Arai et al 1997, Hirsch et al 2000, Li et al 2000, Neptune & Bourne 1997, Neptune et al 1999, Peracino et al 1998, Sotsios et al 1999, Stephens et al 1994, Stephens et al 1997, Wang et al 2014). Small molecule inhibitors and activators of Gβγ have further demonstrated the necessity of Gβγ signaling in chemokine response of leukocytes (Kirui et al 2010, Lehmann et al 2008, Surve et al 2014). The final working hypothesis would implicate the Gαi-GPR module, generated subsequent to receptor activation and prior to heterotrimer reassociation, functioning independently to initiate the formation of an alternative, non-canonical signaling complex distinct from Gβγ that modulates chemokine-directed signaling events (Figure 4.1). A recent report supports this hypothesis by demonstrating that the Gαi – AGS3 complex managed to assemble a Par3 –

aPKC complex through interaction with mInsc which was required for directional migration of neutrophils (Kamakura et al 2013). In this manner, the Gαi – GPR module could function as an independent active signaling entity or a platform for further diverse signal inputs (Blumer & Lanier 2014, Blumer et al 2012, Gonczy 2008). It is also possible that any combination of these hypotheses may be at work concurrently in migrating cells to provide the proper signaling responses to chemoattractants and may be the underlying factor that contributed to the observed deficiency of AGS4-KO neutrophils to respond to inflammatory stimulus *in vivo*. Further research is needed to make direct conclusions as to which of these hypotheses best describes the regulated interaction between Gαi and proteins expressing multiple GPR motifs; however, the work presented in this dissertation further depicts a diverse signaling mechanism for this dynamic complex and biological functions yet described for AGS3 and AGS4 proteins in immunology.

Figure 4.1

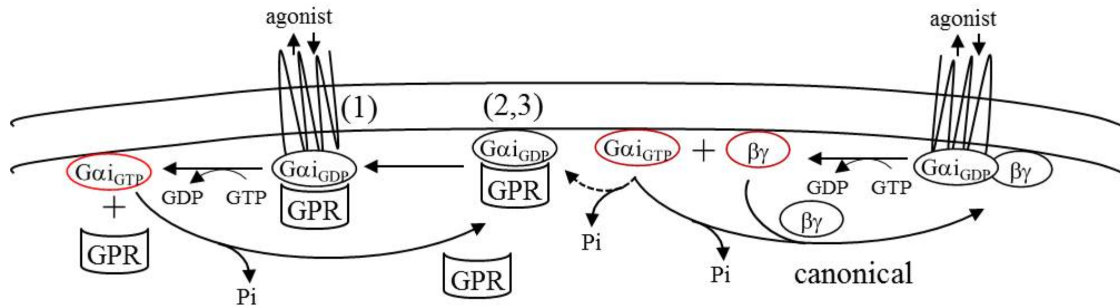


Figure 4.1 Schematic illustration of working hypotheses for the influence of GPR containing proteins on G-protein signal integration

The GαGPR complex may present as direct targets for 7TMR activation. In this scenario, the GPR protein would function analogous to Gβγ in the canonical Gαβγ heterotrimer. Agonist-mediated receptor activation catalyzes nucleotide exchange on GαGPR module to release active $Gαi_{GTP}$ (red outline). Gαi subunits undergo GTP hydrolysis through intrinsic GTPase activity of the Gαi subunit or acceleration of GTPase activity by RGS proteins inactivating the subunit. $Gαi_{GDP}$ can then reassociate with GPR motifs (1). GPR proteins may also influence subunit interactions subsequent to receptor activation of Gαiβγ heterotrimer. In this scenario, agonist-bound receptor catalyzes nucleotide exchange on Gαiβγ heterotrimer to release active $Gαi_{GTP}$ and Gβγ subunits to mediate secondary signals (red outline). GTP hydrolysis of the Gαi subunits inactivates the subunit which can then reassociate with Gβγ or GPR motifs (2). $Gαi_{GDP}$GPR complexes formed during receptor-mediated cycling of G-protein may act to prolong or enhance Gβγ signaling (2). This $Gαi_{GDP}$GPR may also form non-canonical

signaling complexes distinct from Gβγ mediated signaling (e.g., those involved in the regulation of mitotic spindle dynamics and cell polarity (3).

Future directions

Demonstrate the 7TMR – Gαi – GPR interaction results in the formation of a functional ternary complex

My research determined the regulation of Gαi tethered to a 7TMR with GPR proteins to be functionally active in the absence of endogenous G-protein subunits, but further validation of the formation and regulation of this complex is required. One possibility is to track the Gαi – GPR module before and after receptor activation. Using a membrane fractionation assay with the receptor – Gαi2 fusion protein linked with BRET or immunoblotting, one could demonstrate the displacement of AGS3 or AGS4 from the membrane after receptor activation further suggesting the disruption of a Gαi – GPR module by the 7TMR activation similarly demonstrated for untethered Gαi1-YFP untethered constructs (Oner et al 2010a, Oner et al 2013b). Tracking of AGS3 and AGS4 during agonist treatment could be accomplished through fluorescent labeling and real-time microscopy of the GPR-containing proteins. Furthermore, direct physical association of the three components (GPR protein, Gαi, and 7TMR) would further demonstrate Ga-GPR coupling to a 7TMR. Following previously described methodologies, co-immunoprecipitation could be used to isolate all three components simultaneously in the presence or absence of agonist to demonstrate ternary complex formation and regulation by 7TMRs (McCoy et al 2010). To fully determine direct coupling of the Gαi – GPR module to the receptor, a biochemical approach could be used to demonstrate receptor-stimulated nucleotide exchange on Gαi within this complex. A reconstituted

system consisting of purified 7TMR and Gαi subunits pre-assembled with either GPR-containing proteins or Gβγ would be incubated with $GTP\gamma^{35}S$ in the absence or presence of agonist. Agonist-induced $GTP\gamma^{35}S$ binding to Gαi-GPR would confirm direct coupling of the Gαi-GPR module to 7TMRs as a substrate for receptor-catalyzed nucleotide exchange in a manner analogous to Gαβγ heterotrimer.

Define intrinsic differences between AGS3 and AGS4 in the differential regulation of the Gαi-GPR interaction by 7TMRs

My research as well as previous reports have consistently observed that agonist-induced changes in the Gαi – AGS3 interaction are much greater than that seen for Gαi – AGS4 (Chapter 2, Figures 2.1-5) (Oner et al 2010a, Oner et al 2010b), An obvious distinction between AGS3 and AGS4 is the presence of TPR domains. These domains have been implicated in regulatory functions for AGS3 and LGN (Adhikari & Sprang 2003, An et al 2008, Bernard et al 2001, Blumer et al 2003, Oner et al 2010a, Oner et al 2013c, Pan et al 2013, Vural et al 2010); therefore, I initially hypothesized that the presence of these domains may be responsible for the discrepancies observed between AGS3 and AGS4 in the context of 7TMR regulation. Initial experiments using the $\alpha_{2A/D}$AR – Gαi fusion protein BRET platform to compare the regulation of Rluc tagged AGS3-sh and AGS4-sh proteins (which both exhibit truncation of the amino terminus and express three GPR motifs), demonstrated that the interaction of AGS3-sh with Gαi and subsequent regulation was still similar to that observed for AGS3, while AGS4-sh was similar to the full-length AGS4 as well (Chapter 2, Figure 2.5D).

These data would suggest properties intrinsic to the GPR motifs of AGS3 or AGS4 are responsible for imparting their distinct agonist-mediated regulation profiles. Alternatively, intervening residues between the GPR motifs in each protein may also impart a regulatory role on the Gαi interaction with AGS3 versus AGS4. In support of these findings, a single amino acid alteration within the GPR II motif of AGS4 was previously reported to affect Gαi binding (Kimple et al 2004). Additionally, intrinsic differences between Gαi binding to single GPR motifs of AGS3 have demonstrated the importance of the interlinking sequences to specific domains (Adhikari & Sprang 2003). Thus, taking these observations into account, the differential regulation may be due to intrinsic differences between the GPR motifs and/or intervening residues between these domains specific to AGS3 or AGS4. This question could be initially addressed by generating chimeric proteins between AGS3-sh and AGS4 GPR domains. The regulation of such constructs by 7TMRs could shed light on amino acids involved in the distinct regulation of AGS3 versus AGS4 in our BRET experiments.

The diversity of regulation of AGS3 and AGS4 may coincide with distinct functional roles for these proteins. Similar regulation of these complexes by 7TMRs would illustrate a redundant mechanism, while differences suggest a more dynamic signaling process. One could envision AGS proteins functioning as dynamic repositories for Gαi subunits. Under physiological conditions, Gαiβγ heterotrimers are responsible for a large majority of G-protein signaling; however when stressed or under abnormal conditions, the cell could potentially "tap" into these sources of surplus Gαi subunits to maintain effective G-protein signaling. In

this context differential regulation of the Gαi – GPR complex could represent distinct repositories of Gαi where AGS3 has more readily available Gαi subunits for use in the cells while AGS4 has more stringent retention of Gαi subunits for cells to potentially harness during periods of high activity or stress. These dynamically regulated repositories could represent an unexplored mechanism utilized by cells where variations in regulation of the Gαi – AGS3 and Gαi – AGS4 interactions would be advantageous.

Determine if multi GPR-containing proteins can effectively scaffold larger signaling complexes to promote signaling

As previously discussed, proteins containing multiple GPR motifs can effectively dock 2-4 subunits of Gαi simultaneously (Adhikari & Sprang 2003, Bernard et al 2001, Blumer & Lanier 2014, Jahangeer & Rodbell 1993, Kimple et al 2004). Thus, these proteins could potentially scaffold larger receptor complexes each bound to a single Gα subunit. As an initial approach to determine the effect of multi GPR-containing proteins on the formation of larger signaling complexes, I generated a $\alpha_{2A/D}$-AR – Gαi-Rluc fusion protein to be used in concert with the $\alpha_{2A/D}$-AR – Gαi-YFP construct. Increasing levels of AGS3 and AGS4 were able to effectively congregate differentially tagged receptors as determined by BRET measurements, while Q/A mutants that are unable to bind Gαi demonstrated no such effect (Figure 4.2). As an initial follow-up to this experiment, the effect of agonist on the GPR-dependent assembly of these receptor complexes could be determined. Moreover, to further validate the formation of these higher order complexes on the cell surface versus intracellular

domains, time-resolved fluorescence resonance energy transfer (TR-FRET) linked with snap-tag technology can be used. This technique would specifically label the $\alpha_{2A/D}$-AR expressed on the cell surface with FRET capable fluorophores followed by TR-FRET analysis to determine association of the receptors in the presence of increasing levels of GPR proteins as previously used to determine dimerization of other GPCRs (Maurel et al 2008). Although the scaffolding of larger receptor complexes is an interesting potential role for multi-GPR proteins, further investigation into this function is required.

Figure 4.2

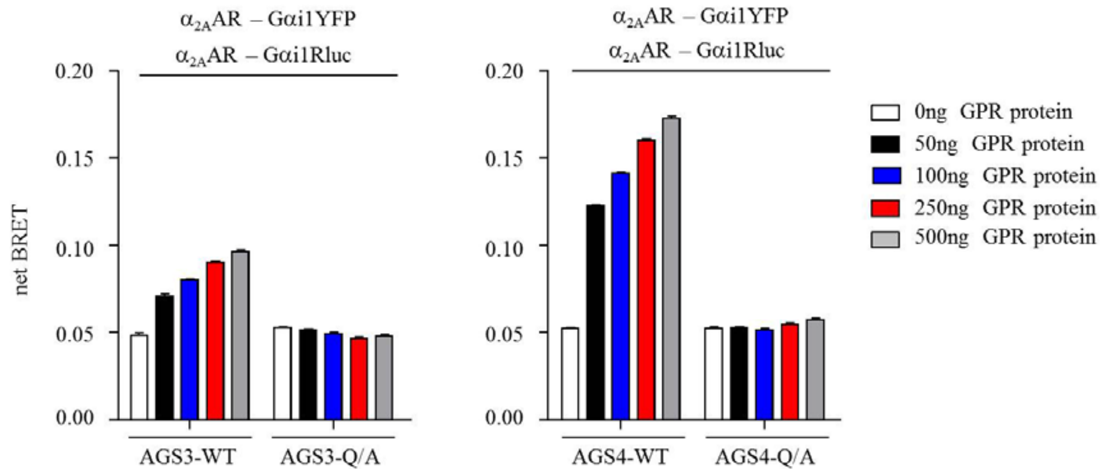


Figure 4.2. GPR-containing proteins scaffold larger receptor signaling complexes

(*Left panel)* Net BRET signals were obtained from HEK293 cells transfected with $\alpha_{2A/D}$-AR–Gαi1YFP (500 ng) and $\alpha_{2A/D}$-AR–Gαi1Rluc (500 ng) with increasing levels of AGS3-WT or AGS3-Q/A (0-500 ng). (*Right panel)* Net BRET signals were obtained from HEK293 cells transfected with $\alpha_{2A/D}$-AR–Gαi1YFP (500 ng) and $\alpha_{2A/D}$-AR–Gαi1Rluc (500 ng) with increasing levels of AGS4-WT or AGS4-Q/A (0-500 ng). Fluorescence and luminescence readings were measured as described in "Chapter 2: Experimental Procedures." All data are expressed as means ± SEM from at least 3 independent experiments with triplicate determinations.

Validate binding regions and functional significance of ARID1b and eEF1d binding AGS4

Identification of binding partners for AGS4 demonstrates potential modulatory functions for AGS4 in alternative signaling pathways. Moving forward, the alternative binding partners of AGS4, ARID1b and eEF1d, require validation. Corroboration of these interactions could be accomplished through generation of GST fusions for ARID1b and eEF1d followed by subsequent co-precipitation studies with lysates of cells expressing AGS4. Even after validation, the region of binding of AGS4 to ARID1b and eEF1d and vice versa would require further exploration. Truncation of AGS4, eEF2, and ARID2 (Figure 4.3) followed by co-immunoprecipitation experiments could determine the regions in which these proteins bind one another. Additionally, BRET experiments could be conducted to further validate these interactions by measuring BRET signals between AGS4 and candidate interacting proteins as well as their effect on the AGS4 – Gαi interaction. Furthermore, screening lysates from immune tissues or cell lines could potentially determine any unique binding partners to AGS4 in the context of leukocytes. With the related protein eEF2 found to bind AGS4 at the carboxy terminal domain where diphtheria toxin is known to inactivate the protein, it would be interesting to determine the effect of AGS4 binding on protein translation (Cao 2005, Van Ness et al 1980). ^{35}S-methionine incorporation into proteins over a period of time in the presence of increasing AGS4 and AGS4-Q/A could demonstrate the effect of AGS4 on protein synthesis. As for the unique interaction of AGS4 with ARID1b, investigation into a potential role of AGS4 in

modulating the endogenous role of ARID1b in transcriptional regulation could exhibit interesting findings. A luciferase reporter assay was recently described to determine the direct effect of inducible ARID1b on c-myc and p21 promoter activity demonstrating both repressive and stimulatory actions of transcription, respectively (Inoue et al 2011). Co-expression of increasing levels of AGS4 or AGS4-Q/A could be monitored for alterations in luciferase activity in this model to determine if the ARID1b component of the SWI/SNF complex can be modulated in part by AGS4 binding. These studies would allow for conclusions to be drawn concerning the function of AGS4 in alternative signaling pathways unexplored as to date.

Figure 4.3

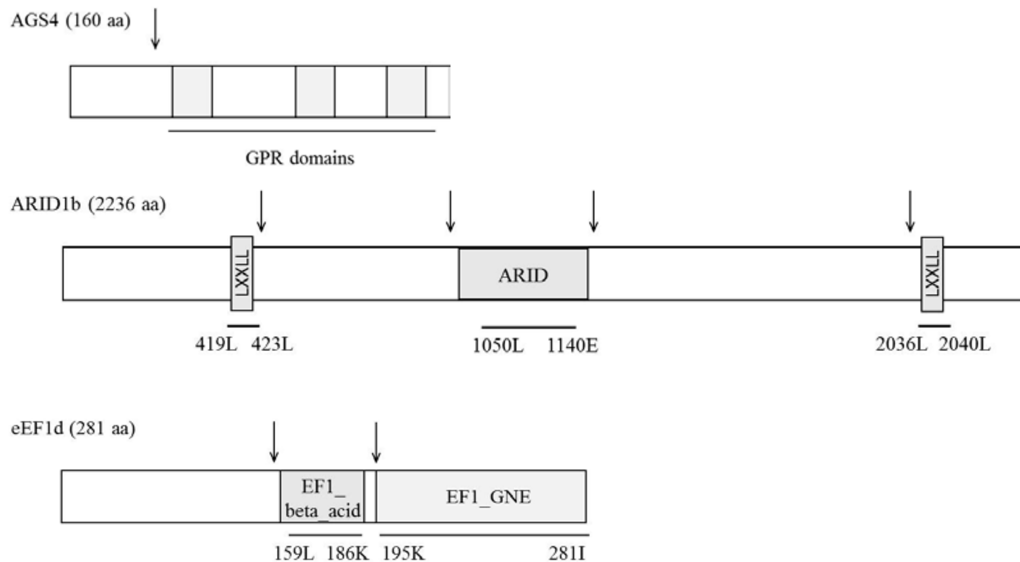


Figure 4.3. Truncation of potential AGS4 interacting proteins

Depicted are schematic representations of AGS4 and potential alternative binding partners of AGS4 identified in my affinity purification screen. Major domains for each protein are identified: 1) GPR domains - G-protein regulatory domains; 2) LXXLL – Nuclear receptor recognition sequence; 3) ARID – AT-rich interaction domain; 4) EF1_beta_acid – Central acidic region related to eEF1b domain; 5) EF1-GNE – Guanine nucleotide exchange domain. Arrows shown are potential sites of truncation to define the region of AGS4 binding to these proteins.

Establish a role for AGS4 phosphorylation in TSLP signal transduction

Earlier reports define post-translational modifications within or proximal to GPR motifs to affect interaction of Gαi subunits with GPR motifs (Adhikari & Sprang 2003, Blumer et al 2003, Hollinger et al 2003, Kimple et al 2004). Building on this notion my dissertation demonstrated that phosphorylation of one (Y108) of the two tyrosine residues expressed by AGS4 effectively decreases the binding of Gαi to the GPR domain. Also recent evidence depicted Y108 being phosphorylated subsequent to TSLP signaling, which my research determined could be potentially mediated by JAK2 and Src kinases activated downstream of TSLP activation (Zhong et al 2012). This is important since TSLP signaling has been shown to be a critical mediator in regulating the immune response in pathologies involving allergies and hypersensitivity, such as asthma and atopical dermatitis (He et al 2008, Liu et al 2007, Redhu et al 2013, Roan et al 2012, Wilson et al 2013, Ziegler 2012, Ziegler et al 2013). Thus, the finding that JAK2 and Src kinases phosphorylate AGS4, potentially through TSLP-mediated activation, begs the question of the regulatory role AGS4 plays on this signaling cascade. To address this possible modulatory function, Jurkat and JAWSII cell lines (representing T-lymphocytes and dendritic/monocyte cells), could be activated by TSLP in the presence of increased levels of AGS4, AGS4Y108F, AGS4Y108D, or AGS4-Q/A to determine the effect of AGS4 phosphorylation on the downstream phosphorylation and subsequent activation of STAT molecules (STAT1, STAT3, and STAT5).

Development of small molecules found to modulate the Gαi – GPR interaction

The role of GPR proteins in various biological systems is intensely investigated through the use of knockout animals (Blumer et al 2008, Branham-O'Connor et al 2014, Giguere et al 2013, Giguere et al 2014, Kwon et al 2012, Lee et al 2010, Regner et al 2011). Development of compounds that block Gαi – GPR would therefore be of great utility in further exploration of the functional role of these proteins. My dissertation represents a step forward in the identification of such compounds, but much more research is required to validate and develop the small molecules presented by this work. Initially, independent validation of the efficacy of these compounds using purified Gαi and AGS3 and AGS4 proteins would be conducted, coupled with immunoblotting or BRET analysis as subsequent readout for disruption of the Gαi – GPR interaction. Furthermore, determination of efficacious concentrations for these potential small molecule modulators would be determined by administering dose responses of the small molecules in our BRET system and in GTPγS binding experiments. Obtaining IC_{50} and EC_{50} values for each compound would assist in the determination of proper dosing to be used on primary cells and potentially in animal models. Secondary screening in primary WT leukocytes would then be conducted to observe if similar defects in chemokine signal integration are recapitulated as were seen in the AGS3-null and AGS4-null animals upon treatment with the small molecule modulators. As a final testament to the potential of these compounds to be developed into therapeutics, I would administer them into mice

subjected to collagen antibody-induced arthritis (CAIA) to simulate rheumatoid arthritis as described previously (Giguere et al 2013, Khachigian 2006). Importantly, GPR proteins are also involved in cells that utilize chemokine receptors CXCR4 and CCR7 for non-canonical measures, such as secondary metastasis in certain malignancies (Cunningham et al 2010, Singh et al 2010). Thus, it would be interesting to determine if administration of these compounds in a xenograft mouse model of inflammatory breast cancer could reduce secondary metastasis (Singh et al 2010).

Determine if AGS3 and AGS4 have functionally redundant roles in leukocytes

Published results from this dissertation and recent studies have connected chemokine signal integration with proteins containing GPR motifs (Branham-O'Connor et al 2014, Cho et al 2000, Giguere et al 2013, Giguere et al 2014, Schmidt et al 2012, van Bodegom et al 2012, Zhong et al 2012). Interestingly, compared to mice lacking Gαi2, the deficiencies seen in this dissertation appear modulatory in nature (Cho et al 2012, Han et al 2005, Hwang et al 2007, Pero et al 2007, Rudolph et al 1995, Skokowa et al 2005, Zarbock et al 2007). Furthermore, it is possible that the effect of loss of AGS3 or AGS4 is masked by the presence of the opposing protein in leukocytes as well as other GPR-containing proteins including LGN/Gpsm2 and RGS14 (Blumer et al 2002, Cho et al 2012, Oliaro et al 2010). Thus, generation of a model deficient in both AGS3 and AGS4 would be of high importance to determine if there is a redundant effect in chemokine-mediated signaling with the simultaneous deletion of both these

proteins. Recently, I have generated these mice through cross breeding of heterozygous mice from Gpsm1$^{+/-}$ and Gpsm3$^{+/-}$ backgrounds (DKO). Initial experiments are promising and demonstrate a potentially synergistic effect of the loss of both AGS and AGS4 as revealed in a dramatic decrease in *ex vivo* neutrophil chemotaxis (Figure 4.4A). Additionally, circulating populations in DKO animals illustrate similar neutropenia and lymphocytosis as seen in AGS4-null mice (Figure 4.4B). Similar experiments to those portrayed in this dissertation may determine that the loss of multiple GPR proteins further exacerbates the phenotypes that were observed with the loss of either AGS3 or AGS4. These animals represent an important tool in the determination of Gαi – GPR function in immunological tissues. Furthermore, the deficiency in chemokine-directed migration observed in AGS3-null, AGS4-null, and DKO animals suggests a fundamental issue with directed motility in these leukocytes. Live cell microscopy of these cells in real time could be used to track the overall speed, velocity, and directionality to chemokine stimuli to determine where the deficiency lies (Kamakura et al 2013). In order to investigate if the functional defects seen in *ex vivo* experiments translates to an *in vivo* phenotype, intravital imaging could be used after laser-induced insult as described previously (Cho et al 2012).

Figure 4.4

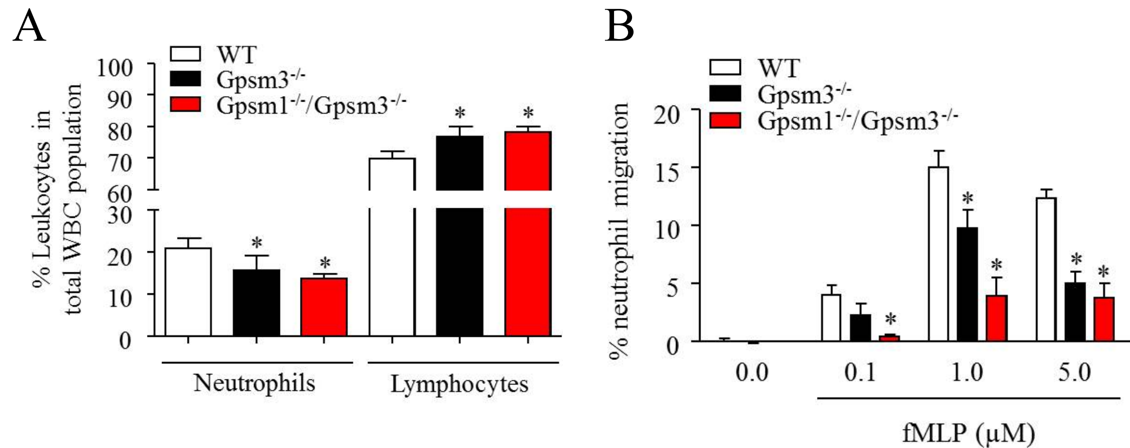


Figure 4.4. Gpsm1$^{-/-}$/Gpsm3$^{-/-}$ demonstrate altered leukocyte populations and substantial reduction in neutrophil chemotaxis

(A) Blood was collected from WT, Gpsm3$^{-/-}$, and Gpsm1$^{-/-}$/Gpsm3$^{-/-}$ mice by cardiac puncture as described in Experimental Procedures (*Chapter 3*). Complete blood cell counts (CBCs) were performed within 2 hr of blood collection using a HemaVet 950 instrument to measure leukocyte levels in each sample. Percent of leukocyte populations in relation to total number of white blood cells was calculated.

(B) Neutrophils from WT, Gpsm3$^{-/-}$, and Gpsm1$^{-/-}$/Gpsm3$^{-/-}$ mice were isolated from freshly harvested bone marrow as described in Experimental Procedures (*Chapter 3*). Isolated neutrophils were loaded in transwell migration chambers with the bottom chamber containing serum-free RPMI with 0.1% BSA and 2mM EDTA in the absence and presence of fMLP (0.1–5 μM). After 3h at 37°C, cells in the bottom chamber were counted, and the percentage of cells migrated was

calculated relative to the input, where the number of cells migrating in the absence of chemokine was subtracted.

Data are represented as the mean +/- S.E. of at minimum 3 independent experiments with at least triplicate determinations (* denotes $p < 0.05$ as compared to WT leukocytes based on Tukey's post hoc test following ANOVA).

Determine if the defective inflammatory response observed for AGS4-null mice is due to a synchronous or independent function of neutrophils and endothelial cells.

This dissertation also demonstrated a possible functional defect in extravasation of neutrophils to a site of inflammation. Indeed, impaired transmigration of other leukocytes has been linked to Gαi2 signaling both in the immunological cells as well as endothelial cells involved in trafficking the leukocytes through to the inflamed tissue (Pero et al 2007, Warnock et al 1998, Wiege et al 2012, Zarbock et al 2007). Thus to investigate the extravasation ability of AGS4-null neutrophils to inflamed tissues, intravital two-photon imaging could be utilized as described in recent reports (Kreisel et al 2010, Li et al 2012). The other interesting possibility is that the deficiency could be in the endothelial cells of AGS4-null mice. Determination of the cell responsible for the defective phenotype observed could be delineated by lethal irradiation of AGS4-null or WT mice (Figure 4.5). After reconstitution with bone marrow from the opposing genotype and subsequent recovery for hematopoietic repopulation, induced peritonitis could demonstrate if the AGS4-null neutrophils or AGS4-null endothelial cells are the source of the defective transmigration (Figure 4.5) (Frasca et al 2000, Jakus et al 2009, von Vietinghoff et al 2010). An alternative explanation to this defective infiltration of neutrophils could be represented by a delayed response to immunological challenge. Therefore, inducing peritonitis and harvesting the inflamed tissues at later time points could determine if the AGS4-

null neutrophils eventually migrate to the IP cavity depicting a latent innate response and not a completely deficient one.

Figure 4.5

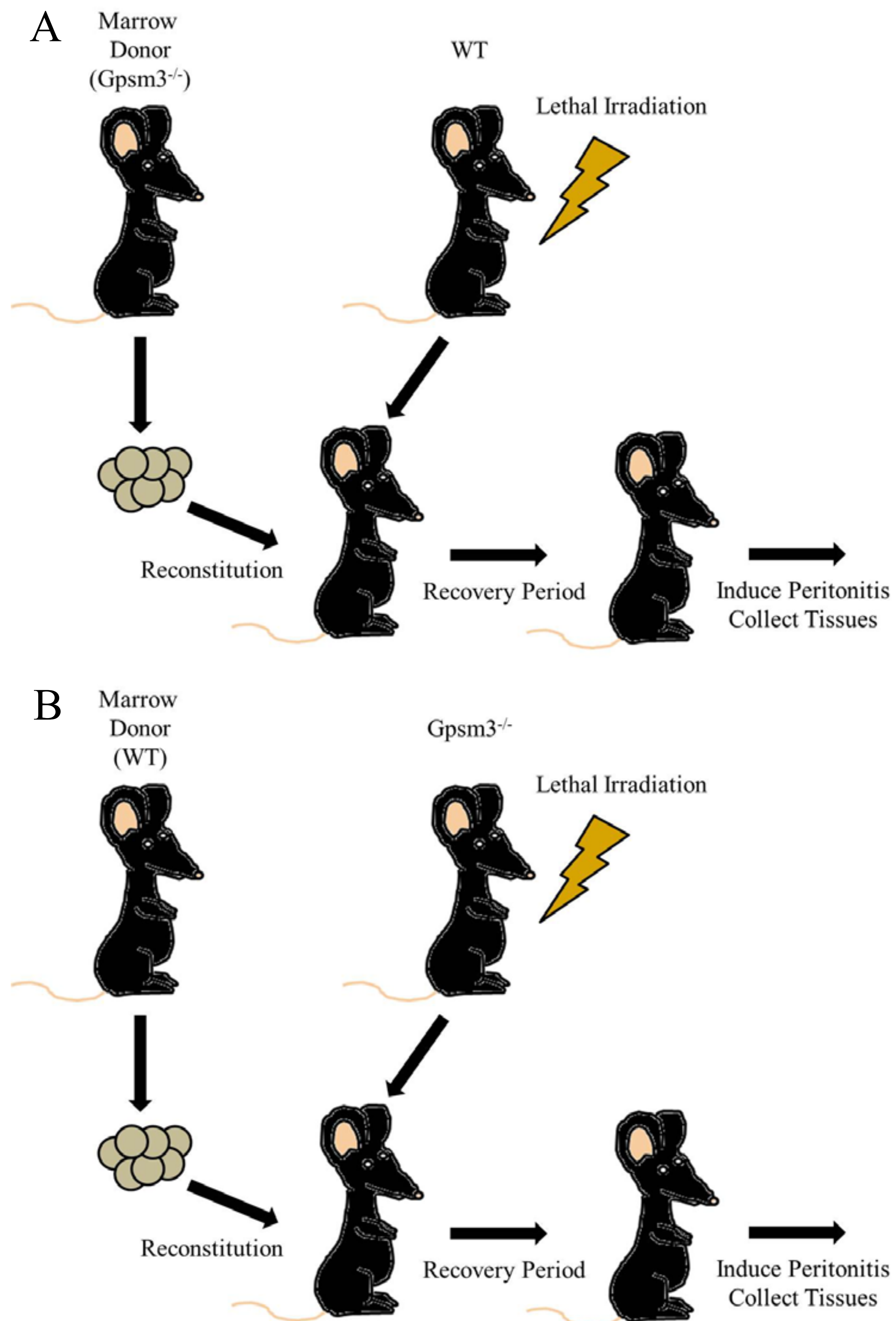


Figure 4.5. Experimental design to determine if neutrophils or endothelial cells are responsible for defective neutrophil infiltration in Gpsm3$^{-/-}$ mice

(A) Gpsm3$^{-/-}$ bone marrow will be isolated as described in Experimental Procedures (*Chapter 3*). WT mice will be lethally irradiated to abolish the endogenous immune system followed by subsequent transplant of the isolated Gpsm3$^{-/-}$ bone marrow as described (Frasca et al 2000, Jakus et al 2009, von Vietinghoff et al 2010). After a sufficient recovery period, mice will have peritonitis induced using 4% thioglycollate. Two hours post-injection, 10 ml of cold, sterile PBS will be used to lavage the intraperitoneal cavity, blood collected by cardiac puncture and femurs processed to harvest bone marrow cells. Samples will have red blood cells lysed and be stained for neutrophil markers, FITC–CD11b and PE–Ly-6G, for flow cytometry analysis.

(B) WT bone marrow will be isolated as described in Experimental Procedures (*Chapter 3*). Gpsm3$^{-/-}$ mice will be lethally irradiated to abolish the endogenous immune system followed by subsequent transplant of the isolated WT bone marrow as described (Frasca et al 2000, Jakus et al 2009, von Vietinghoff et al 2010). After a sufficient recovery period, mice will have peritonitis induced using 4% thioglycollate. Two hours post-injection, 10 ml of cold, sterile PBS will be used to lavage the intraperitoneal cavity, blood collected by cardiac puncture and femurs processed to harvest bone marrow cells. Samples will have red blood cells lysed and be stained for neutrophil markers, FITC–CD11b and PE–Ly-6G, for flow cytometry analysis.

Determine if AGS4 is required for proper clearance of acute infections

Mice of the C57BL/6 background are known to be one of the most highly resistant strains to *Staphylococcus aureus* infections; however, when the innate immune system of the animal is comprised the mice succumb to the infection much more rapidly (Cho et al 2012, McIntyre et al 1989, Verdrengh & Tarkowski 1997, Wiege et al 2013). As a means to determine the extent of the immunodeficiency in AGS4-null mice, *S. aureus* infections would be administered to WT and AGS4-null mice followed by mortality monitoring. Given my encouraging data presented in this dissertation which suggests GPR proteins are important to chemokine signal integration, the future experiments presented above should lay the groundwork for further dissecting the functional roles for GPR proteins in the immune system.

As the culmination of my research, this dissertation demonstrates a diverse array of regulatory mechanisms for the Gαi – GPR module by chemokine receptor regulation and direct coupling of 7TMRs in a manner similar to Gαiβγ heterotrimer. Additionally, my research identified unexpected alternative interacting proteins for AGS4 and phosphorylation of a critical tyrosine residue that may represent unique modulatory functions for this Group II AGS protein. I also defined a biological function for AGS3 and AGS4 in the context of chemokine signal integration in leukocytes of the immune system; furthermore, I determined that AGS4 was required for inflammatory response of neutrophils in intact animals. My dissertation depicts an initial approach to identify compounds

to specifically target the Gαi – AGS4 interaction through a small molecule screening. These small molecule modulators could conceivably be highly valuable as reagents to further investigate the functionality of the Gαi – GPR module or to provide an initial platform for development of potential pathway targeted therapeutics for inflammatory diseases and hematopoietic malignancies.

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Biography

William Gene Robichaux III earned two Bachelor of Science degrees in Microbiology and Chemistry from Nicholls State Univesrity in 2010. He began his graduate training in biomedical sciences at the Medical University of South Carolina in 2010 shortly afterwards joing the Department of Cell and Molecular Pharmacology and Experimental Therapeutics. During his time at MUSC he was nomitted by ASPET at the national Experimental Biology conference for Best Abstract in 2013 and awarded a travel award for his presentation entitled, "Regulation of the AGS4 – Gαi Interaction by Chemokine Receptors and the Non-Receptor Guanine Nucleotide Exchange Factor Ric-8A." At the time of his graduation he has one publication accepted in *Molecular Pharmacology* and another manuscipt currently being drafted for submission. William's work and dissertation were supervised by Dr. Joe B. Blumer.