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Molecular mechanisms of G protein-receptor coupling

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Molecular Mechanisms of G Protein-Receptor Coupling

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Dissertation submitted to the School of Medicine at West Virginia University
in partial fulfillment of the requirements for the degree of

Doctor of Philosophy
in
Pharmacology & Toxicology

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ABSTRACT

Molecular Mechanisms of G Protein-Receptor Coupling

Hongzheng Ma

A variety of extracellular signals are transmitted into the cell interior by interactions with a superfamily of heptahelical cell surface receptors. Heterotrimeric G proteins mediate the signal transduction by coupling these receptors to intracellular effector proteins. The molecular mechanisms in G protein-receptor coupling processes are still not completely understood. In this project, two aspects of G protein-receptor coupling were examined: 1) the G protein-coupling properties of the five human muscarinic acetylcholine receptors (mAChRs); 2) a novel regulatory mechanism by a newly identified G protein signal regulator, Activator of G-protein Signaling 3 (AGS3). To study the regulation of G protein-receptor coupling, an Sf9 cell membrane-based *in vitro* reconstitution system was used, in which purified G protein heterotrimers were reconstituted with individually expressed membrane receptors and their coupling was assessed with radioligand binding assays.

Functional G protein coupling was successfully established for M1, M2, M4 and M5 mAChRs in urea-extracted Sf9 cell membranes. Under the same conditions, M3 mAChRs failed to couple with purified G proteins, indicating they may have a unique G protein signaling mechanism. Within the odd- or even-numbered mAChR groups similar apparent affinities for G protein interactions were observed, however, the odd-numbered mAChRs exhibited higher affinity for G protein heterotrimers than did the even-numbered mAChRs. Differences were also observed among the individual receptor subtypes in their affinity states for the agonist, Oxotremorine-M.

Studies on AGS3 revealed that cytosolic AGS3, but not membrane-associated AGS3, can interfere with receptor-Gi protein coupling. Cytosolic AGS3 can remove Gi α subunits from the plasma membrane and sequester them in the cytosol. Each of the four AGS3 GPR (G protein regulatory) domains was able to interfere with receptor-Gi protein coupling; however, individual domains were less effective than the full-length GPR domain. None of the GPR domains distinguish among the three Gi α subunits but they all interact more weakly with Go α subunits.

These studies demonstrate that five mAChRs have distinct G protein-coupling behaviors in an identical membrane environment and that AGS3 may down-regulate G protein signaling by interfering with receptor coupling. These findings contribute to the understanding of the mechanism and regulation of G protein-receptor coupling.

DEDICATIONS

To my dear wife, Min Wang and my lovely daughter, Grace.

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LIST OF ABBREVIATIONS

5-HT	5-hydroxytryptamine
AC	adenylyl cyclase
AcMNPV	<i>Autographa californica</i> multiple nuclear polyhedrosis virus
AGS	activator of G protein signaling
BCA	bicinchonic acid
CHAPS	3-[(3-cholamidopropyl)dimethylammonil]-1-propanesulfonic acid
ER	endoplasmic reticulum
GAP	GTPase activating protein
GDI	guanine nucleotide dissociation inhibitor
GDP	guanosine diphosphate
GEF	guanine nucleotide exchanging factor
GoLoco motif	Gi/o-Loco interaction motifs
GPCR	G protein coupled receptor
GPR	G protein regulatory
GRK	G protein coupled receptor kinase
GST	glutathione S-transferase
GTP	guanosine triphosphate
GTPγS	guanosine 5'-3- <i>O</i> -(thiotriphosphate)
G protein	heterotrimeric guanine nucleotide binding protein
mAChR	muscarinic acetylcholine receptor
MAP kinase	mitogen-activated protein kinase
MOI	multiplicity of infection
NMS	N-Methyl Scopolamine
Oxo-M	Oxotremorine-M
PI3K	Phosphatidylinositol 3-Kinase
Pins	partner of inscuteable
PKA	protein kinase A
PKC	protein kinase C
PLC	phospholipase C
PVDF	polyvinylidene fluoride
R-G-E	receptor-G protein-effector
RGS	regulator of G protein signaling
SDS-PAGE	sodium dodecylsulfate-polyacrylamide gel electrophoresis
Sf9	<i>Spodoptera frugiperda</i>
TPR	tetratricopeptide repeats

CHAPTER 1: INTRODUCTION

In all eukaryotic organisms, heterotrimeric guanine nucleotide binding protein (G protein) mediated signal transduction is used by cells to respond to a diverse array of extracellular stimuli such as hormones, neurotransmitters, dietary chemicals and sensory stimuli. G proteins mediate the transduction of extracellular signals across the plasma membrane by coupling cell surface receptors to effector proteins. These signal inputs propagate to the intracellular signaling network to control a broad range of cellular responses such as metabolism, contractility, secretion, motility, transcription and growth. Integration of these cellular responses at systemic levels leads to the regulation of many physiological functions such as organismal homeostasis, embryonic development, gonadal development, learning and memory (Neves *et al.*, 2002). Dysfunction of this signaling transduction system has been shown to relate to a variety of human diseases (Spiegel, 1996; Spiegel, 1997; Farfel *et al.*, 1999; Edwards *et al.*, 2000)

The essential roles of the G protein signal transduction system in both normal and pathological states have made its components attractive targets for pharmacological interventions. In fact, more than 40% of all currently prescribed drugs target G protein coupled receptors (GPCRs) (Drews, 2000). Most of these drugs exert their effects on GPCRs either as agonists or as antagonists, but researchers have also been trying to develop new approaches to manipulate this signal transduction process (Akhter *et al.*, 1998; Feldman *et al.*, 2002). Recently, a membrane permeable peptide, designed on the structural basis of receptor-G protein coupling, successfully blocked the thrombin receptor-mediated human platelet aggregation (Covic *et al.*, 2002). Thus, elucidation of

the detailed molecular mechanisms of G protein signaling pathway will not only help us better understand human physiology, but may also provide novel therapeutic strategies to treat human diseases.

Heterotrimeric G protein:

Historical perspectives. The study of heterotrimeric G proteins began over three decades ago in 1971, when Rodbell *et al* noticed that GTP was involved in the activation of adenylyl cyclase by glucagon (Rodbell *et al.*, 1971). Later on, observations from several groups suggested the existence of a GTP-associated protein distinct from the receptor and adenylyl cyclase (Londos *et al.*, 1974; Salomon *et al.*, 1975; Cassel and Selinger, 1976; Maguire *et al.*, 1976). In 1977, Ross and Gilman provided the first direct evidence showing that the GTP-dependent transmembrane signaling pathway was composed of at least three distinct proteins: receptor, G protein and effector (Ross and Gilman, 1977a; Ross and Gilman, 1977b). In 1980, a G protein involved in the stimulation of adenylyl cyclase was first purified by Northup *et al* and shown to have a heterotrimeric structure, which was designated as α , β and γ subunits (Northup *et al.*, 1980). A few years later, the same group purified another G protein, which has an inhibitory effect on adenylyl cyclase (Bokoch *et al.*, 1984). With the use of molecular cloning techniques, numerous G protein subunits have been cloned since 1985 (Simon *et al.*, 1991).

Diversity of G protein subunits. To date over 20 α subunits, 6 β subunits and 12 γ subunits have been identified (Simon *et al.*, 1991; Ray *et al.*, 1995; Watson *et al.*, 1996). Based on amino acid sequence similarity, G protein α subunits are classified into

four major families, α_s , α_i , α_q and α_{12} and the heterotrimeric G proteins are conventionally named after their α subunits. Overall α subunits share at least 40% identity at the amino acid level, with 60-90% identity within individual families (Simon *et al.*, 1991). The α_s family, in which “s” refers to the stimulatory effect on adenylyl cyclases, was discovered first and includes α_s and α_{olf} members. The α_i family including α_{i1} , α_{i2} , α_{i3} , α_{oA} , α_{oB} , α_{t1} , α_{t2} and α_z was initially named for the ability of α_i to inhibit adenylyl cyclases. The α_q family consists of α_q , α_{11} , α_{14} , α_{15} and α_{16} members that can stimulate phospholipase C. The α_{12} and α_{13} were classified into the last family because of their sequence similarity; however, their exact functions have not been well established. In contrast with the α subunits, the amino acid sequences of β subunits are highly conserved. Above 80% of their amino acid sequence are identical with β_5 as an exception, which only has 53% sequence identity to other β subunits (Watson *et al.*, 1996; Clapham and Neer, 1997). The G protein γ subunits are the most divergent ones (Cali *et al.*, 1992). Although they share considerable sequence similarity at their carboxyl termini, their amino terminal sequences are much more different from each other.

G protein α subunits. The α subunits of heterotrimeric G proteins consist of a single polypeptide chain with molecular weight from 39 to 52 kDa. All α subunits can bind and hydrolyze GTP, although their intrinsic rates of GTP hydrolysis vary dramatically (Carty *et al.*, 1990; Linder *et al.*, 1990; Chidiac *et al.*, 1999). Crystallographic studies (Lambright *et al.*, 1994; Sondek *et al.*, 1994; Coleman *et al.*,

1994a; Coleman *et al.*, 1994b; Mixon *et al.*, 1995; Wall *et al.*, 1995; Lambright *et al.*, 1996) reveal that α subunits share common structural features and have significant conformational changes when either the bound guanine nucleotide or the interaction with $\beta\gamma$ subunits is changed (Noel *et al.*, 1993). Briefly, α subunits contain two principal domains. A GTPase domain, which is involved in nucleotide binding and GTP hydrolysis, shares considerable sequence similarity with a superfamily of GTPase proteins including elongation factor EFTu and small G proteins such as Ras, Rab, Rac, Ral, etc (Bourne *et al.*, 1990). The GTPase domain consists of six stranded β -sheets surrounded by six helices ($\alpha 1$ - $\alpha 5$ and αG) (Figure 1). The other is a unique α -helical domain consisting of a long central helix (αA) surrounded by five shorter helices (αB - αF). The guanine nucleotide binds tightly in the cleft between the GTPase and α -helical domains. When bound to GTP, α subunits dissociate from receptors and $\beta\gamma$ subunits to interact with their downstream effector proteins such as adenylyl cyclase, phosphodiesterase, phospholipase C, and ion channels (Gilman, 1987).

G protein $\beta\gamma$ subunits. The $G\beta$ subunits with molecular weight of about 37 kDa consist of two distinct domains, an amino terminal domain, which has a 20-amino acid α -helical structure, and the remainder of the molecule, which has seven repeated sequences (Wall *et al.*, 1995; Lambright *et al.*, 1996; Sondek *et al.*, 1996). The amino terminal α -helical domain associates with $G\gamma$ subunit through a coiled-coil interaction, while the seven repeated sequences, termed as WD-repeats, are made up of small antiparallel β strands that are arranged in a ring to form a propeller-like structure (Lupas *et al.*, 1991;

Figure 1.

Figure 1. Schematic representation of Gα secondary structural domains. The secondary structural domains, which are common to Gα subunits were determined from the crystallographic studies (Lambright *et al.*, 1994; Sondek *et al.*, 1994; Coleman *et al.*, 1994a; Coleman *et al.*, 1994b; Mixon *et al.*, 1995; Wall *et al.*, 1995; Lambright *et al.*, 1996). The shaded regions represent the sequences that form the corresponding secondary structures and are labeled as α for α-helices or β for β-sheets. The underlined are the components of the unique helical domain. *N*, the amino terminus; *C*, the carboxyl terminus.

Sondek *et al.*, 1996). These WD-repeats, composed of approximately 43 amino acids are not unique to G β subunits and are found in a superfamily of proteins with diverse functions (Neer *et al.*, 1994). The G γ subunits are about 75 amino acids long with a molecular weight of about 8 kDa. They are not only divergent in their sequence but also different in their lipid modifications by prenyl groups (Spiegel *et al.*, 1991; Clapham and Neer, 1993; Casey, 1994). The amino terminus of G γ subunits forms a coiled-coil contact with the amino terminal α -helix of G β , and the rest of the molecule extends to the contacting faces of the blades 5, 6 and 7 of the G β propeller structure (Sondek *et al.*, 1996). The multiple contact sites contribute to the tight association of $\beta\gamma$ subunits such that they can only be separated under denaturing conditions. In fact, $\beta\gamma$ subunits act functionally as a monomer, though they are two distinct polypeptides. When freed from G α subunit they can independently or synergistically modulate a variety of effectors such as K⁺ channel, Ca²⁺ channel, phospholipase A2, phospholipase C- β , adenylyl cyclase, PI3K, GPCR kinase, MAP kinase cascade and yeast pheromone response (Clapham and Neer, 1997).

GTPase cycle. In the conventional model (Gilman, 1987; Neer, 1995; Hamm, 1998), G proteins cycle between a GDP-bound inactive state and a GTP-bound active state to transmit signals from cell surface receptors to effectors. As illustrated in Figure 2, when bound to GDP, G α subunits associate with G $\beta\gamma$ subunits to form the inactive heterotrimer. When extracellular stimuli activate GPCRs, GPCRs undergo conformational changes, which enable them to interact with G protein heterotrimers. The

Figure 2.

Figure 2. The GTPase cycle of heterotrimeric G proteins (Adapted from Iiri *et al.*, 1998). The “turn on” step begins when the activated receptor (R^*) interacts with the GDP-liganded heterotrimer ($\alpha_{GDP} \beta\gamma$), catalyzing the GDP dissociation and resulting in a “empty-pocket” state of $R^* \alpha_e \beta\gamma$ complex (“ e ” indicates the empty state of α subunit). The GTP binding to α causes its dissociation from R^* and $\beta\gamma$ subunit. Both α_{GTP} and free $\beta\gamma$ are able to activate their effectors (E_1 , E_2) and initiate signaling cascades. The “turn off” step depicts the GTP hydrolysis (to GDP and inorganic phosphate, P_i) and consequent α_{GDP} and $\beta\gamma$ subunit reassociation.

activated GPCRs then catalyze the dissociation of GDP from G α subunits resulting in a so-called “empty-pocket” complex of liganded receptor and $\alpha\beta\gamma$ heterotrimer and the intracellular GTP consequently binds to the empty G α subunit. Once GTP is bound, G α subunit switches to its active state and dissociates from both receptor and G $\beta\gamma$ subunits. Both G α •GTP and free G $\beta\gamma$ are able to activate a number of effectors, however, the bound GTP will eventually be cleaved back to GDP due to the intrinsic GTPase activity of G α subunits. G α •GDP and free G $\beta\gamma$ then reassociate with each other and the signal is turned off. Thus, the rate of GDP release from G α subunit and the rate of GTP hydrolysis are two key components determining the timing of signal activation and deactivation.

Cellular localization. Unlike transmembrane GPCRs, newly synthesized G protein subunits do not insert into the ER membrane to be translocated to the plasma membrane. In contrast, after synthesis in the cytosol (Rehm and Ploegh, 1997) they have to be modified and assembled before trafficking to the plasma membrane to transmit signals from GPCRs. For example, mutations disrupting the myristoylation of G α_i shift G α_i from the membrane to the cytosol fraction (Jones *et al.*, 1990) and mutations preventing G $\beta\gamma$ interaction with G α_s and G α_q disrupt the membrane targeting of these α subunits (Evanko *et al.*, 2000). Currently there are several possible mechanisms that explain how G protein subunits are assembled and targeted to the plasma membrane. First of all, lipid modifications of G protein α and γ subunits in general facilitate the membrane localization and subunit assembly of heterotrimeric G proteins (Marshall,

1993; Casey, 1994; Wedegaertner *et al.*, 1995; Chen and Manning, 2001). Myristoylation and/or palmitoylation of G α subunits and the prenylation of G γ subunits occur post- or co-translationally in a presumably cytosolic compartment and are believed to provide membrane anchors for the heterotrimeric G proteins. As for N-myristoylation modification, a myristoyl group is co-translationally attached at the amino terminal glycine residue of α subunits and this occurs only for Gi family members, while for palmitoylation a palmitoyl group is post-translationally attached to a cysteine residue near the amino terminus of almost all G α subunits except G α_i . For Gi family members, palmitoylation appears to be sufficient to target their α subunits to the plasma membrane (Shahinian and Silviu, 1995), while N-myristoylation, though not sufficient by itself to target G α_i to the plasma membrane, plays an important role in facilitating the membrane anchorage and the palmitoylation of Gi α (Jones *et al.*, 1990; Mumby *et al.*, 1990; Hallak *et al.*, 1994; Mumby *et al.*, 1994; Mumby and Linder, 1994). N-myristoylation also play a critical role in assembling G α_i with G $\beta\gamma$ subunits (Jones *et al.*, 1990; Linder *et al.*, 1991). Other than G α_i subunits, the majority of G α subunits are not N-myristoylated but they are all palmitoylated at either one site (G α_s and G α_{11}) or two sites (G α_q and G α_{13}) near their amino termini (Chen and Manning, 2001). There was an initial dispute about whether or not palmitoylation is sufficient to ensure a stable membrane anchorage for these α subunits, but Evanko *et al* have demonstrated that G $\beta\gamma$ subunits are required for both membrane anchorage and palmitoylation (Evanko *et al.*, 2000). Although lipid modification of G γ subunits is not necessary for their assembly with G β subunits, it is

required for the productive interaction of G $\beta\gamma$ with G α subunits, receptors and other proteins, and for the correct membrane targeting as well (Casey, 1994; Wedegaertner *et al.*, 1995). All G γ subunits undergo prenylation, in which a 20-carbon isoprenoid geranylgeranyl or a 15-carbon isoprenoid farnesyl group is covalently added to the cysteine residue in the carboxyl terminal “CAAX” box of the G γ subunits.

As partly discussed above, G $\beta\gamma$ subunits are required for G α subunit membrane targeting and stable membrane association (Sternweis, 1986; Wedegaertner *et al.*, 1995; Chen and Manning, 2001). Rehm and Ploegh reported that G β and G γ subunits dimerize immediately upon their synthesis in the cytosol (Rehm and Ploegh, 1997), while Michaelson *et al* demonstrated that G α subunit has to associate with G $\beta\gamma$ on the Golgi before the heterotrimer is translocated to the plasma membrane (Michaelson *et al.*, 2002). G $\beta\gamma$ subunits not only direct the plasma membrane targeting of G α subunits, but also facilitate their palmitoylation (Wang *et al.*, 1999; Evanko *et al.*, 2000; Evanko *et al.*, 2001). There are several lines of evidence showing that the amino terminal region of G α subunit may provide additional membrane anchoring *via* protein-lipid or protein-protein interaction (Busconi *et al.*, 1997; Busconi and Denker, 1997; Gillen *et al.*, 1998). Taken together, the membrane targeting and anchoring of heterotrimeric G proteins may depend on multiple factors including lipid modification, subunit assembly and even direct interaction of G proteins with the plasma membrane.

G protein coupled receptors:

Historical perspectives. After the cloning of human rhodopsin in 1984 and hamster β 2-adrenergic receptor in 1986, it was found that the G protein-coupled “light-receptor” rhodopsin and the “classical” ligand-binding receptor, β 2-adrenergic receptor, share considerable sequence identity (Nathans and Hogness, 1984; Dixon *et al.*, 1986). Based on the physical structure from studies of bacteriorhodopsin (Henderson and Unwin, 1975) and human rhodopsin itself, it was proposed that GPCRs share a common structure consisting of a single-chain peptide with seven stretches of 18-30 hydrophobic amino acid residues that may form membrane-spanning α -helical segments. This concept was rapidly confirmed by the cloning of other GPCRs (Dohlman *et al.*, 1991).

Diversity and classification. In humans, approximate 750 GPCRs have been reported (Watson and Arkininstall, 1994; Vassilatis *et al.*, 2003). These receptors belong to a superfamily of cell surface receptors with seven transmembrane segments and mediate signal transduction *via* heterotrimeric G proteins. Their ligands range from Ca^{2+} ions to small biogenic amines, nucleotides, eicosanoids, peptides and glycoproteins (Ji *et al.*, 1998; Wess, 1998). Based on the sequence similarity, GPCRs can be classified into four major families and they can be further grouped according to their ligand structure and G protein coupling properties (Wess, 1998).

Structure. Although only the crystal structure of rhodopsin has been resolved so far (Palczewski *et al.*, 2000), the seven transmembrane α -helical structure of the other family members has been inferred from cloning, physical, biochemical and theoretical studies (Nathans, 1987; Dohlman *et al.*, 1991; Baldwin, 1993; Pogozheva *et al.*, 1997;

Herzyk and Hubbard, 1998). As shown in Figure 3, the seven α -helical segments are linked by three alternative intracellular (i1-i3) and extracellular loops (o2-o4) with the amino terminus (o1) and the carboxyl terminus (i4) at the extracellular and intracellular side, respectively. If a cysteine residue within the carboxyl terminus is palmitoylated, a fourth intracellular loop may be formed. The seven transmembrane segments are the highly conserved regions among different families and within an individual family while the other regions vary both in their sequences and sizes, which may correlate to their diverse structures and functions. In general, the extracellular segments and the exofacial portions of the transmembrane segments are involved in ligand binding while the intracellular segments participate in the G protein activation and the interaction with other proteins (Dohlman *et al.*, 1991; Schwartz, 1994; Wess, 1997; Ji *et al.*, 1998; Wess, 1998).

Ligand binding and receptor activation. The domains and modes of GPCRs for ligand binding vary greatly as reviewed by Ji *et al.* (Ji *et al.*, 1998). Ligand binding is believed to cause GPCR conformational changes that may be a distinct step from receptor activation depending on the nature of the ligands (i.e. full agonist, partial agonist, competitive antagonist or inverse antagonist). It was proposed that ligand binding may cause relative movements of transmembrane helices, which either stabilize GPCR in inactive conformations (as for antagonists) or shift to active conformations (as for agonists) (Wess, 1997). This idea was supported by direct structural analyses with the fluorescence spectroscopic technique from Kobilka's group (Gether *et al.*, 1995; Gether *et al.*, 1997a; Gether *et al.*, 1997b).

Figure 3.

Figure 3: Schematic presentation of the general structure of GPCRs and terminology. N' and C' represent the amino and carboxyl termini. The seven transmembrane helices are numbered I-VII from left to right; four extracellular segments o1-o4; and four intracellular segments i1-i4.

The models explaining GPCR activation upon ligand binding are still evolving. Currently the majority of the experimental data can be explained by the widely accepted “extended ternary complex model”, which was deduced from the studies of mutations of β 2-adrenergic receptor (Samama *et al.*, 1993). In this model, GPCRs equilibrate between an inactive (R) and an active (R*) conformation. When shifted to the active conformation, GPCRs are capable of coupling to the heterotrimeric G proteins. The conformational change associated with activation may occur spontaneously, or be induced by agonists or certain mutations. The active receptor conformation, R*, couples to the heterotrimeric G proteins and catalyzes the GDP dissociation from the $G\alpha$ subunits, resulting in guanine nucleotide-free G proteins, which in turn stabilize R* in the highest affinity state for agonist binding. This association of agonist/receptor/G-protein is referred to as the ternary complex. This model successfully explained agonist-independent basal G protein activation and mutation-induced “constitutive” G protein activation. However, there are several lines of evidence indicating that the extended ternary model may oversimplify the GPCR behaviors (Krumins and Barber, 1997; Leff and Scaramellini, 1998; Wenzel-Seifert and Seifert, 2000; Lopez-Gimenez *et al.*, 2001). Current results support the existence of multiple activated states of GPCRs that may be induced by agonists with different affinity orders or efficacy orders, or stabilized by heterotrimeric G proteins with different conformations (Chidiac, 1998; Scaramellini and Leff, 1998; Surya *et al.*, 1998). Further evidence also comes from our laboratory with the identification of a G protein chimera, which is inactive in stabilizing receptor in high

affinity agonist binding state but still active in agonist-catalyzed guanine nucleotide exchange (Slessareva *et al.*, 2002).

GPCR-G protein coupling:

Diversity in R-G coupling. A striking characteristic of G protein mediated signaling is the redundancy of receptor subtypes. One extracellular stimulus may exert diverse physiological effects by acting on a variety of receptor subtypes that may have different tissue expression patterns and/or signaling pathways. For biogenic amines as examples, 9 epinephrine receptors, 5 dopamine receptors, 2 histamine receptors, 5 muscarinic acetylcholine receptors and 13 serotonin receptors have been identified by molecular cloning studies and all of them can elicit their responses by coupling to heterotrimeric G proteins (Wess, 1998). By coupling to different heterotrimeric G proteins, these receptors elicit a broad range of actions in response to their endogenous ligands (Hulme *et al.*, 1990; Lucas and Hen, 1995). It is still not well understood if there is any subtle difference in their abilities to recognize endogenous ligands or what may account for the differences in their G protein coupling properties. More puzzlingly, within these receptors and other receptor families multiple subtypes have been shown to couple to the same set of G protein heterotrimers (Gudermann *et al.*, 1996). For example, three muscarinic acetylcholine receptor subtypes (M1, M3 and M5) all couple to Gq proteins upon activation by acetylcholine but elicit different physiological responses (Caulfield and Birdsall, 1998). Therefore, the molecular details of their G protein coupling properties need closer examination to better understand the physiological

actions of a given receptor subtype. Such information will also improve pharmacological intervention of these receptors. Furthermore, as summarized by Gudermann *et al* and exemplified in Figure 4 many receptor subtypes are known to often couple to multiple G protein heterotrimers, which adds more complexity to this signaling system (Gudermann *et al.*, 1997). Extensive efforts have been made to understand how an extracellular stimulus leads to an appropriate response after activating GPCRs and what are the additional components that direct and fine-tune the signal propagation within the intracellular signaling network (see discussion below).

Despite of the fact that many GPCRs can elicit their effects by coupling to multiple G proteins, the majority of GPCRs only interact with a distinct subset or even a specific G protein heterotrimer (Hedin *et al.*, 1993; Raymond, 1995; Gudermann *et al.*, 1996; Wess, 1997; Gudermann *et al.*, 1997). The 20 G α subunits, 6 G β subunits and 12 G γ subunits may theoretically make 1,440 combinations of G $\alpha\beta\gamma$ heterotrimers, however, evidence has indicated that not all the possible combinations can occur (Schmidt *et al.*, 1992; Pronin and Gautam, 1992; Iniguez-Lluhi *et al.*, 1992; Yan *et al.*, 1996). Considering the high homology in the G protein sequences (Simon *et al.*, 1991), it is very interesting to understand how a specific receptor can distinguish among these highly homologous G proteins and especially what is the structural basis determining such a selective interaction. In fact, the knowledge obtained from the studies on the structural basis of selective R-G coupling has been successfully applied to intervene the physiological events *in vivo*. For example, Covic *et al* designed membrane permeable peptides based on the sequences of the third intracellular loop of protease-activated

Figure 4.

Figure 4. Examples of multiple GPCR/G-protein coupling (Adapted from Gudermann *et al.*, 1997). A single receptor subtype may couple to multiple G protein families and individual G proteins may also receive signals from multiple receptors.

receptor 1 or 4, which were determined to be their selective G protein coupling domain. They applied these peptides to target the intracellular interface of the receptors and effectively blocked the thrombine-mediated human platelet aggregation (Covic *et al.*, 2002). Another group used the transgenic approach to target the carboxyl terminal region of Gq α and successfully prevented cardiomyocyte hypertrophy in the experimental animals (Akhter *et al.*, 1998).

Molecular determinants for selective R-G coupling. A huge body of investigation has been performed to study how a selective R-G interaction is achieved at the molecular level. On the GPCR side, numerous studies suggest that the intracellular segments of GPCRs are involved in G protein recognition. Generally speaking, although all GPCRs share a common structural topology, their intracellular segments vary greatly both in size and amino acid sequence. It has been shown that some GPCRs, which share little or no sequence homology in these regions, couple to the same set of G proteins (Hedin *et al.*, 1993). Thus, the G protein coupling profile of a given GPCR can not be deduced solely from its amino acid sequence. However, the general positions of its selective coupling domains could be predicted based on the conserved structural features among GPCRs. As comprehensively reviewed by Wess (Wess, 1998), all four intracellular domains have been indicated to be involved in G protein coupling with the most critical regions being the second intracellular loop, and the amino and carboxyl portions of the third intracellular loop (Wess, 1997). The two most important concepts developed to date are that the selective G protein coupling results from the cooperative

contribution of multiple intracellular regions and that the relative contributions of different intracellular domains vary among individual receptors (Wess, 1998).

On the G protein side, both G β and G γ subunits have been shown to specify receptor-G protein coupling, but most G $\beta\gamma$ complexes appear to function similarly (Phillips and Cerione, 1992). Thus, extensive efforts have been focused on G α subunits. Similar to GPCRs, the selective receptor coupling of G α subunits is also determined by multiple domains. As implicated and supported by the overall crystal structures of G protein (Wall *et al.*, 1995; Lambright *et al.*, 1996), the extreme amino and carboxyl terminal domains of G α subunits are the critical selective coupling determinants from a variety of studies (For reviews, see Conklin and Bourne, 1993; Neer, 1995; Bourne, 1997; Wess, 1998). Evidence also shows that additional domains participate in receptor recognition, such as the α 2 helix- α 2/ β 4 loop (Lee *et al.*, 1995), α 3/ β 5 loop (Grishina and Berlot, 2000) and α 4 helix- α 4/ β 6 loop (Hamm *et al.*, 1988; Onrust *et al.*, 1997; Bae *et al.*, 1997; Natochin *et al.*, 1999; Bae *et al.*, 1999) regions.

Our laboratory has applied a chimeric protein approach to study the molecular basis of R-G protein coupling and our results show that even closely related GPCRs couple to their G proteins differently (Ma *et al.*, 2000). Some closely related GPCRs, although coupled to the same set of G proteins, display different apparent affinities for their coupling to G proteins. Individual receptors use different combinations of multiple G α domains to achieve their selective coupling (Ma *et al.*, 2000; Slessareva and Graber, 2003). Taken together, our studies and those of others suggest that a particular R-G coupling interface is formed by the molecular determinants from both the receptor and G

protein. The R-G coupling interface of each closely related GPCRs or G proteins may vary from each other.

R-G coupling studies using mAChRs as a model:

Muscarinic acetylcholine receptors (mAChRs). Acetylcholine, a classic neurotransmitter, exerts its ionotropic and metabotropic effects via two classes of cholinergic receptors. The metabotropic effects are mediated by the family of muscarinic cholinergic receptors, which belongs to the GPCR superfamily (Wess, 1996; Wess, 1998; Schoneberg *et al.*, 1999). Activation on mAChRs modulates a broad range of physiological functions in human body (Hardman *et al.*, 2001). It was first revealed by ligand binding studies that at least two tissue-specific populations of muscarinic receptors exist (Hammer *et al.*, 1980; Hammer and Giachetti, 1982). To date five members or subtypes of this family (M1-M5) have been identified from pharmacological and molecular cloning studies (reviewed by Caulfield and Birdsall, 1998). Though some subtypes are predominant in certain peripheral tissues, it has been shown that mAChRs display an overlapping expression pattern in tissues based on studies with pharmacological labeling, mRNA detection and immunological approaches, (for reviews, see Hulme *et al.*, 1990; Caulfield, 1993; Eglen *et al.*, 1996).

mAChR pharmacology. The endogenous neurotransmitter, acetylcholine, acts on both nicotinic and muscarinic receptors and is rapidly hydrolyzed by acetylcholinesterase and plasma butyrylcholinesterase. Thus, it has little clinical application. In contrast, enormous efforts have been made to obtain its derivatives from both natural and

chemically synthetical resources, but unfortunately no highly subtype-selective reagent has been found (Eglen and Watson, 1996). Several agonists have relative selectivity based on their functional potencies. For example, (4-Hydroxy-2-butynyl)-1-trimethylammonium-*m*-chlorocarbanilate chloride (McN-A-343) is relatively selective for M1 receptors, but its functional selectivity was probably due to the receptor reserve in the tissue tested (Caulfield, 1993; Eglen and Watson, 1996). Some non-selective agonists such as acetylcholine, carbachol, oxotremorine, oxotremorine M acetate (Oxo-M) are extensively used to characterize the ligand binding properties and functional responses of mAChRs. Compared with mAChR agonists, mAChR antagonists draw much attention because of their usefulness in both receptor pharmacological studies and clinical therapeutics. For examples, non-selective antagonist [³H]N-methylscopolamine (NMS) and [³H]quinuclidinyl benzilate (QNB) are generally used to label mAChR sites either in tissues or in expressed cell lines; atropine (non-selective), pirenzepine (M1 selective), AF-DX 116 (M2 selective), methoctramine (M2 and M4 selective), 4-diphenylacetoxy-N-methylpiperidine methiodide (4-DAMP, M1 and M3 selective), *para*-fluorohexahydrosiladifenidol (*p*-F-HHSiD, M3 selective, himbacine (M2 and M4 selective) and tripitramine (M2 selective) are for characterizing pharmacological and functional properties of mAChR subtypes (Hulme *et al.*, 1990; Eglen *et al.*, 1996).

As extensively reviewed in Goodman&Gilman's "The Pharmacological Basis of Therapeutics" (Hardman *et al.*, 2001), mAChR agonists are typically classified into acetylcholine congeners and natural alkaloids. Due to their non-selective effects on mAChR subtypes, they may cause wide side effects when administrated systemically.

Some agonists are clinically applied to treat smooth muscle disorders in gastrointestinal and lower urinary tract (bethanechol), xerostomia (pilocarpine) and ophthalmological disease such as glaucoma (pilocarpine). mAChR antagonists include the natural alkaloids (atropine, scopolamine) and their derivatives and synthetic congeners. In addition to applications in ophthalmological, gastrointestinal and urinary tract disorders, they may also be used in respiratory disorder (atropine), anesthesia (atropine), cardiovascular and central nervous system, and anticholinesterase and mushroom poisoning.

mAChR-G protein coupling. The mAChR system is a good model for studying the molecular basis of R-G coupling. All five mAChR subtypes mediate their effects via coupling to heterotrimeric G proteins (Hulme *et al.*, 1990). Like other GPCR members, mAChRs share a common three-dimensional topology consisting of seven transmembrane (TM) helices linked by three alternative intracellular (i1-i3) and extracellular (o2-o4) loops (Figure 3) (Wess, 1996). Five mAChRs share high sequence homology (50%) and the sequence homology is even higher (60%) within the odd-numbered mAChRs (M1, M3 and M5) and the even-numbered mAChRs (M2 and M4) subgroups (Hulme *et al.*, 1990; Wess, 1996). Interestingly, not only do the mAChRs in the same subgroup share higher sequence homology but also prefer to couple to the same G protein family members. M1, M3 and M5 mAChRs preferentially couple to the Gq/11 family and primarily modulate PLC- β activity, whereas M2 and M4 mAChRs couple to the Gi/o family members and primarily inhibit adenylyl cyclase activity and activate the inward rectifier K⁺ channel (Wess, 1996; Caulfield and Birdsall, 1998). Therefore, the molecular basis determining the selective G protein coupling between two subgroups and

the possible subtle difference among the same subgroup members have made mAChRs a focus in R-G protein coupling studies. Despite of intensive investigation, the molecular mechanism of their selective G protein coupling is still not completely explained to date.

Structural basis for the selective mAChR-G protein coupling. The G protein-coupling selectivity of mAChRs has been studied using a variety of mutagenesis methods including chimeric receptors, deletions, insertions and site-directed mutations. Previous results suggest that the second and third intracellular segments (i2 and i3) define the G protein-coupling selectivity for mAChRs, especially the amino and carboxyl portions of i3 (hereafter referred to as Ni3 and Ci3, respectively) (Kubo *et al.*, 1988; Shapiro and Nathanson, 1989; Wong *et al.*, 1990; Arden *et al.*, 1992; Kunkel and Peralta, 1993; Blin *et al.*, 1995). By mapping these regions, several amino acid residues conserved in either the M1- or M2-like subgroups are predicted to determine their G protein coupling preferences. For example, within the M1-like subgroup a conserved tyrosine residue in the Ni3 (corresponding to Tyr²⁵⁴ in M3 sequence), a conserved “AALS motif” in the Ci3 (corresponding to Ala⁴⁸⁸, Ala⁴⁸⁹, Leu⁴⁹², and Ser⁴⁹³ in M3 sequence) and four conserved amino acid residues in the i2 loop (corresponding to Ser¹⁶⁸, Arg¹⁷¹, Arg¹⁷⁶, and Arg¹⁸³ in M3 sequence) are critical for their Gq coupling. Similarly, a conserved “VTIL motif” in the Ci3 of the M2-like subgroup (corresponding to Val³⁸⁵, Thr³⁸⁶, Ile³⁸⁹, and Leu³⁹⁰ in M2 sequence) is critical for their Gi/o coupling (Bluml *et al.*, 1994a; Bluml *et al.*, 1994b; Blin *et al.*, 1995; Liu *et al.*, 1995; Burstein *et al.*, 1995). Based on these studies Wess proposed a model that the key residues in the i2, Ni3 and Ci3 regions of mAChRs are predicated to cluster together to form a well-defined G protein binding site, thus

determining their G protein coupling selectivity (Wess, 1998; Kostenis *et al.*, 1999). Based on studies with M2/M5 mutants, Burstein *et al.* proposed a similar model in which the several key residues located in the Ni3 and Ci3 form a G protein coupling pocket composed of a positively charged “lip” and a hydrophobic core (Burstein *et al.*, 1998). Taken together, these data strongly support the idea that multiple intracellular domains of GPCRs act in a cooperative way to contribute to their selective G protein coupling (Wess, 1998).

Problems and remaining questions. A major drawback in previous studies is that effector (E) activity (*i.e.* the generation of second messengers or electrophysiological changes) or even further downstream cellular responses were used as a reporting system to interpret functional R-G interactions. As our knowledge of G protein-mediated signaling pathways has grown, it becomes clear that this linear pattern of signal transduction along a specific “R-G-E” axis is no longer tenable. In contrast, our current view of G protein-mediated signaling pathways suggests a complicated network (Neves *et al.*, 2002). Signals initiated upon ligand binding to GPCRs may converge and diverge at multiple interfaces and effector activities and cellular responses are regulated by many factors at multiple stages (see discussion below). Therefore, effector activity or cellular response may not be the best tool for direct evaluation of the interactions at the R-G interface, especially not for identifying the R-G contact sites.

Reconstitution approaches provide marked advantages in analyzing direct R-G protein coupling. In reconstitution systems, purified or membrane-bound GPCRs are reconstituted with purified G protein subunits with well-controlled concentrations and

stoichiometries (Butkerait *et al.*, 1995; Clawges *et al.*, 1997; McIntire *et al.*, 2002; Windh and Manning, 2002). Thus the R-G coupling step can be separated from other components of the whole signaling pathway in reconstitution. Typically, their functional coupling is analyzed by measuring the enhanced radiolabeled-agonist binding upon the formation of the agonist-R-G ternary complex or the radiolabeled-GTP γ S binding on the α subunits driven by the agonist-occupied receptor (Clawges *et al.*, 1997; Barr *et al.*, 1997; Windh and Manning, 2002). The major advantage of such a reconstitution system is that it provides a direct assay for the functional R-G interaction and the stoichiometry of R-G interaction in a precisely controlled manner. Of note is that other factors that may contribute to selective R-G interaction are eliminated in this artificial assay system (Neubig, 1994; Bohm *et al.*, 1997).

At the molecular level it is still not clear how individual mAChR subtypes with the same G protein coupling preference couple to G proteins and if there is any subtle difference in their G protein coupling properties. For quite a long time mAChRs have been known to mediate the metabotropic effects of the first identified neurotransmitter, acetylcholine. However, the lack of highly subtype-selective agents makes it difficult to define the exact physiological function of individual mAChR subtypes, especially subtypes within the same subgroup and with similar signaling transduction mechanisms. A major problem in developing such selective agents may be the fact that residues predicted to form the ligand-binding cavity are identical among the five subtypes (Wess, 1996). Recent efforts with gene “knock-out” techniques have been made to delineate the physiological roles of individual mAChRs (Hamilton *et al.*, 1997; Gomeza *et al.*, 1999a;

Gomez *et al.*, 1999b; Matsui *et al.*, 2000; Yamada *et al.*, 2001a; Yamada *et al.*, 2001b). These studies provide a wealth of useful information in understanding the physiological roles of a specific mAChR subtype. For example, M1 receptor gene disruption reveals that the non-selective agonist, pilocarpine-induced chronic seizure is specifically mediated by the M1 subtype (Hamilton *et al.*, 1997). In addition to its regulatory roles in cardiac functions that has been well characterized by pharmacological studies, the “knock-out” studies reveal that M2 receptors may also be involved in some muscarinic-dependent central nervous effects such as movement, temperature control and antinociceptive responses (Gomez *et al.*, 1999a). However, some conflicting results were also reported from these gene “knock-out” studies. For example, using the same strain of the M3 mAChR knock-out mice, Matsui *et al* reported that salivation was impaired in these mice while Yamada *et al* observed unchanged salivation (Matsui *et al.*, 2000; Yamada *et al.*, 2001a). In addition to the species variance of a given receptor, the overlapping expression of mAChRs in many tissues and the possible compensatory effects of other receptors (Hulme *et al.*, 1990) should be carefully considered to interpret the results observed in transgenic animals (Caulfield, 1993). Previous studies (Surya *et al.*, 1998; Strange, 1999; Slessareva *et al.*, 2002) indicate that even structurally similar GPCRs may differ in their ability to recognize the same ligand and may display differential agonist binding states stabilized by different G proteins. Thus, in the present study the G protein coupling properties of five closely related mAChRs were analyzed and compared in a reconstitution system with radioligand binding assays.

Regulation of G protein signaling:

Complexity in the regulation of G protein signaling. As discussed above there is a great deal of complexity existing in the GPCR-G protein coupling process. Meanwhile, recent reports on both G protein-independent GPCR signaling pathways (reviewed by Brzostowski and Kimmel, 2001) and GPCR-independent G protein activation (Cismowski *et al.*, 1999; Takesono *et al.*, 1999) make this signal transduction system even more complicated. Thus, the important question prompted is how extracellular stimuli are transduced and integrated *via* the G protein signaling network to initiate appropriate responses. To date many additional regulatory mechanisms and accessory cellular components have been appreciated, which can augment or attenuate signaling strength, optimize signal specificity and integrate cellular responses. The cell-specific distribution and the segregation of specific signaling molecules in intact cells clearly contributes to the specificity observed *in vivo* (Raymond *et al.*, 1993; Neubig, 1994; Li *et al.*, 1995). With regards to GPCRs, continuous stimulation by agonists leads to phosphorylation of most GPCRs by either GPCR kinases (GRKs), which mediate agonist-dependent desensitization, or by second messenger-dependent kinases (PKC and PKA), which mediate agonist-independent desensitization (Bohm *et al.*, 1997; Pitcher *et al.*, 1998; Krupnick and Benovic, 1998). Another class of proteins known as arrestins, are required for GRK-mediated GPCR desensitization and subsequent GPCR internalization (Krupnick and Benovic, 1998). Internalization and downregulation of cell surface GPCR numbers (Bohm *et al.*, 1997) both contribute to the attenuation of GPCR-

initiated signaling. At the level of heterotrimeric G proteins, signal termination was thought to depend solely on the intrinsic GTPase activity of G α subunits. This picture was changed by the identification of a novel class of proteins known as regulators of G protein signaling (RGS), most of which act as GAPs (GTPase activating proteins) to accelerate the GTP hydrolysis (Berman and Gilman, 1998; Hepler, 1999; De Vries *et al.*, 2000a). In contrast with GAPs, other proteins function as GEFs (guanine nucleotide exchanging factors) to stimulate the guanine nucleotide exchange on G α subunits (Luo and Denker, 1999; Cismowski *et al.*, 2000). Accessory regulation also exists for G $\beta\gamma$ subunits, which are regulated by proteins called phosducins (Bauer and Lohse, 1998; Bauer *et al.*, 1998). Recently, a new family of proteins, activators of G protein signaling (AGS) was discovered, which activate G $\beta\gamma$ -mediated signaling in yeast independently of GPCRs (Cismowski *et al.*, 1999; Takesono *et al.*, 1999). Taken together, current information indicates that the signals transmitted *via* GPCR-G protein pathway are regulated at multiple levels by multiple accessory proteins.

Activators of G protein signaling (AGS). Using a functional screen system based on the G $\beta\gamma$ -mediated pheromone response pathway in yeast, Cismowski, Takesono and their colleagues identified a series of proteins, which can activate this pathway in a receptor-deficient genetic background (Cismowski *et al.*, 1999; Takesono *et al.*, 1999). Collectively, these proteins are termed activators of G protein signaling (AGS) for their G protein activation properties, but sequence analysis indicated that these proteins did not share any sequence homology, suggesting that they might have different mechanisms for G protein activation. The first member of this family, AGS1 is found to be a Ras-related

protein, which acts as a GEF for Gi proteins and may antagonize GPCR-initiated signaling (Cismowski *et al.*, 1999; Graham *et al.*, 2002; Takesono *et al.*, 2002). AGS2 selectively interacts with G $\beta\gamma$ but it is not clear whether it actively promotes the subunit dissociation or just simply competes with G α for G $\beta\gamma$ interaction (Takesono *et al.*, 1999). The third member, AGS3 is found in various tissues and has tissue-specific splicing variants (De Vries *et al.*, 2000b; Bernard *et al.*, 2001; Pizzinat *et al.*, 2001). Sequence analysis of AGS3 reveals that it has two distinct domains connected by a linker region (Takesono *et al.*, 1999). Its amino terminal half consists of seven tetratricopeptide repeat (TPR) sequences while the carboxyl half contains four repeated sequences termed as G protein regulatory (GPR) motifs or GoLoco motifs (Siderovski *et al.*, 1999). The TPR sequences have been implicated in its subcellular localization while the GPR sequences are responsible for competing with G $\beta\gamma$ subunit to selectively interact with Gi/o α subunits (Siderovski *et al.*, 1999; Takesono *et al.*, 1999; Bernard *et al.*, 2001; Pizzinat *et al.*, 2001). As reviewed by Kimple *et al.*, the GPR sequence is conserved in a variety of proteins from different species with diverse functions but all of them share the ability to selectively interact with the GDP-bound conformation of the α subunits from the Gi family *via* their GPR sequence(s) (Kimple *et al.*, 2002). Biochemical studies from several groups have demonstrated that the GPR sequence functions as a guanine nucleotide dissociation inhibitor (GDI) to stabilize Gi α subunits in their GDP-bound form (Natochin *et al.*, 2000; Peterson *et al.*, 2000; De Vries *et al.*, 2000b; Bernard *et al.*, 2001; Kimple *et al.*, 2001; Natochin *et al.*, 2001; Pizzinat *et al.*, 2001; Peterson *et al.*, 2002). Recent studies indicate that AGS3-related GPR-containing proteins are involved in cell polarity

and asymmetric cell divisions (Schaefer *et al.*, 2000; Bellaiche *et al.*, 2001; Schaefer *et al.*, 2001; Blumer *et al.*, 2002). However, little is known about the molecular mechanisms of AGS3 in cellular signaling.

Dissertation objectives:

Clearly, many aspects of G protein mediated signal transduction pathways need closer examination. My thesis work addressed two questions in GPCR-G protein coupling mechanisms: 1) Do highly homologous GPCRs couple to their G proteins differently? and 2) how may GPCR-G protein coupling be impacted by a newly identified G protein signaling regulator, AGS3. The *hypotheses* tested were: 1) at molecular level, the closely related mAChRs (Gq/11-coupled mAChRs or Gi/o-coupled mAChRs) couple to their G proteins differently, 2) AGS3 can selectively interfere with Gi/o protein coupling to receptors.

These hypotheses were tested by achieving the following specific aims:

Specific aim 1: To assess and compare the apparent affinities of Gq and Gi proteins for five mAChRs in Sf9 cell membrane-based reconstitution system.

Specific aim 2: To assess and compare the G protein-stabilized high-affinity agonist binding states of five mAChRs.

Specific aim 3: To study the effects of AGS3 on receptor-Gi protein coupling and its potential mechanisms in cellular signaling.

Specific aim 4: To study the selectivity of AGS3-GPR domains for Gi/o proteins.

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CHAPTER 2: METHODS

Materials:

[³H]-Hydroxytryptamine (³H-5-HT, 25.5 Ci/mmol) Binoxalate or Creatinine Sulfate, [³H]-Oxotremorine-M Acetate (³H-Oxo-M, 85.8 Ci/mmol) and [³H]-N-Methyl Scopolamine Chloride (³H-NMS, 70.0 Ci/mmol) were purchased from NEN Life Science Products, *Inc.* (Boston, MA). 3-[(3-cholamidopropyl)dimethylammonil]-1-propanesulfonic acid (CHAPS) was from Calbiochem (San Diego, CA). Atropine Sulfate, N-Methyl Scopolamine Chloride, Oxotremorine-M Acetate (Oxo-M), 5-Hydroxytryptamine (5-HT) and other chemicals were from Sigma-Aldrich *Corp.* (St. Louis, MO). Grace's insect cell culture media were purchased as powder from GibcoBRL Life Technologies (Rockville, MD). Restriction endonucleases, T4 polynucleotide kinase, T4 DNA ligase, *Taq* DNA polymerase, *Pfu* DNA polymerase and M-MLV reverse transcriptase were from New England Biolabs (Beverly, MA), Epicentre Technologies (Madison, WI), USB *Corp.* (Cleveland, OH), PGC Scientifics *Corp.* (Gaithersburg, MD), Stratagene (La Jolla, CA) and GibcoBRL Life Technologies, respectively. Human Gα_q cDNA was obtained from Guthrie cDNA resource center (Sayre, PA). DNA primers were ordered from IDT, *Inc* (Coralville, IA) and DNA/RNA hybrid primers were from Oligos Etc, *Inc* (Wilsonville, OR). QIAprep Spin Miniprep and QIAprep Midiprep kits, Bac-N-Blue Transfection Kit, ProtoBlot AP immunoblotting reagents and BCA Protein Assay Kit were from QIAGEN *Inc.* (Valencia, CA), Invitrogen (Carlsbad, CA), Promega (Madison, WI) and Pierce

(Rockford, IL), respectively. Ni-NTA superflow resin was from QIAGEN *Inc.* Anti-G α i1/2 C-terminal antibody, anti-G α q C-terminal antibody and anti-G β antibody were from Calbiochem-Novabiochem *Corp.* (San Diego, CA), Santa Cruz Biotechnology (Santa Cruz, CA) and NEN Life Science Products (Boston, MA), respectively. Whatman GF/C filters were from Brandel *Inc.* (Gaithersburg, MD) and PVDF membranes were from Millipore *Corp.* (Bedford, MA).

Sf9 cell cultures:

Spodoptera frugiperda (Sf9) insect cells are grown either as monolayer cultures in T25 flasks or plates, or as suspension cultures in spinner flasks. Stock cells are kept as suspension cultures at 1.25×10^6 cells/ml, agitated at 55-60 rpm for small scale cultures (< 500 ml) or 50 rpm for large cultures (> 500 ml) and subcultured every third day. Only cells with at least 95% viability are used for transfection or baculovirus infection. 2×10^6 cells are seeded per T25 culture flask as monolayer cultures for transfection; 1×10^6 cells per 60 mm plate as monolayer cultures for plaque assay; and 3×10^6 cells/ml as suspension cultures for infection. Sf9 cells are grown in FMNH media (Grace's insect cell culture media supplemented with 0.35 g/L NaHCO₃, 3.33 g/L lactalbumin and 3.33 g/L TC-yeastolate, pH6.05) with 10% fetal bovine serum and incubated at 27°C in the incubator with a gas mixture (50% O₂:50% air).

Human mAChRs subcloning and recombinant baculovirus production:

Human M1 and M2 mAChR recombinant baculoviruses were kind gifts from Dr. Elliot Ross (University of Texas Southwestern Medical Center). Human M3 mAChR recombinant baculovirus was kindly provided by Dr. Karl Akerman (Åbo Akademi University, Finland). The production of these viruses was described previously (Parker *et al.*, 1991; Kukkonen *et al.*, 1996).

To produce human M4 mAChR recombinant baculoviruses, the pCD plasmid containing its coding cDNA (Bonner *et al.*, 1987) was first linearized at an upstream Tfi I site and then followed by Mung Bean Nuclease incubation to create blunt ends. The cDNA containing the coding region was excised as a 1.7-kb fragment from the linearized plasmid with Bgl II at its 3'-end and ligated into pVL1393 transfer vector at Sma I and Bgl II sites. To produce human M5 mAChR recombinant baculoviruses, M5 receptor cDNA was excised from its pCD plasmid (Bonner *et al.*, 1988) as a 1.8-kb fragment with Pst I alone and ligated into the Pst I site at the multiple cloning site region of pVL1392 transfer vector. Both constructs were confirmed by restriction analysis and DNA sequencing (All the sequencing confirmations were performed by the Molecular Genetics Instrumentation Facility at University of Georgia). Using Bac-N-Blue Transfection Kit (Invitrogen, CA) each transfer vector was used to co-transfect Sf9 cells with the linearized wild-type AcMNPV DNA to produce recombinant baculoviruses and recombinant viruses were purified following manufacturer's manual.

Preparation of Sf9 cell membranes containing expressed receptors.

As previously described (Clawges *et al.*, 1997), Sf9 cells are infected with recombinant baculoviruses expressing the desired receptor usually at an MOI of 2 for 1 hour. Infected cells are seeded in spinner flasks at 3×10^6 cells/ml, cultured for 40-70 hours with the cell density and viability monitored by microscopy and trypan blue dye and harvested depending on their appearance and density. For harvest, cells are pelleted at 50xg for 5 minutes and washed three times by resuspending and pelleting in ice-cold PBS buffer (47 mM NaCl, 7.3 mM NaH₂PO₄, 2.0 mM CaCl₂, 60 mM KCl, pH 6.2). The final cell pellets are resuspended at 0 - 2.5 g wet weight per 5 ml homogenization buffer (10 mM Tris-Cl, 25 mM NaCl, 10 mM MgCl₂, 1 mM EGTA, pH 8.0 at 4 °C) supplemented at use with 0.1 mM PMSF, 20 µg/ml of benzamidine and 2 µg/ml of each of aprotinin, leupeptin and pepstatin A, snap-frozen in liquid N₂ and stored at -70 °C.

To prepare membranes, harvested cells are thawed in 15x their wet weight of ice cold homogenization buffer with the same protease inhibitors as described above and burst by N₂ cavitation at 600 p.s.i. for 20 minutes. Cavitated cells are centrifuged at 500xg, 4 °C for 10 minutes to remove the unbroken nuclei and cell debris. The supernatant from the low speed spin is centrifuged at 28,000 g, 4 °C for 30 minutes. The second supernatant is discarded and the pellets are resuspended and pooled in 35 ml of HE buffer (5 mM NaHEPES, 1 mM EDTA, pH 7.5) containing the same protease inhibitors as used in the homogenization buffer. The membranes are washed twice, resuspended in the HE buffer without the protease inhibitors at a concentration of 1-3 mg protein/ml, aliquoted, snap-frozen in liquid N₂ and stored at -70 °C.

Expression and purification of G proteins:

All recombinant G protein subunits are purified using Waters 650E Advanced Protein Purification System (Millipore Corp, Milford, MA) at 4°C after expression in Sf9 cells with recombinant baculoviruses. For soluble G protein subunits expression and purification such as G α 1 or G β 1 γ 2, Sf9 cells are infected with recombinant baculoviruses at an MOI of 2 for G α or an MOI of 1 β :1 γ for G β 1 γ 2 and purified as described previously (Graber *et al.*, 1992a; Graber *et al.*, 1992b; Graber *et al.*, 1994). For insoluble G α subunits such as Gq α , Sf9 cells are co-infected with the recombinant baculoviruses to express G α , G β and His-tagged G γ subunits at an infection MOI of 1 α :1 β :1 γ and purified as heterotrimers (Kozasa and Gilman, 1995). Briefly, infected cells are cultured and harvested as described above for receptor expression except that 10 μ M GDP was added to the final homogenization buffer. For purification, harvested cell pellets are thawed in 15x their wet weight of ice-cold homogenization buffer, burst by nitrogen cavitation at 600 p.s.i. for 20 minutes and centrifuged at 100,000xg, 4°C for 60 minutes. The 100,000xg-pellets are first extracted for 1 hour in an extract buffer (50 mM Tris/HCl, pH 8.0 with 10 μ M GDP, 2 mg/ml aprotinin, 20 mg/ml benzamidine, 2 mg/ml leupeptin, 2 mg/ml pepstatin, 0.1 mM PMSF, 5 mM BME and 1% CHAPS) and then centrifuged at 100,000xg, 4°C for 45 minutes. The supernatant containing the solubilized G protein heterotrimers is filtered and loaded into a 30 ml AP-2 DEAE anion exchange column, which is pre-washed with a DEAE column buffer (50 mM Tris, pH 8.0 with 0.7% CHAPS, 5 mM BME and 10 μ M GDP). Proteins are eluted with 4 column volumes

of 0 – 400 mM NaCl linear gradient. Fractions containing G proteins (as indicated by anti-G α immunoblotting in pilot experiments) are pooled, mixed with 2 ml Ni-NTA Superflow resin (used as 4 ml of 50% slurry) and incubated for at least 1 hour before loaded into a 15 ml column. The resin then is washed first with a buffer A (20 mM HEPES, 1 mM MgCl₂, 100 mM NaCl, pH 8.0 with 5 mM Imidazole, 5mM BME, 10 μ M GDP and 0.7% CHAPS), then with a buffer B (buffer A plus 300 mM NaCl), and again with the buffer A. Each washing step is completed when baseline is flat for at least 10 ml. The proteins are eluted with a buffer C (20 mM Hepes, pH 8.0 with 150 mM Imidazole, 1mM MgCl₂, 25 mM NaCl, 5 mM BME and 0.7 % CHAPS). The elute is diluted 3 times with a Q buffer (20 mM HEPES, 2 mM MgCl₂, pH 8.0 with 1mM DTT and 0.7% CHAPS) and loaded into an 8 ml AP-1 QHR15 anion exchange column. Fractions are eluted with 0 – 400 mM linear NaCl gradient. Free G $\beta_1\gamma_{2HIS}$ subunits and G $\alpha_q\beta_1\gamma_{2HIS}$ heterotrimers are eluted within 100 – 125 mM NaCl and 200 - 220 mM NaCl gradients, respectively. The corresponding fractions are pooled, concentrated using Centricon-30 concentrators (Amicon), snap-frozen in liquid N₂ and stored at -70°C. To verify the protein purity, samples from the fractions are resolved by SDS-PAGE, transferred to PDVF membranes and immunoblotted using their corresponding antibodies.

Characterization of expressed receptors with radioligand saturation binding assay:

The mAChR receptor expression levels are characterized by ³H-NMS saturation binding assays. About 5 to 20 μ g membrane protein aliquots are incubated at 25 °C with ³H-NMS in a concentration range of 0.02 to 10 nM in a binding buffer (50mM Tris-HCl,

5mM MgCl₂, 0.5mM EDTA, pH 7.5) for 1.5 hours to achieve equilibrium in a temperature-controlled shaker. The incubations are terminated by filtration over Whatman GF/C filters using a Brandel Cell Harvester. The filters are rinsed thrice with 4 ml ice cold washing buffer (50 mM Tris-Cl, 5 mM MgCl₂, 0.5 mM EDTA, 0.01% NaAzide, pH 7.5 at 4 °C), placed in 4.5 ml CytoScint (ICN Pharmaceuticals, Costa Mesa, CA) and counted to constant error in a scintillation counter. The binding data are fit to a one-site binding model and compared with a two-site binding model using GraphPad Prism software (GraphPad software, CA). Without reconstitution with G proteins, one-site binding is the best-fit model in every assay performed for mAChRs as analyzed by *F*-test. The receptor expression level (Bmax) and the affinity for the radioligand (Kd) are computed with the software and expressed as pmol/mg membrane protein and nM, respectively (Figure 5A). Since there is no radiolabeled antagonist available for the 5-HT receptors, their expression levels are characterized with radiolabeled agonist saturation binding assays. The assays are performed in a similar way as above except that the receptor membranes are first reconstituted with a saturating amount of Gi1 protein in a reconstitution buffer (5 mM NaHEPES, 100 mM NaCl, 5 mM MgCl₂, 1 mM EDTA, 500 nM GDP, 0.04% CHAPS, pH 7.5) prior to the binding (Figure 5B).

Urea-extraction of Sf9 cell membranes expressing mAChRs:

When mAChRs are expressed in Sf9 cell membranes, endogenous G proteins (probably also other peripheral proteins) may hinder the mAChR coupling to the exogenous G proteins added in reconstitution. As shown in Figure 6, on untreated M5

Figure 5. Representative experiment of characterization of receptor expression level with radioligand saturation binding assay. *A.* Sf9 cell membranes (~ 22 µg) expressing M1 receptors were incubated with ³H-NMS in a range of 0.02 – 10 nM at 25 °C for 1.5hr. *B.* Sf9 cell membranes (~10 µg) expressing 5-HT_{1A} receptors were first reconstituted with ~ 20 pmol Gi1 heterotrimer and incubated at 25 °C for 15 minutes. Binding on reconstituted membranes were determined after incubating the membranes with ³H-5-HT in a range of 0.01 – 25 nM at 25 °C for 1.5hr. Data points represent the mean ± SD of triplicate determinations from a representative experiment and the Bmax and Kd values were generated from the best-fitted one- or two-site model determined by *F*-test using GraphPad Prism software.

Figure 5.

Figure 6.

Figure 6. Effects of urea-extraction treatment on M5 mAChR coupling. Sf9 cell membranes (~ 20 µg) before or after treated with 200 nM unlabeled Oxo-M, 25 µM GTPγS and 6 M urea were reconstituted with (solid bars) or without (open and stripped bars) 300 nM Gq proteins at 25°C for 15 minutes. About 6 nM ³H-Oxo-M was incubated with reconstituted membranes in the presence (stripped bars) or absence (open and solid bars) of 50 µM GTPγS. Binding data were normalized as percent of binding on the control membranes (open bars). Each bar represents mean ± SEM from 3 to 6 experiments. *, p<0.01, Tukey's test.

receptor membrane, addition of GTP γ S (striped bar in “Before treatment” group in Fig 6) significantly reduced 3 H-Oxo-M binding as compared to the control (open bar in “Before treatment” group in Fig 6), reflecting the coupling of expressed M5 receptors to the endogenous G proteins in Sf9 cell membranes. Reconstitution with G proteins (solid bar in “Before treatment” group in Fig 6) did not enhance agonist binding, indicating that the exogenous G proteins were unable to stabilize high-affinity agonist binding on M5 receptors in untreated membranes.

To establish the functional coupling between the purified, exogenous G proteins and the mAChRs expressed in Sf9 cell membranes, we adapted a urea-extraction protocol based on the previously published urea-extraction protocols from other groups (Hartman, IV and Northup, 1996; Hellmich *et al.*, 1997; Lindorfer *et al.*, 1998). Briefly, before extraction with a high concentration of urea (6 M) Sf9 cell membranes are pre-treated with a high concentration of agonist (200 nM Oxo-M) and GTP γ S (25 μ M), which are expected to uncouple the endogenous G proteins from the expressed mAChRs. Frozen membranes are thawed on ice, pelleted at 18,000xg at 4 °C for 10 minutes. The membrane pellets are resuspended at a concentration of about 0.5 mg/ml in the binding buffer (50 mM Tris-Cl, 5 mM MgCl₂, 0.5 mM EDTA, pH 7.5) and incubated with 200 nM Oxo-M and 25 μ M GTP γ S in a shaking incubator at 25 °C for 1 hour. After incubation, membranes are pelleted again at 288,000xg, 4 °C for 30 minutes. The resulting pellets are resuspended in a urea-extraction buffer (25 mM HEPES, 1 mM EDTA, pH 7.5 with 6M urea prepared fresh before use) and incubated on ice for 30 minutes. The extracted membranes are washed twice by resuspending in the HE buffer

and centrifuging 30 minutes at 288,000xg, 4 °C. The final pellets are resuspended in the HE at a concentration of 1-3 mg protein/ml, aliquoted, snap-frozen in liquid N₂ and stored at -70 °C. Urea-extracted membranes are also characterized by ³H-NMS saturation binding assay as described above.

Hartman *et al* reported that GTPγS binding was reduced 4 fold on the 5-HT_{2C} receptor membranes after urea-extraction, reflecting the decrease or inactivation of the endogenous G proteins (Hartman, IV and Northup, 1996). Consistent with their GTPγS binding results, our results show that the uncoupling effect of GTγS on the treated membrane was significantly reduced, indicating the decreased coupling between the endogenous G proteins and the M5 receptors in the membrane (compare open and striped bars in “After treatment” group in Fig 6). After treatment, the expressed M5 receptors can be stabilized in the high-affinity agonist binding state by reconstitution with exogenous Gq proteins as reflected by the enhanced agonist binding (solid bar in “After treatment” group in Fig 6). Thus, treating mAChR membranes with high concentrations of unlabeled agonist, GTPγS and urea can clearly reduce endogenous G protein coupling and improve exogenous G protein-stabilized high-affinity agonist binding, which will be a useful tool in establishing the functional GPCR-G protein coupling *in vitro*.

Reconstitution of receptors with exogenous G-proteins or AGS3 protein:

Reconstitution is performed as previously described (Clawges *et al.*, 1997). Briefly, frozen membranes are thawed, pelleted in a refrigerated microcentrifuge (10 minutes, 12,000 rpm) and resuspended at about 5-10 mg/ml in the reconstitution buffer

plus 0.04% or 0.08% CHAPS for 5-HT receptor or mAChR membranes, respectively. G protein subunits are diluted in the same buffer such that the desired amount of subunit is contained in 1-5 μ l. Aliquots of membrane and G proteins are mixed in the presence or absence of indicated additional components. The mixtures are incubated at 25 °C for 15 minutes and then held on ice until the start of the binding assay or pelleted in a refrigerated microcentrifuge at 12,000 rpm, 4 °C for 10 minutes for immunoblotting assay.

To allow soluble, cytosolic AGS3 or exogenous G protein heterotrimers to associate with membranes, membranes containing 5-HT_{1A} receptors are first incubated (25 °C, 15 minutes) with the Sf9 cytosol fractions containing AGS3 or purified G protein heterotrimers, respectively. The membranes are then pelleted in a refrigerated microcentrifuge at 12,000 rpm, 4 °C for 10 minutes and washed thrice with 100 μ l reconstitution buffer. The resulting membranes are then used in a second reconstitution with the indicated additional components as described above.

Radiolabeled agonist binding assay:

To assess functional receptor-G protein coupling, the high-affinity agonist binding on membrane receptors, which is stabilized by the coupling G proteins, is measured with radiolabeled agonist. Based on the extended ternary complex model of GPCR-G protein coupling (Samama *et al.*, 1993), G proteins stabilize GPCRs in the high affinity agonist binding state upon the formation of agonist/receptor/G-protein ternary complex. Thus, using a single, low concentration of radiolabeled agonist near the high-affinity K_d for that

agonist, the G protein-stabilized high affinity agonist binding on membrane receptors when reconstituted with coupling G proteins can be readily detected as enhanced agonist binding compared with the agonist binding on receptors without G protein reconstitution (Clawges *et al.*, 1997; McIntire *et al.*, 2002; Windh and Manning, 2002).

Radiolabeled agonist binding is performed as described (Clawges *et al.*, 1997). Briefly, reconstitution mixtures are diluted 10-fold with the binding buffer prior to the start of the binding assay such that the desired amount of membranes (5 - 25 µg/assay tube) are contained in 50 - 100 µl. Aliquots of diluted membranes are incubated in the presence of a single concentration of radiolabeled agonist near the high affinity K_d of the receptor of interest as indicated in the text. Non-specific binding is determined by addition of 10³-fold or greater excess of unlabeled ligand. Incubation and measurement of radioligand binding are performed as described for ³H-NMS saturation binding assays above.

Analysis of G protein apparent affinities for membrane receptors with radiolabeled agonist binding assay:

Previous results from our laboratory show that four G_i coupled receptors have different apparent affinities for the same G_i protein (Ma *et al.*, 2000). It has not been shown if five mAChRs have different apparent affinities for their coupling G proteins. To assess the apparent affinity of a G protein for a specific receptor, increasing amounts of G protein heterotrimers (typically 10 to 500 nM) are reconstituted with receptor membranes. After reconstitution, high-affinity agonist binding on the reconstituted

membranes is determined with a single, low concentration of radiolabeled agonist as described above. Binding data are fit to a one-site interaction model between receptor and G protein and analyzed with GraphPad Prism software. EC₅₀ values of G protein concentration in stabilizing receptors in high-affinity agonist binding are computed from nonlinear regression analysis and represent the apparent affinity of G proteins for receptors (See Figure 7).

Competition binding assay:

Previous studies indicate that GPCRs may have multiple conformations depending on agonists and G protein coupling (see discussion in Chapter 1). To compare the possible differences in the proportions and the agonist affinities (low and high) of five mAChRs that can be stabilized by their coupling G proteins, competition binding assays are performed for each mAChR-G protein pair and compared with mAChR membranes without G protein reconstitution. Briefly, urea-extracted Sf9 membranes expressing mAChRs are first reconstituted with or without a saturating amount of G proteins. Reconstitution is performed as described above and is diluted 10-fold with the binding buffer prior to the start of binding assay. Aliquots of the diluted reconstitution mixture (100 µl) are incubated with a single concentration (~ 4nM) of radiolabeled non-selective antagonist (³H-NMS) and increasing concentrations of unlabeled agonist (Oxo-M) as a competitor with a range from 10⁻¹ M to 10⁻¹² M in a final volume of 300 µl. The Oxo-M concentrations with duplicate determinations at each point are equally spaced on a log scale. Incubation and measurement of ³H-NMS binding are performed as described

Figure 7.

Figure 7. Representative experiment of G protein concentration titration with radiolabeled agonist binding assay. Aliquots of about 40 μ g urea-extracted Sf9 cell membranes containing M4 mAChRs (0.4 pmol) were reconstituted with increasing amounts (5 – 250 pmol) of Gi1 protein in 25 μ l at 25 $^{\circ}$ C for 15 minutes. The reconstitution mixtures were diluted with binding buffer and then incubated with 6 nM 3 H-Oxo-M in a final volume of 150 μ l. The final concentration of M4 mAChR was 0.8 nM (12 μ g membrane protein) and the Gi1 concentrations were 10-500 nM. Data points were mean $\pm$ SD of triplicate determinations from a representative experiment repeated three times. Agonist binding were fit to a one-site interaction model between receptor and G protein and analyzed with GraphPad Prism software to calculate EC₅₀ values, which represent the apparent affinity between G protein and receptor.

above. Binding data are first fitted into one- and two-site competition models to determine the best-fit model with *F*-test. The dissociation constants (K_i) for Oxo-M and the fractions of receptors in the high-affinity state are then computed from the best-fit curve with the Cheng-Prusoff correction ($K_i = IC_{50}/(1+[radioligand]/K_d)$).

Immunoblot analysis:

To verify and quantitate expressed protein, protein is resolved by 12% SDS/PAGE and transferred to a PVDF membrane (Millipore). The membrane is first probed with appropriate primary antibody for the protein of interest and then visualized with ProtoBlot AP immunoblotting system (Promega). The amount of proteins is analyzed by comparing the band intensities within the linear range of a standard curve prepared from known amounts of purified protein as standards using a FluorChemTM 8000 system (Alpha Innotech Corp.) (see Figure 8 for example). To determine the amounts of protein that associated with membranes during reconstitution, the reconstitution mixtures are pelleted and washed three times as described above. The washed pellets or one-tenth volume of the first supernatant (for proteins in the membrane and cytosol fractions, respectively) are electrophoresed, transferred to PVDF membranes and probed with appropriate primary antibodies (see Figure 9 for example). The antibody detection, visualization and quantitation are performed with a ProtoBlot AP kit as described above.

Figure 8.

Figure 8. Quantitation of AGS3 expressed in Sf9 cell membranes. Purified GST-GPR proteins and the Sf9 cell membranes prepared from recombinant AGS3 baculovirus-infected cells were mixed with Laemmli buffer and boiled for 5 minutes prior to loading to 12% SDS-PAGE gels. After electrophoresis, proteins were transferred to PDVF membranes, probed and visualized as described in the text. Band densities were analyzed using a FluorChemTM 8000 system and the amounts of AGS3 proteins were determined from the linear range of a standard curve from the known amounts of purified GST-GPR. The amounts of GST-GPR in lane 1-6 were 5, 10, 2, 1, 0.5 and 0.1 pmol, respectively while the amounts of total Sf9 cell membrane proteins were 2.30, 1.15, 1.15 and 0.575 μ g in lane 6-10, respectively.

Figure 9.

Figure 9. Concentration- and Gβγ-dependent membrane association of Giα subunit. Aliquots of Sf9 cell membranes (~30 μg) were reconstituted with increasing amounts (0-250 pmol, lane 2-8) of Gi1α subunits in the presence (+Gβγ, upper panel) or absence (-Gβγ, lower panel) of equal molar Gβγ subunits at 25 °C for 15 minutes. After reconstitution, membranes were pelleted, washed and resuspended in Laemmli buffer. The Gi1α subunits associated with the membranes were resolved by 12% SDS-PAGE gels, transferred, probed and visualized as described in the text. Lane 1 was loaded with 1.0 pmol Gi1α as standard. Data show that Giα subunits associate with the membrane in a concentration-dependent pattern in reconstitution and that this association is greatly impaired in the absence of Gβγ subunits.

Protein assay:

Protein concentrations are assayed routinely by the BCA method with the BCA Protein Assay Kit from Pierce (Rockford, IL) using bovine serum albumin as standard. Protein samples are solubilized in 0.5 N NaOH:5% SDS and samples containing reducing reagents such as β -mercaptoethanol or Dithiothreitol in the buffer are first incubated with 0.2 M iodoacetamide at 60 °C for 15 minutes with vented plastic caps to eliminate their reducing effects that will interfere with the assay. Absorbance of samples at 562 nm is performed in a Spectronic Genesys 5 spectrophotometer (Milton Roy Com.) and protein concentration is determined by the linear regression analysis with GraphPad Prism software (GraphPad Software, CA).

Data analysis:

All data computation and statistical analyses are performed using GraphPad Prism software (GraphPad Software, CA). All experiments were repeated the indicated number of times and each data point within experiments are obtained from duplicate or triplicate samples. Data are represented as mean $\pm$ SD determined from single experiment or mean $\pm$ SEM from multiple experiments. Binding and dose-response curve are fitted using non-linear regression analysis and *F*-test. Student *t*-test, one-sample *t*-test, one-way or two-way ANOVA and appropriate post tests are used to compare differences as indicated in the text.

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CHAPTER 3. RESULTS

Section 1:

Human muscarinic acetylcholine receptors exhibit distinct G protein coupling behaviors in membranes from Sf9 cells

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Running title: Comparison of mAChR-G protein coupling

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Abbreviations:

5-HT, 5-hydroxytryptamine; **CHAPS**, 3-(3-cholamidopropyl)dimethylammonil)-1-propanesulfonic acid; **CHO** cells, Chinese hamster ovary cells; **G protein**, heterotrimeric guanine nucleotide binding protein; **GPCRs**, G protein coupled receptors; **GTP γ S**, guanosine 5'-3-*O*-(thiotriphosphate); **mAChR**, muscarinic acetylcholine receptor; **MOI**, multiplicity of infection; **NMS**, N-Methyl Scopolamine; **Oxo-M**, Oxotremorine-M; **Sf9**, *Spodoptera frugiperda*.

Abstract

The G protein coupling of five human muscarinic acetylcholine receptors (mAChRs) was studied in an Sf9 cell based reconstitution system, in which Sf9 cell membranes expressing individual mAChR subtypes were reconstituted with purified Gq and Gi1 proteins. Functional coupling was assessed by the enhancement of high-affinity agonist binding using radioligand binding assays. Urea-extraction of the Sf9 cell membranes containing the expressed mAChRs greatly improved their coupling with purified G proteins. Functional G protein coupling was established for M1, M2, M4 and M5 receptors in reconstitution. However, functional G protein coupling could not be established for M3 receptors. Similar apparent affinities were observed for Gq and Gi interactions with M1 and M5 receptors, which were significantly stronger than the interactions of Gi1 with M2 and M4 receptors. M2 and M4 receptors did not interact with Gq. The affinity for the muscarinic agonist Oxotremorine-M also varied among the receptors in both the coupled and uncoupled states. Thus, the five closely related mAChRs displayed different agonist binding and G protein coupling behaviors in an identical membrane environment.

Introduction

The metabotropic responses of acetylcholine are mediated by a family of muscarinic acetylcholine receptors (mAChRs). With molecular cloning studies, five mAChR subtypes have been identified (Kubo *et al.*, 1986; Bonner *et al.*, 1987; Peralta *et al.*, 1987; Bonner *et al.*, 1988). Though some subtypes are predominant in certain peripheral tissues, accumulating evidence suggests an overlapping distribution pattern of mAChRs in many tissues. For example, all five subtypes are detected in central nervous tissues (See Caulfield and Birdsall, 1998 for a recent review). Five mAChRs all belong to the G protein coupled receptor (GPCR) superfamily and elicit their cellular responses *via* coupling to heterotrimeric G proteins upon activation. Based on their G protein coupling profiles and signal transduction mechanisms, five mAChRs can be further classified into two subgroups. The odd-numbered subtypes (M1, M3 and M5) preferentially couple to phosphoinositide hydrolysis and calcium mobilization *via* Gq/11 proteins while the even-numbered subtypes (M2 and M4) typically couple to the inhibition of adenylyl cyclase and activation of potassium channel *via* Gi/o proteins (Caulfield and Birdsall, 1998).

Given that multiple mAChRs share the same G protein coupling preference and similar signal transduction mechanisms, it is of interest to understand the functional significance of these closely related subtypes. Intensive study in a variety of systems indicates that mAChRs may differentially couple to G proteins (Peralta *et al.*, 1988; Buck and Fraser, 1990; Wang and el Fakahany, 1993; Burford *et al.*, 1995a). A great number of studies attempting to define the specific roles of individual subtypes are compromised by the heterogeneity of mAChRs in tissues, variations in expression systems and the lack of

highly subtype-selective reagents (Caulfield, 1993; Eglen and Watson, 1996; Wess, 1998). At the biochemical level, elucidation of the detailed G protein coupling mechanisms of each mAChR by reconstitution of the individual components will help distinguish the functional significance of the individual subtypes.

The aim of the present work was to compare the G protein coupling properties of five closely related mAChRs using a membrane-based reconstitution approach. *In vivo* evidence indicates that GPCRs are outnumbered by G proteins (Neubig, 1994; Post *et al.*, 1995). Jakubik and colleagues studied the effects of different GPCR-G protein ratios on their coupling properties and suggested that a constant ratio between GPCRs and G proteins might retain in native cells (Jakubik *et al.*, 1998). Earlier work from our laboratory indicates that four closely related 5-HT₁ receptors can distinguish themselves by the affinities for their coupling G proteins (Clawges *et al.*, 1997). Clearly, GPCR-G protein stoichiometry may be a characteristic aspect of G protein mediated signal transduction. For mAChRs, it is not known if these aspects of G protein coupling vary among the individual subtypes.

The extended ternary complex model has been widely used to explain GPCR-G protein coupling (Samama *et al.*, 1993). In this model, GPCRs shift between two conformational states: an inactive, uncoupled state with low agonist binding affinity and an active, G-protein-coupling state with high agonist binding affinity. However, an increasing number of observations indicate that this model needs revision and that GPCRs may exhibit multiple conformation states depending on the identities of agonists and the coupling of G proteins (Strange, 1999). Akam *et al* have shown that the G

proteins activation by mAChRs not only depends on receptor subtype but also depends on the nature of agonists (Akam *et al.*, 2001). In this work, we investigated if a specific mAChR subtype could have different affinity states for the same agonist when interacting with different G proteins and if this feature is different among five mAChRs.

Our results show that five mAChRs displayed different G protein coupling behaviors when examined by the formation of the high affinity agonist binding state in radioligand binding assays. The Gi-coupled M2 and M4 mAChRs displayed similar apparent affinities for Gi1 heterotrimers and did not interact with Gq heterotrimers. The Gq-coupled M1 and M5 receptors had similar apparent affinities for both Gi1 and Gq heterotrimers. However, although the M3 receptor is coupled to Gq-mediated responses in tissues, it did not exhibit a high affinity agonist binding state with either Gi1 or Gq heterotrimers in the Sf9 cell membranes. Differences were also observed among the individual mAChR subtypes in their high and low affinity states for the agonist Oxotremorine-M. Thus, when placed in an identical membrane environment the individual mAChR subtypes exhibit distinct G protein coupling behaviors.

Materials and Methods

Materials. [^3H]-Oxotremorine-M Acetate (^3H -Oxo-M, 85.8 Ci/mmol) and [^3H]-N-Methyl Scopolamine Chloride (^3H -NMS, 70.0 Ci/mmol) were purchased from NEN Life Science Products, *Inc.* (Boston, MA). 3-[(3-cholamidopropyl)dimethylammonil]-1-propanesulfonic acid (CHAPS) was from Calbiochem (San Diego, CA). Atropine Sulfate, Oxotremorine-M Acetate (Oxo-M) and other chemicals were from Sigma-Aldrich *Corp.* (St. Louis, MO). Restriction endonucleases and Mung Bean Nuclease were from New England Biolabs (Beverly, MA). T4 DNA ligase was from USB *Corp.* (Cleveland, OH). Bac-N-Blue Transfection Kit and BCA Protein Assay Kit were from Invitrogen (Carlsbad, CA) and Pierce (Rockford, IL), respectively.

Production of recombinant baculoviruses expressing human mAChRs. Recombinant baculoviruses expressing human M1 and M2 mAChR were kind gifts from Dr. Elliot Ross (University of Texas Southwestern Medical Center). Human M3 mAChR recombinant baculovirus was kindly provided by Dr. Karl Akerman (Åbo Akademi University, Finland). The production of these viruses was described previously (Parker *et al.*, 1991; Kukkonen *et al.*, 1996).

To produce human M4 mAChR recombinant baculoviruses, the pCD plasmid containing its coding cDNA (Bonner *et al.*, 1987) was first linearized at a Tfi I site (103 nucleotides upstream of the initiation codon) and then followed by Mung Bean Nuclease incubation to create blunt ends. The cDNA containing the coding region was excised as a 1.7-kb fragment from the linearized plasmid with Bgl II at its 3'-end and ligated into

pVL1393 transfer vector at its Sma I and Bgl II sites. To produce human M5 mAChR recombinant baculoviruses, its cDNA was excised from its pCD plasmid (Bonner *et al.*, 1988) as a 1.8-kb fragment with Pst I and ligated into pVL1392 transfer vector at its Pst I site. Both constructs were confirmed by restriction analysis and DNA sequencing. Using Bac-N-Blue Transfection Kit (Invitrogen, CA) each transfer vector was co-transfected into Sf9 cells with the linearized wild-type AcMNPV DNA to produce recombinant baculoviruses and recombinant viruses were purified following manufacturer's manual.

Expression and preparation of human mAChRs in Sf9 cell membranes. Each mAChR was expressed in Sf9 cells using recombinant baculoviruses expressing the desired receptor subtype. Sf9 cells were infected, cultured, harvested and the membranes were prepared as described (Clawges *et al.*, 1997). Protein concentrations were analyzed with the BCA Protein Assay Kit from Pierce (Rockford, IL) using bovine serum albumin as standard.

Urea-extraction of Sf9 cell membranes containing the expressed mAChRs. To establish functional coupling between the purified G proteins and the mAChRs expressed in Sf9 cell membranes, we adapted a urea-extraction protocol based on previous reports from others (Hartman, IV and Northup, 1996). Briefly, 28,000 x g postnuclear membranes prepared as described (Clawges *et al.*, 1997) were resuspended at a concentration of about 0.5 mg/ml in a binding buffer (50 mM Tris-Cl, 5 mM MgCl₂, 0.5 mM EDTA, pH 7.5) and incubated with 200 nM Oxo-M and 25 μM GTPγS in a shaking

incubator at 25 °C for 1 hour. After incubation, membranes were pelleted at 288,000 x g, 4 °C for 30 minutes and then resuspended in a urea-extraction buffer (25 mM HEPES, 1 mM EDTA, pH 7.5 and 6M urea added freshly at use) at about 0.6-0.75 mg/ml and incubated on ice for 30 minutes. The extracted membranes were washed twice by resuspending in a HE buffer (5 mM NaHEPES, 1 mM EDTA, pH 7.5) and centrifuging at 288,000 x g, 4 °C for 30 minutes. The final pellets were resuspended in the HE at a concentration of 1-3 mg protein/ml.

Expression and purification of G proteins. Recombinant G protein subunits were also expressed in Sf9 cells using recombinant baculoviruses. Gi1 α subunits were purified as described (Bae *et al.*, 1999). Gq proteins were purified as detergent-extracted G $\alpha_q\beta_1\gamma_2$ heterotrimers from Sf9 cells co-infected with the recombinant baculoviruses to express Gq α , G β_1 and His-tagged G γ_2 subunits at an infection MOI of 1 α :1 β :1 γ (Kozasa and Gilman, 1995). Briefly, a 100,000 x g-pellet from cell homogenates was first extracted for 1 hour in an extract buffer (50 mM Tris/HCl, pH 8.0 with 10 μ M GDP, 2 mg/ml aprotinin, 20 mg/ml benzamidine, 2 mg/ml leupeptin, 2 mg/ml pepstatin, 0.1 mM PMSF, 5 mM BME and 1% CHAPS) and then centrifuged again at 100,000xg, 4°C for 45 minutes. The resulting supernatant was subject to sequential chromatography on AP-2 DEAE anion exchange, Ni-NTA Superflow resin and AP-1 QHR15 anion exchange columns. Free G $\beta_1\gamma_{2HIS}$ subunits and G $\alpha_q\beta_1\gamma_{2HIS}$ heterotrimers were eluted at 100 - 125 mM and 200 - 220 mM NaCl gradients, respectively.

Reconstitution of G proteins with mAChR-containing Sf9 cell membranes.

Reconstitution was performed as previously described (Clawges *et al.*, 1997) except that 0.08% CHAPS was included in the reconstitution buffer (5 mM NaHEPES, 100 mM NaCl, 5 mM MgCl₂, 1 mM EDTA, 500 nM GDP, 0.08% CHAPS, pH 7.5). G protein subunits were diluted in the same buffer such that the desired amount of subunit is contained in 1-5 μ l. Aliquots of membrane and G proteins were mixed and incubated at 25 °C for 15 minutes and then held on ice until the start of the binding assay.

Radioligand binding assays. For [³H]-NMS saturation binding assays, 5-20 μ g membrane aliquots were incubated with [³H]-NMS in a concentration range of 0.02 to 10 nM. For high-affinity [³H]-Oxo-M binding assays and [³H]-NMS competition binding assays, membranes were first reconstituted with purified G proteins as indicated in the text at 25 °C for 15 minutes. The reconstitution mixtures were diluted 10-fold with binding buffer prior to the start of the binding assay such that the desired amount of membranes (5 - 25 μ g/assay tube) were contained in 50 - 150 μ l. Aliquots of diluted membranes were incubated with a single concentration (~5 nM) of [³H]-Oxo-M for the high-affinity [³H]-Oxo-M binding assays; and a single concentration (~ 3 nM) of [³H]-NMS and increasing concentrations of unlabeled Oxo-M as a competitor with a range from 10⁻¹ M to 10⁻¹² M for the [³H]-NMS competition binding assays. Non-specific binding was determined by addition of 10³-fold or greater excess of atropine. The binding experiments were all performed in the binding buffer at 25 °C for 1.5 hours to achieve

equilibrium in a temperature-controlled shaker. The determination of binding was carried out as described (Clawges *et al.*, 1997).

Data analysis. The [^3H]-NMS saturation binding data were fit to one- and two-site binding models and compared by *F*-test. Without reconstitution with G proteins, one-site binding was the best-fit model in every assay performed for mAChRs in both the treated and non-treated Sf9 cell membranes. Thus, the receptor expression level (B_{max}) and the affinity for [^3H]-NMS (K_d) were computed from the one-site binding model with the GraphPad software and expressed as pmol/mg membrane protein and nM, respectively. To assess the apparent affinities between mAChRs and their coupling G proteins, the [^3H]-Oxo-M binding data were fit to a one-site interaction model between receptor and G protein and analyzed with GraphPad Prism software to calculate EC_{50} values, which represent the apparent affinity between G protein and receptor. [^3H]-NMS competition binding data were first fitted into one- and two-site competition models to determine the best-fit model with *F*-test. The dissociation constants (K_i) for Oxo-M and the fractions of receptors in the high-affinity state were then computed from the best-fit curve with the Cheng-Prusoff correction.

Results

Expression and characterization of mAChRs in Sf9 cell membrane. Five recombinant human muscarinic receptors were expressed in Sf9 cells using recombinant baculoviruses containing their cDNAs. The postnuclear Sf9 cell membrane fractions containing the expressed mAChRs were prepared and characterized by [^3H]-NMS saturation binding assay as described in “Methods”. The [^3H]-NMS binding sites on these membranes ranged from 2.2 to 33.2 pmol/mg membrane protein. The dissociation constants (K_d) for [^3H]-NMS binding were between 0.25 nM and 1.44 nM (Table 1), which were in good agreement with those obtained on muscarinic receptors expressed in native tissues, transfected mammalian cell lines and Sf9 cells (Buckley *et al.*, 1989; Hulme *et al.*, 1990; Dorje *et al.*, 1991; Dong *et al.*, 1995; Kukkonen *et al.*, 1996). Previous studies have demonstrated that urea-extraction of the membranes is required for reconstitution of functional coupling to Gq (Hartman, IV and Northup, 1996; Hellmich *et al.*, 1997; Lindorfer *et al.*, 1998; McIntire *et al.*, 2002). Consistent with previous studies, urea-extraction of the Sf9 cell membranes containing expressed mAChRs removed about 60% of the total membrane protein. However, the relative density of the mAChRs, expressed as pmol/mg membrane protein, was only significantly increased for M4 receptors. For each muscarinic receptor, urea-extraction did not significantly alter the dissociation constant for [^3H]-NMS binding ($p < 0.05$, t test) (Table 1).

High-affinity agonist binding on mAChR membranes. Based on the well-accepted ternary complex model (Samama *et al.*, 1993), GPCRs can be stabilized in their high-affinity agonist binding state by their coupling G proteins. Thus, using a single, low concentration of agonist which is near the high-affinity K_d of the agonist, functional receptor-G protein coupling may be readily detected as enhanced agonist binding on the receptors (Clawges *et al.*, 1997; McIntire *et al.*, 2002; Windh and Manning, 2002). To assess the functional coupling between the expressed muscarinic receptors and G proteins, we reconstituted the Sf9 cell membranes containing individual expressed muscarinic receptors with at least a 100-fold molar excess of purified G protein heterotrimers. As shown in Figure 1, without urea-extraction, addition of G proteins significantly enhanced agonist binding on M2 receptors (solid bar in the “Non-extracted” group, 5.42 fold over the control binding, $p < 0.05$), but only poorly enhanced agonist binding on M1 receptors (1.29 fold over the control binding, $p < 0.05$). Addition of G proteins was unable to enhance agonist binding on the other muscarinic receptors (M3, M4 and M5). When G protein coupled receptors are expressed in recombinant cells, endogenous G proteins are often able to couple with the expressed receptors. As shown in Figure 1, in the presence of 50 μ M GTP γ S, agonist binding was significantly reduced on all the “Non-extracted” receptor membranes except the M3 receptor membrane (striped bars in “Non-extracted” groups), indicating that some of the expressed muscarinic receptors coupled with endogenous G proteins in Sf9 cell membranes. The agonist binding was reduced about 40% on M1 and M5 receptor membranes and about 86% on M2 and M4 receptor membranes (Figure 1).

Several groups have successfully applied high concentrations of urea to reduce endogenous G protein coupling and improve functional coupling between membrane expressed receptors and purified G proteins in reconstitution (Hartman, IV and Northup, 1996; Hellmich *et al.*, 1997; Lindorfer *et al.*, 1998; Lim and Neubig, 2001). As shown in Figure 1, after the Sf9 cell membranes were treated with urea-extraction, addition of G proteins significantly improved the high-affinity agonist binding on both M1 and M2 receptors (6.64 vs. 1.29 fold for M1 and 175.37 vs. 5.42 fold for M2, respectively, $p<0.05$). On the extracted membranes containing M4 and M5 receptors, addition of G proteins significantly enhanced agonist binding to these receptors (7.64 fold for M4 and 5.29 fold for M5 of their controls, $p<0.05$). However, after urea-extraction, addition of G proteins still could not stabilize the high-affinity agonist binding on the M3 receptors. Addition of GTP γ S did not reduce agonist binding on the extracted M3 and M4 receptor membranes ($p>0.05$) but still could significantly reduce agonist binding on the extracted M1, M2 and M5 receptor membranes (18%, 25% and 12% less than the controls, respectively, $p<0.05$) (Figure 1). Compared with the non-extracted membranes, this uncoupling effect was significantly decreased in the extracted membranes (compare striped bars in “Non-extracted” and “Extracted” groups).

G protein concentration-dependent high-affinity agonist binding on reconstituted membrane mAChRs. To further characterize the G protein coupling properties of five muscarinic receptors (mAChRs), we assessed apparent affinities of G proteins for five mAChRs. Urea-extracted Sf9 cell membranes containing individual mAChR subtypes

were reconstituted with increasing concentrations of Gq or Gi1 proteins. The high-affinity agonist binding on these receptors stabilized by G proteins at different concentrations were determined with a single, low concentration (~ 5 nM) of [3 H]-Oxo-M. As shown in Figure 2, the G protein-stabilized high-affinity agonist binding on 4 out of 5 mAChRs tested exhibited a G protein concentration-dependent pattern. The EC_{50} for G proteins was determined by fitting the binding data to a one-site interaction model between G proteins and receptors and represents the apparent affinity of the receptor for the G protein heterotrimer. As shown in Figure 2A, the M1 and M5 mAChRs had EC_{50} values of 65.6 ± 0.4 and 87.8 ± 19.2 nM for Gq, respectively, which are not significantly different ($p > 0.05$). Consistent with the results in Figure 1, the M3 receptor could not be stabilized in the high-affinity agonist binding state by Gq protein even at a very high concentration (313 nM). Under the same conditions, Gq was also unable to enhance agonist binding to urea-extracted M2 and M4 receptor membranes (data not shown).

When reconstituted with Gi1 (Figure 2B), an EC_{50} of 412.3 ± 5.5 and 423.4 ± 51.3 nM was obtained for M2 and M4 receptors, respectively, which are not significantly different ($p > 0.05$). Enhanced agonist binding was also observed for M1 and M5 receptors when reconstituted with Gi1 protein (Figure 2C) with EC_{50} values of 97.8 ± 40.29 nM and 45.0 ± 20.8 nM, respectively. Interestingly, the EC_{50} values with which M1 and M5 receptors interact with Gi1 and Gq do not differ significantly, however, the M1 and M5 receptors interact with G proteins with significantly higher affinity than do the M2 and M4 receptors. Again, the M3 receptor was unique in that Gi1, even at a concentration of 400 nM, did not enhance high-affinity agonist binding.

Competition analysis of G protein stabilized agonist affinity states on reconstituted mAChRs. Agonist affinities of five mAChRs in urea-extracted Sf9 cell membranes were investigated with competitive inhibition of [³H]–NMS binding by non-labeled Oxo-M. Without G protein reconstitution, competition binding data were consistent with a one-site competition model for all five mAChRs (Figure 3, control curves). As summarized in Table 2, without G protein reconstitution, four mAChRs (M1, M2, M4 and M5) have the same low affinity for Oxotremorine-M (Ki-Low) ($p>0.05$, Tukey’s test). However, the M3 receptor has a significantly lower affinity for Oxotremorine-M than the other receptors ($p<0.05$, Tukey’s test).

When reconstituted with high concentrations of Gq proteins (more than 600-fold molar excess over receptors), the competition binding curves fit a two-site model best for M1 and M5 receptors but fit a one-site model best for M2, M3 and M4 receptors. About 18% of both M1 and M5 receptors could be stabilized in the high-affinity agonist binding state (Table 2). The proportions of receptors in the high-affinity state and the high-affinity dissociation constants (Ki-High) were not significantly different between M1 and M5 receptors ($p>0.05$, *t* test) (Table 2). For the low agonist affinities (Ki-Low) among five mAChRs in Gq reconstitutions, only M3 receptor was different from others ($p<0.05$, Tukey’s test) (Table 2).

All four mAChRs could be stabilized in the high-affinity agonist binding state by Gi1 reconstitution and their binding curves fit a two-site model best (Figure 3 and Table 2). However, the receptor proportions that could be stabilized in the high-affinity agonist

binding state by Gi1 were different among them ($M4 > M2 = M5 > M1$) (Table 2). Again, for the low-affinity state with Gi1 reconstitution, only the Ki-Low from M3-Gi1 reconstitution was different from those of others ($p < 0.05$, Tukey's test) (Table 2). The high-affinity dissociation constants (Ki-High) did not differ among M1, M2 and M5 receptors, but the Ki value for the M4 receptor was significantly greater than the others (Ki-High, 92.35 nM, $p < 0.05$, Tukey's test) (Table 2).

Discussion

The baculovirus expression system has been a useful tool to study GPCR-G protein coupling. It has been used to express high levels of GPCRs and/or G proteins (Graber *et al.*, 1992; Graber *et al.*, 1994; Kozasa and Gilman, 1995; Windh and Manning, 2002). The muscarinic acetylcholine receptors (mAChRs) expressed in Sf9 cells have been reported to retain their structural integrity, ligand binding pharmacology and G protein coupling properties (Parker *et al.*, 1991; Dong *et al.*, 1995; Vasudevan *et al.*, 1995; Kukkonen *et al.*, 1996; Weill *et al.*, 1997). In the present study, we expressed five human mAChRs in Sf9 cells. Although the five mAChRs were expressed and characterized under the same conditions, their [³H]–NMS binding sites ranged from 2.2 to 33.2 pmol/mg protein in a 28,000 x g postnuclear membrane preparation (Table 1). The M2 and M3 mAChR binding sites were expressed at higher levels in comparison with other three mAChRs. A similar difference between M1 and M2 mAChR binding sites was reported using the same baculoviruses (Parker *et al.*, 1991). The reason for the difference in their expression levels is not well understood, but Parker *et al* suggests that it may be due to both the nucleotide and amino acid sequence of individual GPCRs. Despite their different expression levels in Sf9 cells, all five mAChRs displayed a single affinity state for [³H]–NMS binding with K_d values of 0.25 nM to 1.44 nM. These values were in close agreement with those from previous studies using native tissues, transfected mammalian cells as well as Sf9 cells (Buckley *et al.*, 1989; Hulme *et al.*, 1990; Dorje *et al.*, 1991; Dong *et al.*, 1995; Kukkonen *et al.*, 1996).

Previous studies indicate that mAChRs display differential G protein coupling properties not only between Gi- and Gq-coupled mAChRs but also among mAChRs coupled to the same G protein. In this study, we examined the potential difference in the G protein coupling properties of five closely related mAChRs in a Sf9 cell based reconstitution system. Based on the extended ternary complex model (Samama *et al.*, 1993), the high-affinity agonist binding on mAChRs stabilized by their coupling G proteins was measured to assess the functional coupling between G proteins and mAChRs. As shown in Figure 1, when the purified G proteins reconstituted with the (non-extracted) membranes containing the M1 and M2 mAChRs, an enhanced agonist binding level was detected, which was well explained by the model. However, such a functional coupling was not established for the M3, M4 and M5 mAChRs in the (non-extracted) Sf9 cell membranes. To circumvent this problem, we adapted a urea-extraction protocol based on previous reports. After urea-extraction, about 60% total membrane proteins were lost. In contrast with the reports by Northup's group (Hartman, IV and Northup, 1996), urea-extraction did not significantly increase the relative abundance of [³H]-NMS labeled mAChR sites except for M4 receptor membranes. It is not known if this resulted from the removal of receptor proteins from the membrane or the inactivation of some receptor binding sites. As reported by Parker *et al.*, it seems that not all receptor proteins expressed in Sf9 cell membranes could be labeled by radioligand from the comparison of immunoblot and radioligand labeling data (Parker *et al.*, 1991). For each mAChR subtype expressed in Sf9 cell membranes, urea-extraction did not significantly alter their binding affinity for [³H]-NMS (Table 1). However, their endogenous G protein

coupling was significantly decreased (Figure 1). Hartman *et al* reported that the GTP γ S binding level was reduced 75% on the extracted Sf9 cell membranes, reflecting the removal or inactivation of the endogenous G proteins (Hartman, IV and Northup, 1996). Consistent with their results, ours showed that urea-extraction also decreased the endogenous G protein-stabilized, high-affinity, agonist binding on the extracted membranes (Figure 1). More importantly, urea-extraction greatly improved the mAChR coupling with the purified G proteins added in reconstitution (Figure 1). Thus, the extracted mAChR-containing Sf9 cell membrane combined with radiolabeled agonist binding assay may provide an alternative tool to analyze mAChR-G protein coupling.

Intriguingly, G protein-stabilized, high-affinity, agonist binding was not observed for M3 mAChR in either the extracted or the non-extracted Sf9 cell membranes (Figure 1 and 2). Competition binding analysis revealed that the M3 receptor only showed a relatively low agonist binding affinity regardless of G protein reconstitution (Figure 3 and Table 2). The functional G protein coupling of M3 receptors have been demonstrated when using intact Sf9 cells infected with recombinant baculoviruses expressing M3 receptors. For example, Kukkonen *et al* showed that stimulation of M3 receptor could increase phosphoinositide hydrolysis and Ca⁺⁺ mobilization (Kukkonen *et al.*, 1996) and Vasudevan *et al* showed M3 receptor activation could couple to K⁺ channel in a pertussis toxin-sensitive way (Vasudevan *et al.*, 1992). Thus, it appears that M3 receptor may functionally couple to both Gq and Gi proteins in intact Sf9 cells. In contrast, Guo *et al* showed that stimulation of Gq-fused M3 receptors in Sf9 cell membrane could not catalyze guanine nucleotide exchange on Gq (Guo *et al.*, 2001). Taken together, these

results suggest that the functional M3-G protein coupling may require additional cellular component(s) that may be eliminated during membrane preparation. As in the conventional G protein signaling model (Gilman, 1987; Neer, 1995), it was believed that activation of GPCRs upon agonist binding may catalyze the guanine nucleotide exchange on G α subunits, resulting in the dissociation of GTP-bound G α from G $\beta\gamma$ subunits, which are both able to interact with downstream effectors. Based on accumulating evidence, Chidiac questioned the generality of this model and proposed three possible mechanisms explaining GPCR-G protein-Effector (R-G-E) coupling process (Chidiac, 1998). One of these mechanisms proposes the existence of stable R-G-E complexes, possibly containing more than one copy of each component. Though direct evidence is needed to verify this model, current evidence indicates the existence of oligomeric receptors (Hebert and Bouvier, 1998), receptor-G protein complexes (Rodbell, 1980) (Wreggett and Wells, 1995) and effectors (Tang *et al.*, 1995), suggesting the possibility of such complexes. For M3 mAChRs, Zeng and Wess' study showed that M3 receptors were able to form dimers and multimers independently of agonist interaction (Zeng and Wess, 1999). As shown in the present study, the G protein coupling properties of M3 receptors is clearly different from other two Gq-coupling mAChRs (M1 and M5) in an identical environment. Upon reconstitution with either Gq or Gi1 proteins, the high-affinity agonist binding state could be restored for both M1 and M5 receptors but not for M3 receptors. Thus, in future studies it will be very interesting to test whether or not addition of effectors (such as PLC- β in the M3-Gq protein signaling cascade) could restore high-affinity agonist binding on M3 receptors with our approach presented here.

Verification of this issue may provide strong evidence for the stable R-G-E complex model proposed by Chidiac (Chidiac, 1998).

Quantitation of G protein signaling components *in vivo* reveals that GPCRs are outnumbered by excessive G proteins in the plasma membrane (Post *et al.*, 1995). Kenakin *et al* discussed that the alteration in GPCR-G protein stoichiometry in heterologous systems may produce abnormal GPCR-coupling properties compared with *in vivo* situations, while Neubig emphasized the accessibility between GPCRs and G proteins in native cells may be a regulating factor for GPCR-G protein coupling (Neubig, 1994; Kenakin, 1997). Consistent with these conclusions, an earlier work from our laboratory showed that the agonist binding on 5-HT receptors was enhanced in a G protein concentration-dependent manner, suggesting that the strength in GPCR signaling may depend on the amount of available G proteins (Clawges *et al.*, 1997). In addition, for four different 5-HT₁ receptor subtypes that can couple to the same G protein, their difference in G protein coupling could be distinguished by their apparent affinities for G protein (Clawges *et al.*, 1997). For mAChR subtypes, although promiscuous G protein coupling has been observed, their selective G protein coupling is more predominant. For example, Offermanns *et al* reported that M1, M2 and M3 receptors could activate Gi1 proteins when expressed in transfected HEK 293 cell membranes; however, much higher agonist concentrations were needed for Gi1 activation by M1 and M3 receptors in comparison with M2 receptors (Offermanns and Schultz, 1994). Provided that mAChRs have the identical access to G proteins, would their interaction with G protein be different at the biochemical level? To address this question, we assessed the apparent affinity of G

proteins in stabilizing the high-affinity agonist binding on mAChRs. Our data showed that M2 and M4 receptors could not couple to Gq protein in the urea-extracted Sf9 cell membranes but they had a similar apparent affinity for Gi1 protein coupling (Figure 2B). Similar apparent affinities were observed for M1 and M5 receptors in their interactions with either Gq or Gi1 proteins (Figure 2A&C). These results are in good agreement with the well-established G protein coupling preferences for mAChRs by other approaches (Caulfield and Birdsall, 1998). In general, the even-numbered muscarinic receptors (M2 and M4 receptors) predominantly couple to pertussis toxin-sensitive Gi/o proteins, while the odd-numbered muscarinic receptors (M1, M3 and M5 receptors) preferentially couple to pertussis toxin-insensitive Gq/11 proteins. However, this G protein coupling selectivity is not absolute but relative. For example, Burford *et al* demonstrated that, when expressed in CHO cells, M1 and M3 receptors also could stimulate GTP γ S binding to G proteins in a pertussis toxin-sensitive way (Burford *et al.*, 1995b). Our results show that both M1 and M5 receptors can be stabilized in high-affinity agonist binding state by Gi1 proteins. In addition, they seem to have a stronger interaction with Gi1 than M2 and M4 receptors do. However, M3 receptors clearly have different G protein-coupling properties from those of M1 and M5 receptors as discussed above.

In competition binding assays (Figure 3 and Table 2), without G protein reconstitution, only a single, low-affinity agonist binding state was observed for all five mAChRs in the urea-extracted Sf9 cell membranes, which can be well explained by the ternary complex model. For Gi-coupled M2 and M4 receptors, their high-affinity agonist binding states were stabilized when reconstituted with Gi1 proteins but not Gq proteins,

confirming that they are Gi-selective coupled receptors. Similarly, a high-affinity agonist binding state was observed for M1 and M5 receptors when reconstituted with Gq proteins as well as with Gi1 proteins. When reconstituted with Gi1 proteins, M1 and M5 receptors have similar agonist binding affinities but M2 and M4 receptors are significantly different in their high binding affinities (Ki-High) for Oxo-M.

M3 receptors not only had one agonist affinity state regardless of Gq or Gi1 reconstitution but its Ki at low-affinity state for Oxo-M was much higher than others (at least 10-fold). It was different from the previous observations by others. For example, using radioligand binding assays on the S9 cell membranes without urea-extraction, Dong *et al* reported that less than 10-fold difference in ligand binding affinity was observed among a variety of antagonists and agonists including Oxotremorine (Dong *et al.*, 1995). It may suggest that agonist binding properties of M3 receptors may be dramatically affected by the membrane-environment and the availability of other cellular components.

As discussed by others, caution should be taken when interpreting the results obtained from heterogeneous system due to varied receptor-G protein stoichiometry, membrane environment and signaling component repertoire in different systems (Neubig, 1994; Kenakin, 1997). Experimental conditions in reconstitution may not exactly reflect the situations *in vivo*, but do provide a basis to study the functional differences among closely related GPCRs. As presented in this study, our data clearly demonstrated that five G protein coupled muscarinic receptors could potentially be stabilized in different ligand binding affinity states by the same G protein population in an identical environment.

In summary, five closely related mAChRs can be distinguished in their G protein coupling properties at the biochemical level. M1 and M5 receptors couple to both Gq and Gi1 proteins but only Gi1 protein coupling was observed for M2 and M4 receptors in reconstitution. For M1 and M5 receptors, their apparent affinities were not different for either Gq or Gi1 coupling. Similarly, for M2 and M4 receptors, they were not different in their apparent affinities for Gi1 coupling but could not couple to Gq. Between the odd-numbered and even-numbered muscarinic receptors, it appears that M1 and M5 receptors have a stronger interaction for Gi1 coupling. Interestingly, M3 receptors could not couple to either Gq or Gi1 proteins in reconstitution, suggesting that it may employ a unique G protein coupling mechanism. In conclusion, our data demonstrate that the five closely related muscarinic receptors exhibit different agonist binding and G protein coupling behaviors in an identical membrane environment.

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Table 1. Saturation binding parameters of five human mAChRs expressed in Sf9 cell membranes with or without urea extraction.

Recombinant human mAChRs were expressed in Sf9 cells with baculoviruses and the membranes were prepared with or without 6 M urea extraction as described in “Methods”. 10 – 25 µg membrane protein per tube was used in binding assay in the presence of 0.02 – 10 nM [³H]-NMS in a final volume of 500 µl. Kd values (nM) were generated by fitting the data to one-site model and expressed as mean ± SEM with the number of preparations indicated in the parentheses.

Receptor	M1	M2	M3	M4	M5
Non-extracted	0.25 ± 0.10 (5)	0.72 ± 0.05 (3)	0.83 ± 0.05 (3)	0.56 ± 0.38 (3)	0.38 ± 0.08 (7)
Extracted	0.27 ± 0.05 (10)	0.67 ± 0.12 (3)	1.44 ± 0.27 (7)	0.45 ± 0.17 (2)	0.64 ± 0.11 (4)

Table 2. Competition binding parameters of reconstituted Sf9 cell membranes containing individual mAChR subtypes.

Inhibition of [^3H]NMS (3 nM) binding on the reconstituted Sf9 cell membranes containing individual mAChR subtypes was determined and analyzed as indicated in Figure 3. Binding parameters were computed from the best-fit competition model and represented as Mean $\pm$ SEM from the number of independent experiments shown in the parentheses.

Subtype	Reconstitution	R-High (%) ^a	Ki-Low (μM)	Ki-High (nM)
M1	Control		17.16 $\pm$ 2.43 (3)	
	Gq	18.00 $\pm$ 2.30 (2)	14.33 $\pm$ 2.93 (2)	14.09 $\pm$ 12.2 (2)
	Gi1	12.85 $\pm$ 3.85 (2)	6.05 $\pm$ 2.15 (2)	8.91 $\pm$ 8.89 (2)
M2	Control		8.60 $\pm$ 2.20 (2)	
	Gq		6.11 $\pm$ 0.01 (2)	
	Gi1	38.49 $\pm$ 5.61 (3)	3.27 $\pm$ 0.87 (3)	2.59 $\pm$ 2.15 (3)
M3	Control		188.7 $\pm$ 37.52 (3)	
	Gq		135.3 $\pm$ 8.90 (2)	
	Gi1		125.1 $\pm$ 26.98 (3)	
M4	Control		11.25 $\pm$ 3.82 (3)	
	Gq		4.72 $\pm$ 1.77 (3)	
	Gi1	70.89 $\pm$ 3.34 (3)	16.34 $\pm$ 2.14 (3)	92.35 $\pm$ 7.53 (3)
M5	Control		8.08 $\pm$ 1.98 (2)	
	Gq	18.37 $\pm$ 1.58 (3)	17.41 $\pm$ 9.20 (3)	2.88 $\pm$ 2.52 (3)
	Gi1	33.78 $\pm$ 0.10 (2)	10.46 $\pm$ 2.24 (2)	0.16 $\pm$ 0.05 (2)

^a percentage of receptors stabilized in high-affinity agonist binding state by the indicated G protein.

Figure Legends

Figure 1. Determination of high-affinity agonist binding on mAChR-containing Sf9 cell membranes stabilized by purified G proteins in reconstitution. Urea-extracted or non-extracted Sf9 cell membranes containing individual mAChR subtypes were reconstituted in the presence (solid bars) or absence (open and striped bars) of at least 100-fold molar excess of G proteins as indicated. After reconstitution, the membranes were diluted in binding buffer with (striped bars) or without (open and solid bars) 50 μ M GTP γ S and the high-affinity agonist binding were determined in the presence of a single concentration ($\sim$ 5 nM) of 3 H-Oxo-M. The binding data were normalized as folds over the binding on the control membranes (open bars) and expressed as Mean $\pm$ SEM from at least three separated experiments. *a*, $p < 0.05$ compared with the control within each group; *b*, $p < 0.05$ compared with the corresponding binding within the non-extracted group.

Figure 2. G protein concentration dependence of high-affinity agonist binding on reconstituted mAChR membranes. Aliquots of urea-extracted Sf9 cell membranes expressing individual mAChR subtypes were reconstituted with increasing concentrations of their coupling G proteins. After reconstitution, membranes were diluted in binding buffer and incubated with a single concentration ($\sim$ 5 nM) of 3 H-Oxo-M in a final volume of 150 μ l. Non-specific binding was determined in the presence of 0.6 mM atropine. The final concentrations of G protein in the binding ranged from 0 to $\sim$ 600 nM and receptor concentrations were 0.95 nM, 0.85 nM, 1.80nM, 1.25 nM and 0.90 nM for M1 to M5

receptors, respectively. Data points were from a representative experiment with triplicate determinations. The apparent affinity was represented as EC₅₀ for G proteins, at which the G protein-stabilized high-affinity agonist binding reached the half-maximal level. EC₅₀ values were generated by fitting data into a one-site interaction model between G protein and receptor and expressed as Mean $\pm$ SEM from 2-8 independent experiments as indicated in the parentheses.

Figure 3. Inhibition of [³H]NMS binding on the reconstituted Sf9 cell membranes containing individual mAChRs by Oxo-M.

Sf9 cell membranes containing individual mAChR subtypes were reconstituted with control buffer (● control) or Gq proteins (Δ Gq or ■ Gi1). After dilution in binding buffer, inhibition of [³H]NMS (3 nM) binding on the reconstituted membranes was determined with increasing concentrations (10⁻¹ to 10⁻¹² M) of unlabeled Oxo-M. The final concentrations in binding of G proteins were 300 nM Gq or 200 nM Gi1. M1-M5 receptor concentrations were 0.5nM, 0.5nM, 0.3 nM, 0.3 nM and 0.4nM, respectively. Binding data were normalized as percent of maximum and each point represented average value from 2 – 4 separate experiments with standard error less than 10%. After fitting the data to one- or two-site competition model, the inhibition curves were generated from the best-fit model determined by *F* test. Oxo-M affinities (K_i-low and K_i-high) and the receptor fractions in the high-affinity binding state were computed from the best-fit model corresponding with GraphPad software and summarized in Table 2.

Figure 1.

Figure 2.

Figure 3.

CHAPTER 3: RESULTS

Section 2:

Influence of Cytosolic AGS3 on Receptor G-Protein Coupling[†]

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Abbreviations and Textual Footnotes— The abbreviations used are: **5-HT**, 5-hydroxytryptamine; **AGS3**, activator of G protein signaling 3; **CHAPS**, 3-(3-cholamidopropyl)dimethylammonil)-1-propanesulfonic acid; **GDI**, guanine nucleotide dissociation inhibitor; **GoLoco motif**, Gi/o-Loco interaction motifs; **GPCRs**, G protein coupled receptors; **GPR**, G protein regulatory; **GST**, glutathione S-transferase; **MOI**, multiplicity of infection; **Pins**, partner of inscuteable; **PVDF**, polyvinylidene fluoride; **SDS-PAGE**, sodium dodecylsulfate-polyacrylamide gel electrophoresis; **Sf9**, *Spodoptera frugiperda*; **TPR**, tetratricopeptide repeats.

Abstract

Activator of G protein signaling 3 (AGS3) activates the $G\beta\gamma$ mating pathway in yeast independently of heptahelical receptors. It competes with $G\beta\gamma$ subunits to bind GDP-bound $G_i/o\alpha$ subunits *via* four repeated G protein regulatory (GPR) domains in the carboxyl terminal half of the molecule. However, little is known about the functional role of AGS3 in cellular signaling. Here the effect of AGS3 on receptor-G protein coupling was examined in an Sf9 cell membrane-based reconstitution system. A GST-AGS3-GPR fusion protein containing the four individual AGS3-GPR domains inhibits receptor coupling to $G\alpha$ subunits as effectively as native AGS3 and more effectively than GST fusion proteins containing the individual AGS3-GPR domains. While none of the GPR domains distinguished among the three $G_i\alpha$ subunits, both individual and full length GPR domains interacted more weakly with $G_{o\alpha}$ than $G_i\alpha$. Cytosolic AGS3, but not membrane-associated AGS3, can interact with $G_i\alpha$ subunits and disrupt their receptor coupling. Immunoblotting studies reveal that cytosolic AGS3 can remove $G_i\alpha$ subunits from the membrane and sequester $G_i\alpha$ subunits in the cytosol. These findings suggest that AGS3 may down-regulate heterotrimeric G protein signaling by interfering with receptor coupling.

Heterotrimeric G proteins, located on the cytoplasmic face of the plasma membrane, are widely used to transduce extracellular signals through heptahelical GPCRs to intracellular signaling networks. According to the widely accepted ternary complex model (1-3), agonist occupied GPCRs stimulate the release of GDP and binding of GTP to the G α subunit of a heterotrimer. The conformational changes associated with GTP binding dissociate the ternary complex and both the GTP-bound G α subunit and G $\beta\gamma$ dimer are then free to modulate downstream effectors including enzymes and ion channels. This signaling is in part terminated by GTP hydrolysis and subunit reassociation. A diverse array of proteins distinct from GPCRs, G-proteins and effectors are now known to regulate this signaling process at multiple levels (4-7). Among the newest family of accessory proteins are the Activators of G protein Signaling, or AGS proteins (8;9). A member of this family, AGS3, belongs to a novel class of proteins containing G protein regulatory (GPR) motifs, also known as GoLoco motifs (10). AGS3 has four repeated GPR sequences in its carboxyl-terminal half and seven tetratricopeptide repeat (TPR) sequences in its amino-terminal half. AGS3, *via* its GPR domains, selectively interacts with the GDP-bound conformation of alpha subunits of the Gi/o family (9;11-15) and effectively competes with G $\beta\gamma$ subunits for binding to G α (13).

Studies in *Drosophila melanogaster*, *Caenorhabditis elegans* and several cultured cell lines indicate that GPR containing proteins related to AGS3 are involved in cell polarity and asymmetric cell division (16-20), but little is known about the influence of AGS3 and related proteins on signal processing by GPCRs. Since AGS3 can potentially disrupt G protein heterotrimers and stabilize the GDP-bound conformation of G α

subunits, we hypothesized that AGS3 might interfere with receptor-G protein coupling. To examine the effect of AGS3 on receptor-G protein coupling, we used an Sf9 cell membrane based reconstitution system. Relatively few GPCRs expressed in Sf9 cells are coupled to endogenous G proteins (21-24). Reconstitution of the expressed receptors with appropriate, purified, exogenous G proteins couples the majority of the expressed receptors. This functional receptor-G protein coupling is readily detected as an enhanced level of agonist binding using a radiolabeled agonist at concentrations near the high-affinity K_D of the receptor (21;24). The abilities of cytosolic AGS3, membrane-associated AGS3, and individual GPR domains to interfere with receptor-G protein coupling were assessed in reconstitution assays.

In this study, we report that cytosolic AGS3, but not membrane-associated AGS3, interferes with 5-HT_{1A} receptor-Gi/o protein coupling. In interactions with Gi/o α subunits, the GPR domain alone is as effective as the native AGS3 protein. Although all four individual AGS3-GPR domains bind Gi α more tightly than Go α , they do not distinguish among the three Gi α subunits. Taken together, our results demonstrate that AGS3 affects receptor-G protein coupling by dissociating G α subunits from the membrane and sequestering G α subunits in the cytosol.

Experimental Procedures

Expression and purification of proteins. Recombinant $G_{i1,2,3}\alpha$ and $G_{o}\alpha$ and $G\beta_1\gamma_2$ subunits were purified after expression in Sf9 cells using recombinant baculoviruses as described (25;26). Cytosolic AGS3 was prepared as a 100,000 g extract from Sf9 cells infected at an MOI of 2 with recombinant baculoviruses encoding AGS3 (9). The infected Sf9 cells were then cultured, harvested and fractionated as previously described (21) except that harvested cells were thawed in 5× (rather than 15×) their wet weight of ice-cold homogenization buffer (10 mM Tris-Cl, pH 8.0 at 4°C, 25 mM NaCl, 10 mM $MgCl_2$, 1 mM EGTA, 1 mM DTT, 0.1 mM PMSF, 20 μ g/ml of benzamidine and 2 μ g/ml of each of aprotinin, leupeptin and pepstatin A). The 100,000 g crude cytosol was concentrated to a final protein concentration of 5-7 mg/ml in a pressurized Amicon Cell using a YM-30 membrane. Control cytosol was prepared from uninfected Sf9 cells in the same fashion. The GST-AGS3 fusion proteins containing all four AGS3-GPR domains, GST-AGS3-GPR (Pro⁴⁶³-Ser⁶⁵⁰) or individual AGS3-GPR domains, GST-AGS3-GPR-I (Pro⁴⁶³-Glu⁵⁰¹), GST-AGS3-GPR-II (Ser⁵¹⁶-Leu⁵⁵⁵), GST-AGS3-GPR-III (Gly⁵⁶³-Thr⁶⁰²), and GST-AGS3-GPR-IV (Thr⁶⁰²-Ser⁶⁵⁰) were expressed and purified as previously described (13). Protein concentrations were determined with the bicinchonic acid method (Pierce Chemicals).

Preparation of Sf9 cell membranes containing expressed 5-HT_{1A} receptors.

To prepare membranes containing 5-HT_{1A} receptors, Sf9 cells were infected with recombinant baculoviruses expressing 5-HT_{1A} receptor at an MOI of 2. To prepare membranes containing both 5-HT_{1A} receptors and AGS3, Sf9 cells were co-infected with

recombinant baculoviruses expressing 5-HT_{1A} receptor and AGS3 at MOIs of 1 and 3, respectively. Sf9 cells were cultured and harvested; membranes were prepared and analyzed to determine 5-HT_{1A} receptor numbers as described (21) except that membranes co-expressing AGS3 were washed once rather than thrice during preparation.

Reconstitution of Sf9 cell membranes with purified G-proteins.

Reconstitution was performed as previously described (21). Briefly, 50 µg membrane protein and 1-2 µl containing the desired amount of G protein heterotrimer were resuspended in reconstitution buffer plus 0.04% CHAPS (Calbiochem) prior to the addition of the indicated additional components. The mixtures were incubated at 25°C for 15 minutes and then held on ice until the start of the binding assay, or pelleted in a refrigerated microcentrifuge at 12,000 rpm, 4°C for 10 minutes for immunoblotting assay.

To allow soluble, cytosolic AGS3 or exogenous G protein heterotrimers to associate with membranes, membranes containing 5-HT_{1A} receptors were first incubated (25°C, 15 minutes) with cytosol containing AGS3 or purified G protein heterotrimers, respectively. The membranes were then pelleted in a refrigerated microcentrifuge at 12,000 rpm, 4°C for 10 minutes and washed thrice with 100 µl reconstitution buffer. The resulting membranes were then used in a second reconstitution with the indicated additional components as described above.

[³H]-5-HT binding assay. Just prior to the start of the binding assay the reconstitution mixture was diluted 10-fold with binding assay buffer (50 mM Tris/HCl, 5 mM MgCl₂, 0.5 mM EDTA, pH 7.5) such that the desired amount of membranes (~15

μg/assay tube) was contained in 50-100 μl. High affinity agonist binding was measured with 1-2 nM [³H]-5-HT (25.5Ci/mmol, Perkin Elmer), which is near the high affinity K_D of 5-HT_{1A} receptor, in a final volume of 150-300 μl. Non-specific binding was determined in the presence of a million-fold excess of unlabeled 5-HT. Incubation was performed at 25°C for 1.5 hours in a temperature controlled shaker and terminated by filtration over Whatman GF/C filters (Brandel Inc.) using a Brandel Cell Harvester (Brandel Inc.). The filters were rinsed thrice with 4 ml ice cold washing buffer (50 mM Tris-Cl, 5 mM MgCl₂, 0.5 mM EDTA, 0.01% sodium azide, pH 7.5), placed in 4.5 ml CytoScint (ICN Pharmaceuticals) and counted to constant error in a scintillation counter. The binding data were analyzed using GraphPad PRISM (GraphPad Software).

Preparation of rat brain membranes. One rat brain was homogenized on ice in a Dounce glass homogenizer with 40 ml lysis buffer (5 mM Tris, pH 7.4, 5 mM EDTA, 5 mM EGTA, and protease inhibitors) for 8 strokes. The homogenate was centrifuged at 1,000 g for 10 minutes to remove debris. The resulting supernatant was centrifuged at 40,000 g for 30 min at 4°C. The resulting pellet was homogenized (in 40 ml) and centrifuged (at 40,000 g) four times: twice in high salt buffer (50 mM Tris, pH 7.4, 0.6 mM EDTA, 5 mM MgCl₂, 500 mM NaCl) and twice in no salt buffer (50 mM Tris, pH 7.4, 0.6 mM EDTA). The resulting pellet was resuspended in 1 ml no salt buffer using a glass homogenizer.

Immunoblot analysis. To determine the amount of AGS3 in membrane or cytosolic fractions, 0.5 μg protein was resolved by 12% SDS/PAGE and transferred to a PVDF membrane (Millipore). The membrane was probed with AGS3 antisera P-32 (13)

and visualized with ProtoBlot AP immunoblotting system (Promega). The levels of AGS3 were estimated by comparing the band intensities with known amounts of purified GST-AGS3-GPR (Pro⁴⁶³-Ser⁶⁵⁰) fusion protein standard using FluorChemTM 8000 system (Alpha Innotech Corp.). To determine the amounts of protein that associated with membranes during reconstitution, the reconstitution mixtures were pelleted and washed three times as described above. The washed pellets or one-tenth volume of the first supernatant were electrophoresed, transferred to PVDF membranes and probed with anti-G $\alpha_{i1/2}$ (Calbiochem), anti-G β antibody (NEN Products) and AGS3 antisera P-32, respectively, as indicated in the text. The antibody detection and visualization were performed with a ProtoBlot AP kit as described above. Band intensities were analyzed with FluorChemTM 8000 system (Alpha Innotech Corp.) and quantified within the linear range of a standard curve prepared from known quantities of Gi1 α or GST-AGS3-GPR (Pro⁴⁶³-Ser⁶⁵⁰).

To perform the fractionation experiments depicted in Figure 5, rat brain membranes were incubated with homogenization buffer (10 mM Tris-Cl, pH 8.0 at 4 °C, 25 mM NaCl, 10 mM MgCl₂, 1 mM EGTA, 1 mM DTT, 0.1 mM PMSF, 20 μ g/ml of benzamidine and 2 μ g/ml of each of aprotinin, leupeptin and pepstatin A), control cytosol, or AGS3 transfected cytosol for 30 minutes at room temperature, rotating. The incubation mixture was then centrifuged at 100,000 *g* for 30 minutes at 4°C. The resulting supernatant was termed the 100,000 *g* supernatant (S). The remaining pellet was resuspended in no salt buffer then centrifuged at 12,000 rpm in a microcentrifuge for 30 minutes. The resulting pellet was termed the 100,000 *g* pellet (P). Samples were then

mixed with Laemmli buffer and boiled prior to loading on 10% SDS-PAGE gels. Proteins were transferred to PVDF, immunoblotted with G α specific antiserum 976 and visualized with ECL reagents (PerkinElmer Life Sciences, Inc., Boston, MA).

Results

Membrane-expressed AGS3 does not interfere with receptor-Gi protein coupling— Protein-protein interaction studies indicate that AGS3 can bind GDP-containing $G_{i\alpha}$ subunits (9;13), and thus membrane localized AGS3 might disrupt G-protein heterotrimers by interactions with $G_{i\alpha}$ and thereby interfere with their coupling to receptors. To test this possibility, membranes were prepared from Sf9 cells expressing 5-HT_{1A} receptors alone or co-expressing AGS3. In both crude membrane preparations, 5-HT_{1A} receptors were expressed at 3.5-5.5 pmol/mg membrane protein as determined by saturation radioligand binding. In preparations co-expressing AGS3, AGS3 was present in the membranes at a 518 molar excess over 5-HT_{1A} receptors as quantitated by immunoblotting.

Membrane preparations with and without co-expressed AGS3 were reconstituted with increasing amounts of G_{i1} heterotrimers to stabilize the high affinity agonist binding state of the receptors. As shown in Figure 1A, the high affinity [³H]-5-HT binding to both membranes reached the maximum level at a G_{i1} concentration of about 85 nM. Interestingly, there was no right-ward curve shift observed for the membranes with co-expressed AGS3 even though it was present in a 1.2-fold molar excess over the highest concentration of G protein used. The EC₅₀ values for G_{i1} were not significantly different between the two membrane preparations, 6.07 ± 0.58 nM for the membranes expressing 5-HT_{1A} receptors alone and 6.53 ± 1.44 nM for the membranes with co-expressed AGS3 ($p > 0.05$, student t test). These results suggest that the membrane-expressed AGS3 does not interfere with receptor-Gi protein coupling. Additional evidence is shown in Figure

1B. Reconstitution with Gi1 did not alter the amount of AGS3 in the membrane fraction (panel B, lane 7 vs. 8, $p > 0.05$, one-sample t -test, $n = 3$) and equivalent amounts of Gi1 associated with membranes expressing or not expressing AGS3 (panel B, lane 4 vs. 8, $p > 0.05$, one-sample t -test, $n = 3$). Taken together, these results indicate that even when present in excess over receptors and G proteins, membrane-expressed AGS3 does not interfere with receptor-Gi1 protein coupling.

Cytosolic AGS3 interferes with receptor-Gi protein coupling by sequestering $G_{i\alpha}$ and blocking its membrane-association during reconstitution—Although a subpopulation is found loosely associated with a membrane fraction, AGS3 is primarily localized in the 100,000 g supernatant of native tissue lysates and various transfected cell lines (13;15;20). To determine if cytosolic AGS3 interferes with receptor-Gi protein coupling, a 100,000 g crude cytosol preparation was obtained from Sf9 cells infected with a recombinant baculovirus expressing AGS3. As a control, a similar cytosol preparation was obtained from uninfected Sf9 cells. As shown in Figure 2A, the cytosol preparation containing AGS3 significantly inhibited 5-HT_{1A} receptor-Gi1 protein coupling but lost its activity after being heated at 99 °C for 5 minutes. In contrast, the control cytosol preparation, reconstituted at the same total protein concentration, did not alter agonist binding significantly, demonstrating the specificity of the AGS3 effect.

Cytosolic AGS3 may interfere with receptor-G protein coupling by inhibiting the membrane association of heterotrimeric G-proteins or by stabilizing a conformation of $G_{i\alpha}$ -GDP that is not capable of interacting with receptors. We addressed this issue by first determining the effect of cytosolic AGS3 on the membrane-association of Gi1 α and

Gβγ subunits in immunoblotting experiments. Cytosolic AGS3 blocks the membrane-association of Gi1α subunits, but has no effect on the membrane association of Gβγ subunits (Figure 2B lane 10 vs. 13). These data indicate that cytosolic AGS3 sequesters Gi1α subunits during reconstitution and prevents their association with the membrane thus interfering with receptor coupling to G-proteins.

Cytosolic AGS3 associates with the membrane and loses its ability to interfere with receptor-G protein coupling—A portion of the cytosolic AGS3 associates with Sf9 cell membranes in a concentration-dependent manner regardless of the presence of receptors in the membrane (data not shown). Although the nature of this association is not understood, to rule out the possibility that the inactivity of the membrane associated AGS3 expressed in Sf9 cells shown in Figure 1 was due to denaturation or aggregation, it was important to know if the cytosolic AGS3 retains its ability to interfere with receptor-G protein coupling following membrane association. To accomplish this, membranes expressing 5-HT_{1A} receptors were first pre-incubated with or without sufficient cytosol containing AGS3 to allow an excess of AGS3 over receptors to associate with the membrane. After thorough washing the membranes were reconstituted with or without Gi1 heterotrimers and receptor coupling was assessed by measuring high affinity agonist binding.

As shown in Figure 3A, equivalent amounts of AGS3 remained associated with the membranes following the pre-incubation whether or not the membranes were subsequently reconstituted with Gi1 (compare AGS3 band in lanes 5 and 6). Figure 3A also shows that the pre-incubation with the AGS3 containing cytosol did not alter ($p >$

0.05, one-sample *t*-test, *n* = 3) the amount of Gi1 α that associated with the membranes during reconstitution (compare Gi1 α band in lanes 4 and 6). Quantitation of the immunoblots indicates that the AGS3 that remained associated with the membranes was in 29-fold molar excess over receptors and 69-fold molar excess over reconstituted Gi1 α . As shown in Figure 3B, the partition of cytosolic AGS3 to the membrane did not alter high-affinity agonist binding to the receptors following reconstitution with Gi1. Taken together, the results shown in Figures 2 and 3 suggest that although cytosolic AGS3 can prevent receptor-G protein coupling during reconstitution, it does not do so once it associates with the membrane.

Cytosolic AGS3 interacts with membrane-associated Gi1 α subunits—In a physiological setting G proteins are associated with the inner face of the plasma membrane. To understand if cytosolic AGS3 could interact with membrane-associated Gi α subunits and interfere with their coupling to receptors, Sf9 cell membranes expressing 5-HT_{1A} receptors were first reconstituted with Gi1 heterotrimers to allow their association with the membrane. The membranes were washed three times to remove unassociated Gi1 heterotrimers and incubated with crude Sf9 cell cytosol containing expressed AGS3 or with an equivalent amount of Sf9 cell cytosol from uninfected cells or buffer as controls. As an additional control, membranes were reconstituted with or without Gi1 and not subjected to a second incubation. The amount of Gi1 α that remained in the membranes following these treatments was determined by immunoblotting. The lanes from a representative immunoblot repeated four times are shown in Figure 4A. As shown in lane 4, no Gi1 α was detected in the membrane pellet

without reconstitution with exogenous Gi1. As shown in lane 5, an estimated 1.75 pmol Gi1 α subunits associated with 33 μ g membrane protein following the first round reconstitution with Gi1 heterotrimers. Pelleting, washing, and a second reconstitution with either control buffer (lane 6) or 245 μ g of cytosolic proteins from normal Sf9 cells (lane 8) did not significantly decrease the amount of membrane-associated Gi1 α subunits, indicating a strong association of G proteins with the membrane. Interestingly, lane 7 shows a significant decrease in membrane-associated Gi1 α following the addition of the AGS3 containing cytosol in the second reconstitution ($p < 0.05$, Tukey's Test, $n = 4$).

The high affinity agonist binding to membranes following these treatments from a representative experiment repeated 3 times with similar results are shown in the bar graph in Figure 4B. The binding data are consistent with the immunoblotting data, and also indicate that the association of G proteins with the membrane during reconstitution is quite strong. Control binding to membranes lacking exogenous G proteins (vertically striped bar in Figure 4B) was less than all other groups ($p < 0.01$, Tukey's Test, $n = 3$). Pelleting, washing, and the second incubation with control buffer did not lower the high affinity agonist binding, which reflects receptor-G protein coupling (compare the white versus black bars in Figure 4B). However, incubation with both control and AGS3 containing cytosol led to a significant decrease ($p < 0.01$, Tukey's Test, $n = 3$) in high affinity agonist binding (grey and horizontally striped bars in Figure 4B). In titration experiments the IC_{50} for inhibition of high affinity agonist binding of the AGS3 cytosol was significantly lower than the IC_{50} of the control cytosol (4.6 ± 0.7 vs. 34.6 ± 6.8 ng/ μ l,

n = 2, $p < 0.05$, data not shown) indicating the significant contribution of the cytosolic AGS3 protein. Thus, the results show that cytosolic AGS3 can dissociate $G_{i1}\alpha$ subunits from the membrane and interfere with receptor coupling. The nature of the inhibitory activity in the normal Sf9 cell cytosol is unknown but may represent an endogenous GPR-containing protein. The AGS3 antibody cross-reacts with several faint bands present in the normal Sf9 cytosol (Figure 4, lanes 12 and 13). Though it did not reach the level of statistical significance, the normal Sf9 cell cytosol did slightly reduce high affinity agonist binding, and this effect was reversed upon boiling (Figure 2A). To demonstrate that cytosolic AGS3 is capable of dissociating endogenous G proteins from a native membrane, a preparation of rat brain membranes was incubated in the presence of buffer, 40 μ g of control cytosol from uninfected Sf9 cells and 40 μ g of cytosol from Sf9 cells expressing AGS3 (0.3 pmol/ μ g protein). As shown in Figure 5, only the AGS3 containing cytosol was able to redistribute a significant amount of $G_i/o\alpha$ to the 100,000 g supernatant following fractionation of the incubation mixtures. Clearly AGS3 is capable of extracting $G_i/o\alpha$ subunits from their native membrane environment.

Selective inhibition of receptor-Gi protein coupling by GST-AGS3-GPR fusion proteins—The general domain structure of mammalian AGS3 is shown in Figure 6A. The carboxyl terminal GPR domain of AGS3, which consists of four homologous repeated motifs, is known to bind $G_{i\alpha}$ subunits (9;13) but little is known about possible functional roles for the amino-terminal TPR domain. The role of each individual GPR motif and their relative selectivity for specific G-proteins is also poorly understood. The latter issues were addressed using GST fusion proteins that contain all four GPR motifs

or individual GPR motifs. The GST-AGS3-GPR (Pro⁴⁶³-Ser⁶⁵⁰) fusion protein inhibited 5-HT_{1A} receptor coupling to all four G-proteins tested (Figure 6B). The IC₅₀ for G_o is significantly different from the values obtained with G_i ($p < 0.05$, Neuman-Keuls Test). The native cytosolic AGS3 protein exhibited an IC₅₀ of 29 ± 6 nM ($n = 3$) for G_{i1} (data not shown) indicating that the presence of amino terminal TPR domains did not alter the apparent affinity of the GPR domains for G_i.

Each of the four individual AGS3-GPR domains inhibited 5-HT_{1A} receptor-G protein coupling (Figure 6C), although not to the same extent as the complete AGS3-GPR domain ($p < 0.05$, Tukey's Test). Two-way ANOVA of the inhibition of 5-HT_{1A} receptor coupling to different G proteins by individual AGS3-GPR domains indicates that all four AGS3-GPR domains interact more weakly with G_o than with G_i proteins ($p < 0.01$), while there is no significant difference among individual AGS3-GPR domains for their interactions with G_{i/o} proteins ($p > 0.05$). These results suggest that the individual GPR domains do not differ from one another in their interactions with G α subunits and that all of them interact more weakly with G_o α than with the G_i α subunits.

Discussion

The recent discoveries of diverse proteins that interact with various components of heterotrimeric G-protein mediated signaling pathways provide additional insights into the regulation of this signaling process. In particular, the identification of the AGS family of proteins suggests that inputs other than GPCRs are capable of activating these pathways. AGS3 was initially identified as a receptor-independent activator for G protein signaling in a yeast-based functional screen (9). Sequence analysis reveals that AGS3 has four repeated GPR motifs (also termed GoLoco motifs) in the carboxyl-terminal half of the protein. This GPR sequence is conserved among various proteins from different species. Although these proteins have diverse functions, they share the ability to bind α subunits of the Gi family *via* their GPR domains. Biochemical studies from different groups have demonstrated that the GPR motif acts as a GDI to stabilize Gi α subunits in a GDP-bound form (11-15;27-29).

Recent studies indicate that GPR containing proteins are involved in cell polarity and asymmetric cell division (16-20) but little is known about the physiological role of AGS3 in GPCR signaling. The effects of AGS3 on receptor-G protein coupling and potential mechanisms were the subjects of this study. We hypothesized that because G $\alpha\beta\gamma$ heterotrimers are required on the cytoplasmic face of the plasma membrane for coupling with heptahelical GPCRs, AGS3 might interfere with receptor-Gi protein coupling since AGS3 can disrupt G protein heterotrimers by competing with G $\beta\gamma$ to bind GDP bound Gi α . The majority of cellular AGS3 is found in the cytosol but a small portion is associated with membrane fractions (13;15;20). In a cellular context either

fraction might be capable of disrupting G protein heterotrimers and interfere with receptor coupling. Our results indicate that membrane localized AGS3 does not appear to interact with G α subunits and has no effect on the reconstitution of receptor-G protein coupling (Fig 1). To exclude the possibility that the membrane expressed AGS3 failed to block receptor coupling because it was aggregated or denatured, we demonstrated that cytosolic AGS3, which has the ability to block reconstitution of receptor-G protein coupling (Figure 2), lost its ability to do so following association with the membrane fraction (Figure 3). Our data do not exclude the possibility of a signaling mechanism that would allow membrane associated AGS3 to interact with G α subunits and modulate receptor coupling. Although it is not well understood how AGS3 associates with the membrane, the amino-terminal TPR domains have been implicated in subcellular localization (11) perhaps through an as yet undefined mammalian binding partner. Interestingly, the subcellular localization of a short form of AGS3, lacking the TPR domains, may be determined in part by a regulated interaction with G α (11). However, Yu and colleagues (30) reported that both carboxyl-terminal GPR and amino-terminal TPR domains are required for the apical membrane targeting of Pins, an AGS3/LGN ortholog from *Drosophila*.

The localization of G protein heterotrimers at the plasma membrane involves a complex interplay among several processes including post-translational modifications, subunit assembly and direct protein-membrane interactions (31-40). It is clear that assembly with G $\beta\gamma$ is required for localization of G α at the plasma membrane (33;35;37). A recent model proposes that all three G protein subunits are synthesized in

the cytosol on free polysomes and that G α and G $\beta\gamma$ associate on the cytosolic face of the Golgi prior to transport from an endosome to the plasma membrane (37). Since AGS3 can compete with G $\beta\gamma$ subunits to bind G α subunits, cytosolic AGS3 might disturb the assembly of G $\beta\gamma$ with G α subunits and thereby decrease the amount of G protein heterotrimer at the plasma membrane. As reviewed by Geyer and Wittinghofer (41), the GDIs for small G proteins generally complex with small G proteins in the cytosol and prevent their translocation to the membrane. Our results suggest the possibility of a similar role for AGS3 with heterotrimeric G proteins. In reconstitution assays, AGS3 in a cytosol fraction binds G α subunits and prevents the membrane association of G α , but not G $\beta\gamma$ (Figure 2). Cytosolic AGS3 actually interacted with membrane-associated G α subunits, dissociating them from the membrane and interfering with receptor coupling (Figure 4). While disruption of G protein heterotrimers is sufficient to inhibit receptor coupling in a reconstitution system, in a cellular context there must be an appropriate signal regulating the access of AGS3 to membrane localized G proteins. In the few existing studies on cellular functions of related GPR containing proteins it is clear that the distributions and functions of these proteins are regulated by signals during the cell cycle (18-20).

The AGS3 carboxyl-terminal domain containing four repeated GPR motifs is the region responsible for binding G α subunits (13). The carboxyl-terminal GPR domain is as potent as full-length AGS3 in interactions with G α subunits. Our results also confirm a weak interaction between AGS3-GPR and G α subunits (12;13;15). While it is tempting to interpret the reduced apparent affinity for G α in terms of selectivity, it is

perhaps more likely that the reduced affinity ensures that only appropriate amounts of AGS3 and G α form complexes in a cellular setting. For example, in brain membranes G α is by far the most abundant G α subunit (42;43).

It is clear that amino acids within both the GPR domain and the G α subunit contribute to the selectivity of the interaction. Peterson *et al.* have defined critical amino acids within the core GPR domain and demonstrated differential effects on interactions with G α vs. G α (27) while the crystal structure of G α_{i1} -GDP bound to the RGS14 GPR motif peptide (44) and functional studies with chimeric G proteins (45) demonstrate that the helical domain of G α subunits contribute to the selectivity of G α -GPR interactions. However, for proteins such as Pins, LGN and AGS3 that contain multiple GPR motifs the possibility of selectivity for different Gi/o α subunits has not been previously addressed. Interestingly, our data indicate that although all four individual AGS3 GPR motifs interact more strongly with G α than G α , they do not discriminate among the three G α subunits (Figure 6B). Each AGS3-GPR motif can bind G α subunits and the complete GPR domain actually binds multiple subunits at the same time suggesting a possible scaffolding function for the complete AGS3-GPR domain (13). Our results are consistent with this interpretation and show that each AGS3-GPR domain can interfere with receptor-G protein coupling but that at equal concentrations (near the IC₅₀ for GST-AGS3-GPR (Pro⁴⁶³-Ser⁶⁵⁰), the complete GPR domain inhibits coupling to a greater extent than any individual GPR domain (Figure 6C).

In summary, our data show that AGS3 can interfere with receptor-G protein coupling by disrupting G $\alpha\beta\gamma$ heterotrimers in the cytosol as well as by removing G α

subunits from the plasma membrane. The disruption of G protein heterotrimers might also serve to initiate G $\beta\gamma$ -mediated signaling events. Similarly, there may be as yet unappreciated roles in cellular signaling for the AGS3-G α -GDP complex. Interestingly, membrane associated AGS3 appears not to interact with G α subunits, although our study does not exclude the possibility that such interactions may require an initiating signal.

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Figure Legends

Figure 1. Effect of membrane expressed AGS3 on receptor-G protein coupling and association of Gi1 α with the membrane. (A) Sf9 cell membranes expressing 5-HT_{1A} receptors (110 fmol) or co-expressing AGS3 (110 fmol receptor, 57 pmol AGS3) were reconstituted with increasing concentrations of Gi1 heterotrimers (2-42 pmol) in 17 μ l. Following dilution into binding buffer, high affinity agonist binding was determined with 1 nM [³H]-5-HT and normalized as the percent of maximal binding achieved with a saturating amount of Gi1 (85 nM). Final conditions in the binding assay were 0.2 nM receptor with or without 104 nM AGS3 and the indicated concentrations of Gi1 in 150 μ l. Data are the mean $\pm$ SEM from 3 independent experiments. The EC₅₀ values for Gi1 were not significantly different for membranes expressing 5-HT_{1A} receptors or co-expressing AGS3 ($p > 0.05$, student t test). (B) Sf9 cell membranes (50 μ g, ~225 fmol receptor) expressing 5-HT_{1A} receptors (lanes 1-4) or co-expressing AGS3 (lanes 5-8) were reconstituted with 50 pmol of Gi1 heterotrimers in a volume of 20 μ l. The amounts of Gi1 α and AGS3 associated with the soluble and membrane fractions were estimated by immunoblotting. For each membrane condition the lanes contained: **T**: 10% of the reconstitution before pelleting; **S**: 10% of the supernatant after pelleting in a refrigerated microcentrifuge; **cP**: the entire membrane pellet (after washing 3x) from control membranes without reconstitution with Gi1; **P**: the entire membrane pellet (after washing 3x) from membranes reconstituted with Gi1. An estimated 2.5 pmol of Gi1 α and 90 pmol of AGS3 remained associated with the membranes as determined by densitometry

in comparison with standards. In three separate experiments there was no significant difference in either the amount of AGS3 associated with the membrane in the presence or absence of Gi1 or the amount of Gi1 α associated with membrane in the presence or absence of AGS3.

Figure 2. Effect of cytosolic AGS3 on receptor-G protein coupling and association of Gi1 with the membrane. (A) Sf9 cell membranes expressing 5-HT_{1A} receptors (655 fmol) were reconstituted with 2.75 pmol Gi1 heterotrimers and 71 μ g of crude cytosol from Sf9 cells expressing AGS3 (23 pmol) or from uninfected Sf9 cells in 32 μ l. Following dilution into binding buffer, high affinity agonist binding was determined with 1 nM [³H]-5-HT. Final conditions in the binding assay were 1 nM receptor, 4.2 nM Gi1 with or without 35 nM AGS3 in 200 μ l. Binding to membranes in the absence of exogenous G proteins was 360 ± 8 fmol/mg in the experiment depicted (data not shown). The open bar represents control binding to membranes reconstituted with Gi1 without either Sf9 cell cytosol. The black bars represent binding to membranes reconstituted with Gi1 in the presence of the indicated cytosol fractions. The striped bars represent cytosol fractions that were boiled prior to reconstitution. Data are the mean $\pm$ SD of triplicate determinations from a representative experiment that was repeated three times with similar results. Only the non-boiled AGS3 cytosol was different from the Gi1 control (*: $p < 0.01$, Tukey's Test). (B) Membranes expressing 5-HT_{1A} receptors were reconstituted as in (A) with the indicated components. The G-protein subunits associated with the soluble and membrane fractions were estimated by immunoblotting. The lane labeled I

contained 1 pmol Gi1 heterotrimer (40 ng Gi1 α and 36 ng G β 1) as standards. For each reconstitution the lanes contained: **T**: 10% of the reconstitution before pelleting; **S**: 10% of the supernatant after pelleting in a refrigerated microcentrifuge; **P**: the entire membrane pellet (after washing 3x). Immunoblots were repeated 3 times with similar results.

Figure 3. Association of cytosolic AGS3 with membranes and its effect on receptor-G protein coupling. Sf9 cell membranes expressing 5-HT_{1A} receptors (655 fmol) were pre-incubated with or without cytosolic AGS3 (64 pmol) in 40 μ l to allow AGS3 to associate with the membranes. Following pre-incubation the membranes were pelleted, washed and resuspended. Pre-incubated membranes were reconstituted with or without 5.5 pmol Gi1 heterotrimers in 33 μ l. **(A)** The amounts of Gi1 α and AGS3 associated with the soluble and membrane fractions from membranes pre-incubated and reconstituted under the indicated conditions were estimated by immunoblotting. The lanes contained: **T**: 10% of the reconstitution before pelleting; **S**: 10% of the supernatant after pelleting in a refrigerated microcentrifuge; **cP** and **P**: the entire membrane pellet (after washing 3x). Lanes 9 and 10 represent 10% of the total and supernatant from the first pre-incubation with AGS3 cytosol and should not be compared with lanes 7 and 8 respectively which are following the second incubation with Gi1. An estimated 19 pmol of AGS3 (lanes 5 and 6) and 275 fmol Gi1 α (lanes 4 and 6) remained associated with the membrane pellets. In three separate experiments there was no significant difference in either the amount of AGS3 associated with the membrane in the presence or absence of

Gi1 or the amount of Gi1 α associated with membrane in the presence or absence of AGS3. **(B)** Membranes treated as in (A) were diluted in binding buffer and high affinity agonist binding was determined with 1.0 nM [3 H]-5-HT. The bars correspond to lanes 3-6 in panel A. Final conditions in the binding assay were 1 nM receptor with or without 8.5 nM Gi1 and with or without 29 nM AGS3 in 200 μ l. Data are the mean $\pm$ SD of triplicate determinations from a representative experiment that was repeated three times with similar results.

Figure 4. Effect of cytosolic AGS3 on membrane-associated Gi1. Sf9 cell membranes expressing 5-HT $_{1A}$ receptors (620 fmol) were reconstituted with or without 35 pmol Gi1 heterotrimers in 45 μ l. After pelleting and washing an estimated 1.75 pmol of Gi1 α remained associated with the membranes reconstituted with Gi1. Membranes reconstituted with Gi1 were incubated a second time with buffer, 245 μ g cytosolic proteins from Sf9 cells expressing AGS3 (+**AGS3**, 78 pmol total), or 245 μ g cytosolic proteins from uninfected Sf9 cells (+**NSf9**) in a volume of 45 μ l. **(A)** The amounts of Gi1 α and AGS3 associated with the soluble and membrane fractions from membranes reconstituted as described above were estimated by immunoblotting. The lanes contained: **T**: 10% of the reconstitution before pelleting; **S**: 10% of the supernatant after pelleting in a refrigerated microcentrifuge; **cP** and **P**: the entire membrane pellet (after washing 3x). In four separate experiments the amount of Gi1 α (expressed as pmol/mg membrane protein) in lane 7 was less than that in lanes 5, 6 and 8 ($p < 0.05$, Tukey's test) while the amount in lanes 5 and 6 did not differ from one another ($p > 0.05$, Tukey's

test). **(B)** Membranes treated as described above were diluted into binding buffer and high affinity agonist binding was determined with 1.5 nM [3 H]-5-HT. Final conditions in the binding assay were 0.6 nM receptor in the presence or absence of 2 nM Gi1. The vertically striped bar represents binding to control membranes without exogenous G proteins while the black bar represents binding to membranes after a single reconstitution with Gi1. The white, grey, and horizontally striped bars represent binding to membranes after the second incubation in the presence of buffer, cytosol containing 84 nM AGS3 (final concentration), or control cytosol from uninfected Sf9 cells, respectively. Binding in the Gi1 controls (black and white bars) is not significantly different ($p > 0.05$, Tukey's test). The * indicates $p < 0.01$ vs. +Buffer (Tukey's test) while the # indicates $p < 0.05$ vs. +NSf9 (Tukey's test). Data are the mean $\pm$ SD of triplicate determinations from a representative experiment repeated 3 times with similar results.

Figure 5. Effect of cytosolic AGS3 on subcellular distribution of Gi/ α from native rat brain membranes. Rat brain membranes (10 μ g) were incubated in 50 μ l with buffer, 40 μ g of crude cytosol protein from uninfected Sf9 cells (**NSf9**) or from Sf9 cells expressing AGS3 (**AGS3**) at 0.3 pmol/ μ g. After incubation the mixtures were fractionated into a 100,000 g supernatant (**S**) and 100,000 g pellet (**P**). Lane 1 contained 2.5 μ g of membrane protein without incubation. Lanes 8 and 9 (**AGS3 –membrane**) are supernatant and pellet fractions from 40 μ g AGS3 containing cytosol incubated without the addition of membranes. After SDS-PAGE membrane transfers were immunoblotted

for G-proteins using the G α specific antisera 976. The immunoblot is representative of results obtained in four independent experiments.

Figure 6. Effect of GST-AGS3-GPR domains on receptor-G protein coupling. (A)

The general domain structure of mammalian AGS3 is shown with striped boxes for the amino-terminal TPR domains and grey boxes for the carboxyl-terminal GPR domains known to interact with G protein α subunits. **(B)** Sf9 cell membranes expressing 5-HT_{1A} receptors (187 fmol) were reconstituted with the indicated Gi/o proteins (4.25 pmol) in 17 μ l. Increasing concentrations (0.1–1,163 pmol) of GST-AGS3-GPR (Pro⁴⁶³-Ser⁶⁵⁰) fusion protein containing all four individual AGS3-GPR domains were added to the reconstitution mixtures. Following dilution with binding buffer, high affinity agonist binding was determined with 1 nM [³H]-5-HT binding and expressed as the percent maximum binding in the absence of GST-AGS3-GPR (Pro⁴⁶³-Ser⁶⁵⁰) for each G protein. Final conditions in the binding assay were 0.4 nM receptor, 8 nM G protein and the indicated concentrations (0.2–2,281 nM) of GST-AGS3-GPR in 150 μ l. The data shown are the mean $\pm$ SD of triplicate determinations from a representative experiment and the IC₅₀ values are the mean from the indicated number of independent experiments. The IC₅₀ for G α_o is significantly different from the others ($p < 0.05$, Tukey's test). **(C)** Sf9 cell membranes expressing 5-HT_{1A} receptors (655 fmol) were reconstituted with 9 pmol individual Gi/o proteins and 250 pmol of the indicated GST-AGS3 fusion proteins in 17 μ l. Following dilution into binding buffer, high affinity agonist binding was determined

with 2 nM [3 H]-5-HT. The bars represent the percent inhibition by the GST-AGS3 fusion proteins relative to the maximum binding observed after reconstitution with the indicated G proteins in the absence of GST-AGS3 fusion proteins. Data are the mean $\pm$ SEM from two independent experiments. For each G protein, the inhibition by the complete AGS3-GPR domain is greater than any individual AGS3-GPR domains ($p < 0.05$, Tukey's test). Two-way ANOVA indicates that there is no significant difference among individual AGS3-GPR domains for their interactions with G proteins although the inhibition with all four AGS3-GPR is least with Go ($p < 0.01$).

Figure 1.

Figure 2.

Figure 3.

Figure 4.

A.

B.

Figure 5.

Figure 6.

CHAPTER 4. DISCUSSION

The overall objective of this project is to investigate molecular mechanisms in GPCR-G protein coupling. Two basic aspects of GPCR-G protein coupling were studied in the present work. One was to examine the potential G protein coupling differences among closely related muscarinic receptors; the other was to explore a novel regulatory mechanism in GPCR-G protein coupling.

A variety of extracellular stimuli cause cellular responses by interacting with a superfamily of cell surface receptors known as G protein coupled receptors (GPCRs). GPCRs convey the extracellular signals into the intracellular signaling network by coupling to heterotrimeric G proteins. Upon interaction with signal molecules, GPCRs undergo conformational changes, which enable them to interact with heterotrimeric G proteins. Although it has been studied for many years, the exact molecular mechanism governing the GPCR-G protein coupling process is still not completely understood. A variety of models have been developed in an attempt to explain the mechanism of ligand-receptor and receptor-G protein interactions. An early model proposed that G protein activation was initiated upon the formation of an agonist/receptor/G-protein (ARG) ternary complex (De Lean *et al.*, 1980). However, it could not explain the observed basal activities in the absence of agonist. Based on the studies of a mutated β 2-adrenergic receptor, this ternary complex model was extended by including the concept of a constitutively active state of the receptor (Samama *et al.*, 1993). This extended ternary

complex model proposed that GPCRs could spontaneously isomerize between an inactive, resting conformation and an active conformation that is capable of interaction with G proteins. The basal activity and the mutation-induced constitutive activity of GPCRs could be well explained by the formation of the active receptor conformation. Agonists and G proteins were believed to stabilize GPCRs in the active conformation. In recent years, accumulating evidence indicates that this model is still not enough to explain the exact nature of agonist/receptor/G-protein interactions. The concept of multiple active receptor conformations has rapidly emerged. Several models have been proposed to incorporate the current observations. For example, Kenakin proposed an “agonist-trafficking” model, in which an agonist by its own can induce or select the specific receptor conformation with which it can interact (Kenakin, 1995); while Leff *et al* introduced a “three-state” receptor model, in which one inactive and two active receptor states were assumed to exist (Leff *et al.*, 1997).

It is now realized that actions of ligands on receptor-G protein coupling may depend on the nature of every component involved. In a recent review, Strange discussed the possible difference between multiple receptor active states and a single receptor active state that might be stabilized to different extents by different agonists (Strange, 1999). He further suggested that the organization of receptors and G proteins may also affect the receptor-G protein coupling properties. As pointed out by Neubig (Neubig, 1994) and Chidiac (Chidiac, 1998), receptors and G proteins in cell membranes may exist in a higher order array. Kenakin discussed the effects of stoichiometry between receptor and G proteins on the ligand-receptor interaction, indicating that alteration in the

receptor-G protein stoichiometry in recombinant receptor system may lead to abnormal ligand binding parameters as compared with those obtained from native tissues (Kenakin, 1997). Chidiac further pointed out that not only the interaction between receptors and G proteins but also the interactions within receptor/G-protein/effector complexes can affect the agonist binding to receptors (Chidiac, 1998). In summary, a specific receptor-G protein coupling process may be affected by multiple factors such as the nature of agonists, specificity of receptors, G proteins and effectors, arrangement of each component and the stoichiometry among them. Therefore, examination of each aspect of receptor-G protein coupling is needed to define the exact mechanisms for a specific receptor function and also may reveal the functional significance of the redundancy for GPCR subtypes and multiplicity of G protein subunits.

In this project, the G protein-coupling properties of five closely related muscarinic acetylcholine receptors (mAChRs) were examined in an attempt to define the potential difference in their signaling mechanisms. An Sf9 cell membrane based reconstitution system was employed to allow well-controlled stoichiometry among receptors and G proteins. An interesting finding was that M3 receptors were significantly different from other mAChRs in their G protein coupling mechanisms in an identical membrane environment (Chapter 3, Section 1). With reconstitution, the functional and selective coupling between the purified G proteins and mAChRs expressed in Sf9 cell membranes were well-established for all other mAChRs except M3 receptors as assessed by G protein-stabilized high-affinity agonist binding. The functional G protein coupling for four mAChRs (M1, M2, M4 and M5) could be explained by the extended ternary

complex model such that addition of G proteins could stabilize receptors in a high-affinity state for agonist binding. However, the G protein behavior of M3 receptors did not fit this model. As previously reported, M3 receptor retained its G protein coupling function and pharmacological properties when assessed in the intact Sf9 cells but lost its G protein coupling ability in the membrane preparations (Vasudevan *et al.*, 1992; Kukkonen *et al.*, 1996; Guo *et al.*, 2001). This may suggest that the functional G protein coupling of M3 receptors may require additional cellular components.

Since no direct *in vivo* evidence has shown that G α and G $\beta\gamma$ subunits can actually dissociate from each other upon activation by active GPCRs, it was speculated that the G protein subunits do not have to go through the “GTPase cycle” (See Figure 2 in Chapter 1) to convey the signals from receptors to effectors (Chidiac, 1998). Thus, how signals are transduced along this pathway may depend on the types and amounts of the R-G-E entities and additional determinants, which may include cell architecture and accessory proteins. As proposed by Chidiac, three possible mechanisms may be applied to explain how signals propagate along the R-G-E axis. Agonist interaction may promote the formation and the dissociation of R-G-E complexes or may interact with stable R-G-E complexes. Supporting evidence showed that receptors, G proteins and effectors were all able to segregate into oligomeric complexes *in vivo* (Post *et al.*, 1995; Huang *et al.*, 1997; Oh and Schnitzer, 2001; Gazi *et al.*, 2002) and their organization in cells appears in a higher order (Neubig, 1994; Chidiac, 1998). In this study, we noticed the inability of M3 receptors to interact with G proteins in an isolated environment. Unlike other mAChRs, M3 receptors probably employ a distinct G protein coupling mechanism, for example, it

may need the co-existence of effectors (or maybe other accessory proteins) in order to functionally couple to G proteins. The reconstitution system set up for M3 receptors will likely serve as a useful tool to screen the possible, additional components required for M3-G protein coupling. It will be of interest to test if the addition of effectors such as PLC- β could restore the functional coupling between M3 receptor and Gq protein. Verification of this issue will provide strong evidence for the possible “stable R-G-E complexes” model as proposed by Chidiac (Chidiac, 1998).

It has been noticed that the stoichiometry between GPCRs and G proteins can affect their interactions. For example, receptors at high concentrations can activate G protein in the absence of agonists and different R-G ratios had different effect on their ligand binding properties as assessed in transfected cell lines or in reconstitution systems (Gudermann *et al.*, 1996). As demonstrated from *in vivo* studies, receptors were found to be outnumbered by G proteins and the accessible receptor and G protein repertoires appear to be limited in microdomains in cells (Neubig, 1994; Post *et al.*, 1995; Oh and Schnitzer, 2001). For GPCRs with highly homologous subtypes such as mAChRs, when they have the access to the same G protein population, will their interactions with G proteins be different? Understanding of this mechanism will be helpful to define the functional significance of a specific receptor subtype. Jakubik *et al* reported that different R-G ratios in reconstitution had different effects on G protein activation and receptor ligand binding properties and that the densities of receptors and G proteins had little effect on ligand binding if their ratios were kept constant (Jakubik *et al.*, 1998). Therefore, they concluded that *in vivo* R-G ratios may also remain constant even though

they cluster in the cell microdomains. In this study, we examined the apparent affinities of G proteins for their interactions with mAChRs. The high-affinity agonist binding on mAChRs was not only dependent on the nature, but also the concentration, of the G proteins. For example, M1 and M5 receptors could be stabilized in a high-affinity agonist binding state by both Gq and Gi1 proteins in a concentration dependent manner (Figure 2 in Chapter 3, Section 1). However, Gi1 was less effective than Gq in stabilizing high-affinity agonist binding. Gq-coupled M1 and M5 receptors had similar apparent affinities for Gq while Gi-coupled M2 and M4 receptors had similar apparent affinities for Gi1. However, the apparent affinities for a same G protein such as Gi1 appear to be different between the Gq- and Gi-coupled mAChRs. These results indicate that the difference in their apparent affinities may correlate to their G protein coupling selectivity.

As for Gi coupling, previous studies showed that the saturating responses could be achieved with picomolar concentrations of purified Gi or nanomolar concentrations (<30 nM) of purified G $\beta\gamma$ subunits when examining the K⁺ channel activation using isolated membrane patches from either atrial cells or endocrine GH3 cells (Yatani *et al.*, 1987; Codina *et al.*, 1987; Cerbai *et al.*, 1988). In previous studies from our laboratory, nanomolar concentrations of G protein heterotrimers were shown to be able to saturate the high-affinity agonist binding on 5-HT receptors and with EC₅₀ values around 10 nM (Clawges *et al.*, 1997). In this study, Gi-coupled M2 and M4 receptors required several hundred nanomolars of G protein heterotrimers to achieve their saturating response. A significant difference in the mAChR reconstitution was that urea-extraction of receptor membranes was required for their coupling to exogenous G proteins. We showed that

urea-extraction dramatically improved the functional coupling between the membrane mAChRs and the purified G proteins in reconstitution and reduced the endogenous G protein coupling in the membranes. However, the apparent affinities for G protein coupling were also altered after urea-extraction. For non-extracted M2 receptor membranes, the EC_{50} of Gi1 protein was about 47 nM (Slessareva, 2003), which was much lower than the 412 nM required for the extracted M2 receptor membrane. An unsolved question is why urea-extraction dramatically changed the apparent affinities between Gi-coupled mAChRs. The possible explanation could be that the extraction released the restraints for the access of M2 receptors to the added G proteins such as the removal (or inactivation) of the endogenous G proteins or other possible peripheral proteins. Consequently, the proportion of M2 receptors that was available for G protein interactions increased. As reported by Hartman and colleagues, after urea-extraction of Sf9 cell membrane, 60-70% of the membrane-associated proteins were removed and the GTP γ S binding on the extracted membranes was reduced by 75% (Hartman, IV and Northup, 1996). However, Lim and Neubig reported that urea-extraction only partially removed G $\beta\gamma$ but not Gi α subunits from the membrane and that the uncoupling effect by urea was due to the inactivation of the endogenous G proteins. Studies from our laboratory indicate that only a small portion of Gq-coupled receptors couple to the endogenous G proteins (Slessareva, 2003). Thus, the removal or inactivation of endogenous G proteins can not completely explain the successful establishment of the functional coupling between the muscarinic receptors and the exogenous G proteins after urea-extraction. Another possibility is that the lipid membrane environment was changed

so that more receptors could be stabilized in the high-affinity state by the exogenous G proteins. Further investigation is necessary to explore the effects of urea-extraction on the membrane proteins and membrane architecture. It should be kept in mind that the biochemical characteristics revealed in reconstitution studies may not reflect those *in vivo* situations. However, five mAChRs clearly displayed different biochemical features in an identical environment and their selective G protein coupling profiles were in good agreement with those obtained from a variety of systems. They exhibited differences not only in their apparent affinities for G protein interactions but also in their agonist binding states when coupled to G proteins. Our data showed that M3 receptors only had a low affinity state regardless of the G protein reconstitution (Figure 3 in Chapter 3 Section 1). M1 and M5 receptors were not different in their binding parameters when reconstituted with Gq proteins, but were different when reconstituted with Gi1 proteins. For Gi-coupled M2 and M4 receptors, their agonist binding affinities were not different when they were reconstituted with control buffer or Gq proteins. When reconstituted with Gi1 proteins, M4 receptors had a lower agonist binding affinity but a higher proportion of receptors that were in high-affinity state as compared with M2 receptors. Therefore, even for the same agonist, mAChRs displayed subtype- and G-protein-specific affinity states. Taken together, these results indicate that the intrinsic G protein coupling properties vary among different GPCRs that even share high similarities in their sequences, structures and signal transduction pathways.

Another aim of this project was to study a novel regulatory mechanism in GPCR-G protein coupling. It has now been appreciated that G protein mediated signal transduction pathways are subject to regulation at multiple levels. To specify appropriate cellular responses upon GPCR activation, many accessory proteins and a variety of mechanisms have been employed in addition to those confined to the agonist-receptor-G protein interface as discussed above.

The actions of agonists on GPCRs are usually rapidly attenuated. Some accessory proteins have been identified to regulate the GPCR attenuation process, including GRKs (G-protein receptor kinases), PKA (protein kinase A), PKC (protein kinase C), arrestins, GRPs (GPCR phosphatases) and recoverin. GRKs are a family of protein kinases that recognize the agonist-occupied, activated GPCRs and disrupt their coupling to G proteins by phosphorylating GPCRs (see Pitcher *et al.*, 1998 for review). Another family of proteins called arrestins is also involved in GRK-mediated signal termination (Krupnick and Benovic, 1998). They can interact with phosphorylated GPCRs and direct the subsequent GPCR internalization. In addition to agonist-dependent phosphorylation by GRKs, some second messenger dependent kinases such as PKA and PKC also participate in GPCR phosphorylation and desensitization, but in an agonist-independent way (Lefkowitz, 1993). GRK-phosphorylated GPCRs may be dephosphorylated by specific GPCR phosphatases (GRP) (Palczewski *et al.*, 1989; Pitcher *et al.*, 1995). A unique protein called recoverin has been found to regulate photoreceptor, rhodopsins (Dizhoor *et al.*, 1991). Besides these regulatory proteins, internalization and down-regulation of cell surface GPCRs also serve as important regulatory mechanisms in attenuating GPCR

mediated signaling (Bohm *et al.*, 1997). As discussed above, the specific expression, organization and stoichiometry of the primary components in G protein signaling pathway all influence the specific signaling initiated on GPCRs.

Heterotrimeric G protein activity is also subject to regulation by multiple processes. G proteins switch to their active state upon the guanine nucleotide exchange on their α subunits. Proteins called GEFs (guanine nucleotide exchange factors) stimulate the guanine nucleotide exchange on G α subunits (Luo and Denker, 1999; Cismowski *et al.*, 2000). Once GTP is bound to G α subunits, the intrinsic GTPase activity of G α subunits catalyzes GTP hydrolysis to ensure the termination of signaling. A superfamily of proteins, RGS (regulators of G protein signaling) have been found to act as GAPs (GTPase activating proteins) to accelerate the GTP hydrolysis (Berman and Gilman, 1998; Hepler, 1999). Recently, a novel class of proteins has been identified, which can activate G protein signaling independently of GPCRs. (Cismowski *et al.*, 1999; Takesono *et al.*, 1999). In yeast with a GPCR-defect genetic background, these proteins were found to activate the G $\beta\gamma$ -mediated growth and mating pathway. Collectively, they were called activators of G protein signaling (AGS). A member of this family, AGS3 has been found to belong to a novel class of proteins containing G protein regulatory (GPR) motifs (also termed GoLoco domains). Although these proteins have diverse functions, they share the ability to bind G α subunits of the Gi family *via* their GPR domains (De Vries *et al.*, 2000; Natochin *et al.*, 2000; Peterson *et al.*, 2000; Bernard *et al.*, 2001; Kimple *et al.*, 2001; Natochin *et al.*, 2001; Pizzinat *et al.*, 2001; Peterson *et al.*, 2002).

Little is known of the functional role of AGS3 in cellular signaling. Biochemical studies showed that AGS3 could effectively compete with G $\beta\gamma$ subunits for binding to Gi α subunits (Bernard *et al.*, 2001). Since G protein signaling requires a G protein heterotrimer at the inner surface of the plasma membrane and AGS3 can disrupt the G protein heterotrimer, we hypothesized that AGS3 might interfere with receptor coupling to Gi proteins. In this study, we examined the effects of AGS3 on receptor-Gi protein coupling using a membrane based reconstitution system. Previous work from our laboratory successfully established the functional coupling between purified Gi proteins and 5-HT_{1A} receptors expressed in Sf9 cell membranes (Clawges *et al.*, 1997). It was shown that the functional G protein coupling of 5-HT_{1A} receptors required the presence of complete Gi protein heterotrimers. Therefore, it provides an effective system to test the effects of AGS3 on functional receptor-Gi protein coupling. Our results from both high-affinity agonist binding assays and immunoblot assays indicated that AGS3 can sequester Gi α subunits in the cytosol fraction, thus decreasing the amounts of G protein heterotrimers in the membrane (Figure 2 in Chapter 3, Section 2). The receptor-G protein coupling was consequently inhibited. Consistent with other studies (Bernard *et al.*, 2001), AGS3 did not affect the G $\beta\gamma$ subunit membrane partition. However, when AGS3 was expressed in the membrane, it was unable to interact with Gi protein. After the functional, soluble, AGS3 partitioned in the membrane, it also lost its activity to interact with Gi α , indicating that the function of AGS3 is affected by its membrane interaction. The remaining questions are how AGS3 associates with the membrane and how its membrane association may regulate its function in a cellular setting. Pizzinat *et al* speculated that the

amino terminal half of AGS3 molecule, which contains seven repeated TPR (tetratricopeptide repeat) sequences, might determine its subcellular localization (Pizzinat *et al.*, 2001). However, another group reported that both the carboxyl-terminal GPR and amino-terminal TPR domains are required for the apical membrane targeting of Pins, an AGS3/LGN ortholog from *Drosophila* (Yu *et al.*, 2002). With regard to the inability of membrane AGS3 to sequester $G_{i\alpha}$, it can not be excluded that unidentified signaling inputs may initiate the interaction of membrane-partitioned AGS3 with $G_{i\alpha}$ subunits.

On the other hand, the majority of cellular AGS3 is in the cytosol fraction and appears to co-localize with some unidentified subcellular microdomains (De Vries *et al.*, 2000; Bernard *et al.*, 2001). Considering that G protein heterotrimers are translocated to the plasma membrane after synthesis in the cytosol, it may suggest that the function of AGS3 is related to the $G_{i\alpha}$ trafficking. In fact, the GDIs for small G proteins generally complex with small G proteins in the cytosol and prevent their translocation to the plasma membrane. Our results suggest the possibility of a similar role for AGS3 with heterotrimeric G proteins. When G protein heterotrimers were incubated with AGS3 in the cytosol, AGS3 completely blocked the membrane-partition of $G_{i\alpha}$ subunits with no effect on $G_{\beta\gamma}$ membrane-partition. In a cellular setting, this role might have two possible outcomes, either resulting in inhibition of the signaling mediated by G protein heterotrimers at the plasma membrane as observed in our reconstitution studies, or resulting in up-regulation of $G_{\beta\gamma}$ -specific signaling as observed in the yeast-based functional studies (Takesono *et al.*, 1999). However, it can not be excluded that the resulting AGS3- $G_{i\alpha}$ -GDP complex may also be a functional entity in cells. Furthermore,

our data showed that cytosolic AGS3 not only disrupted the membrane partition of Gi α but also dissociated Gi α from the membrane (Figure 4 in Chapter 3, Section 2). Therefore, it is possible that AGS3 may regulate the basal activity in G protein signaling by balancing the available amounts of G protein heterotrimers at the plasma membrane or the cellular AGS3 level may be up-regulated in response to unidentified signaling inputs that will lead to the down-regulation of G protein signaling at the plasma membrane. Future studies are required to confirm the physiological relevance of AGS3 in a cellular setting. It would be interesting to test if AGS3 and Gi α may mutually interfere with their subcellular distribution in co-expression experiments. Clearly our studies provide new insights and starting points for the possible functions of AGS3 in regulating G protein-mediated signaling.

Sequence analysis reveals that the AGS3 molecule has four GPR domains. This GPR sequence is the unique feature of a variety of proteins with diverse functions and conserved in different species (Kimple *et al.*, 2002). These proteins contain one to four GPR sequences. For proteins containing multiple GPR sequences, GPR sequences are repeated in tandem arrays. Characterization of GPR sequences reveals that it acts as a GDI for the α subunits from Gi protein family (De Vries *et al.*, 2000; Natochin *et al.*, 2000; Peterson *et al.*, 2000; Bernard *et al.*, 2001; Kimple *et al.*, 2001; Natochin *et al.*, 2001; Pizzinat *et al.*, 2001). GPR domains selectively interact with GDP-bound Gi α units and inhibit the GDP dissociation from Gi α subunits. Bernard *et al* reported that the four GPR domains of AGS3 were all able to compete with G $\beta\gamma$ for Gi α binding (Bernard *et al.*, 2001). Consistent with that work, our results showed that the four GPR domains were

also able to interfere with receptor-Gi protein coupling (Figure 6 in Chapter 3, Section 2). In Bernard's report, it was noticed that the full-length GPR domain could bind multiple copies of Gi α subunits at the same time (Bernard *et al.*, 2001), suggesting a possible scaffolding function for the complete GPR domain. Our results indicated that the full-length GPR domain was more effective than individual GPR domains, which was consistent with Bernard's interpretation. However, the effects of the full-length GPR in inhibition of receptor-Gi coupling did not exactly correlate with numbers of GPR domains when compared with individual GPR domains at equal concentrations (Figure 6 in Chapter 3, Section 2). Possibly, the spatial arrangement of four GPR domains may have an impact on their stoichiometry in Gi α interactions. In contrast to the conclusion of Kimple and colleagues (Kimple *et al.*, 2002), our results showed that GPR domains also could interact with Go α , although with a lower affinity than those for other Gi α subunits (Figure 5 & 6 in Chapter 3, Section 2). Considering the fact that Go α is by far the most abundant G α subunit in brain membranes (Sternweis and Robishaw, 1984; Huff *et al.*, 1985) and that AGS3 is enriched in brain tissues (De Vries *et al.*, 2000; Bernard *et al.*, 2001; Pizzinat *et al.*, 2001), it is perhaps more likely that this weak interaction ensures that only appropriate amounts of AGS3 and Go α form the complexes in a cellular setting.

In summary, the present work studied the difference in the G protein coupling properties of five closely related muscarinic receptors, the effects of AGS3 on receptor-G protein coupling and its possible molecular mechanisms in regulating G protein signaling. Our results indicate that at biochemical level five mAChRs displayed different G protein

coupling behaviors. Among them, M3 receptors may have a unique G protein coupling mechanism. AGS3 can selectively interfere with receptor coupling to Gi/o proteins. Cytosolic AGS3 can remove Gi α from the membrane and sequester Gi α in the cytosol, suggesting that it may down-regulate heterotrimeric G protein signaling by interfering with receptor coupling.

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APPENDIX I.

CONSTRUCTION OF CHIMERIC G PROTEIN α SUBUNITS

Introduction

Heterotrimeric G proteins mediate the signaling transduction across the plasma membrane from a superfamily of heptahelical cell surface receptors (GPCRs). A striking characteristic in this signal transduction system is the redundancy of each component. Highly conserved sequence and structure features exist in both heterotrimeric G proteins and GPCRs. Thus, an important question is how selective interactions between G proteins and GPCRs are achieved to ensure appropriate responses. Two most important concepts developed so far are that the selective G protein coupling results from the cooperative contribution of multiple intracellular regions and that the relative contributions of different intracellular domains vary among individual receptors (Wess, 1998).

A great number of studies have been done to determine the structural basis of G proteins for their selective coupling to GPCRs. Although both G β and G γ subunits have also been shown to directly interact with GPCRs (Phillips and Cerione, 1992; Heithier *et al.*, 1992; Kisselev *et al.*, 1994; Kisselev *et al.*, 1995; Taylor *et al.*, 1996; Azpiazu *et al.*, 1999; Ernst *et al.*, 2000), most G $\beta\gamma$ complexes appear to function similarly (Clapham and Neer, 1997). Extensive efforts have been made on G α subunits because it has been demonstrated that G α subunits are the primary determinant of heterotrimeric G proteins for their selective coupling to GPCRs. In attempt to delineate the G α subunit domains that determine their selective coupling to the five highly homologous muscarinic acetylcholine receptors (mAChRs), our laboratory designed, constructed and applied a variety of chimeric G α subunits. As recently reported by Slessareva (Slessareva and

Graber, 2003), a unique 6 amino acid extension at the amino terminus and 35 amino acids at the carboxyl terminus of Gq α subunit was critical for its selective coupling to muscarinic M1 receptors. When both of these regions on Gq α displaced those on Gi1 α subunit, the resulting chimeric Gi1 α , Gi1/q-6N35C (see Figure 1 A&B), lost its coupling to M2 receptors but gained coupling to M1 receptors. When either the amino or carboxyl terminus was replaced alone, none of the resulting chimeras (Gi1/q-6N and Gi1/q-35C) was able to gain M1 receptor coupling, indicating that both regions are required for M1 receptor coupling. To test the generality of these regions on G α subunits in their selective mAChR-coupling, we constructed a series of chimeric G α subunits in pair as depicted in Figure 1 A&B and summarized below.

Methods

The construction of the Gi1-based chimeras, Gi1/q-35C and Gi1/q-6N35C has been described (Slessareva and Graber, 2003). To further define the critical role of the short amino terminal region on G α subunits in determining selective coupling to muscarinic receptors, we designed another Gi1-based chimera, Gi1/q-37N35C, in which the amino terminal region was extended to cover the whole α N helix was replaced (see Figure 1A&B). To construct this Gi1/q-37N35C chimera, the coding region for the N-terminal 37 amino acid residues of G α q was amplified with PCR using human G α q cDNA as a template. An upstream Nco I site and a silent Dra III site at the 37th amino acid residue position were introduced into the forward and reverse primers, respectively (See below).

Forward primer: 5' — CTTGGTACCATGGATGACTCTCGAG

Nco I

Reverse primer: 5' — CAGTTTTAGATCACGACGTGCGTCC

Dra III

The G α i1 internal fragment from the 32nd to 217th amino acid residue were also amplified with PCR by using the Gi1/q-35C construct as a template to take advantage of a unique Bam HI site at the 217th amino acid residue position. A Dra III site and a Bam HI site were introduced into the forward and reverse primer, respectively.

Forward primer: 5' — ATGGAGAAAAAGCACGTCGTGAGG

Dra III

Reverse primer: 5' — CAAAGCAGTGGATCCACTTCTTCC

Bam HI

After digestion with their corresponding restriction enzymes, the two PCR products were ligated into the pVLKD-Gi1/q-6N35C construct (Slessareva and Graber, 2003), which had the Nco I -- Bam HI fragment removed before ligation.

As the counterparts of Gi-based chimeras, three Gq-based chimeras were also constructed. To construct Gq/i1-31N25C, three DNA fragments were amplified with PCR using human G α q and rat G α i1 cDNAs as templates, correspondingly:

- 1) the coding region for the N-terminal 31 amino acid residues of G α i1 was amplified with a forward primer containing an upstream Bam HI site and a reverse primer containing a silently introduced Xma I site at the 31st amino acid residue position;

Forward primer (fp1): 5' -- CATCGCTAGCGGATCCAT

Bam HI

Reverse primer (rp1): 5' -- GATTTCCCGGGCTGCCTT

Xma I

- 2) the coding region for the N-terminal 38th to the –25th amino acid residues of G α q was amplified with the following primers:

Forward primer (fp2): 5' -- GATATACCCCGGGAGCTCAA

Xma I

Reverse primer (rp2): 5' -- gcgcATGTGAAGTGGGAGTA

(The lower cases in the reverse primer above indicate the RNA components in this hybrid DNA/RNA primer. The RNA sequence was used as a template to obtain its DNA complementary sequence by reverse transcriptase and then was hydrolyzed with 0.5 N NaOH to generate a 3' DNA overhang for the following ligation step)

A silent Xma I site was introduced at the 38th amino acid residue position and a 3' DNA overhang at the -25th amino acid residues position was generated with the DNA overhang cloning method (Coljee *et al.*, 2000);

- 3) the coding region for the C-terminal 25 amino acid residues of G α i1 with a 3' DNA overhang DNA overhang at the -25th amino acid residue position and a downstream Pst I site was amplified with the following primers:

Forward primer (fp3): 5' -- gcgcAACGGATACGAAGAAT

Reverse primer (rp3): 5' -- CTACGCGTCTGCAGAGCTTA
Pst I

(The lower cases in the forward primer above indicate the RNA components in this hybrid DNA/RNA primer.)

The second and the last PCR fragments were first ligated at their complementary 3'-overhang sites and PCR-amplified with the primers fp2 and rp3. After digestion with Xma I and Pst I, this jointed fragment was ligated into pVL1393 vector together with the first PCR fragment, which was pre-digested with Bam HI and Xma I before ligation.

The construction of other two Gq-based chimeras began with the construct of pVL1393-Gq, in which the G α q cDNA was excised from its pCDNA3.1⁺ vector (obtained from Guthrie cDNA resource center, Sayre, PA) at Nhe I and Pst I sites and ligated into pVL1393 vector at Xba I and Pst I sites. Nhe I and Xba I sites are compatible but both are destroyed after ligation. The Gq/i1-25C construct was obtained by replacing the Eco RI--Pst I fragment of the pVL1393-Gq with that from the Gq/i1-31N25C construct. The Gq/i1-5N25C was constructed by first removing the 11 N-terminal amino

acid coding region from the Gq/i1-25C at an upstream Bam HI site and an Eco NI site located at the 12th residue. The removed fragment was then replaced with an oligonucleotide linker coding the 5 N-terminal amino acid residues of G α i1.

Oligonucleotide linker:

```
5'—GATCCTAGGATGGGCTGCACACTGA ---3'
3'---- GATCCTACCCGACGTGTGACTC --5'
```

All constructs were confirmed by restriction analysis and DNA sequencing before they were used to produce recombinant baculoviruses.

Future studies

The availability of the chimeric G α constructs described above has made a basis for further delineating of the structural determinants of G proteins for their selective mAChR (muscarinic acetylcholine receptor) coupling and examining the generality of the defined domains in future experiments. For M1 and M2 receptors, the proper context on both amino and carboxyl terminal regions of G α subunits has been shown to be sufficient to specify their coupling from the studies on a chimeric G α subunit, Gi1/q-6N35C (Slessareva and Graber, 2003). Our preliminary data show that this chimera can also gain the coupling to M5 receptors and lose the coupling to M4 receptors. Thus, by further characterizing these domains and testing their generality in mAChR-G protein coupling, the common structural requirements for the selective G protein-mAChR coupling may be revealed or the structural determinants may be identified that can distinguish mAChR subtypes, which share the same G protein coupling selectivity.

A potential problem could hinder the characterization of Gq-based chimeras (Gq/i1-25C, Gq/i1-5N25C and Gq/i1-31N25C, Figure 1) with purified proteins in reconstitution. Initial characterization of their expression in Sf9 cells indicated that these chimeras are largely found in the membrane fraction as are the wild type Gq α subunits. As reported previously (Kozasa and Gilman, 1995; Slessareva and Graber, 2003), possible contamination by the endogenous Gi proteins may occur during protein purification from the membranes. Receptor coupling of the endogenous Gi protein can be abolished with pertussis toxin treatment, which can catalyze the ADP-ribosylation at a

carboxyl terminal cysteine residue in Gi/o α subunits (corresponding to the cysteine residue at -4 position of Gi1 α in Figure 1B) and uncouple Gi/o protein from GPCRs (West, Jr. *et al.*, 1985). Since the ADP-ribosylation site is present in these chimeras, the gain of their Gi activity is unlikely to be distinguished from the endogenous Gi contamination by the pertussis toxin treatment. Thus, alternative approaches may be needed to examine their coupling selectivity. For example, an alanine can be used to replace the cysteine residue by point mutation in order to get rid of the ADP-ribosylation site. Or, their selective receptor coupling activity can be examined in whole-cell assay system. In fact, preliminary data (not shown) from our collaborators showed a robust response in testing the M2 receptor coupling to the Gq-based chimera, Gq/i1-5N25C using a whole-cell based assay system, in which the M2 receptor coupling activity is amplified by a reporter gene. However, such a response was not observed for the M2 coupling to another Gq-based chimera, Gq/i1-31N25C. In addition, when the amino terminal region in the Gi1/q-6N35C was extended to cover the α N helix region (Figure 1A&B), the resulting chimera, Gi1/q-37N35C lost its activity as both Gi1 and Gq (data not shown). Taken together, these data suggest that the short region (5 residues in Gi1 and 11 residues in Gq) at G α amino terminus is critical in determining their selective coupling to mAChRs. Thus, it will be interesting to test the critical role of this sort region in G protein-mAChR selective coupling in future experiments and it will also be interesting to test if this role may apply for other GPCRs.

Figure 1. Schematic representation of G α i1, G α q and their chimeras and sequence alignment of their amino and carboxyl terminal regions. *1A.* To explore the structural determinants of selectivity in receptor-G protein coupling, chimeric G α subunits comprised of various regions of G α i1 and G α q were constructed. Numbers at the end of each α subunit structure indicate the total amino acid residues. The bottom diagram depicts the secondary structural domains common to G α subunits. *1B.* Amino acid sequence alignments at the amino (upper panel) and carboxyl (lower panel) termini of G α i1 and G α q α subunits. The letters in bold indicate the different amino acid residues between these two α subunits. The shaded regions represent the sequences that form the corresponding secondary structures (α -helix or β -sheet) as indicated at the top portions in each panel. The numbers point to the positions of the amino acid residues counted either from the amino terminus or the carboxyl terminus. *N'*, the amino terminus; *C'*, the carboxyl terminus.

Figure 1A.

Figure 1B.

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APPENDIX II

Closely related G protein coupled receptors use multiple and distinct domains on G protein α subunits for selective coupling

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Abbreviations and Textual Footnotes—¹The abbreviations used are: GPCRs, G protein coupled receptors; GTP γ S, guanosine 5'-3-*O*-(thio)triphosphate; OXO-M, Oxotremorine-M; 5-HT, Hydroxytryptamine; CCPA, chloro-N⁶-cyclopentyladenosine, R-PIA, R-phenylisopropyl adenosine.

ABSTRACT

The molecular basis of selectivity in receptor-G protein coupling has been explored by comparing the abilities of G protein heterotrimers containing chimeric G α subunits, comprised of various regions of Gi1 α , Gt α and Gq α , to stabilize the high affinity state of serotonin, adenosine and muscarinic acetylcholine receptors. The data indicate that multiple and distinct determinants of selectivity exist for individual receptors. While the A1 adenosine receptor does not distinguish between Gi1 α and Gt α sequences, the 5-HT_{1A} and 5-HT_{1B} serotonin and M2 muscarinic receptors can couple with Gi1 but not Gt. It is possible to distinguish domains that eliminate coupling and hence are defined as “critical”, from those that impair coupling and hence are defined as “important”. Domains within the N terminus, α 4 helix, and α 4 helix- α 4/ β 6 loop of Gi1 α are involved in 5-HT and M2 receptor interactions. Chimeric Gi1 α /Gq α subunits verify the critical role of the C terminus in receptor coupling, however, the individual receptors differ in the G α amino acids that are required for coupling. Furthermore, the EC₅₀ for interactions with Gi1 differ among the individual receptors. These results suggest that coupling selectivity ultimately involves subtle and cooperative interactions among various domains on both the G protein and the associated receptor as well as the G protein concentration.

INTRODUCTION

A large number of diverse seven transmembrane spanning cell surface receptors mediate signaling to a variety of intracellular effectors by coupling to the heterotrimeric guanine nucleotide-binding regulatory proteins (G proteins) (Pierce *et al.*, 2002). The mechanisms responsible for selectivity in G protein mediated signaling pathways are not fully understood (Wess, 1998; Albert and Robillard, 2002). Although it is known that at the molecular level the selectivity in G protein-receptor coupling is determined by amino acid sequences of both receptor and G protein, the individual amino acids involved in this selective recognition have not been completely identified. Different receptor systems and different methodologies indicate that the in the G α subunit C terminus and α 5 helix (Hamm *et al.*, 1988; Conklin *et al.*, 1993; Gilchrist *et al.*, 1998; Natochin *et al.*, 2000), N terminus and α N helix (Hamm *et al.*, 1988; Hepler *et al.*, 1996; Kostenis *et al.*, 1997; Swift *et al.*, 2000), α 4 helix and α 4/ β 6 loop (Bae *et al.*, 1997; Natochin *et al.*, 1999; Blahos *et al.*, 2001), α 2 helix and α 2/ β 4 loop (Lee *et al.*, 1995), α 3/ β 5 loop (Grishina and Berlot, 2000), α N/ β 1 loop (Blahos *et al.*, 2001) and amino acids 110-119 from the α helical domain (Krieger-Brauer *et al.*, 1999) are involved in receptor coupling selectivity. Some of these domains contact the receptor directly, while others regulate receptor coupling selectivity indirectly by playing a role in nucleotide exchange. Despite the fact that many of the receptor interacting domains have been identified, the relationship between receptor subtypes and G α domains involved in receptor coupling has not been clearly established. Thus, it is difficult to predict which

G α domains will be utilized by a specific receptor. Here we propose that individual receptors recognize specific patterns formed by amino acids of G α thus making G protein interface look different for different receptors. The C terminus of G α is a well accepted receptor recognition domain, which contacts receptors directly (Conklin and Bourne, 1993). Although individual C terminal amino acids important for receptor coupling have been identified in several G α subunits, the specific G α amino acids participating in receptor recognition may differ among receptors. The α 4 helix- α 4/ β 6 loop domain, first described as an effector domain, has been shown to be important for 5-HT_{1B} receptor coupling to Gi1 (Bae *et al.*, 1997). Later it was demonstrated that Gln304 and Glu308 in the α 4 helix of Gi1 α are important for 5-HT_{1B} receptor coupling (Bae *et al.*, 1999). However the generality of the role for the α 4 helix- α 4/ β 6 loop domain in receptor coupling selectivity has not been determined.

Gi1 α and Gt α are closely related G α subunits, which belong to the Gi/o class of G protein α subunits, share 68% homology, and have nearly identical overall structures. Although the 5-HT_{1B} receptor discriminates between Gi1 and Gt (Skiba *et al.*, 1996; Bae *et al.*, 1997) the fact that their C termini are identical render Gi1 α /Gt α chimeras useless for exploring the role of this domain in receptor coupling. However, the extreme C terminus of Gq α differs from that of Gi1 α by four amino acids, while their α 5-helices differ by additional nine amino acids. Thus Gi1 α /Gq α chimeras are ideal for studying the role of this domain in coupling. Since several different GPCRs¹ can couple to the same G protein, we wanted to test the hypothesis that individual receptors utilize slightly

different domains on G α subunits to achieve coupling. G protein-receptor coupling selectivity may also be regulated at the level of G protein concentration. In fact, Clawges *et al.* demonstrated that 5-HT_{1A} and 5-HT_{1B} receptors distinguish themselves by the affinity with which they interact with G proteins (Clawges *et al.*, 1997). Therefore we also wanted to test the generality of this mechanism with different receptors. Here we compare the coupling behavior of four Gi/o-coupled receptors (5-HT_{1A} and 5-HT_{1B} serotonin, A1 adenosine and M2 muscarinic) by reconstituting them with G protein heterotrimers containing native or chimeric G α 's composed of G α i1, G α t and G α q. Our data demonstrate that selective coupling between Gi1 and the members of Gi/o-coupled receptor family is directed by multiple and distinct G α domains and is regulated at the level of G protein concentration.

EXPERIMENTAL PROCEDURES

Materials. [^3H]-Oxotremorine-M Acetate ([^3H]-OXO-M) (85.8 Ci/mmol), [^3H]-Hydroxytryptamine Binoxalate ([^3H]-5-HT) (25.5 Ci/mmol) and [^3H]-Chloro-N⁶-cyclopentyladenosine ([^3H]-CCPA) (30 Ci/mmol) were from New England Nuclear Life Science Products, Inc. (Boston, MA). Atropine Sulfate, 5-Hydroxytryptamine (5-HT) and R-phenylisopropyl adenosine (R-PIA) were from Sigma-Aldrich Corporation (St. Louis, MO). Adenosine deaminase was from Roche Molecular Biochemicals (Indianapolis, IN). The BCA Protein Assay reagents were from Pierce (Rockford, IL). All other chemicals were from Sigma-Aldrich Corporation (St. Louis, MO) or EMD Biosciences (formerly Calbiochem-Novabiochem Corporation; San Diego, CA).

Expression and purification of proteins. The expression and purification of the G α_{i1} and G $\beta\gamma$ subunits was as previously described (Graber *et al.*, 1992a; Graber *et al.*, 1992b). The chimeric G α_{i1} /G α_t subunits were constructed, expressed in *E. coli* and purified as described (Skiba *et al.*, 1996). The Gi1/Q3C, Gi1/Q5C and Gi1/Q11C chimeras were made from pHis₆G α_{i1} using the silent BamHI site introduced at amino acid position 212 (Skiba *et al.*, 1996). The pHis₆G α_{i1} cDNA was amplified by PCR reaction with primer oligonucleotides containing the desired mutations. The PCR products were digested with BamHI and HindIII, and the BamHI-HindIII fragment was used to replace the corresponding fragment from pHis₆G α_{i1} . To construct Gi1/Q35C, the C-terminal portion of a Gq α cDNA was amplified by PCR reaction, followed by digestion with BglII and HindIII. The digested PCR fragment was inserted into the BglII and HindIII sites of

the Chi13 plasmid (Bae *et al.*, 1997). Functional characterization of all bacterial subunits included GTP γ S binding, AlF $_4^-$ -dependent conformational change (measured as an increase in intrinsic tryptophan fluorescence) or binding to the cGMP phosphodiesterase γ subunit (Skiba *et al.*, 1996; Bae *et al.*, 1997; Bae *et al.*, 1999).

Preparation of Sf9 membranes containing expressed receptors. Sf9 cells were infected with a recombinant baculovirus expressing the desired receptor, cultured and harvested as previously described (Graber *et al.*, 1992b). To prepare membranes, harvested cells were thawed in 15x their wet weight of ice cold homogenization buffer (10 mM Tris-Cl, pH 8.0 at 4 °C, 25 mM NaCl, 10 mM MgCl $_2$, 1 mM EGTA, 1 mM DTT, 0.1 mM PMSF, 20 μ g/ml of benzamidine and 2 μ g/ml of each of aprotinin, leupeptin and pepstatin A) and burst by nitrogen cavitation (600 psi, 20 minutes). Cavitated cells were centrifuged at 4 °C for 10 minutes at 500 x g to remove the unbroken nuclei and cell debris. The supernatant from the low speed spin was centrifuged at 4 °C for 30 min at 28,000 x g. The supernatant was discarded and the pellets were resuspended and pooled in 35 ml of HE buffer (5 mM NaHEPES, 1 mM EDTA, pH 7.5) containing the same protease inhibitors as used in the homogenization buffer. Adenosine receptor HE buffer included 100 mM NaCl in addition to the above components. The membranes were washed twice in HE, resuspended in the same buffer at a concentration of 1-3 mg protein/ml, aliquoted, snap frozen in liquid nitrogen, and stored at -70 °C.

Reconstitution of receptors with exogenous G-proteins. Frozen membranes were thawed, pelleted in a refrigerated microcentrifuge (10 min, 12,000 rpm) and resuspended at about 10 mg/ml in a reconstitution buffer consisting of 5 mM NaHEPES, 100 mM

NaCl, 5 mM MgCl₂, 1 mM EDTA, 500 nM GDP, 0.04% CHAPS (0.08% CHAPS for M2 receptor), pH 7.5. G protein subunits were diluted in the same buffer such that the desired amount of subunit was contained in 1-5 µl. Typically, 1-2 µl of G protein subunits were added to 40 µl of membrane suspension, the mixture was incubated at 25 °C for 15 minutes and held on ice until the start of the binding assay.

Radioligand binding. Just prior to the start of the binding assay the reconstitution mixture was diluted 10-12 fold with binding assay buffer appropriate to the receptor of interest such that the desired amount of membranes (5-25 µg/assay tube) were contained in 10-50 µl. Binding buffer for 5-HT and M2 receptors was 50 mM Tris, 5 mM MgCl₂, 0.5 mM EDTA, pH 7.5. Binding buffer for A1 adenosine receptor was 10 mM HEPES, 5 mM MgCl₂, 1 mM EDTA, pH 7.4. Radioligand binding in the affinity shift assay was determined in the presence of the [³H]-OXO-M for M2 muscarinic receptor, [³H]-5-HT for 5-HT serotonin receptors and [³H]-CCPA for A1 adenosine receptor. Adenosine deaminase was added to the [³H]-CCPA solution at 12 µg/ml in binding buffer. Non-specific binding was determined by addition of 1000-fold excess of unlabeled ligand– 5-HT for 5-HT receptors, atropine sulfate for M2 receptor and R-PIA for A1 receptor. Incubations were for times sufficient to achieve equilibrium in a temperature controlled shaker (1 hr for M2 receptor, 1.5 hrs for 5-HT receptors, 2 hrs for A1 receptor) and were terminated by filtration over Whatman GF/C filters using a Brandel Cell Harvester. The filters were rinsed thrice with 4 ml ice cold 50 mM Tris-Cl, 5 mM MgCl₂, 0.5 mM EDTA, 0.01% NaAzide, pH 7.5 at 4 °C, placed in 4.5 ml CytoScint (ICN Pharmaceuticals, Costa Mesa, CA) and counted to constant error in a scintillation counter. For reconstitution of

high affinity agonist binding in affinity shift assays, a single concentration of radioligand near the high affinity K_D of the receptor of interest was used in a final volume of 150 μ l. [3 H]-5-HT radioligand purity was monitored by HPLC or TLC using an appropriate mobile phase. Radioligands were repurified or replaced when the radiochemical purity fell below 85%.

Affinity shift activity assay. The Sf9 cell membranes expressing individual receptors were reconstituted with saturating amounts of native or chimeric Gi1 protein heterotrimers (≥ 25 nM or 40-400 fold molar excess over receptors) to achieve the maximal specific binding during the binding assays. Because the magnitude of the affinity shifts observed with native Gi1 protein heterotrimers varied significantly among the individual receptors affinity shift activity was normalized to Gi1 activity and expressed as % affinity shift activity, which is (Chimera Reconstituted Binding - Control Binding/Gi1 Reconstituted Binding - Control Binding) x100.

Analysis of the data. Data analysis was done using the GraphPad Prism software package (GraphPad Software, San Diego, CA). For affinity shift assays, triplicate determinations were used within each experiment and experiments were repeated 3 or more times. Data represent the mean $\pm$ SEM from multiple experiments. One-way ANOVA with Tukey's multiple comparison post test was used to compare the activities of chimeras.

RESULTS

Previously we have shown that amino acids 299-318 and 1-219 of Gi1 α are molecular determinants of 5-HT_{1B} receptor coupling (Bae *et al.*, 1997) and that two amino acids in the α 4 helix of Gi1 α (Gln304 and Glu308) are especially important for 5-HT_{1B} receptor coupling (Bae *et al.*, 1999). The goal of the present study was to examine the generality of these findings among closely related members of the Gi/o-coupled receptor family. Our general strategy involves reconstitution of purified G proteins containing chimeric α subunits with receptors expressed in Sf9 insect cell membranes and comparison of the abilities of these chimeric G proteins to stabilize the high affinity agonist binding state of the receptors in an affinity shift activity assay. In the present study we compared the coupling behavior of four different Gi/o-coupled receptors: 5-HT_{1A} and 5-HT_{1B} serotonin receptors, M2 muscarinic receptors and A1 adenosine receptors.

Affinities of individual receptors for G proteins- First we determined the concentration of G proteins in the binding assay that produced the maximum affinity shift for each receptor. Increasing amounts of G protein heterotrimers were reconstituted with individual receptors and EC₅₀ values for reconstitution of high affinity agonist binding were determined. The data indicate that A1, 5-HT_{1A}, 5-HT_{1B} and M2 receptors have different EC₅₀ values for Gi1 (Figure 1). A1 receptors have the highest apparent affinity (0.4 nM) and M2 receptors have the lowest apparent affinity (47 nM) for the Gi1 heterotrimer. 5-HT receptors have intermediate EC₅₀ values of 3.7 nM and 16.2 nM for

the 5-HT_{1A} and 5-HT_{1B} receptors respectively. Titration experiments similar to those shown in Figure 1 were used to determine the concentration of chimeric G proteins needed to saturate affinity shift activities with individual receptors. For individual receptors, the EC₅₀ values of the active chimeras were not significantly different and even high concentrations (>600 nM) of inactive chimeras did not have affinity shift activity (data not shown). All affinity shift activities were determined with saturating concentrations of G proteins.

Affinity shift activity of chimeric G α subunits- Figure 2 depicts the secondary structures of the Gi1 α /Gt α chimeras used in this study. All of these chimeras have been previously described and were used to study Gi1 α domains involved in 5-HT_{1B} receptor coupling (Skiba *et al.*, 1996; Bae *et al.*, 1997; Bae *et al.*, 1999). Figure 3, in which 100% activity corresponds to the affinity shift activity of Gi1, shows the per cent affinity shift activity of Chi2, Chi3, Chi6, Chi13 and Chi21. Chi6 was constructed as a soluble analog of Gt α and has the same functional properties as Gt α (Skiba *et al.*, 1996). Chi6 is primarily Gt α in character as it includes N- terminal amino acids 1-215 and C- terminal amino acids 295-350 of Gt α with the amino acids corresponding to 216-294 from Gi1 α to maintain solubility. In this region there are just 26 amino acids that differ between Chi6 and Gt α . As shown in Figure 3, Chi6 was inactive with 5-HT_{1A}, 5-HT_{1B} and M2 muscarinic receptors. Earlier experiments with native transducin demonstrated it also failed to couple with the 5-HT receptors (Clawges *et al.*, 1997). In contrast, the data in Figure 3 demonstrate Chi6 was 74% active with the A1 adenosine receptor, indicating that A1 adenosine receptor doesn't discriminate well between Gt and Gi1 sequences.

Although the activity of Chi6 with the A1 adenosine receptor was significantly lower ($p < 0.001$) than the activity of Gi1, the magnitude of the difference was too small to be of use in identifying the precise domains responsible for the reduced activity. However, the inability of the 5-HT_{1A}, 5-HT_{1B} and M2 muscarinic receptors to couple with Chi6 allowed us to use additional chimeras containing less Gt α sequence to more precisely identify the domains required for coupling.

We first examined whether the N- terminal or C- terminal portion of Gi1 α was critical for receptor coupling. Chi21 has N- terminal amino acids 1-215 of Gt α with the rest of the molecule Gi1 α sequence (Figure 2). Chi21 was fully active with the A1 adenosine receptor, indicating that the A1 receptor does not distinguish between N-terminal amino acid sequences of Gi1 α and Gt α (Figure 3). The activity of Chi21 with 5-HT_{1A}, 5-HT_{1B} and M2 receptors was significantly ($p < 0.001$) reduced (44%, 57% and 42% respectively, Figure 3) demonstrating that amino acids 1-219 of Gi1 α contain an important determinant of Gi coupling with these receptors. Chi2 has the C- terminal amino acids 295-350 of Gt α with the rest of the chimera Gi1 α sequence (Figure 2). Figure 3 demonstrates that amino acids 299-354 of Gi1 α contain residues critical for 5-HT_{1A}, 5-HT_{1B} and M2 receptor coupling because the affinity shift activity of Chi2 with these receptors (2%, 9% and 23% respectively) was not significantly different from Chi6 activity. In contrast, Chi2 was fully active with A1 adenosine receptors supporting our conclusion that A1 adenosine receptor does not distinguish well between Gi1 α and Gt α sequences. To further evaluate the role of amino acids 299-354 of Gi1 α in 5-HT and M2 receptor coupling we tested two additional chimeras, Chi3 and Chi13 (Figure 2). Chi3

has amino acids 299-319 of Gi1 α replaced with the corresponding amino acids of Gt α (amino acids 295-315) while Chi13 has the 35 C-terminal amino acids of Gi1 α replaced with the corresponding amino acids of Gt α . As shown in Figure 3, the affinity shift activities of Chi3 show that amino acids 299-319 of Gi1 α (α 4-helix and α 4/ β 6-loop) are critical for 5-HT_{1A}, 5-HT_{1B} and M2 receptor coupling, but not for A1 adenosine receptor coupling. In contrast, Chi13, with six amino acids variant from Gi1 α , was active with all four receptors indicating that the 35 C-terminal amino acids of Gi1 α and Gt α are functionally interchangeable in coupling these receptors. Nevertheless, the significantly ($p < 0.01$) reduced activity of Chi13 (85.9%) with the M2 receptor and the significantly ($p < 0.01$) increased activity with both 5-HT_{1A} (128%) and 5-HT_{1B} (124.5%) receptors suggest subtle differences in the coupling mechanism of these receptors. The role of the extreme C-terminus of Gi1 α cannot be evaluated with these chimeras because the eight C-terminal amino acids of Gi1 α and Gt α are identical.

Role of the α 4-helix and α 4/ β 6-loop of Gi1 α in receptor coupling- In order to investigate α 4- α 4/ β 6 region of Gi1 α in more detail we used several additional chimeras to subdivide this region (Figure 4). Chi22 has the α 4 helix of Gi1 α replaced with that from Gt α while Chi25 has the α 4/ β 6 loop of Gi1 α replaced with that from Gt α . Chi23 has the α 4/ β 6 loop of Gi1 α replaced with that from Gt α and also switches the Glu in Gi1 α at the end of the α 4 helix for the Leu found in Gt α . Chi24 has the central part of the α 4/ β 6 loop with two variant amino acids switched between Gi1 α and Gt α . These chimeras were fully active with the A1 adenosine receptor (data not shown), supporting

our conclusion that the A1 receptor does not use the $\alpha 4$ - $\alpha 4/\beta 6$ region to distinguish between Gt and Gi1 (see Figure 3). Figure 5 shows the affinity shift activity of these chimeras with 5-HT_{1A}, 5-HT_{1B} and M2 receptors. Chi22 had low affinity shift activity with all three receptors indicating that a critical determinant of coupling selectivity for these receptors is located in the $\alpha 4$ helix of Gi1 α (Figure 5). For the 5-HT_{1B} receptor, the activity of Chi22 was significantly higher than the activity of Chi3 ($p < 0.01$), indicating that the $\alpha 4/\beta 6$ loop may also play a role in 5-HT_{1B} receptor coupling. This conclusion is supported by the Chi25 activity with the 5-HT_{1B} receptor (73%), which was significantly ($p < 0.001$) lower than the activity of Gi1 (100%). However, Chi25 was 91% as active with M2 muscarinic receptor) which was not significantly different ($p > 0.05$) from Gi1 activity) and was 121% as active with the 5-HT_{1A} receptor (which was significantly ($p < 0.001$) higher than Gi1). Clearly the $\alpha 4/\beta 6$ loop is utilized differently by these receptors. Chi24 was fully active with all three receptors (Figure 5) which suggests that the reduced activity of Chi25 with the 5-HT_{1B} receptor is due to the replacement of Asp309 by Glu at the beginning of the $\alpha 4/\beta 6$ loop (Figure 4). Figure 5 also shows the affinity shift activity of Chi23 was significantly reduced ($p < 0.001$) compared with the activity of both Gi1 and Chi25 for all three receptors. Chi23 differs from Chi25 by just one amino acid (replacement of Glu308 from Gi1 α for Leu from Gt α) indicating that Glu308 is important for coupling to 5-HT_{1A}, 5-HT_{1B} and M2 receptors. Taken together, the data indicate that the $\alpha 4$ helix (Glu308 in particular) is important for all three receptors, and that the $\alpha 4/\beta 6$ loop (probably Asp309) is also important for 5-HT_{1B} receptors.

Defining individual amino acids in the $\alpha 4$ - $\alpha 4/\beta 6$ region of $G_{i1}\alpha$ – To prove the role of Glu308 in receptor coupling and also to study the role of other amino acids in the $\alpha 4$ - $\alpha 4/\beta 6$ region of $G_{i1}\alpha$ we used chimeras in which amino acids Ala301, Gln304, Cys305, Glu308, Lys312 and Thr316 of $G_{i1}\alpha$ were replaced individually or in combinations with the corresponding amino acids of $G_{t\alpha}$. All of the mutants used here have been previously described (Bae *et al.*, 1999). First we studied the role of these amino acids with a loss of function assay. Mutants in which amino acids of $G_{i1}\alpha$ were replaced individually or in combinations with the corresponding amino acids of $G_{t\alpha}$ would be expected to exhibit reduced affinity shift activities if these amino acids were important for coupling. Replacement of Ala301 with Asn did not reduce activity ($G_{i1}A301N$, Figure 6) demonstrating that Ala301 is not important for coupling any of the receptors tested. When Gln304 was changed to Lys ($G_{i1}Q304K$, Figure 6) activity with 5-HT_{1A} and M2 receptors was significantly ($p<0.001$) reduced, but as reported previously (Bae *et al.*, 1999), this single amino acid replacement did not significantly reduce affinity shift activity with 5-HT_{1B} receptors (Figure 6). The activity of $G_{i1}C305V$ shows that Cys305 is important for M2 muscarinic receptors (67% activity, $p<0.001$) but not important for either 5-HT receptor (Figure 6). Glu308 is an important amino acid for all three receptors as the $G_{i1}E308L$ mutant displays 62%, 73% and 61% of activity with 5-HT_{1A}, 5-HT_{1B} and M2 receptors respectively ($p<0.001$) (Figure 6). Lys312 and Thr316 are not important for coupling these receptors and the increased activity of $G_{i1}K312M$ and $G_{i1}T316V$ with the 5-HT_{1A} receptor ($p<0.001$) is consistent with the increased activity of Chi25 with this receptor.

Data obtained with three double mutants (Gi1Q304K/C305V, Gi1Q304K/E308L, Gi1C305V/E308L) and a triple mutant (Gi1Q304K/C305V/E308V) support the conclusions drawn from the point mutants (Figure 6). The activity of the Gi1Q304K/E308L mutant was lower than the activity of either Gi1Q304K or Gi1E308L for all receptors supporting the importance of both Gln304 and Glu308 in receptor coupling. The role of Cys305 in M2 receptor coupling is supported by the observation that the activity of Gi1Q304K/C305V mutant was significantly lower than the activity of the Gi1Q304K mutant ($p<0.05$). Furthermore, the activity of the triple mutant (Gi1Q304K/C305V/E308V) was the lowest of all with the M2 receptor, supporting the idea that Gln304, Cys305 and Glu308 are all important for M2 receptor coupling. On the other hand, the conclusion that Cys305 is not important for coupling the 5-HT_{1A} and 5-HT_{1B} receptors is supported by the observations that the 304/305 and 305/308 double mutants have similar activities with these receptors as the Q304K and E308L single mutants and the 304/305/308 triple mutant is similar in activity to the 304/308 double mutant with these receptors.

Gain of function assays, in which amino acids from Gi1 α replaced those from Gt α in Chi22 were used to confirm the role of the amino acids identified in the loss of function assay. The data in Figure 7 demonstrate that substituting back Ala301 does not lead to gain of function with any of the receptors tested, supporting the conclusion that Ala301 of Gi1 α is not important for receptor coupling. Substituting back Gln304 (Chi22K300Q) resulted in significant ($p<0.001$) gain of activity with 5-HT_{1B} receptors which is in contrast to the absence of a loss of activity with 5-HT_{1B} receptors when

Gln304 was mutated to Lys in Gi1 α . Similarly, substituting back Cys305 in the Chi22V301C mutant also resulted in significant ($p<0.05$) gain of activity with 5-HT_{1B} receptors. The precise reasons for these anomalies are unknown but may be related to the actual role of these amino acids in the context of their neighbors. Substituting back Glu308 alone (Chi22L304E) resulted in a gain of affinity shift activity of 48% with 5-HT_{1A} receptors ($p<0.001$), 38% with 5-HT_{1B} receptor ($p<0.001$) but only 17% ($p>0.05$) with M2 receptors. However, when both Gln304 and Glu308 were substituted back into Chi22 sequence (Chi22K300Q/L304E), a full gain of activity was observed with 5-HT_{1A} and 5-HT_{1B} receptors, as Chi22K300Q/L304E activity was not significantly different from activity of Gi1 (100%). The gain of function with M2 receptors was significant (45% gain of activity, $p<0.001$), though still less than the activity of Gi1. Taken together, the data indicate that Gln304 and Glu308 of Gi1 α are important for 5-HT_{1A}, 5-HT_{1B} and M2 receptor coupling, and that Cys305 of Gi1 α is important for M2 receptor coupling in addition to Gln304 and Glu308.

Role of C terminus of Gi1 α in receptor coupling- Alignment of the C-terminal sequences of Gi1 α and Gt α indicates that their extreme eight C-terminal amino acids are identical (Figure 8). Because numerous studies have indicated the C terminus of G α plays a significant role in receptor coupling, we decided to investigate the role of C terminus of Gi1 α in 5-HT_{1A}, 5-HT_{1B}, A1 and M2 receptor coupling using Gi1 α /Gq α C-terminal chimeras. As shown in the sequence alignments in Figure 8, the extreme C-terminus of Gq α differs from that of Gi1 α in just four amino acids. Loss of function experiments may demonstrate partial or complete loss of activity. As shown in Figure 9,

replacement of just two of these amino acids with those from Gq α in the Q3C mutant significantly lowers the affinity shift activity with all four receptors. The nearly complete loss of affinity shift activity (0.3% and 11.2%, respectively) with 5-HT_{1B} serotonin and A1 adenosine receptors suggests that these amino acids are critical for coupling, while the more modest decrease in activity (65% and 68% activity, respectively) with the 5-HT_{1A} and M2 receptors suggest these amino acids are important, but not critical, for coupling. Substitution of the five C-terminal amino acids of Gi1 α with those from Gq α eliminates coupling with the A1 adenosine receptor while substitution of 11 C-terminal amino acids are required for complete loss of 5-HT_{1A} receptor coupling (Figure 9). These data indicate that the 5-HT_{1A}, 5-HT_{1B}, A1 adenosine and M2 muscarinic receptors differ in their utilization of the C-terminal amino acids of Gi1 α for coupling.

DISCUSSION

G protein-receptor coupling can be regulated by a variety of mechanisms (Wess, 1998; Albert and Robillard, 2002). At the G protein-receptor interface, the selectivity of coupling is regulated by the amino acid sequences of both receptor and G protein. By comparing the coupling mechanism of four closely related receptors to the same G proteins, we found that receptors use multiple domains on G α to achieve selective coupling. Coupling selectivity is also regulated by the G protein concentration as demonstrated by the significant differences among the EC₅₀ values for Gi1-receptor interactions. This suggests that in living cells the expression levels of specific G protein subunits may regulate receptor coupling preferences.

At the level of G α domains, the major difference we found is that the A1 adenosine receptor does not discriminate well between Gi1 α and Gt α sequences. In contrast, the 5-HT and M2 receptors couple with Gi1 but fail to couple with Gt. This selectivity allowed us to use Gi1 α /Gt α chimeras to define domains on Gi1 α important for coupling with these receptors. Our findings indicate that amino acids especially important for receptor coupling are located in the α 4 helix. In addition, the 5-HT_{1B} receptor may require Asp309 at the beginning of α 4/ β 6 loop for optimal coupling. The corresponding amino acid in Gt α is Glu305, and while both are negatively charged, glutamate is one -CH₂ group bigger than aspartate. Thus replacement of aspartate with glutamate may decrease 5-HT_{1B} receptor coupling because of the change in the size of the receptor interacting surface on G α . In addition, we demonstrated that within the α 4

helix- $\alpha 4/\beta 6$ loop region of $G_{i1}\alpha$ the amino acids that are involved in receptor coupling differ slightly among the receptors. While all three receptors utilize Gln304 and Glu308, the M2 receptor also uses Cys305 and the 5-HT_{1B} receptor may use Asp309. Interestingly, interaction of the 5-HT_{1A} receptor with the K312M mutant actually leads to an increased affinity shift. This increase in affinity shift activity may represent tighter coupling of the receptor with the chimera. Other investigators have also demonstrated the importance of this region of $G\alpha$ in receptor coupling. Natochin *et al.* demonstrated the role of Arg310 and Asp311 in interaction of $G_{t\alpha}$ with rhodopsin (Natochin *et al.*, 1999). Blahos *et al.* demonstrated that $\alpha 4-\alpha 4/\beta 6-\beta 6-\alpha 5$ region of $G_{\alpha 16}$ is important but not critical for interaction with metabotropic glutamate receptor 8 (Blahos *et al.*, 2001). In contrast, the work of Grishina and Berlot shows that $\alpha 4/\beta 6$ loop of $G_{\alpha s}$ is not important for interactions with $\beta 2$ adrenergic receptor (Grishina and Berlot, 2000). Using gain of function experiments, Ho and Wong demonstrated that incorporation of $\alpha 4/\beta 6$ loop of $G_{\alpha z}$ into a $G_{\alpha t}$ backbone was not sufficient for δ -opioid receptor coupling (Ho and Wong, 2000). Taken together, these results support the idea that even if different receptors recognize the same general domain on $G\alpha$ subunits, the specific amino acids involved in receptor interactions may be different.

Another region of $G_{i1}\alpha$ important for 5-HT and M2 receptor coupling is the N terminus, as affinity shift activity with Chi21 was lower than with G_{i1} for these receptors. According to the literature, the amino acids that bind to the receptor map to approximately positions 1-30 of the α subunits (Hamm *et al.*, 1988). This region, which includes the N terminus and the αN helix, contains the most differences between

Gi1 α and Gt α with 15 variant amino acids compared with just 9 variants from amino acids 31 to 219. Another significant difference between Gi1 α and Gt α is that the α N helix of Gt α is 4 amino acids shorter than the α N helix of Gi1 α . Thus it is possible that amino acids 1-30 are important but not critical for 5-HT and M2 receptor coupling.

Although the C terminus of G α subunits is postulated to directly contact the receptor and mediate receptor coupling selectivity, our data show that the specific amino acids involved in this recognition differ among the receptors studied. Cys351 (position -4), Gly352 (position -3) and Phe354 (position -1) in Gi family members have been shown to be important for mediating selectivity of receptor coupling (reviewed in) (Wess, 1998). Gain of function studies with Gq/i chimeras (Conklin *et al.*, 1993; Conklin *et al.*, 1996) indicate that five C terminal amino acids of Gi are sufficient for coupling to A1 and M2 receptors while three C terminal amino acids of Gi are not enough for A1 receptor coupling (Conklin *et al.*, 1993). Although so far it has not been possible to successfully solve the structure of the G α C terminus in the context of the whole molecule (the C terminus is disordered in the crystal), the structure of the C-terminal undecapeptide of Gt α bound to activated rhodopsin has been resolved by NMR spectroscopy (Koenig *et al.*, 2002). In this C-terminal decapeptide, the first eight residues form an α helix which is terminated by an α_L type C-cap (Aurora *et al.*, 1994) with C terminal glycine (Gly348 in Gt α , Gly352 in Gi1 α) in the center of the reverse turn (Kisselev *et al.*, 1998). Thus the observation that in case of A1 receptor three C terminal amino acids of Gi1 α are critical in the loss of function experiments but five C terminal amino acids are required to gain coupling may be explained by the fact that this

α_L C-cap, which is disrupted in Gi1/Q3C chimera, is required for A1 receptor coupling. This is probably also true for the 5-HT_{1B} receptor. Our M2 receptor data indicate that although this α_L C-cap structure is important, it is not critical for receptor coupling. For the 5-HT_{1A} receptor three C terminal amino acids of Gi1 α are important while amino acids at the positions -4 and -5 (Asp350 and Cys351) are not important (activities of Gi1/Q3C and Gi1/Q5C are the same). Gi/Q5C and Gi/Q11C are different in three amino acids, which are probably involved in 5-HT_{1A} receptor coupling. Some additional amino acids involved in 5-HT_{1A} receptor coupling are located in the α_5 helix (see Figure 8) as evident from the activity of Gi1/Q35C chimera. Taken together, our results support the idea that different receptors may recognize a specific pattern of amino acids which form receptor recognition surfaces.

Figure 10 depicts the structure of the G $\alpha_{i1}\beta_1\gamma_2$ G protein heterotrimer. Six amino acids from the C terminus and four amino acids from the N terminus are missing in this structure (Wall *et al.*, 1995). The domains of Gi1 α discussed herein are surface exposed and located on the G protein surface that is presumed to face the receptor. They are therefore available for receptor coupling. However, while some amino acids may be involved in coupling by making direct contact with receptors, others may be involved indirectly by playing a role in guanine nucleotide exchange. Amino acids Glu304, Glu308 and Asp309 are surface exposed and so are also available for receptor coupling. Molecular modeling indicates that Gi1Q304K, Gi1E308L and Gi1304/308 mutations alter the surface potential (Bae *et al.*, 1999), while Gi1D309E mutation alters steric interactions because Glu is one CH₂ group larger than Asp (water-accessible surfaces of

native Gi1 and Gi1D309E were constructed and superimposed in Insight II; not shown). Therefore, structural considerations are consistent with our conclusions. In summary, here we demonstrated that four closely related Gi/o coupled receptors distinguish themselves by the affinity with which they interact with Gi1 and by their use of multiple domains of Gi1 α for selective coupling.

Figure 1.

FIGURE 1. Concentration dependence of Gi1 in affinity shift assays for individual Gi1-coupled receptors. Sf9 cell membranes expressing the indicated Gi1-coupled receptors were reconstituted with increasing concentrations of Gi1 heterotrimer. The affinity shift activities, percent of Gi1 for each receptor were fit to a single-site interaction between receptor and G protein. Saturation was achieved for each receptor, however for visual purposes the curves have been extended to a common endpoint. Shown are the data from representative experiments. EC₅₀ data are the mean ± SEM from 3 or more independent experiments.

Figure 2.

FIGURE 2. Secondary structure of Gα subunits. Numbers above the chimeric structures indicate the junction points of Gαt and Gαi1 sequences and refer to the amino acid positions in Gαt. Numbers for the wild type forms of Gαt and Gαi1 represent their total amino acid residues. The bottom diagram depicts the secondary structural domains common to Gα subunits.

Figure 3.

FIGURE 3. Functional coupling of receptors to the indicated Gi1/Gt chimeras. Sf9 cell membranes expressing individual receptors were reconstituted with the indicated chimeric Gα and βγ subunits. Data represent the affinity shift activities, percent of Gi1 as mean ± SEM from 3 or more independent experiments for each receptor. Exogenous G proteins were present in 40-200 fold molar excess over receptors during reconstitution to achieve the maximal specific binding during the binding assays.

Figure 4.

FIGURE 4. Primary sequence alignment of the $\alpha 4$ - $\alpha 4/\beta 6$ loop region of Gi1 α and Gt α . The boxes indicate the regions of Gi1 α that were substituted with the corresponding sequences from Gt α to generate the indicated Gi1 α /Gt α chimeras.

Figure 5.

FIGURE 5. Functional coupling of receptors to the indicated Gi1/Gt chimeras. Sf9 cell membranes expressing individual receptors were reconstituted with the indicated chimeric G α and $\beta\gamma$ subunits. Data represent the affinity shift activities, percent of Gi1 as mean $\pm$ SEM from 3 or more independent experiments for each receptor. Exogenous G proteins were present in 40-200 fold molar excess over receptors during reconstitution to achieve the maximal specific binding during the binding assays.

Figure 6.

FIGURE 6. Functional coupling of receptors to the indicated Gi1α point mutants.

Sf9 cell membranes expressing individual receptors were reconstituted with the indicated chimeric Gα and βγ subunits. Data represent the affinity shift activities, percent of Gi1 as mean ± SEM from 3 or more independent experiments for each receptor. Exogenous G proteins were present in 40-200 fold molar excess over receptors during reconstitution to achieve the maximal specific binding during the binding assays.

Figure 7.

FIGURE 7. Functional coupling of receptors to the indicated Chi22 point mutants.

Sf9 cell membranes expressing individual receptors were reconstituted with the indicated chimeric Gα and βγ subunits. Data represent the affinity shift activities, percent of Gi1 as mean ± SEM from 3 or more independent experiments for each receptor. Exogenous G proteins were present in 40-200 fold molar excess over receptors during reconstitution to achieve the maximal specific binding during the binding assays.

Figure 8.

FIGURE 8. Sequence alignment of 35 C terminal amino acids of **Gtα**, **Gi1α** and **Gqα**. The sequences of **Gtα** and **Gqα** are compared to **Gi1α** sequence. Depicted in bold are amino acids of **Gtα** and **Gqα** that are different from corresponding amino acids of **Gi1α**.

Figure 9.

FIGURE 9. Functional coupling of receptors to the indicated Gi1/Gq chimeras. Sf9 cell membranes expressing individual receptors were reconstituted with the indicated chimeric Gα and βγ subunits. Data represent the affinity shift activities, percent of Gi1 as mean ± SEM from 3 or more independent experiments for each receptor. Exogenous G proteins were present in 40-400 fold molar excess over receptors during reconstitution to achieve the maximal specific binding during the binding assays.

Figure 10.

FIGURE 10. Spacefilling representation of the G $\alpha\beta 1\gamma 2$ heterotrimer. The image was generated using GRASP (developed by A. Nicholls and B. Honig, Columbia University) with coordinates from Wall et al. (Wall *et al.*, 1995). The α subunit is shown in metallic blue and the $\beta\gamma$ dimer is in yellow. The regions involved in receptor coupling as discussed herein are the $\alpha 4$ -helix and $\alpha 4/\beta 6$ -loop in *pink*, Gln304 in *red*, Glu308 in *green*, Asp309 in *blue*, the C-terminus in *cyan*, and the N-terminus and αN -helix in *gray*.

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APPENDIX III

STABILIZATION OF THE GDP-BOUND CONFORMATION OF G α BY A PEPTIDE

DERIVED FROM THE G-PROTEIN REGULATORY MOTIF OF AGS3

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ABSTRACT

The G-protein regulatory (GPR) motif in AGS3 was recently identified as a region for protein binding to heterotrimeric G-protein α subunits. To define the properties of this ~20 amino acid motif, we designed a GPR consensus peptide and determined its influence on the activation state of G-protein and receptor coupling to G-protein. The GPR peptide sequence (28 amino acids) encompassed the consensus sequence defined by the four GPR motifs conserved in the family of AGS3 proteins. The GPR consensus peptide effectively prevented the binding of AGS3 to $G_{i\alpha 1,2}$ in protein interaction assays, inhibited $GTP\gamma S$ binding to $G_{i\alpha}$ and stabilized the GDP-bound conformation of $G_{i\alpha}$. The GPR peptide had little effect on nucleotide binding to $G_{o\alpha}$ and brain G-protein indicating selective regulation of $G_{i\alpha}$. Thus, the GPR peptide functions as a guanine nucleotide dissociation inhibitor for $G_{i\alpha}$. The GPR consensus peptide also blocked receptor coupling to $G_{i\alpha}\beta\gamma$ indicating that although the AGS3-GPR peptide stabilized the GDP-bound conformation of $G_{i\alpha}$, this conformation of $G_{i\alpha}GDP$ was not recognized by a G-protein coupled receptor. The AGS3-GPR motif presents an opportunity for selective control of $G_{i\alpha}$ - and $G\beta\gamma$ -regulated effector systems and the GPR motif allows for alternative modes of signal input to G-protein signaling systems.

INTRODUCTION

The G-protein regulatory (GPR) motif or GoLOCO repeat is a ~20 amino acid domain found in several proteins that interact with and/or regulate G-proteins (1,2). Such proteins include the activator of G-protein signaling AGS3, the AGS3-related protein PINS in *Drosophila melanogaster*, two members of the RGS family of proteins and three proteins (LGN, Pcp2 and Rap1GAP) isolated in yeast two hybrid screens using $G_{i\alpha}$ or $G_{o\alpha}$ as bait. Rat AGS3 was isolated in a yeast-based functional screen designed to identify receptor-independent activators of heterotrimeric G-protein signaling (1). The AGS3-related protein PINS is required for asymmetric cell division of neuroblasts in *D. melanogaster* and it is found complexed with Gi/Go (3,4), but neither the signal input or output for this complex are known. Some insight as to how PINS may regulate Gi/Go is provided by studies with AGS3 (1). In the yeast-based system, AGS3 selectively activated $G_{i\alpha 2}$ and $G_{i\alpha 3}$, but it did not function in $G_{s\alpha}$, $G_{\alpha 16}$ or $G_{\alpha 1}$ genetic backgrounds. The action of AGS3 as a G-protein activator in the yeast-based system was independent of nucleotide exchange as it was not antagonized by overexpression of RGS4 and it was still observed following replacement of $G_{i\alpha 2}$ with $G_{i\alpha 2}$ -G204A, a mutant that is deficient in making the transition to the GTP-bound state (1,5). Both of these manipulations effectively prevent receptor-mediated activation of G-protein signaling in the yeast system and block the action of AGS1, which was isolated in the same screen and apparently behaves as a guanine nucleotide exchange factor for heterotrimeric G-proteins (5,6). These data indicate that the interaction of AGS3 with G-

protein influences a unique control mechanism within the activation /deactivation cycle of heterotrimeric G-proteins.

AGS3 exists as a 650 amino acid protein enriched in brain and a 166 amino acid protein (AGS3-SHORT) enriched in heart (1).^{1,2} The 650 amino acid protein consists of two functional domains defined by a series of seven amino terminal tetratricopeptide repeats (TPR) and four carboxyl terminal GPR motifs. The shorter variant enriched in heart contains at least three GPR motifs but lacks the TPR domains.² Site directed mutagenesis, protein interaction studies and subcellular localization experiments indicated that the GPR motifs of AGS3 were likely responsible for binding G-protein, whereas the TPR domain is a site for binding of regulatory proteins (1,3,4).¹ AGS3 preferentially binds to G_{α} in the presence of GDP (1). AGS3-GPR effectively competed with $G\beta\gamma$ subunits for binding to G_{α} and inhibited $GTP\gamma S$ binding to $G_{\alpha}1$.¹ Such an activity likely has significance in a number of aspects of G-protein mediated signaling events and presents a novel opportunity to control the basal activity of G-protein signaling as well as influence receptor-mediated activation of G-protein. These observations also raise many interesting questions relative to basic aspects of G-protein structure/function and alternative modes of regulation and functional roles for G-protein signaling systems in the cell. To address these issues, we generated a series of peptides based upon the consensus GPR motif in AGS3 and evaluated their effects on the nucleotide binding properties of G_{α} . A 28 amino acid GPR peptide effectively blocked the interaction of AGS3 with G_{α} and inhibited $GTP\gamma S$ binding to G_{α} by a mechanism that involved stabilization of the GDP-bound conformation of G_{α} . Thus, the AGS3-GPR

peptide is actually a guanine nucleotide dissociation inhibitor (GDI) for the G_i family of proteins. The GPR consensus peptide also blocked receptor coupling to $G_{i\alpha\beta\gamma}$ indicating that although the AGS3-GPR peptide stabilized the GDP-bound conformation of $G_{i\alpha}$, this conformation of $G_{i\alpha}GDP$ was not recognized by a G-protein coupled receptor.

EXPERIMENTAL PROCEDURES

Materials

[³⁵S]GTPγS (1250 Ci/mmol), [³H]GDP (29.6 Ci/mmol) and [³H] 5-HT (21.8 Ci/mmol) were purchased from Dupont/NEN (Boston, MA). Peptides were synthesized and purified by Bio-Synthesis, Inc. (Lewisville, TX) and peptide mass verified by matrix-assisted laser desorption ionization mass spectrometry. Guanosine diphosphate (GDP), guanosine 5'-O-(3- thiotriphosphate (GTPγS) and 5-hydroxy tryptamine (5-HT) were obtained from Sigma (St. Louis, MO). Acrylamide, bis-acrylamide, Bio-rad protein assay kits and sodium dodecyl sulfate were purchased from Bio-Rad (Hercules, CA). Ecoscint A was purchased from National Diagnostics (Manville, NJ). CytoScint was purchased from ICN Pharmaceuticals (Costa Mesa, CA). Thesit (polyoxyethylene-9-lauryl ether) was obtained from Boehringer-Mannheim (Indianapolis, IN). Polyvinylidene difluoride (PVDF) membranes were obtained from Pall Gelman Sciences (Ann Arbor, MI). Nitrocellulose BA85 filters were purchased from Schleicher and Shuell (Keene, NH). Whatman GF/C FP200 filters were purchased from Brandel Inc.(Gaithersburg, MD). Purified bovine brain G-protein was kindly provided by Dr. John Hildebrandt (Department of Pharmacology, Medical University of South Carolina) (7). All other materials were obtained as described elsewhere (1,8).

Protein interaction assays The GPR domain of AGS3 (P463-S650) containing the four GPR motifs was generated as a glutathione-S-transferase fusion protein by polymerase

chain reaction (PCR) using the full length cDNA of AGS3 as a template. The AGS3-P463-S650 segment was also cloned into the pQE-30 vector (Qiagen, Valencia, CA) to generate an amino-terminal HIS-tagged protein. HIS-tagged AGS3 was expressed in and purified from bacteria using a nickel affinity matrix (ProBond Resin, Invitrogen – Carlsbad, CA). The HIS-tagged AGS3 was eluted from the matrix with imidazole and desalted by centrifugation as with the GST fusion protein (1). The interaction of GST-AGS3-GPR and HIS-tagged AGS3-GPR with G-proteins was assessed by protein interaction experiments using purified G-protein as previously described (1). $G_{i\alpha 1-3}$ and $G_{o\alpha}$ were purified from Sf9 insect cells infected with recombinant virus as described (8). All purified G-proteins used in these studies were isolated in the GDP-bound form and G-protein interaction assays contained 10 μ M GDP. The GST-AGS3-GPR fusion protein or HIS-tagged AGS3-GPR (300 nM) was incubated with purified G-protein (75 nM) for 1 hr at 24°C in a total volume of 250 μ l. Glutathione-sepharose or nickel ProBond Resin (12.5 μ l packed resin) was added and the mixture rotated at 4 °C for 20 min after which the affinity matrix was pelleted and washed three times with 500 μ l of incubation buffer (20 mM Tris-HCl, pH 7.5, 0.6 mM EDTA, 1 mM DTT, 70 mM NaCl, 0.01% Thesit). Proteins retained on the matrix were solubilized in 2x Laemmli loading buffer and separated by electrophoresis on denaturing, 10% polyacrylamide gels. Proteins were transferred to PVDF membranes for immunoblotting. Each blot was checked by immunoblot with AGS3 antisera to verify equal loading of fusion proteins.

A separate series of protein interaction experiments was designed to determine if the $G_{i\alpha}$ complexed with AGS3 contained bound GDP. $G_{i\alpha 1}$ (100 nM) was loaded with

^3H -GDP (0.5 μM , 2.0×10^4 dpm/pmol) by incubation for 20 min at 24°C in binding buffer (50 mM Hepes-HCl pH 7.5, 1 mM EDTA, 1 mM dithiothreitol, 50 μM adenosine triphosphate and 10 $\mu\text{g/ml}$ bovine serum albumin). The ^3H -GDP loaded $\text{Gi}\alpha 1$ was incubated with 300 nM GST or GST-AGS3-GPR in the presence and absence of 10 μM GPR peptide and processed as described above. The washed resin containing bound proteins was transferred to vials for measurement of ^3H -GDP by liquid scintillation spectroscopy.

GTP γ S binding assays GTP γ S binding assays were generally conducted as described (9). G-proteins (100 nM) were preincubated for 20 min at 24°C in the presence and absence of GPR peptides. Binding assays (duplicate determinations) were initiated by addition of 0.5 μM GTP γ S (4.0×10^4 dpm/pmol) and incubations (total volume = 50 μl) continued 30 min at 24°C . Both preincubations and GTP γ S binding assays were conducted in binding buffer containing 2 mM MgCl_2 . Reactions were terminated by rapid filtration through nitrocellulose filters (S&S BA85) with 4 x 4 ml washes of stop buffer (50 mM Tris-HCl, 5 mM MgCl_2 , 1 mM EDTA, pH 7.4, 4°C). Radioactivity bound to the filters was determined by liquid scintillation counting. Nonspecific binding was defined by 100 μM GTP γ S.

GDP dissociation assays $\text{Gi}\alpha 1$ (100 nM) was loaded with ^3H -GDP (0.5 μM , 2×10^4 dpm/pmol) by incubation for 20 min at 24°C in binding buffer without MgCl_2 . Forty five μl aliquots of the preincubation mixture ($\sim 500,000$ dpm) were then added to incubation

tubes containing 10 μ l of vehicle or peptide and samples incubated for 30 minutes at 24°C. Two sets of tubes were set up for each time point to be analyzed. Each set contained duplicate samples for determination of total binding, nonspecific binding or binding in the presence of peptide. For each time point, one set of tubes served as an internal time control whereas the other set received added GTP γ S or GDP to initiate dissociation. Data are expressed as % of control where control represents the level of 3 H-GDP binding at each time point in the set of tubes that did not receive added nucleotide to initiate dissociation. The amount of 3 H-GDP bound following the 20 min preincubation (~30,000 dpm) was identical to that observed at the 30 min incubation time point following addition of vehicle or peptide. 3 H-GDP dissociation was initiated by addition of GTP γ S or GDP in a volume of 5 μ l (final concentration - 100 μ M). Reactions were terminated at specified time points by rapid filtration through nitrocellulose filters (S&S BA85) with 4 x 4 ml washes of stop buffer. Radioactivity bound to the filters was determined by liquid scintillation counting. Nonspecific binding was defined by 100 μ M GDP.

High Affinity Agonist Binding Sf9 cell membranes expressing 5-HT_{1A} receptors were reconstituted with G $\alpha\beta\gamma$ and high affinity agonist binding measured with 3 H-5-HT as previously described (8,10). Membrane aliquots (100 μ g membrane protein, 85 nM receptor) were preincubated for 15 minutes at 25°C with G-proteins (2125 nM G $\alpha\beta\gamma$) with or without GPR peptides in a total volume of 17 μ l (reconstitution buffer - 5 mM NaHEPES, 100 mM NaCl, 5 mM MgCl₂, 1 mM EDTA, 500 nM GDP, 0.04% CHAPS,

pH 7.5). The reconstitution mixtures were then diluted 10-fold with binding buffer (50 mM Tris-HCl, 5 mM MgCl₂, 0.5 mM EDTA, pH 7.5) and 50 µl added to binding tubes (total volume = 150 µl) containing 2 nM ³H 5-HT. The final concentrations of receptor, G-protein and peptide in the binding tubes were 2.8 nM, 70.8 nM and 114 µM, respectively. Non-specific binding was determined in the presence of 100 µM 5-HT. Binding reactions were incubated at 25 °C for 1.5 hr and terminated by filtration over Whatman GF/C FP200 filters using a Brandel Cell Harvester. The filters were rinsed thrice with 4 ml ice cold washing buffer (50 mM Tris-Cl, 5 mM MgCl₂, 0.5 mM EDTA, 0.01% sodium azide, pH 7.5 at 4 °C), placed in 4.5 ml CytoScint and counted to constant error in a scintillation counter.

RESULTS AND DISCUSSION

The ~20 amino acid GPR motif is repeated four times in AGS3 related proteins, with the exception of the three repeats found in the *Drosophila* protein PINS (Fig 1). Alignment of the four GPR repeats from five species revealed a GPR consensus sequence (Fig 1). The GPR consensus sequence is characterized by the upstream negative charge (EE) and hydrophobic cluster (FF), L/M10, L/I11, Q15, S/A16, R18, M/L19 and the DDQR sequence at the carboxyl end of the motif. Helical wheel and Chou-Fasman analysis indicated that this region is capable of existing as an amphipathic helix. Each of the GPR motifs illustrated in figure 1 possess a varying number of proline residues just after and in some cases before the core consensus sequence, which may exert an important influence within the overall organization of the four GPR motifs. As part of an effort to define the structural basis of the interaction of AGS3 with $G_{i\alpha}$ and the functional consequences of this interaction, we asked if a peptide containing this consensus sequence effectively interacted with $G_{i\alpha}$.

The core GPR consensus sequence was bracketed by additional residues (three – amino terminus, five - carboxyl terminus) derived from AGS3-GPR-IV and the carboxyl terminus was amidated (Fig 1). The 28 amino acid GPR consensus peptide completely blocked the binding of $G_{i\alpha 1}$ or $G_{i\alpha 2}$ to GST-AGS3-GPR with an IC_{50} of ~200 nM (Fig 2A,B). The GPR consensus peptide also inhibited $GTP\gamma S$ binding to $G_{i\alpha 1}$ and $G_{i\alpha 2}$ (IC_{50} ~200 nM) (Fig 2C,D) consistent with the preferential binding of AGS3 to $G_{i\alpha}$ in the presence of GDP (1). The inhibitory effect of the GPR consensus peptide on $GTP\gamma S$

binding was selective for $G_{i\alpha}$ as it only minimally effected nucleotide binding to $G_{o\alpha}$ or brain G-protein (Fig 2D). The activity of the GPR consensus peptide in both the protein interaction assays and $GTP\gamma S$ binding assays was lost upon substitution of F for the highly conserved R23 (Fig 2B,C,D). However, substitution of A for the invariant Q15 did not alter the activity of the GPR peptide (Fig 2B)³. Similar results were obtained when these amino acid substitutions were made in the context of GST-AGS3 fusion protein which contained the terminal 74 amino acids of AGS3 including part of GPR III and all of GPR IV (1).¹

We then addressed the mechanism by which the GPR consensus peptide inhibited $GTP\gamma S$ binding to $G_{i\alpha 2}$ and determined the effect of the GPR motif on receptor coupling to G-protein. The inhibition of $GTP\gamma S$ binding to $G_{i\alpha}$ by the GPR consensus peptide may reflect a reduction in the rate of nucleotide exchange. Indeed, the rate of GDP dissociation was markedly diminished in the presence of the GPR consensus peptide (Fig 3A).⁴ The R23F mutation, which eliminated the effectiveness of the peptide to block interaction of AGS3 with $G_{i\alpha}$ and $GTP\gamma S$ binding to $G_{i\alpha}$, also did not alter GDP dissociation (Fig 3B). The inhibition of GDP dissociation by the GPR consensus peptide suggests that the GPR motif is stabilizing the GDP-bound conformation of $G_{i\alpha}$. To address this issue we evaluated the interaction of GST-AGS3-GPR with $G_{i\alpha 2}$, which had been preloaded with 3H -GDP. Subsequent analysis of the G-protein complexed with AGS3-GPR on the glutathione affinity matrix indicated that the nucleotide binding site of G-protein bound to AGS3 indeed contained GDP (Fig 3B). $G_{i\alpha_{GDP}}$ binding to AGS3-GPR was blocked by the GPR consensus peptide (Fig 3B) consistent with the ability of

this peptide to inhibit interaction of GST-AGS3-GPR with $G_{i\alpha 1/2}$ as detected by immunoblotting (Fig 2A,B).

The stabilization of the GDP-bound conformation of $G_{i\alpha}$ by the GPR consensus peptide indicates that the AGS3-GPR motif can influence subunit interactions by interfering with $G\beta\gamma$ binding to $G_{i\alpha}$.¹ This apparent effect may account for the results obtained in protein interaction assays using GST-AGS3-GPR and brain lysates, where $G\beta\gamma$ is absent from the AGS3- $G_{i\alpha}$ complex (1). The influence of the GPR motif on subunit interactions would have significant implications for signal processing. First, interaction of the AGS3-GPR motif with $G\alpha\beta\gamma$ would release $G\beta\gamma$ for regulation of downstream signaling events, while stabilizing $G\alpha_{GDP}$ (1). Such a mode of signal input may be of utility where there is a need for selective regulation of $G\beta\gamma$ -sensitive effectors. The time frame for termination of such a signaling event (i.e. reassociation of $G\beta\gamma$ with $G_{i\alpha_{GDP}}$) likely differs from that of a more typical signaling event in which there has been an exchange of nucleotide bound to $G_{i\alpha}$ and signal termination involves GTP hydrolysis along with subunit reassociation. A second implication of stabilization of $G\alpha_{GDP}$ by a GPR domain is related to receptor G-protein coupling. We addressed this issue experimentally using a membrane assay system where receptor - G-protein coupling is reflected as high affinity binding of agonists. The high affinity binding of agonist observed upon reconstitution of the membrane-bound 5-HT₁ receptor with $G_{i\alpha\beta\gamma}$, was inhibited by addition of the GPR peptide (Fig 4). This action of the GPR peptide was not observed with the R23F peptide and was selective for Gi versus Go (Fig 4).^{4,5}

The influence of the single amino acid substitutions on the bioactivity of the GPR both within the context of a short peptide and a GST fusion protein containing an additional 74 amino acids of AGS3 sequence strongly suggest a relatively discrete and specific surface interaction with $G_i\alpha$ (Fig 2) (1).¹ Helical wheel projections and 3-D models (Swiss PDB viewer) indicated that when the GPR consensus peptide is fixed in an α helical conformation, the F8, A12, Q15, M19 and R23 residues are on the same face of the helix. On this face of the helix is a hydrophobic sector defined by F8, A12 and M19, which is bounded by polar residues, which may be involved in charge pairing to residues in $G_i\alpha$. As was the case for the R23F substitution, disruption of this hydrophobic sector by substitution of R for F8 also resulted in a loss of activity for the GST-AGS3-GPR fusion protein in GTP γ S binding and protein interaction assays (1).¹ Thus, either extension (R23F substitution) or shortening (F8R) of the hydrophobic sector on this face of the helix resulted in a loss of bioactivity for the GPR motif. In contrast, strengthening of this hydrophobic sector by substitution of A for Q15 did not alter the activity of the GPR peptide. These data indicate an important role for a spatially constrained hydrophobic stretch of ~ 16.6 angstroms that is key for peptide interaction with $G_i\alpha$.

The inability of receptor to productively couple to $G\alpha_{GDP}$ -GPR is of interest. The $G\alpha_{GDP}$ conformation stabilized by the GPR peptide may differ from that stabilized by $G\beta\gamma$ in such a manner that the receptor cannot recognize $G\alpha$. Indeed, the orientations of the amino and carboxyl domains of $G_i\alpha_1$, which are important interactions sites with receptor, are quite different in the $G_i\alpha_{GDP}$ and $G_i\alpha_{GDP}\beta\gamma$ structures (11-13). In addition

to such differences in the structural orientation of $G_{i\alpha}$ domains interacting with receptor, it is likely that receptor contact points on $G\beta\gamma$ also play a role in receptor-mediated activation of guanine nucleotide exchange (14-18). Alternatively, the receptor may indeed interact with the $G_{\alpha_{GDP}}$ -GPR complex, but this interaction stabilizes a receptor conformation with low affinity for agonist (19). Ultimately, one may think of the $G_{\alpha_{GDP}}$ -GPR complex as a type of dimeric G-protein, and it is not clear what might provide “signal input” to such a complex.

Although, the GPR motif is present in several proteins that interact with G_{α} and/or regulate nucleotide binding/hydrolysis (1,2), these proteins have different and often opposing effects on the activation state of G-protein (20,21).^{1,4} *Pcp2*, which contains two GPR motifs based upon this consensus sequence, actually appears to increase the dissociation of GDP from $G_{o\alpha}$ (21). Thus, there are either subtle differences in this motif or other residues outside of this motif that play a key role in the specific functional output gendered by interaction of the GPR motif with G_{α} . Of note is the selective effects of the AGS3 GPR peptide for $G_{i\alpha}$ versus $G_{o\alpha}$ in both nucleotide binding assays and the analysis of receptor coupling to G-proteins. Further dissection of the structural basis for this selectivity will provide clues as to the site of interaction of the GPR peptide with $G_{i\alpha}$ and the mechanism by which it stabilizes the GDP-bound conformation. One prominent area of sequence divergence between $G_{o\alpha}$ and $G_{i\alpha}$ encompasses switch IV, a region implicated in the formation of $G_{i1\alpha_{GDP}}$ multimers observed by X-ray crystallography (11).

The role of AGS3 as a GDI is an unexpected concept for heterotrimeric G-proteins although such proteins serve similar regulator roles for ras-related G-proteins. Proteins containing the AGS3-GPR motif may promote dissociation of $G\alpha$ and $G\beta\gamma$ in the absence of nucleotide exchange and present an opportunity for selective control of $G\alpha$ -and $G\beta\gamma$ -regulated effector systems. GPR-containing proteins likely play a role in regulating basal activity of G-protein signaling systems in the cell and provide alternative modes of signal input to G-protein signaling systems which may either augment, complement or antagonize G-protein activation by GPCRs.

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FOOTNOTES

¹ Bernard, M., Peterson, Y.K., Chung, P. and Lanier, S.M. Submitted.

² Pizzinat, N, Takesono, A. and Lanier SM. Submitted.

³ Peterson YK and Lanier SM. Unpublished observations.

⁴ Natochin M, Lester B, Peterson YK, Bernard ML, Lanier SM and Artemyev NO. A GST-AGS3 fusion protein containing two or four GPR motifs inhibited Gt $\alpha\beta\gamma$ activation by light-activated rhodopsin and this involved inhibition of GDP dissociation from G α . Submitted.

⁵ Ma H, Bernard ML, Lanier SM and Graber SG. A GST-AGS3 fusion protein containing four GPR motifs also blocked the high affinity binding of agonist observed upon reconstitution of the membrane-bound 5-HT₁ receptor with Gt $\alpha\beta\gamma$ and exhibited a higher potency than the GPR peptide. Unpublished observations.

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FIGURE LEGENDS

Figure 1. Alignment of the GPR motifs found in AGS3 and related proteins. The overall domain structure of AGS3 (650 amino acid protein) is indicated at the top of the figure. The hashed boxes represent the TPR domain. The GPR domains of rat AGS3 (AAF08683), human AGS3 (CAB55951), the *D. melanogaster* PINS protein (AAF36967), the *C. elegans* protein (CE) (AAA81387) and the *Tetraodon nigroviridis* (puffer fish) protein (AL338846) were aligned by PILEUP (University of Wisconsin GCG program) and visual adjustment. A consensus amino acid was defined by the presence of an amino acid or closely related residue in all four GPR repeats.

Figure 2. Influence of GPR peptides on the interaction of GST-AGS3GPR with Gi α and GTP γ S binding to Gi α . A,B) The carboxyl region of AGS3 (P463-S650) containing all four GPR repeats was generated as a HIS-tagged (A) or GST fusion (B) protein for protein interaction assays as described in “Experimental Procedures”. All interactions were done in the presence of 10 μ M GDP and the input lanes represent one tenth of the G-protein used in each interaction assay. A) Gi α 1 (75 nM) was incubated with 300 nM HIS-tagged AGS3-GPR in the absence and presence of increasing amounts of the GPR peptide after which bound Gi α was isolated on a nickel affinity matrix and samples processed for immunoblotting with Gi α antisera. The blot in the upper panel of A was stripped and reprobed with AGS3 antisera to provide internal controls for protein loading. B) Gi α 2 (75 nM) was incubated with 300 nM AGS3-GPR or GST in the

presence and absence of 100 μ M GPR consensus peptide, GPR peptide Q15A or GPR peptide R23F. The immunoblots presented in A and B are representative of three experiments. C,D) GTP γ ³⁵S binding to G-proteins (100 nM) was measured in the absence and presence of peptides as described in “Experimental Procedures”. Data are expressed as the percent of specific binding ($\sim$ 5 pmol) observed in the absence of peptide and represent the means $\pm$ SEM derived from three experiments. The concentration of peptides in (D) is 10 μ M.

Figure 3. Stabilization of the GDP-bound conformation of Gi α 2 by the GPR consensus peptide. A) ³H GDP dissociation from Gi α 2 (100 nM). Gi α 2 was loaded with ³H-GDP and dissociation initiated by addition of GTP γ S (100 μ M) as described in “Experimental Procedures”. Data are expressed as the percent of specific binding observed in control samples for each time point that did not receive 100 μ M GTP γ S. The peptide concentration was 10 μ M. B) Gi α 2 (100 nM) was loaded with ³H-GDP and incubated with 300 nM GST or GST-AGS3-GPR in the presence and absence of 10 μ M GPR consensus peptide and processed for protein interactions as described in “Experimental Procedures”. The proteins bound to the glutathione affinity matrix were eluted and the amount of bound GDP measured by liquid scintillation spectroscopy. Data in A and B are presented as the means $\pm$ SEM derived from three experiments.

Figure 4. Influence of GPR peptides on receptor interaction with G-proteins. Sf9 cell membranes expressing 5-HT_{1A} receptors were reconstituted with G-proteins in the

presence and absence of GPR peptides as described in “Experimental Procedures”. The final concentration of peptide was 114 μ M. Radioligand binding assays used a concentration of ^3H -5HT near the K_d for the high affinity, Gpp(NH)p sensitive binding site (8,10). The control bar indicates the amount of agonist binding observed in the absence of added G-protein. Data are presented as the mean $\pm$ SEM from 4 independent experiments.

Figure 1.

Figure 2.

Figure 3.

Figure 4.

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