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MECHANISTIC PATHWAYS AND BIOLOGICAL ROLES FOR RECEPTOR-INDEPENDENT ACTIVATORS OF G-PROTEIN SIGNALING

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Abstract

Signal processing via heterotrimeric G-proteins in response to cell surface receptors is a central and much investigated aspect of how cells integrate cellular stimuli to produce coordinated biological responses. The system is a target of numerous therapeutic agents, plays an important role in adaptive processes of organs, and aberrant processing of signals through these transducing systems is a component of various disease states. In addition to GPCR-mediated activation of G-protein signaling, nature has evolved creative ways to manipulate and utilize the $G\alpha\beta\gamma$ heterotrimer or $G\alpha$ and $G\alpha\beta\gamma$ subunits independent of the cell surface receptor stimuli. In such situations, the G-protein subunits ($G\alpha$ and $G\alpha\beta\gamma$) may actually be complexed with alternative binding partners independent of the typical heterotrimeric $G\alpha\beta\gamma$. Such regulatory accessory proteins include the family of RGS proteins that accelerate the GTPase activity of $G\alpha$ and various entities that influence nucleotide binding properties and/or subunit interaction. The latter group of proteins includes receptor independent activators of G-protein signaling or AGS proteins that play surprising roles in signal processing. This review provides an overview of our current knowledge regarding AGS proteins. AGS proteins are indicative of a growing number of accessory proteins that influence signal propagation, facilitate cross talk between various types of signaling pathways and provide a platform for diverse functions of both the heterotrimeric $G\alpha\beta\gamma$ and the individual $G\alpha$ and $G\alpha\beta\gamma$ subunits.

Keywords

G-proteins; Activators of G-protein signaling; nucleotide exchange; signal transduction; G-protein signaling modulators

1. INTRODUCTION

Signal processing via heterotrimeric G-protein proteins generally involves an initial input sensed by a cell surface GPCR leading to conformational changes in receptor subdomains then transfer this signal to a G-protein, promoting exchange of GTP for GDP and subunit

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dissociation or rearrangement allowing $G\alpha$ and $G\alpha\beta\gamma$ to regulate a number of downstream signaling molecules. Multiple discoveries over the last several years have forced us to broaden our perspective on the role of G-proteins as “signaling switches” recognizing 1) that these entities are regulating intracellular events independent of their role as transducers for GPCRs, 2) the $G\alpha$ and $G\alpha\beta\gamma$ subunits may function independently of each other and 3) the existence of accessory proteins that provide unexpected modes of signal input in this context.

The discovery of alternative modes of regulation of G-proteins and unexpected functional roles for these proteins resulted from a confluence of several independent lines of investigation. A biochemical approach built upon data suggesting cell-specific differences in signal transfer from R to G, the partial purification of a putative non-receptor G-protein activator from extracts of NG108-15 cells and the identification of other non-receptor proteins that could influence the activation state of G-proteins (see Cismowski and Lanier, 2005). An extension of this line of investigation led to the development of a functional yeast-based screen for mammalian entities that activated G-protein signaling in the absence of a receptor (Cismowski et al., 1999; Takesono et al., 1999). In parallel with these studies was the initiative of several labs to search for $G\alpha$ and $G\alpha\beta\gamma$ binding partners in yeast two hybrid screens, (see Table III in Sato et al., 2006a) and the realization that G-protein subunits were associated with intracellular organelles (Stow et al., 1991; Wilson et al., 1994). Interspersed with these biochemical approaches was the realization that there were changes in signal processing through G-protein signaling systems that occurred independent of any obvious changes in receptor number or G-protein expression levels suggesting additional undefined regulatory mechanisms.

Another line of investigation evolved out of the study of asymmetric cell division in *D. melanogaster* neuroblasts and sensory organ precursor cells in parallel with the *C. elegans* embryo. Gotta and Arhinger reported that $G\beta\gamma$ regulated the orientation of the mitotic spindle in *C. elegans* in the one-cell embryo (Gotta and Arhinger, 2001). In addition, $G\alpha$ and a protein containing a G-protein regulatory (GPR) motif (a signature feature of Group II AGS proteins discussed in detail later in this review) interacting with $G\alpha$, were identified as regulators of asymmetric cell division in a large scale RNAi-based functional screen (Kamath et al., 2003). Parallel studies in *D. melanogaster* also led to the identification of key players involved in this biological process (Betschinger and Knoblich, 2004). One of these key players was Pins, which contains four GPR motifs and is an ortholog of the Group II AGS proteins AGS3 and LGN discussed later in this review. Pins and its binding partner Insc influence the positioning of cell fate determinants to generate asymmetry and are important for the stability and targeting of protein complexes that transmit polarity information at the apical cortex of the neuroblast to orient the mitotic spindle. Notably, Pins was identified as a binding partner of $G\alpha$ (Bellaiche et al., 2001; Parmentier et al., 2000; Schaefer et al., 2001; Schaefer et al., 2000; Yu et al., 2000).

Thus we had the confluence of biochemical data indicating unexpected modes of regulation for heterotrimeric G-proteins and data in model organisms implicating $G\alpha$ and $G\beta\gamma$ in control of asymmetric cell division. One of the many interesting aspects of the signaling role played by G-proteins in the asymmetric cell division in the model organisms was that the process was apparently an intrinsically regulated event independent of a cell surface receptor. This initiated a lot of discussion in the literature about the implications of such a functional role for G-proteins, how this process is regulated and what G-proteins might be involved with in the cell independent of their well-characterized role as transducers from cell-surface GPCRs.

The investigations alluded to above revealed four major concepts that have altered our basic concepts of G-protein signaling: 1) $G\alpha$ and $G\alpha\beta\gamma$ are processing signals within the cell distinct from their role as transducers for cell surface receptors; 2) such signals involve previously unrecognized functional roles for heterotrimeric G-protein subunits; 3) $G\alpha$ and $G\beta\gamma$ may exist

complexed with alternative binding partners independent of the classical $G\alpha\beta\gamma$ heterotrimer; and 4) the G-protein activation/deactivation cycle may be regulated independent of nucleotide exchange.

This review focuses on the group of proteins defined in a yeast-based functional screen as receptor-independent activators of G-protein signaling or AGS proteins. The goal of the review is to highlight concepts evolving from the discovery of alternative modes of G-protein regulation via AGS proteins, to discuss various unresolved issues in the field and to provide information on the current status of our knowledge regarding functional roles of AGS proteins. The reader is referred to other reviews for a broader discussion of additional G-protein regulators and a more detailed discussion of the discovery of AGS proteins and their initial characterization along with a more extensive listing of citations (Blumer et al., 2005; Cismowski and Lanier, 2005; Sato et al., 2006a).

2. STRATEGY TO IDENTIFY RECEPTOR-INDEPENDENT ACTIVATORS OF G-PROTEINS

A series of experimental approaches were developed in our laboratory and others to identify novel proteins that directly regulate the activation state of G-proteins. One approach involved the influence of cell and tissue extracts on nucleotide binding to purified G-proteins and a second approach utilized a yeast-based functional screen (Cismowski et al., 2002; Cismowski et al., 1999; Ribas et al., 2002a; Takesono et al., 1999). The first approach resulted in the partial purification and characterization of the NG108-15 G-protein activator (Sato et al., 1996). This protein activates both heterotrimeric brain G-protein and free $G\alpha$ and exhibits mechanistic properties that are distinct from receptor-mediated activation of G-protein (Ribas et al., 2002b). The second approach involved an expression cloning system utilizing the pheromone response pathway in *S. cerevisiae* (Cao et al., 2004; Cismowski et al., 2002; Cismowski et al., 1999; Sato et al., 2006b; Takesono et al., 1999).

The pheromone response pathway in *S. cerevisiae* involves the pheromone receptor as a typical GPCR and a heterotrimeric G-protein with $G\beta\gamma$ coupling to a kinase cascade that regulates mating behavior and growth. Building upon the knowledge base for this system and the diversity of experimental tools developed in this genetically tractable organism, a yeast strain lacking the pheromone receptor and containing a modified mammalian $G\alpha_3$ subunit was used to rapidly screen mammalian cDNAs for their ability to activate the pheromone response pathway in the absence of a receptor using a readout of inducible growth (Cismowski et al., 2002; Cismowski et al., 1999; Takesono et al., 1999). $G\beta\gamma$ subunit is the primary transducer in this signaling pathway activating the kinase cascade that forms the core of the pheromone response. This system is referred to in this review as the yeast-based functional assay and it was used to screen a panel of mammalian cDNA libraries (human heart, liver, brain and prostrate leiomyosarcoma, rat brain and transient ischemic myocardium, NG108-15 neuroblastoma) for entities that promoted the readout of growth. Secondary epistasis analysis allowed the identification of a group of cDNAs that required $G\beta\gamma$ for their bioactivity and this group of cDNAs were termed Activators of G-protein Signaling (AGS) (Table 1). In some cases, the AGS cDNA encodes a previously identified protein or gene product as indicated in Table 1.

2.1 PROTEINS IDENTIFIED AS RECEPTOR-INDEPENDENT ACTIVATORS OF G-PROTEIN SIGNALING

The different cDNA libraries screened to date yielded 17 distinct cDNAs that promoted growth in the yeast functional screen. Ten of the isolated cDNAs require $G\beta\gamma$ to promote growth in the yeast screen and are therefore classified as activators of G-protein signaling as defined

above (Figure 1). Two of the isolated cDNAs exhibit homology to polyA-RNA binding proteins and likely exert a regulatory influence on growth downstream of G-proteins and the kinase cascade within the pheromone response pathway, whereas an additional five cDNAs are under investigation.

Although the end readout of growth for the AGS proteins reflects augmented G $\beta\gamma$ signaling, this may be achieved by various mechanisms. AGS proteins may act in the yeast system to: 1) promote G-protein activation as a guanine nucleotide exchange factor; 2) interfere with subunit interactions during basal cycling of nucleotide exchange and hydrolysis; 3) promote subunit dissociation or rearrangement independent of nucleotide exchange; 4) influence trafficking of G-protein subunits; and/or 5) bind to G-protein subunits co-translationally so that heterotrimeric G $\alpha\beta\gamma$ is not formed. While this strategy isolated cDNAs defined as receptor independent activators of G-protein signaling, the proteins could conceivably also function as scaffolding proteins for G-protein subunits, effectors for G $\beta\gamma$, chaperones for G-protein subunits and/or alternative binding partners for G-protein subunits independent of classical heterotrimeric G $\alpha\beta\gamma$. The AGS proteins isolated in this functional screen are actually part of a larger class of accessory proteins for G-protein signaling systems that are the subject of investigation in many laboratories (Sato et al., 2006a).

2.2 PROPERTIES OF AGS PROTEINS IN YEAST-BASED FUNCTIONAL SCREEN AND G-PROTEIN SUBUNIT BINDING

The AGS proteins exhibited distinct properties in terms of G-protein regulation as determined in the yeast-based functional screen and by biochemical approaches even though they were isolated in the same basic functional screen (Figure 2). AGS proteins interact with different G-protein subunits and/or conformations, exhibit selectivity for different G α subunits in the yeast functional screen, utilize different mechanisms to regulate the G-protein activation/deactivation cycle, and/or exhibit different subcellular locations.

The properties of the different AGS proteins were initially studied in the yeast functional screen using yeast strains with different mammalian G α subunits in combination with other known G-protein regulators and also in biochemical assays with purified proteins. The mechanism of G-protein activation by AGS1 appears similar to that of a GPCR in that it was inactive in yeast strains expressing G204A G α_2 and antagonized by overexpression of RGS4 (vida infra) and RGS5 (Cismowski et al., 2002; Cismowski et al., 1999; Takesono et al., 1999). *In vitro*, AGS1 interacted with G α but not G $\beta\gamma$ and it increased GTP γ S binding to purified G-protein. G α_2 G204A behaves as a dominant negative G α subunit by virtue of its predicted low affinity for GTP and it is incapable of supporting signal propagation by an activated receptor in the yeast functional assay. In contrast, AGS2-8 were functional in the G204A G α_2 genetic background and in the presence of overexpressed RGS4. Thus, AGS2-8 differed from AGS1 and apparently activated heterotrimeric G-protein signaling by a mechanism independent of nucleotide exchange (Bernard et al., 2001; Cao et al., 2004; Cismowski et al., 1999; Sato et al., 2006b; Takesono et al., 1999) (Figure 2). AGS3-6 were active in yeast strains expressing mammalian G α_2 and G α_3 but not G α_s or G α_{16} . In contrast, AGS2 and AGS7-10 were active in yeast strains expressing G α_2 , G α_3 , G α_s or G α_{16} suggesting that their action involved an interaction with G $\beta\gamma$, which was common to each of the yeast strains. AGS1-6 bind to G α subunits, whereas AGS2 and 8 bind G $\beta\gamma$ (Cao et al., 2004; Sato et al., 2006b; Takesono et al., 1999). The AGS proteins may also interact at least transiently with intact G $\alpha\beta\gamma$ heterotrimer. Based upon these properties, AGS1-10 are functionally divided into three groups. Although AGS1 is the only AGS protein in Group I, this classification scheme provides a logical platform for further discussion and experimentation. AGS9 (Rpn10) and AGS10 (Gao) exhibit properties in the yeast screen similar to AGS2, 7 and 8 in that they do not exhibit selectivity for different G α strains and are included at this stage in Group III AGS proteins (Table 1, Figure

2). However, the interaction of AGS9 with G-protein subunits has not been investigated and how Gao is actually functioning in this system is also not clear.

Other than the presence of the GPR motif in the Group II AGS proteins, there are no generally conserved structural features for these proteins even though they were all isolated in the same functional screen. This is reflected in the diversity of mechanisms for regulation of G-protein signaling and the distinct functional roles associated with different AGS proteins (Figure 3).

3. GROUP 1 AGS PROTEINS

AGS1 was first discovered as a dexamethasone-inducible, ras-related cDNA in AtT20 cells and termed DexRas (Kempainen and Behrend, 1998) and subsequently named RASD1 by the HUGO Gene Nomenclature Committee. AGS1 was also identified in yeast two hybrid screens (Fang et al., 2000; Tu and Wu, 1999) and as a regulated cDNA in various other “discovery” platforms (Table 2). Among the superfamily of small G-proteins, AGS1 is most closely related (~60%) to Rhes (Ras homolog enriched in striatum) /TEM (Tumor endothelial marker) 2/RASD2 and exhibits a lower but likely significant sequence similarity to Rap proteins. AGS1 contains the conserved Ras-like motifs with additional extensions at the amino and carboxyl termini (Figure 2). The C-terminal extension of AGS1 contains several basic amino acids as well as a terminal consensus sequence for farnesylation.

3.1 BIOCHEMICAL CHARACTERIZATION OF AGS1 AND SIGNAL PROCESSING

While several studies focused on the role of AGS1 in signal transduction as described below, we have relatively little information on the biochemical properties of AGS1 in terms of guanine nucleotide binding and hydrolysis, regulatory binding partners, subcellular distribution and posttranslational modifications. Several amino acids in AGS1 within key domains for nucleotide binding/hydrolysis differ from their counterparts in Ras (Figure 4A). Mutation of residues in Ras corresponding to those different residues in AGS1 leads to constitutive activity of Ras (Valencia et al., 1991), suggesting that AGS1 may be constitutively active. A similar situation may operate for the small G-protein rnd3 (Foster et al., 1996; Nobes et al., 1998) (Figure 4). AGS1 is indeed active in the yeast-based assay and in transfected COS cells without any apparent “stimuli”. A mutation in PM1 (G31V) renders AGS1 inactive in several assay systems and it is postulated that this is due to altered nucleotide binding properties but this has not been experimentally proven. The equivalent of Gln61 in Ras is Asn82 in AGS1 and mutating Gln61 to any residue except Glu results in a dominant active Ras. Amino acids 33 and 80 in AGS1 are also similar to those conferring constitutive activity to Ras proteins upon mutation. However, it is difficult to make firm conclusions on these points as other aspects of structure may compensate in unexpected ways. Mutation of A178 to V in AGS1, part of the conserved SAK motif in Ras, was reported to be constitutively active (Graham et al., 2001).

GST-AGS1 isolated from yeast or bacteria does not readily bind GTP γ S (Cismowski et al., 2000) except at fairly high concentrations of nucleotide and perhaps in the presence of a GEF (Fang et al., 2000). Side-by-side comparison of GST-AGS1 and the small G-protein GST-Cdc42 purified following expression in yeast indicated that AGS1 bound less nucleotide and that GTP binding predominated compared to parallel experiments with Cdc42. AGS1 exhibited a GTPase rate similar to that observed for GST-Cdc42 and suggesting that it may prefer the GTP bound state and that the lower amount of bound nucleotide reflects the hydrolysis of GTP and/or a lower affinity for GDP relative to Cdc42 (Cismowski et al., 2000). The nucleotide binding and hydrolysis properties of AGS1 may be quite different in mammalian systems due to the potential presence of regulatory proteins. Indeed, AGS1 expressed in HEK293 or AtT20 cells is predominantly GDP-bound (Fang et al., 2000; Graham et al., 2001).

The signaling mechanisms of AGS1 and its cellular role remain incompletely understood. While several studies report the involvement of AGS1 in signal transduction, there is not a clear consensus on its signaling mechanism. AGS1 is implicated in a variety of signaling pathways including cAMP, Erk1/2 and NMDA-mediated signaling and as a Ras family member it likely has signaling roles independent of heterotrimeric G-protein signaling.

The influence of AGS1 on G-protein signaling is complex. Certainly the yeast-based functional screen and initial biochemical studies indicate G α regulation by AGS1 (Cismowski et al., 2000; Cismowski et al., 1999). The activity of AGS1 in the yeast functional assay is dependent upon nucleotide exchange on G α and it is selective for G α 2 and G α 3. AGS1, purified as a GST fusion protein following expression in *S. cerevisiae*, increased GTP γ S binding to purified G α 1 and G α 2 as well as purified brain G-protein heterotrimer. Thus, in this regard it differs mechanistically from a GPCR that couples only to heterotrimeric G-protein as well as from the GEF Ric 8A, which only behaves as a GEF for free G α , but it is similar to the partially purified NG108-15 G-protein activator (Ribas et al., 2002b; Tall et al., 2003). Several other non-receptor accessory proteins also promote nucleotide exchange on G-proteins (Sato et al., 2006a) providing an additional impetus to the concepts of receptor independent signaling mechanisms for G-proteins developed in this review. However, Hiskens recently reported that in yeast two-hybrid assays, AGS1 specifically interacted with G β 1 but not G α 2 and that AGS1 expressed in HEK cells coimmunoprecipitated with G β (Hiskens et al., 2005). The explanation for these disparate results is not clear, but may reflect differences in the nucleotide bound states of G α or AGS1 in the different systems.

In mammalian cells, transfected AGS1 activated the ERK1/2 pathway and this was blocked by pertussis toxin pretreatment or cotransfection with Gat, which binds released G $\beta\gamma$ (Cismowski et al., 2000), though it cannot be ruled out that Gat also binds AGS1 preventing activation of G $\alpha\beta\gamma$. The magnitude of inhibition of Erk1/2 by transfected AGS1 may be system-dependent (Graham et al., 2002; Nguyen and Watts, 2006, *vide infra*). AGS1 also inhibits cAMP accumulation in response to forskolin or a constitutive active G α (Graham et al., 2004), which is also consistent with regulation of G α by AGS1. A role in regulating G-protein signaling events is also indicated by the ability of transfected AGS1 to block sensitization of adenylyl cyclase mediated by receptors coupled to G α (Nguyen and Watts, 2006). The influence of AGS1 on G-protein regulated effectors such as adenylyl cyclase is further complicated by the ability of AGS1 to inhibit PKC δ (Nguyen and Watts, 2006) and thus the outcome of various studies regarding AGS1 regulation of the effector AC is likely dependent upon the type of AC expressed in the cell of study. For example, transfected AGS1 inhibited phorbol ester stimulation of AC2 but did not alter Gas-mediated regulation of AC2 and this was attributed to an action of AGS1 on PKC δ (Nguyen and Watts, 2006).

On the other hand, AGS1 transfection blocked GPCR-mediated regulation of GIRK channels and Erk1/2 (Graham et al., 2002; Takesono et al., 2002), but not the inhibition of cAMP elicited by activation of receptors coupled to G α (Nguyen and Watts, 2005). Based upon the interaction of AGS1 with G α and the data from the yeast-based functional assay discussed above, AGS1 may uncouple receptor from G-protein. AGS1 and a GPCR may “compete” for the available pool of G-proteins or perhaps an AGS1-initiated signal alters receptor – G-protein coupling (Graham et al., 2002; Takesono et al., 2002). Interaction of AGS1 with G $\beta\gamma$ might also explain these data as Erk1/2 and GIRK channels are G $\beta\gamma$ effectors (Hiskens et al., 2005). The AGS1-related protein Rhes blocked GPCR-G α mediated regulation of effectors, but not that regulated by receptors coupled to G α (Vargiu et al., 2004).

Following up on their initial isolation of AGS1 as a binding partner of CAPON, Snyder's laboratory has developed a very interesting story indicating that AGS1 is nitrosylated in response to nNOS activation by NMDA receptors via CAPON docking and that this

nitrosylation, which occurs on cysteine 11 in AGS1, accelerates nucleotide exchange on AGS1 and subsequent coupling to downstream effectors regulating iron transport and neuronal toxicity (Cheah et al., 2006; Jaffrey et al., 2002). The activation of AGS1 by NMDA receptors may provide an interface to heterotrimeric G-protein signaling systems as the NMDA mediated activation of Erk1/2 was inhibited by pertussis toxin (Chandler et al., 2001), which ADP ribosylates Gi/Go α subunits and blocks the effect of AGS1 in COS cells (Cismowski et al., 2000; Graham et al., 2002). One exciting functional connection for the work of Snyder's laboratory on NMDA-mediated activation of AGS1 is provided by the studies of AGS1^{-/-} mice generated by Cheng et al indicating that these mice exhibit changes in the NMDA-mediated influence on circadian rhythm (Cheng et al., 2004).

In addition to its ability to regulate heterotrimeric G-proteins, AGS1 likely has functions independent of G-proteins involving more typical effectors for Ras-related proteins. AGS1 expression in some but not all cell types causes growth inhibition and this was not blocked by pertussis toxin (Vaidyanathan et al., 2004). The core effector domain of Ras (28FVDEYDPTIEDSY⁴⁰) is similar to the corresponding region in AGS1 and Rhes (Figure 4), although there are notable differences. The AGS1 related protein Rhes actually binds the Ras binding domain of PI3K but not that of Raf (Vargiu et al., 2004). A patent issued on AGS1 as a hypoxia-induced cDNA presents data indicating that AGS1 does not bind the Ras binding domain of Raf.¹

Thus, much work remains to be done to define the signaling role of AGS1. Most studies to date on this topic involve the use of transfected overexpression systems, which may complicate data interpretation and studies involving RNAi mediated knockdown of AGS1 may shed light on some of the issues in the field. The availability of AGS1^{-/-} mice will also be of importance as these studies go forward (Cheng et al., 2004).

3.2 REGULATION OF AGS1

As basic information regarding the biochemistry, tissue distribution and signaling mechanisms of AGS1 has accumulated, the question remains as to its biological role in health and disease. Observations of note in this regard are the dramatic, tissue-specific, upregulation of AGS1 by dexamethasone in cultured cells and the intact animal (Brogan et al., 2001; Kemppainen and Behrend, 1998; Kemppainen et al., 2003) *vide infra*, the growth suppression observed upon AGS1 overexpression in some but not all cell types, the down-regulation of AGS1 (RASD1) in breast ductal carcinoma *in situ* (Abba et al., 2004), and changes in AGS1 mRNA during the circadian cycle in specific brain areas (Table 2). Glucocorticoids have diverse effects in the body and certainly play important roles in development and cell differentiation and it is interesting to suggest that some of their effects may be due to induction of AGS1 expression (Graham et al., 2001). AGS1 is also up-regulated in rat renal isografts from brain dead donors, which is consistent with its earlier isolation as a blood-loss inducible cDNA from kidney.¹ Cell dessication and hypertonicity also increase AGS1 expression (Huang and Tunnacliffe, 2004). AGS1 mRNA is widely distributed with enrichment in the brain and both AGS1 and Rhes/RASD2 mRNA are increased following acute amphetamine (Harrison and LaHoste, 2006).² Overall these data suggest a role of AGS1 in the response to cellular or tissue stress.

If AGS1 is constitutively active, then the "signal input" to AGS1 may simply travel through transcriptional regulation as AGS1 mRNA is clearly upregulated by glucocorticoids and various stress-related stimuli. Translational control mechanisms may also be operative. If

¹Mammalian Blood Loss-Induced Gene, KD312, Amgen US patent 6,462,177

²Schwendt, M., Chandler, L.J., Blumer, J.B., Lanier, S.M., and McGinty, J.F. Expression of Activator of G-protein Signaling AGS1/ DexRas1 in the Rat Brain Increases During Development and in Response to Acute Amphetamine. (2005) Abstract, Society of Neuroscience, San Diego, CA.

AGS1 is not constitutively active, then how is it regulated? One postulate concerns the activation through nitrosylation discussed above. It is also likely that there are other proteins that influence the nucleotide binding and hydrolysis properties of AGS1, which would provide a path for signal input to AGS1.

4. GROUP II AGS PROTEINS

Group II AGS proteins are defined by the presence of at least one G-protein regulatory (GPR) motif, a 20-25 amino acid cassette that serves as a docking site for Gi/oα-GDP and Gtα (Figures 2 and 5) (Bernard et al., 2001;Blumer et al., 2005;Peterson et al., 2000;Sato et al., 2006a;Takesono et al., 1999).³ The GPR motif is also found in Pcp2/L7, RGS14, Rap1GapII and WAVE1 (see Blumer et al., 2005;Sato et al., 2006a reviews for a broader discussion of the individual GPR proteins; Song et al., 2006). AGS3 and AGS5 (LGN, mPINS) are the most extensively studied members of this group and contain four GPR motifs down stream of a series of TPR motifs (Figure 2). AGS3-5 were subsequently named G-protein signaling modulator (GPSM) 1-3, respectively by the HUGO Gene Nomenclature Committee. AGS6 encodes RGS12 (Chatterjee and Fisher, 2000). AGS5 is widely expressed, whereas expression of AGS3 and AGS4 are more restricted (Blumer et al., 2002;Cao et al., 2004;Pizzinat et al., 2001;Takesono et al., 1999). AGS3 is also expressed as a short form that consist of only the GPR domain (AGS3-Short) that exhibits an expression profile different from the full length protein (Pizzinat et al., 2001).

GPR motifs interact with Gα1-3, Gαt and exhibit lower and varying affinities for Gαo (Blumer et al., 2005). Individual GPR motifs may differ in their selectivity for individual Gαi isoforms (McCudden et al., 2005;Mittal and Linder, 2004;Shu et al., 2006) and regions outside of the core GPR motif can influence G-protein isoform selectivity (Adhikari and Sprang, 2003;Kimple et al., 2002;Kimple et al., 2004). AGS3-6 and other GPR-containing proteins provide an unexpected mode of regulation for signals involving Gi and Go G-proteins that is distinct from that achieved with the super family of GPCRs (Blumer et al., 2005;Sato et al., 2006a). The interaction of GPR motifs with Gαi/o stabilizes the GDP-bound conformation of Gα and interferes with Gβγ for binding to Gα (Blumer et al., 2005). The ability of a protein with multiple GPR motifs to bind an equivalent number of multiple Gαi/o at any given moment presents an unusual scaffolding potential for organizing a signaling cassette (Adhikari and Sprang, 2003;Bernard et al., 2001;Tall and Gilman, 2005). It will be of great interest to define the structural aspects of a multiple GPR motif protein in which each motif is docked with a Gα subunit. Such a structure might reveal an interaction of Gα subunits within the complex that would perhaps provide insight for their organization in other contexts.

Functional studies with GPR proteins indicate a wide range of functional roles including an involvement in cell division, neuronal outgrowth, the biology of craving, autophagy and GIRK channel regulation (Bowers et al., 2004;Du and Macara, 2004;Guan et al., 2005;Lechler and Fuchs, 2005;Pattingre et al., 2003;Ron and Jurd, 2005;Sanada and Tsai, 2005;Sans et al., 2005;Wiser et al., 2006;Yao et al., 2005). The GPR motif may also represent a target for therapeutic manipulation and structure activity relationships have been defined (Figure 5) (Cao et al., 2004;Kimple et al., 2002;Peterson et al., 2002). The GPR motif consists of a core 20-25 amino acid sequence in which specific residues and spatial relationships within this sequence play an important role in the interaction with Gα (Figure 5) (Peterson et al., 2002). Additional sequences outside of this core motif may influence selectivity for different Gα subunits and the relative affinities of individual GPR motifs for Gα isoforms (Adhikari and Sprang, 2003;Cao et al., 2004;Kimple et al., 2002;Kimple et al., 2004;McCudden et al., 2005;Mittal and Linder, 2004;Willard et al., 2006a;Willard et al., 2006b). The following

³The GPR motif is also known as the GoLoco motif (Siderovski et al., 1999).

sections will discuss current thoughts regarding GPR-mediated regulation of G-proteins, the regulation of selected GPR proteins and their role in cell division.

4.1 GPR PROTEINS AND SIGNAL PROCESSING

With the discovery of an entity that behaves as a GDI for $G\alpha$ and apparently activates heterotrimeric G-protein signaling in yeast independent of nucleotide exchange, the field was presented with an unexpected mode of G-protein regulation. The GPR motif may either promote dissociation of $G\alpha\beta\gamma$ subunits, interfere with $G\beta\gamma$ binding to $G\alpha$, or a preformed GPR- $G\alpha$ complex may await an incoming signal from a guanine nucleotide exchange factor that would initiate a $G\alpha$ GTP or GPR regulated signaling cascade. One hypothesis based upon available data is that the GPR motif interacts with heterotrimeric $G\alpha\beta\gamma$ and actively accelerates subunit dissociation (Bernard et al., 2001; Ghosh et al., 2003). GPR peptides enhance the rate of dissociation of a preformed heterotrimer and the enhanced rate of G protein subunit dissociation by GPR peptides was comparable to that of a known activator of G proteins, AMF (Ghosh et al., 2003). These data indicate that GPR proteins are not simply acting by competing for $G\beta\gamma$ binding $G\alpha$ GDP and preventing reassembly of dissociated G-protein subunits. Indeed, several $G\beta\gamma$ binding peptides that dock at the $G\alpha\beta\gamma$ interface and compete for $G\alpha$ binding do not accelerate subunit dissociation (Davis et al., 2005; Ghosh et al., 2003).

The structure of the GPR RGS14 GoLoco peptide with $G\alpha i 1$ suggests that the binding site for the peptide does not directly bind to the $G\beta\gamma$ contact sites on $G\alpha$ and that the binding site for a GPR motif (switch II/ $\alpha 3$ helix binding pocket) on $G\alpha$ is accessible in the heterotrimer $G\alpha\beta\gamma$ (Kimple et al., 2002) (Figure 6). It is hypothesized that conformational differences in switch II resulting from GPR motif binding results in subunit dissociation via a mechanism analogous to the GTP or AlF_4^- dependent conformational changes in the switch regions of the α subunit that contribute to subunit dissociation or rearrangement (Lambright et al., 1994; Wall et al., 1995). Such a process would lead to guanine-nucleotide exchange independent dissociation or rearrangement of the $G\alpha$ and $G\beta\gamma$ subunits. Thus, in the context of heterotrimeric G-proteins, the GPR motif would “release” $G\beta\gamma$ for down stream signaling events. The docking of a GPR motif on $G\alpha$ would also delay reassociation of subunits during the basal or stimulated activation-deactivation cycle of heterotrimeric G-protein. Such a regulatory process would add additional diversity to this much studied signaling pathway.

Functional studies concerning the role of G-proteins and GPR proteins in asymmetric cell division in *D. melanogaster* and the developing rodent nervous system are consistent with a GPR protein acting upstream of heterotrimeric G-protein and promoting subunit dissociation (Sanada and Tsai, 2005; Schaefer et al., 2001; Yu et al., 2005). At present, it is not clear if a similar model of GPR-mediated activation of signaling operates in asymmetric cell division observed in the *C. elegans* embryo (Afshar et al., 2004; Bellaiche and Gotta, 2005; Couwenbergs et al., 2004; Srinivasan et al., 2003). The influence of GPR proteins on G-protein regulated ion channels has also been investigated. In HEK293 cells and *X. laevis* oocytes expressing GIRK1/2, LGN and GPR peptides activated basal $G\beta\gamma$ dependent K^+ currents and siRNA knockdown of LGN decreased basal GIRK currents in primary neuronal cultures (Wiser et al., 2006). The GPR containing protein Pcp2/L7 also modulated receptor regulation of Cav2.1 calcium channels expressed in *X. laevis* oocytes (Kinoshita-Kawada et al., 2004), but did not modify basal current. On the other hand, perfusion of a GPR peptide into AtT-20 cells did not activate basal native K^+ currents leading the authors to conclude that the GPR peptide could not dissociate G protein heterotrimers in cells (Webb et al., 2005). The explanation for these differences is not clear, but may relate to the importance of accessibility of G-proteins and placement of the GPR protein in various cellular environments. Inducible expression of Pcp2/L7 in PC12 cells elicited cell differentiation and this was blocked by the β -adrenergic receptor kinase $G\beta\gamma$ binding domain consistent with the GPR protein Pcp2/L7 promoting subunit

dissociation (Guan et al., 2005). However, the effect of Pcp2/L7 in PC12 cells was also blocked by pertussis toxin, which may indicate the involvement of a receptor-mediated event. It is not known if $G\alpha$ -GDP complexed with a GPR protein is a substrate for pertussis toxin as is the case for the heterotrimeric $G\alpha\beta\gamma$.

Another mechanism by which GPR proteins may integrate into G-protein signaling is by existing as a complex with $G\alpha$ GDP totally independent of heterotrimer as initially suggested by Takesono et al (Takesono et al., 1999). Indeed, subpopulations of GPR proteins and $G\alpha$ -GDP are co-immunoprecipitated from cell extracts (Bernard et al., 2001). Of course, this leads to several questions. Where does this complex exist within the cell and how is its formation regulated? Is there a GEF for a GPR- $G\alpha$ GDP complex that functions in a manner similar to that of a GPCR for classical $G\alpha\beta\gamma$? If there is a GEF for such a complex then what are the down stream effectors for either the GPR protein or $G\alpha$ GTP? Studies by Tall and Gilman implicate Ric-8A as one such factor (Tall and Gilman, 2005). Ric-8A was isolated in a *C. elegans* genetic screen for entities that conferred resistance to inhibitors of acetylcholinesterase (Miller et al., 2000) and in yeast two-hybrid screens with $G\alpha$ (Tall et al., 2003). Purified mammalian Ric-8A increased $GTP\gamma S$ binding to $G\alpha i1$ bound to AGS3-short, LGN-short, full length LGN and a complex of LGN-NuMA (Tall and Gilman, 2005) contrasting with an earlier report suggesting that *C. elegans* Ric-8A did not effectively act as a GEF for a reconstituted $G\alpha$ GDP-GPR1/2 complex (Afshar et al., 2004). These differences may be due to the observation by Tall and Gilman that the myristoylated $G\alpha i1$ complexed with a GPR protein was a much better substrate for Ric-8A as compared to unmyristoylated $G\alpha i1$ (Tall and Gilman, 2005). Ric-8A promotes nucleotide exchange on $G\alpha q$, $G\alpha i1$ and $G\alpha o$, whereas the Ric-8B isoform may augment $G\alpha_{olf}$ signaling (Von Dannecker et al., 2005).

Functional studies regarding GPR motifs and G-protein-mediated regulation of various events in combination with biochemical approaches are beginning to provide a better operational understanding of this regulatory process. Although several lines of investigation support the role of Group II AGS proteins and related GPR proteins as receptor-independent activators of G-proteins, the role of GPR proteins in the processing of signals from GPCRs is not resolved. Overexpression of GPR proteins in various formats does not alter the ability of a $G\alpha$ -coupled GPCR to inhibit the elevation of cellular cAMP elicited by forskolin or $G\alpha s$ -coupled GPCRs (Sato et al., 2004). Overexpression of AGS4 also did not alter the $G\beta\gamma$ -mediated regulation of phospholipase C $\beta 2$ (Cao et al., 2004). On the other hand, LGN modulates receptor-mediated regulation of GIRK channels (Wiser et al., 2006) and GPR-mediated modulation of receptor signaling may be operative in the function of AGS3 in addiction and craving (Bowers et al., 2004; Yao et al., 2005). A key control factor may be the positioning of the interacting proteins in the right place at the right time within the cell and such regulation may not be operative in a straightforward heterologous overexpression system. On the other hand, there is stronger evidence for a role of these proteins in the "adaptation" capability of GPCR systems. The sensitization of $G\alpha s$ regulation of adenylyl cyclase observed with prolonged activation of a $G\alpha$ -coupled GPCR is blocked by overexpression of AGS3 (Sato et al., 2004). AGS3 also plays an important role in the adaptive neuronal events associated with the biology of craving or desire, with some components mimicked with a GPR motif peptide (Bowers et al., 2004; Yao et al., 2005).

In addition to the GPR peptides, a variety of approaches have also identified other peptide motifs and chemical compounds that influence the nucleotide binding, nucleotide hydrolysis and subunit interactions for heterotrimeric G-proteins (Bonacci et al., 2006; Cismowski and Lanier, 2005; Gilchrist et al., 2002; Hessling et al., 2003; Ja et al., 2005). Using an mRNA display strategy, Ja and Roberts identified a peptide sequence that binds $G\alpha$ GDP (Ja et al., 2005; Ja and Roberts, 2004). Although the GPR motif was used as a starting point for the mRNA display strategy, the structural and mechanistic properties of the isolated peptide appear

distinct from the GPR peptide (Ja et al., 2005; Ja and Roberts, 2004; Willard and Siderovski, 2006). The $G_{i\alpha}$ GDP binding peptide KB-752, identified in a phage display using $G_{i\alpha}$ -GDP, alter nucleotide binding properties of purified $G_{i\alpha}1$ and attenuates adenylyl cyclase (Johnston et al., 2005a; Johnston et al., 2005b). The X-ray crystal structure of the KB-752 peptide bound to $G_{i\alpha}1$ indicates that the peptide also binds in a cleft between switch II and the $\alpha 3$ helix altering the conformation of residues in switch II (Johnston et al., 2005b), as is the situation for the GPR-GoLoco RGS14 peptide (Kimple et al., 2002). However, the GPR peptide acts as a GDI and KB-752 acts as GEF for $G_{i\alpha}$. A key difference between these peptides is that the GPR motif also contacts the helical domain and introduces an arginine into the GDP binding site directly stabilizing GDP binding while KB-752 has a much more limited spatial interaction restricted to the switch II/ $\alpha 3$ cleft (Johnston et al., 2005b; Kimple et al., 2002). The accumulating information regarding discrete targets on the G-protein subunits provides a potential platform for developing therapeutics that directly influence G-protein signaling by modulating nucleotide binding or hydrolysis, subunit interactions or by interfering with regulatory input from receptor-independent activators of G-protein signaling.

4.3 REGULATION OF GROUP II AGS PROTEINS

One fundamental unresolved question is how GPR motif-containing proteins regulate their interactions with $G_{i\alpha}$ subunits. One working hypothesis is that binding partners influence the subcellular distribution and/or the conformation of Group II AGS proteins such that their interaction with $G_{i\alpha}$ is constrained within a specified microenvironment. This is certainly operative in the role these proteins play in asymmetric cell division discussed in the next section. Several GPR containing proteins possess other protein interaction modules that could serve to bind regulatory proteins influencing trafficking of the GPR protein or the interaction of GPR motifs with G_{α} subunits. This is perhaps best illustrated for AGS3 and LGN, which contain a series of TPR motifs upstream of the GPR domain (Figures 2 and 7). TPR domain-interacting proteins for AGS3 and LGN have been identified that influence the subcellular localization of AGS3 or LGN and/or the interaction of AGS3 and LGN with $G_{i\alpha}$ subunits (Tables 3 and 4) (Figure 7). One interesting example of this regulatory function is NuMA, a nuclear protein that during mitosis organizes the minus ends of microtubules at the spindle poles. NuMA was identified as an LGN-interacting protein by a yeast two-hybrid screen using the LGN-TPR domain as bait (Du et al., 2001). Not only does NuMA appear to target LGN to the spindle poles, it also appears to induce a conformational change in LGN that allows it to more readily bind $G_{i\alpha}$ subunits at the cell cortex (Du and Macara, 2004).

A number of interactions observed for Pins in *Drosophila* are evolutionarily conserved for its mammalian counterparts AGS3 and LGN with interactions mediated by their TPR and/or linker domains (Tables 3 and 4). With respect to the above discussion of the LGN-NuMA interaction, a NuMA homolog in *Drosophila*, Mud, was recently identified and demonstrated to bind Pins and regulate mitotic spindle orientation in neuroblasts (Bowman et al., 2006; Izumi et al., 2006; Siller et al., 2006). Analogous to the Insc-Pins interaction in *Drosophila* neuroblasts, LGN and AGS3 bind mInsc, the long sought-after mammalian homolog of *Drosophila* Insc, which modulates the orientation of the mitotic spindle and thus the cell fate of retinal progenitors and basal epidermal cells (Izaki et al., 2006; Lechler and Fuchs, 2005; Zigman et al., 2005). *Drosophila* Pins also binds discs-large (Dlg), a member of the MAGUK family of proteins, to control mitotic spindle orientation in sensory organ precursor cells (Bellaiche et al., 2001). Analogously, LGN interacts with members of the mammalian PSD-95 family of MAGUK proteins, namely SAP102, PSD-95, and SAP97/hDlg (Sans et al., 2005). It is unknown whether the LGN-hDlg interaction influences mitotic spindle positioning in mammalian cells; however, LGN interaction with SAP102, in conjunction with $G_{i\alpha}$ subunits, modulates trafficking of NMDA receptor subunits to the plasma membrane and increases the density and size of dendritic spines in hippocampal neurons (Sans et al., 2005). Additional

LGN binding partners include Lethal giant larvae 2 (Lgl2), which appears to connect LGN to the aPKC-Par6 complex in dividing HEK293 cells (Yasumi et al., 2005), Ha-Ras (Marty et al., 2003).

In yet another evolutionarily conserved interaction, both AGS3 and Pins bind to the serine-threonine kinase LKB1 (DmLKB1 in *Drosophila*) which regulates oocyte polarity in *Drosophila* and is a tumor suppressor in mammals (Blumer et al., 2003; Martin and St Johnston, 2003). LKB1 was also recently identified as the upstream kinase for AMPK, a key regulator of energy homeostasis (Hawley et al., 2003; Woods et al., 2003). The role of AGS3 in these apparently diverse functions of LKB1 remains to be determined. LKB1 phosphorylates the GPR domains of both AGS3 and LGN, and phosphorylation of a conserved serine residue within the GPR motif blocks its interaction with G α , thus implicating phosphorylation as a regulatory mechanism to modulate GPR - G-protein interaction (Blumer et al., 2003; Hollinger et al., 2003).

In addition to the regulatory role of binding partners, the expression of both AGS3 and LGN is developmentally regulated. The levels of LGN appear to change with the cell cycle (Du and Macara, 2004; Whitfield et al., 2002). The relative expression of AGS3 mRNA encoding the full length protein or the shorter version lacking the TPR motifs in heart changes during the course of development (Pizzinat et al., 2001). Whereas, the expression of LGN in different brain areas of the rat is fairly stable with aging, the levels of AGS3 decline (Blumer et al., 2002; Sans et al., 2005). AGS3 is upregulated in the brain in a rat model of craving following cocaine exposure and this upregulation is important for adaptive neuronal processes (Bowers et al., 2004).

4.4 ROLE OF GPR PROTEINS IN CELL DIVISION

While diverse functional roles for GPR proteins are observed as noted above and discussed in detail elsewhere, we will discuss only one of these which has matured quite rapidly over the last several years. Asymmetric cell division resulting in daughter cells of different cell fates, is one mechanism for achieving cell type diversity during development and was the area of investigation in which functional roles for GPR proteins and the unexpected roles of G-proteins were first appreciated (Bellaiche et al., 2001; Gotta and Ahringer, 2001; Parmentier et al., 2000; Schaefer et al., 2001; Schaefer et al., 2000; Yu et al., 2000).

The asymmetry of the division is derived not only from the different sizes of the daughter cells, but also, and perhaps more importantly, it involves the asymmetric localization of cell fate determinants which are specifically inherited by each daughter cell. Asymmetric cell division has been extensively studied in the model organisms *Drosophila melanogaster* and *C. elegans* (for review, see (Bellaiche and Gotta, 2005; Betschinger and Knoblich, 2004; Wang and Chia, 2005). In either case, asymmetric cell division employs the use of protein interaction cassettes to coordinate the transfer of polarity information to the dividing cell to specify the plane of division and simultaneously target cell fate determinants to the correct location for each daughter cell. In *Drosophila* neuroblasts, protein complexes containing Bazooka/Par-3, atypical PKC, and Par-6 are tethered to Pins-G α complexes at the apical cortex by Insc. This complex is conserved in mammalian asymmetric divisions of the developing epidermis (Lechler and Fuchs, 2005) and atypical PKC-Par6-LGN complexes were also observed in mitotic HEK293 cell lysates (Yasumi et al., 2005). Correct targeting of this complex to the apical cortex of *Drosophila* neuroblasts is essential for asymmetric cell division to occur, and many of the proteins within this complex contribute to its overall stability.

In *C. elegans* one-cell zygotes, interaction of the GPR motif-containing proteins GPR1/2 with G α at the posterior cortex is critical for the proper asymmetric positioning of the mitotic spindle and this GPR motif-G protein interaction regulates differential cortical pulling forces on the

spindle (Afshar et al., 2004;Colombo et al., 2003;Couwenbergs et al., 2004;Gotta et al., 2003;Srinivasan et al., 2003). In contrast to AGS3, AGS5/LGN and Pins, which have up to three (Pins) or four (AGS5/LGN, AGS3) GPR motifs, GPR1/2 has only one GPR motif. There is no obvious protein in higher organisms with a structural organization similar to GPR1/2. The molecular mechanism by which the spindle pulling forces might be regulated by GPR proteins and Gi/o α subunits is not yet defined, although recent data indicate a role for both an RGS protein and a non-receptor guanine nucleotide exchange factor, Ric-8 (Afshar et al., 2004;Couwenbergs et al., 2004;Hess et al., 2004). In *D. melanogaster*, the interactions between Pins, G-proteins and Ric-8 also play a key role in asymmetric division of neuroblasts and sensory organ precursor cells (Betschinger and Knoblich, 2004;David et al., 2005;Hampoelz et al., 2005;Wang et al., 2005).

The role of the mammalian GPR proteins LGN and AGS3, which are 66% homologous, or G-proteins in cell division and polarity in higher organisms is an area of intense investigation. Recent reports indicate roles for these proteins in asymmetric cell division of neuronal precursors, retinal progenitors and cells of the developing epidermis (Lechler and Fuchs, 2005;Sanada and Tsai, 2005;Zigman et al., 2005) and cell division in the absence of polarity cues (Du and Macara, 2004;Kaushik et al., 2003). Certainly, LGN and Gi α localize to subcellular structures (i.e. centrosomes, spindle pole, midbody, kinetochores) involved in control of cell division (Figure 8) (Blumer et al., 2002;Blumer et al., 2006;Crouch et al., 2000;Du and Macara, 2004;Du et al., 2001;Fuja et al., 2004;Kaushik et al., 2003). Altering the expression level of LGN in cells causes defects in chromosomal segregation, mitotic spindle organization, and cell cycle progression, but the precise mechanism for these effects are undefined (Du et al., 2001;Kaushik et al., 2003). Overexpression of LGN and Gi α causes aberrant metaphase chromosome alignment (Du and Macara, 2004) and a repositioning of the spindle poles closer to the cell cortex (Blumer et al., 2006), which may reflect alterations in the orientation and/or in the pulling forces on the mitotic spindle. AGS3 influences spindle positioning during asymmetric division of mammalian neuronal progenitors via a process involving G $\beta\gamma$ (Sanada and Tsai, 2005). Knockdown of AGS3 in the neocortex of E15 mouse embryos alters the spindle orientation of neuronal progenitor cells from apical-basal to planar divisions resulting in an increase in the number of differentiated neurons at the expense of neuronal progenitors (Sanada and Tsai, 2005). Similarly, the AGS3 and LGN binding protein mInsc influences positioning of the mitotic spindle in the developing rat retina and developing mouse epidermis (Lechler and Fuchs, 2005;Zigman et al., 2005). Collectively, these observations clearly indicate roles for both AGS3 and LGN in mitotic spindle organization in mammalian asymmetric cell division and cell divisions that occur in the absence of obvious polarity cues.

The interaction of LGN with NuMA mentioned in the preceding section is an important component of the mechanism by which GPR proteins can regulate cell division. LGN binding to NuMA influences NuMA localization to the cortex where it is speculated to influence mitotic spindle positioning by modulating pulling forces on astral microtubules at the cortex via its interaction with the dynein/dynactin complex (Du and Macara, 2004). It should be noted that the LGN binding site on NuMA overlaps with its tubulin binding site and blocks NuMA binding to microtubules (Du et al., 2002). Tall and Gilman demonstrated that Gi α bound to LGN-NuMA complexes serve as a substrate for the guanine nucleotide exchange factor Ric-8, and that after nucleotide exchange on Gi $\beta\alpha$ has occurred, NuMA is released from LGN (Tall and Gilman, 2005). One obvious conclusion from these data is that Gi α is not merely a membrane “tether” for LGN-NuMA complexes as has been suggested (Du and Macara, 2004), but may act as a switch, allowing a “ratcheting” of NuMA-mediated pulling forces on astral microtubules at the cortex by rapid cycling of nucleotide exchange on Gi α and temporary release of NuMA from LGN as previously proposed (Tall and Gilman, 2005). An RGS protein may be a component of such a cycle for mammalian cells as determined for RGS7 in the integrated

control of spindle pulling forces via G-proteins and Ric-8 in *C. elegans* (Hess et al., 2004). RGS14, which contains both an RGS and a GPR domain, is an attractive possibility for this role, as it is localized to spindle poles and the cell cortex during mitosis and also binds microtubules (Cho et al., 2005; Martin-McCaffrey et al., 2004; Martin-McCaffrey et al., 2005; Shu et al., 2006).

One major outstanding question in the field is exactly how force is exerted on the spindle in order to reorient it relative to the complex of specified polarity determinants described above and the mechanisms by which G-proteins, the GPR-containing proteins AGS3 and AGS5/LGN and other accessory proteins for G-proteins interplay to influence these events.

5. GROUP III AGS PROTEINS

As discussed earlier in the yeast-based functional assay, Group II and Group III AGS proteins are both active in the G204AG α 2 background and are not antagonized by overexpression of RGS5, which accelerates signal termination. However, Group II proteins bind G α , whereas the Group III proteins AGS2 and AGS8 bind G $\beta\gamma$. AGS10 or Go α also obviously binds G $\beta\gamma$. However, the mechanisms by which the Group III AGS proteins activate G-protein signaling in the yeast functional screen and function in mammalian signaling systems are not yet fully understood. As more information becomes available, it is likely that members of this loosely defined Group III will exhibit different mechanisms of action in terms of their ability to lead to the end readout of G $\beta\gamma$ -dependent growth in the yeast functional screen. One mechanism by which the Group III proteins may act is by an interaction with G $\beta\gamma$ and the remainder of this section focuses on this concept.

A central idea for both Group II and III AGS proteins is that they alter α - $\beta\gamma$ subunit interactions releasing free $\beta\gamma$ subunits that activate the yeast MAP kinase signaling system in the yeast functional assay. As discussed above, GPR containing AGS proteins may activate $\beta\gamma$ signaling in yeast by altering the conformation of the G α -switch II and either promoting subunit dissociation/rearrangement or compromising G $\beta\gamma$ subunit binding leading to accumulation of free G $\beta\gamma$ (Ghosh et al., 2003; Kimple et al., 2002; Peterson et al., 2000). In this scenario the free G $\beta\gamma$ subunits are not hindered with respect to target binding. A similar mechanism for Group III proteins that bind directly to G $\beta\gamma$ is more difficult to understand. Binding of Group III AGS proteins to the G α subunit switch II-binding region on G $\beta\gamma$, to compete for α subunit interactions, would simultaneously occlude an important effector binding interface on G $\beta\gamma$. Thus we are presented with two possible considerations. First, the AGS protein in this group binds to a site on G $\beta\gamma$ other than the G α subunit switch II-binding region on G $\beta\gamma$ and exerts allosteric influences on subunit interactions to “release” G $\beta\gamma$ for downstream signaling. Second, the AGS protein binds to the G α subunit switch II-binding region on G $\beta\gamma$ and conformationally reveal a secondary effector regulation site. In either scenario, the AGS proteins would dissociate from G $\beta\gamma$ or remain bound to G $\beta\gamma$ when it interacts with an effector. This is further discussed below from the perspective of what is known about effector interactions with G $\beta\gamma$ subunits.

5.1 EFFECTOR RECOGNITION BY G PROTEIN $\beta\gamma$ SUBUNITS - “HOTSPOTS” AND BEYOND

Two major ideas concerning effector recognition by G $\beta\gamma$ subunits have emerged in the past 10 years. One is that a key interaction surface on G $\beta\gamma$ normally occluded by G α subunits, mediates interactions with multiple effectors (Ford et al., 1998; Li et al., 1998). This region has been characterized as a “hot spot” that accommodates binding to structurally diverse effectors (Scott et al., 2001). “Hot Spots” are surfaces on proteins that by virtue of steric adaptability and the ability to form multiple types of chemical interactions are particularly well suited for mediating the energetics of diverse protein-protein interactions (Clackson and Wells, 1995; DeLano, 2002). During G-protein subunit dissociation or rearrangement this surface is thought to

become exposed and allow binding and regulation of effectors (Hamm, 1998). A second view based on mutagenic and structural evidence from a variety of laboratories is that other surfaces of G $\beta\gamma$ subunits outside the “hot spot” are involved in interactions with different effectors and target proteins. For example, site directed mutagenesis of amino acids on the surface and sides of the G β subunit propeller structure that are not involved in interaction with G α subunits, are required for activation of PLC β 2, but not adenylyl cyclase (Panchenko et al., 1998). Chemical crosslinking and mutagenesis studies indicate an interaction of PLC β 2 with the amino terminus of G β subunits, which does not overlap with the G α subunit binding site (Yoshikawa et al., 2001). Yeast G β and G γ subunits also possess putative effector binding sites that do not map to the “hot spot” (Leberer et al., 1992).

Direct structural evidence for effector contacts with G $\beta\gamma$ outside of the “hot spot” in G α is limited since only two of the many G $\beta\gamma$ subunit binding proteins, other than α subunits, have been co-crystallized with $\beta\gamma$ subunits. One is phosducin, a G $\beta\gamma$ binding protein in the visual signal transduction system (Gaudet et al., 1996). Comparison of the phosducin structure with the heterotrimer structure reveals that the phosducin binding site on G $\beta\gamma$ overlaps extensively, but not completely, with the binding site for G α . On the other hand, in the recently solved structure of G $\beta\gamma$ subunits in a complex with G protein coupled receptor kinase 2 (GRK2), the main amino acid contacts of G $\beta\gamma$ with the pleckstrin homology domain of GRK2 were similar to those contacting G α (Lodowski et al., 2003). Overall, data suggest that effectors bind to $\beta\gamma$ subunits at common core interaction site, but that alternate sites exist on $\beta\gamma$ that could be important for binding and/or activation of effectors and could be exploited by Group III AGS proteins to propagate G protein signaling.

5.2 ACTIVATION OF G PROTEIN $\beta\gamma$ SUBUNIT SIGNALING BY G PROTEIN $\beta\gamma$ BINDING PEPTIDES

Some insight into the possible mechanisms of action for those members of the Group III AGS proteins interacting with G $\beta\gamma$ could be perhaps gleaned by considering how a novel class of G $\beta\gamma$ binding peptides activates G protein signaling. Peptides that bind to the G protein “hot spot” were recently identified in a random peptide phage display screen (Scott et al., 2001). One of these peptides SIRK, has unique properties in that it binds directly at the α -subunit switch II binding site on G $\beta\gamma$ and actively promotes subunit dissociation (Davis et al., 2005; Scott et al., 2001). In addition, SIRK blocked activation of PLC β and PI3K *in vitro* by G $\beta\gamma$, yet stimulated G $\beta\gamma$ -regulated ERK activation in intact cells. One interpretation of these data is that the peptide occupied the “hot spot” on G $\beta\gamma$ blocking PLC β and PI3K interactions, but effector sites on G $\beta\gamma$ outside the “hot spot” were used for ERK activation. Virtual docking of chemical compounds based upon the structure of the peptide-G $\beta\gamma$ structure led to the generation of small molecules selectively altering G $\beta\gamma$ signaling pathways in cells (Bonacci et al., 2006). This suggests that if Group III AGS proteins indeed occupied the “hot spot” on G $\beta\gamma$, there may be an additional mechanism for regulation of effectors that bind to regions outside of the “hot spot”.

Initial data available for the action of AGS8 provides a further platform for development of this concept. AGS8 was discovered as a receptor-independent activator of G-protein signaling upregulated during transient cardiac ischemia (Sato et al., 2006b). Initial characterization of AGS8 indicated that it bound to G $\beta\gamma$ subunits. Of particular interest, in transfected mammalian cells, AGS8 produced a small activation of PLC β 2, but completely reversed the inhibitory effect of G α on G $\beta\gamma$ -mediated activation of PLC β 2. One interpretation of these data is that AGS8 prevents binding of G α to the “hot spot” for effector interaction on G $\beta\gamma$ but yet does not impede G $\beta\gamma$ -mediated stimulation of PLC β 2, perhaps through an effector binding site distinct from the “hot spot”.

6. AGS AND RELATED PROTEINS IN SIGNAL ADAPTATION AND DISEASE

The regulatory mechanisms operational with AGS proteins reveal unexpected diversity in the “G-switch” signaling mechanism and cellular functions regulated by G-proteins. This flurry of activity over the last 5-6 years has opened up new avenues for therapeutic manipulation of G-protein signaling and the expanded role that G-proteins may play in disease or tissue adaptation. Many questions remain to be addressed including the following. What controls the activity of AGS proteins? Are $G\alpha$ and $G\beta\gamma$ playing a role within the cell independent of heterotrimer formation by complexing with alternative binding partners? If so, what provides signal input to such a complex and what is “downstream” of $G\alpha$ and $G\beta\gamma$ functioning in this context? Are AGS proteins functioning as a component of an unexpected intracellular signaling network (Slessareva et al., 2006; Sorkin and Von Zastrow, 2002)? Do AGS and related proteins play a role in GPCR signaling as modulators or as organizational structures within a larger signal transduction complex?

The regulatory mechanisms operational for AGS proteins also may relate to other evolving concepts regarding G-protein signaling systems and the dynamics of G-protein activation and deactivation. Recent FRET based analyses suggest that G-protein heterotrimers may not physically dissociate upon activation by certain receptors (Bunemann et al., 2003; Frank et al., 2005), but rather undergo a ill-defined rearrangement of subunits to allow binding of effectors and other regulators. Indeed, a covalently linked α - $\beta\gamma$ fusion protein is signaling competent in yeast consistent with the idea that physical subunit dissociation is not necessarily required for G protein signaling (Klein et al., 2000). This concept is also supported by the kinetic coupling model for the action of RGS proteins indicating that in the presence of RGS proteins, GTP hydrolysis is too rapid to allow dissociation of $G\alpha$ and $G\beta\gamma$ prior to effector activation (Ross and Wilkie, 2000). An interesting thought was presented by Ugur and Onaran with their observation that the β_2 -adrenergic receptor could activate adenylyl cyclase without causing nucleotide exchange on $G\alpha$ (Ugur et al., 2005).

From the perspective of disease and therapeutics, several thoughts emerge. AGS1 may be of particular interest in terms of cell growth and differentiation and an expanded analysis of AGS1 mutants that may be associated with disease states would be of value. The GPR motif present in Group II AGS proteins has presented itself as a therapeutic target (Bowers et al., 2004) in modulating desire and given the role of GPR proteins in cell division, a similar strategy may be of value in manipulating stem cell propagation or uncontrolled cell growth. Recent success with small molecules targeting $G\beta\gamma$ (Bonacci et al., 2006), suggests that the mechanisms of $G\beta\gamma$ regulation by the Group III AGS proteins may provide an additional platform for expansion of this strategy.

Finally, it is likely that additional receptor-independent activators of G-protein signaling will surface as the yeast-based functional screen is expanded. AGS proteins that are upregulated in disease states or as part of a tissue adaptation program can likely be identified by screening a panel of cDNA libraries generated from human disease tissue in the yeast-based discovery platform. Proof of concept with this approach is provided by Sato et al (Sato et al., 2006b) and the functional screen of cDNA libraries from rat ischemic myocardium and a prostate leiomyosarcoma (Cao et al., 2004; Sato et al., 2006b).

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Abbreviations

AGS	Activators of G-protein signaling
AMPK	adenosine monophosphate activated protein kinase
cAMP	cyclic 3' 5'-adenosine monophosphate
CHO	Chinese hamster ovary
ERK	extracellular signal-regulated kinase
FRET	fluorescence energy transfer
GAP	GTPase-activating protein
GDI	guanine nucleotide dissociation inhibitor
GEF	guanine nucleotide exchange factor
GDP	guanosine -5'-diphosphate
GIRK	G-protein regulated inwardly rectifying potassium channel
GPCR	G-protein coupled receptor

GPSM	G-protein signaling modulator
GPR	G-protein regulatory
GRK	G-protein coupled receptor kinase
GST	glutathione-S-transferase
GTP	guanosine -5'-triphosphate
GTPγS	guanosine 5'-3- <i>O</i> -(thio)triphosphate
HEK	human embryonic kidney
Inscuteable	Insc
Lgl	lethal giant larvae
MAP	mitogen-activated protein
MAGUK	membrane associated guanylate kinase domain
MBP	maltose binding protein
NMDA	N-methyl-D-aspartic acid
nNOS	neuronal nitric oxide synthase
NuMA	nuclear mitotic apparatus protein
PAR	partitioning defective
Pcp2	purkinje cell protein-2
Pins	Partner of Inscuteable
PKC	protein kinase C
PLC	phospholipase C

PSD-95	post synaptic density 95
RGS	regulator of G-protein signaling
Ric	resistant to inhibitors of cholinesterase
SAP	synapse associated protein
SH3	src homology 3 domain
shRNA	short hairpin RNA
TPR	tetratricopeptide repeat

Figure 1. Properties of AGS proteins in the yeast based functional screen

The details of the yeast-based functional screen are described elsewhere (Cismowski and Lanier, 2005; Cismowski et al., 2002; Cismowski et al., 1999).

Figure 2. Diversity among functional domains in AGS proteins

Schematic diagrams of Group I-III AGS proteins are shown with functional domains highlighted. Domains are as predicted by SMART, PFAM or as described (Takesono et al., 1999). Ras, ras domain - VWA-A, von Willebrand factor A domain - Fn3 - fibronectin type 3 domain, UIM - Ubiquitin-interacting motif, Trip13 - thyroid receptor interacting protein 13, AAA-ATPase - AAA-ATPase domain, TPR – tetratricopeptide repeat. GPR – G-protein regulatory motif.

Figure 3. Functional roles of AGS proteins

A

	PM1 _	PM3	G2 _	G3	CAAX
H-ras	10 GA GG VGKSA	57 DTAG Q	116 NKCD	143 ETS A K	186 CVLS
AGS1	31 GSSK V GKTA	78 DTSG N	144 NKGD	174 EIS A K	278 CVIS

B

Figure 4. Comparison of key domains in Ras and AGS1

Human H-Ras and AGS1 were aligned using the ClustalW program (MacVector™ 7.0) and manual adjustment. A) The three conserved GDP/GTP binding motifs (G1-G3), phosphate-magnesium binding motifs (PM1-PM3) and CAAX motifs were compared. G2 and PM2 regions are identical in the two proteins and are not indicated in this figure. Mutation of amino acids in red result in constitutively active Ras. Residues in Ras that when mutation lead to dominant negative activity are indicated by the single red overlines (S17, D119 in Ras). B) Alignment of the Ras effector domain with the corresponding region of AGS1 and Rhes. Identical residues are indicated by asterisk. This figure is adapted from the thesis of Dr. Govindan Vaidyanathan (2004).

Figure 5. Structure-activity relationships of a consensus GPR peptide for inhibiting GTPγS binding to purified Gia1

A) Identification of the core GPR motif. Amino acid sequence of GPR peptide and truncated GPR peptides. Consensus amino acids are depicted in red. The right column portrays GTPγS binding (500 nM with 2 mM MgCl₂) to Gia₁ (100 nM) measured in the absence and presence of GPR peptides (100 μM). B) Sequence alignment and IC₅₀ values for mutated GPR peptides in GTPγS binding assays. Conserved amino acids are depicted in red and mutated amino acids are colored green. Effect of increasing concentrations of competing peptides on GTPγS (500 nM with 2 mM MgCl₂) binding to Gia₁ (100 nM). Data are expressed as the percent of specific binding (~1 pmol) observed in the absence of added peptide and are expressed as the mean ± S.E of two experiments with duplicate determinations. Inactive - inhibition of GTPγS binding was less than 30% at peptide concentrations of 100 μM. Modified from Peterson et al (Peterson et al., 2002).

Figure 6. Accessibility of the GPR-GoLoco binding site on $G\alpha_i$ within the context of heterotrimeric $G\alpha\beta\gamma$

Left Panel - The model was created by performing a structural carbon-carbon alignment of the $G\alpha_i$ subunit in the G protein heterotrimer structure solved by Wall et al (Wall et al., 1995) with the structure of $G\alpha_i$ complexed with the GPR-GoLoco peptide derived from RGS14 (Kimple et al., 2002). The $G\alpha_i$ subunit from the heterotrimer structure was removed to illustrate how $G\alpha_i$ complexed with the GPR-GoLoco peptide would be positioned relative to the $G\beta\gamma$ subunit suggesting that the binding site for peptide binding is accessible in the heterotrimer. Right panel - Comparison of the conformation of $G\alpha$ switch II in the GPR-Goloco bound (red) or $G\beta\gamma$ bound (green) structures. $G\alpha K210$ and $G\alpha E216$ are involved in direct electrostatic interactions with $G\beta\gamma$ in the heterotrimer structures.

Figure 7. Schematic of interacting proteins for AGS3 and AGS5/LGN
See Tables 3 and 4 for additional information.

Figure 8. Localization of AGS5/LGN during the cell cycle

CHO cells were synchronized to the entry of mitosis and subsequently allowed to enter the cell cycle and following fixation at increasing times, the cells were stained with antibodies against LGN (green - left panel) or G α 3 (green - right panel) and β -tubulin (red), and DNA stained with DAPI (blue). The yellow signal reflects overlap of the green and red signals. The figure is modified from Blumer et al. 2006.

Table 1**AGS cDNAs isolated in functional screens for receptor independent activators of G-protein signaling**

cDNA libraries were screened in yeast strains CY1141 (G α 3), CY8342 (G α s) and CY9603 (G α 16) (Cao et al., 2004; Cismowski et al., 1999; Sato et al., 2006; Takesono et al., 1999). GPR – G-protein regulatory motif. Unless indicated otherwise (i.e. C-terminal...) the full length cDNA was isolated in the functional screen.

Activator of G-protein Signaling	Protein or gene names in database or literature
AGS1	GROUP I DexRas1, RASD1
AGS3	GROUP II GPSM1 (C-terminal 278 amino acids - 4 GPR motifs) (C-terminal 74 amino acids – 1 GPR motif)
AGS4	G18.1b, GPSM3 (3 GPR motifs)
AGS5	LGN, GPSM2, mPINS (C-terminal 322 amino acids – 4 GPR motifs)
AGS6	RGS12 (C-terminal 365 amino acids - 1 GPR motif)
AGS2	GROUP III Tctex-1
AGS7	Thyroid receptor interacting protein-13 (TRIP13)
AGS8	KIAA1866 (C-terminal 372 amino acids)
AGS9	Rpn10
AGS10	G α_o

Table 2

Interventions or treatments that regulate AGS1 mRNA expression.

Stress or intervention	Induction or modification	Citation
Alcohol exposure	Down-regulated by embryonic exposure to alcohol	(Hard et al., 2005)
Amphetamine	Increased expression in rat brain	Footnote #2
Blood loss	Identified as blood loss inducible cDNA in rat kidney	Footnote #1
Breast cancer	Downregulated in breast cancers	(Abba et al., 2005; Porter et al., 2003)
Ciradian rhythm	Identified as a cDNA in suprachiasmatic nucleus that cycles with circadian rhythm	(Takahashi et al., 2003)
Dessication stress	Increased by > 300 fold	(Huang and Tunnacliffe, 2004)
Dexamethasone	Identified as a dexamethasone inducible cDNA in AtT-20 corticotroph cells	(Brogan et al., 2001; Kemppainen and Behrend, 1998; Kemppainen et al., 2003)
Hypertonicity stress	Increased by 20 to >100 fold	(Huang and Tunnacliffe, 2004)
Melanoma	1 of 13 CG islands in putative promoters methylated in melanoma cell lines	(Furuta et al., 2006)
Rat renal isografts from brain dead donors	Increased > 6 fold	(Kusaka et al., 2005)

Table 3**Proteins other than Gα that bind AGS3**

Co-IP – coimmunoprecipitation.

AGS3 interacting protein	Identification of interaction	Location of interaction	Functional role of the interaction	Citation
LKB1	Y2H with AGS3- TPR+linker domain	C-terminus of LKB1 interacts with linker of AGS3	LKB1 phosphorylates the GPR domain of AGS3 and LGN; phosphorylation of GPR motif inhibits interaction with G α	(Blumer et al., 2003)
Frmpd1	Y2H with AGS3- TPR+linker domain	C-terminus of Frmpd1 interacts with TPR domain of AGS3	Unknown	An et al., unpublished observation
mInsc	GST and MBP affinity matrix; co-IP	Unknown	Unknown	(Zigman et al., 2005)

Table 4

Proteins other than Gα that bind AGS5/LGN

Co-IP – coimmunoprecipitation.

LGN interacting protein	Identification of interaction	Location of interaction	Functional role of the interaction	Citation
NuMA	Y2H with LGN- TPR domain (aa 93-133)	LGN-TPR II interacts with NuMA (aa 1878-1910)	Interferes with NuMA binding to tubulin in vitro; LGN recruits NuMA to the cell cortex during mitosis; NuMA binding facilitates LGN binding to Gii; localization of LGN to spindle poles is disrupted upon NuMA knockdown	(Du and Macara, 2004; Du et al., 2001; Du et al., 2002)
PSD-95 family of MAGUK proteins (SAP102, SAP97/Discs-large, PSD95)	Y2H with SAP102 SH3- GK domain; Y2H with LGN- linker +GPR domain; co-IP	GK domain of PSD-95 MAGUK proteins bind linker domain of LGN	Increase trafficking of NMDA receptor subunits to the plasma membrane; increase number and size of dendritic spines in hippocampal neuronal cultures	(Sans et al., 2005); Blumer et al., unpublished observation
mInsc	GST and MBP affinity matrix; co-IP	mInsc N- terminus (aa1-101) binds LGN; binding site on LGN unknown	Unknown; may influence targeting of LGN to plasma membrane during mitosis; mInsc shRNA results in defects in mitotic spindle orientation in rat retinal explants	(Lechler and Fuchs, 2005; Zigman et al., 2005)
Lgl	Y2H with full length Lgl; confirmed with GST pull down and reciprocal co-IP	Lgl-C terminus (aa 636-1015) binds LGN-TPR (aa 1- 365); includes half of the LGN linker domain	Formation of PAR complex observed with LGN, Lgl, Par-6 and aPKC in mitotic HEK cells; Lgl siRNA blocks LGN localization to the plasma membrane, mis-localizes NuMA and causes defects in chromosomal segregation during mitosis	(Yasumi et al., 2005)
Ha-Ras	Co-IP in trasnfected HEK293T cells	Wild-type and G12V Ha-Ras bind LGN- TPR +linker (LGN aa 1- 400)	Unknown	(Marty et al., 2003)