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Critical Amino Acids of the Gα₂ Subunit Helical Domain in *Dictyostelium discoideum*

Steven Martin Rauch

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**CRITICAL AMINO ACIDS OF THE G α 2 SUBUNIT HELICAL
DOMAIN IN *Dictyostelium discoideum***

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A THESIS

Submitted in Partial Fulfillment of the

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Master of Science

(in Biochemistry)

The Graduate School

The University of Maine

December, 2002

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An Abstract of the Thesis Presented
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In *Dictyostelium discoideum* organism, the G α 2 subunit of the heterotrimeric G-protein signaling complex plays a pivotal role during the aggregation stage in the *Dictyostelium* life cycle. The biochemical functions of the G-protein complex include separation of the G-protein coupled receptor from the G-protein subunits, GDP displacement by GTP in the G α subunit, separation of the G α monomer from the $\beta\gamma$ complex, GTP hydrolysis to GDP, activation of adenylyl cyclase as a downstream effector, and activation of guanylyl cyclase as a separate downstream effector. Upon release from the heterotrimer, the $\beta\gamma$ subunits lead to downstream activation of the membrane bound adenylyl cyclase A. The role of the G α 2 subunit is not well defined other than acting as a regulated GTPase to terminate the signal. To further define the role of the G α 2 subunit, a previously constructed library of random mutations in the G α 2 subunit was screened for aggregation negative mutants expressing the

Gα2 protein. Mutants were reconfirmed as chemotaxis negative. One of these mutants, N74D, has an unusual phenotype. After stimulation with the extracellular ligand cAMP, the affinity of the cAMP specific G-protein coupled receptor to this ligand remained high, as in the unactivated state, and the receptor was unable to activate the G-protein. This mutation is in the Gα2 subunit helical domain, the function of which is not well understood. The N74D mutant provides some insight regarding the mechanistic role of the helical domain in G-protein signaling.

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1. INTRODUCTION

1.1 Heterotrimeric G-Proteins

As the study of cellular biochemical signaling progresses, an intricate and complex network of pathways is being uncovered. One of the first components identified of the many signaling proteins now known was the heterotrimeric G-protein (reviewed in Gilman, 1987). This family of proteins has been found participating in a vast array of physiologic functions ranging from hormonal signaling to vision and olfactory function to modulating cell division and differentiation (reviewed in Hamm, 1998; and Sprang, 1997). The heterotrimeric G-proteins function at an early branch point in signaling cascades, being closely associated with the family of cell membrane receptors known as G-protein coupled receptors (GPCR). These receptors share a structure of 7 transmembrane domains with associated intracellular and extracellular loop domains that provide much of the specificity of extracellular ligand binding and intracellular G-protein association (reviewed in Bourne, 1997). Ligand binding induces a conformational change in the receptor which is transmitted to the associated intracellular G-protein and induces activity of this next component in the signaling cascade (reviewed in Wess, 1997).

The heterotrimeric G-protein consists of three subunits, the $G\alpha$ subunit and the $\beta\gamma$ subunits. The latter two subunits, although coded by separate genes, are functionally a single unit as the β and γ subunits remain bound together in all but denaturing conditions (Schmidt *et al.*, 1992). In the resting state of the ligand-free receptor, also a high affinity

state for the extracellular ligand (Gilman, 1987), the three subunits are bound together and the $G\alpha$ subunit has a molecule of GDP bound tightly in its guanine nucleotide recognition site. As a heterotrimer, the $\beta\gamma$ subunits stabilize the GDP-bound conformation of the $G\alpha$ subunit (Wall *et al.*, 1995; Lambright *et al.*, 1996; Phillips and Cerione, 1992). The complex of the heterotrimer with the receptor mutually prevents exposure of surfaces on the $G\alpha$ subunit and the $\beta\gamma$ subunits that will later interact with effector proteins (Sprang, 1997). Upon ligand induced receptor conformational change, several events happen rapidly. The receptor acts as a guanine nucleotide exchange factor (GEF) for the $G\alpha$ subunit (reviewed in Bourne, 1997). The GDP is replaced by a separate GTP molecule with the kinetics of this reaction driven, at least in part, by the higher concentration of GTP within the cell (Gilman, 1987). As a GEF, the receptor may stabilize the nucleotide-empty transitional state of the $G\alpha$ subunit. The receptor conformational change and GDP/GTP exchange disrupt the stability of the heterotrimer and the $\beta\gamma$ subunits dissociate. The significant $G\alpha$ subunit conformational change on activation by GTP, mostly seen in the switch II region, may be the main cause of this disruption as switch II is the main contact surface between the $G\alpha$ and $\beta\gamma$ subunits.

The $G\alpha$ subunit has three conformations. One is the inactive receptor bound state with GDP in the guanine nucleotide binding pocket. Upon GTP binding, the $G\alpha$ subunit assumes a different conformation at its switch regions largely due to the ionic influence of the γ phosphate of GTP and a required magnesium ion (Noel *et al.*, 1993; Lambright *et al.*, 1994; Coleman *et al.*, 1994; Mixon *et al.*, 1995; reviewed in Sprang, 1997). This activated conformational state exists until the γ phosphate of GTP is hydrolyzed. After

hydrolysis a third conformational state occurs, that of the inactive GDP-bound $G\alpha$ subunit. With the switch regions now disordered, the $\beta\gamma$ subunits bind in a way which increases affinity between the $G\alpha\beta\gamma$ complex and the receptor.

At completion of the initial signaling step, the G-protein complex has dissociated from the receptor and has divided into the $G\alpha$ -GTP subunit and the $\beta\gamma$ subunits, each of which now have separate roles in signaling. The receptor at this point has a low affinity for its ligand which persists until re-association with the G-protein complex. The $G\alpha$ -GTP complex has a low affinity for the receptor and will not re-associate until hydrolysis of the γ phosphate releases the GTP-induced conformational change. The $\beta\gamma$ subunits interact with a large number of downstream effector proteins (reviewed in Clapman and Neer, 1997). The $G\alpha$ subunit also interacts with downstream effectors but has the additional function of controlling re-association with the $\beta\gamma$ subunits by regulated GTP hydrolysis.

The intrinsic GTPase activity of the $G\alpha$ subunit alone is relatively slow, on the order of 3-5 GTP molecules/min effecting GTP γ phosphate hydrolysis (Gilman, 1987). Regulators of G protein signaling (RGS) proteins serve as GTPase activating proteins (GAPs) to increase the rate of hydrolysis and thus shorten the duration of the 'on' state of G-protein signaling. The mechanism involves GAP proteins binding to the $G\alpha$ subunit such that the enzymatic transitional state of the hydrolysis reaction is stabilized (Privé *et al.*, 1992; Berman, 1996; reviewed in Berman, 1998). The amino acids required to carry out the hydrolysis of the GTP γ phosphate are supplied by the $G\alpha$ subunit itself. This is different than the GTPase activity of the monomeric G proteins where the GAPs provide residues in the catalytic pocket (Sprang, 1997). Some downstream effector proteins act as GAPs in a

manner similar to the RGS proteins (Berstein *et al.*, 1992; Arshavsky *et al.*, 1994; Biddlecome *et al.*, 1996). The helical domain of the α subunit, described later, may play a role in GTPase activation as well.

The activated α -GTP subunit does not necessarily stay associated with the membrane. The C-terminus of the γ subunit and the N-terminus of many of the α subunits are lipid modified (reviewed in Sprang, 1997), are in close proximity to each other in the heterotrimer (Wall *et al.*, 1995; Lambright *et al.*, 1996), and may be anchored to the membrane at the same focus near the GPCR (Wedegaertner *et al.*, 1993). Recent experiments with green fluorescent protein (GFP) tagged α subunits suggest this subunit can also move into the cytosol where it would be able to interact with cytosolic effectors as well (Yu and Rasenick, 2002). Upon GTP hydrolysis, the α subunit regains affinity for the $\beta\gamma$ subunits and this complex then can re-associate with the receptor. This terminates the signaling activity of both the α and $\beta\gamma$ subunits. With the receptor now returned to a state of high ligand affinity, the continued presence or absence of the receptor's ligand can be detected intracellularly by reactivation or quiescence of heterotrimeric G-protein activity.

1.2 α Subunit Structure and Activity

The monomeric small G-proteins and the α subunit of the heterotrimeric G-proteins have a similar core structure (Jurnak, 1985; La Cour *et al.*, 1985). This core, termed the G domain, involves α -helix and β -sheet structural elements along with the guanine nucleotide-binding and γ -phosphate hydrolysis catalytic motifs. The central structural element is a six stranded gradually twisting β -sheet, surrounded around its center by five α -

helices (Figure 1). These are termed α 1-5 helices and β 1-6 strands starting after the initial N-terminal helix. Dispersed between structural elements are five loops involved in the binding and catalytic functions that are shared between all G-proteins (Bourne et al, 1991; reviewed in Sprang, 1997) (Figure 2). The P-loop is located in a short connecting region between the β 1 strand and the α 1 helix. The highly conserved GXXXXGK(S/T) sequence is the binding site of the α and β phosphates of GDP/GTP. Figure 3 aligns the G domain conserved sequences of this and the other loops involved in guanine nucleotide binding and hydrolysis. Between the α 1 helix and the next core G domain loop, switch I, lies a 130 amino acid segment which is found only in the heterotrimeric $G\alpha$ subunits and is termed the helical domain (Figure 4). This consists of one long and five short helical elements, α A thru F and will be discussed later. Following this are two flexible loops termed switch I and switch II. They are structurally held in proximity to each other at one end of the two antiparallel β 2 and β 3 strands and are located on the surface of the $G\alpha$ subunit during its activated monomeric state. Between switch I and II lies the γ phosphate of GTP and it is here where the conformational change between the GDP and GTP bound forms of the $G\alpha$ subunit is effected (Figure 5). In the GTP bound conformation, the conserved threonine residue in switch I (G-2 loop; T185 in *Dictyostelium* $G\alpha$ 2) hydrogen bonds with the γ phosphate of GTP and ionically coordinates with a magnesium ion to interact with the conserved DXXG(Q) sequence of switch II. The α 2 helix is located just C-terminal to switch II and orients differently between GDP and GTP conformations. The conserved glutamine residue just adjacent to the DXXG sequence is critical for hydrolytic activity (Q208 in $G\alpha$ 2) along with a conserved arginine residue at the N-terminal area of

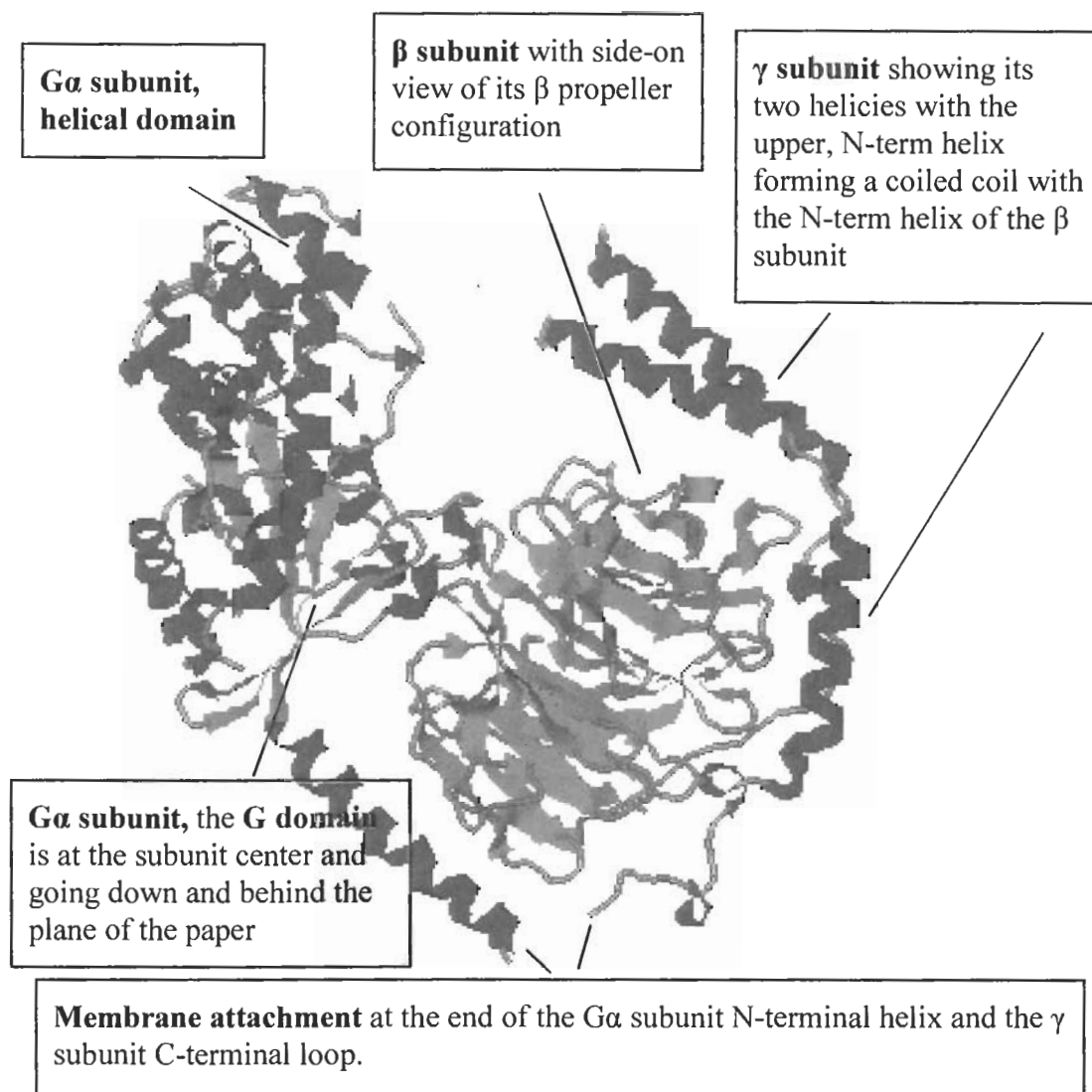


Figure 1. The Heterotrimeric G Protein; Gα, β, and γ subunits. In this view, the membrane is at the bottom where lipid modifications of the Gα subunit N-terminus and the γ subunit C-terminus provide anchoring points near the GPCR. In dark pink are the α helix structural elements and in dark yellow are the β strands. These colors, representing structural α helix and β strand, are maintained throughout the thesis. This G-protein heterotrimer contains a Gat/Gai chimera (mostly Gat) and a GDP molecule. It is taken from crystal data submitted by Lambright *et al.*, 1996 and available as a .pdb file. The Internet site is The Protein Data Base (www.rcsb.org/pdb/) and the Rasmol program is Version 2.7.1.1, also available from the same Internet site.

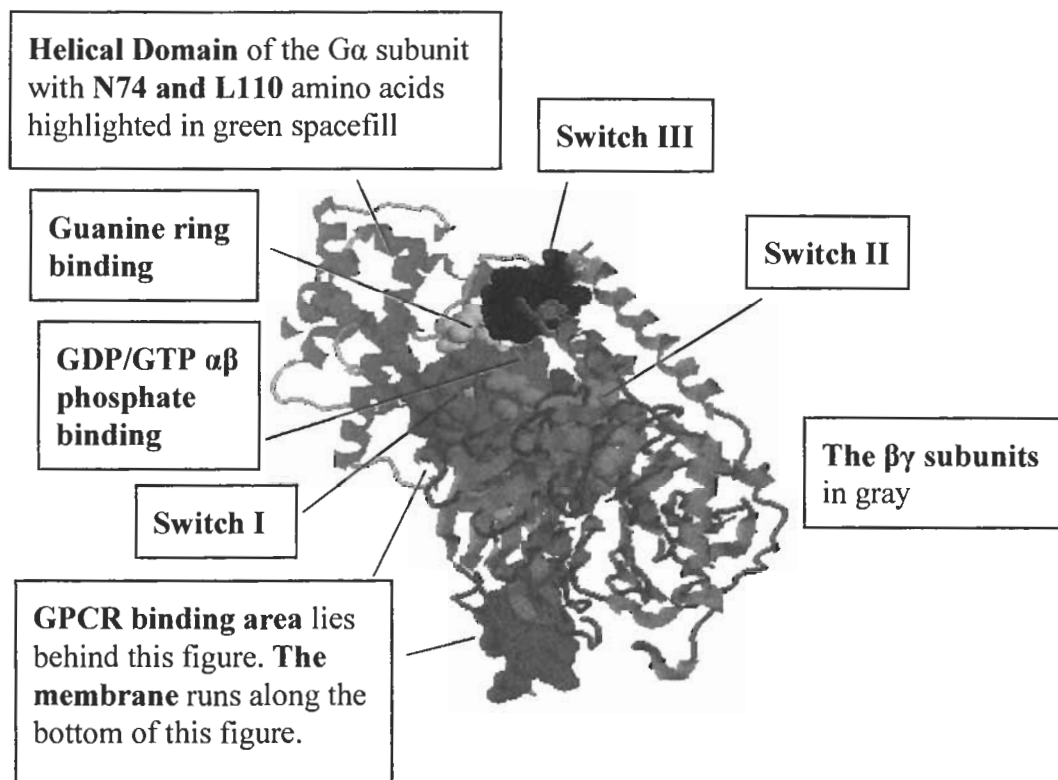


Figure 2. The $G\alpha$ subunit G-domain. This view is Figure 1 rotated approximately 90 degrees such that the view is ‘through’ the $\beta\gamma$ subunits onto the switch regions. On the $G\alpha$ subunit: Switch I is in red spacefill, Switch II is in gold spacefill, and Switch III is in black spacefill. Receptor binding areas are in blue spacefill. The guanine ring binding area is in yellow spacefill, mostly located behind this view. The magenta spacefill in the middle is the location of GDP/GTP α and β phosphate binding. The amino acids which are the main subject of this thesis are in green spacefill. The $\beta\gamma$ subunit is gray. During the resting ‘off’ state, the $\beta\gamma$ subunit covers Switch II entirely and sterically blocks Switches I and III from binding proteins of all but the smallest size. The receptor would be located adjacent to the blue spacefill areas on the opposite face from this view. The exact structure of the GPCR bound to the G-protein has not been determined. The receptor cytoplasmic loops II and III are relatively small (see Figure 24) and would not be expected to interact much beyond the blue spacefill highlighted area. These color schemes are maintained through the figures in this paper.

Figure 3. G-domain conserved nucleotide binding motifs. The critical amino acids involved in the function of each binding motif are highlighted in bold. Gat is the subunit represented from crystal data in the figures throughout this paper.

Gα subunit	starting amino acid	P loop (GDP/GTP α and β phosphate binding)
------------	---------------------	-----------------------------------------------

Gai	40	G A G E S G K S
Gao	40	G A G E S G K S
Gas	47	G A G E S G K S
Gat	36	G A G E S G K S
Gα2	38	G A G E S G K S
Gaz	40	G T S N S G K S
Gaq	40	G T G E S G K S

Gα subunit

Switch I

Switch II

Gai	178	R V K T T G I	198	L F D V G G Q R
Gao	179	R V K T T G I	199	M F D V G G Q R
Gas	201	R V L T S G I	221	M F D V G G Q R
Gat	174	R V K T T G I	194	M F D V G G Q R
Gα2	182	R V M T R G V	202	L V D V G G Q R
Gaz	179	R D M T T G I	199	M V D V G G Q R
Gaq	177	R V P T T G I	197	M V D V G G Q R

Gα subunit

Guanine nt ring binding

Guanine nt ring binding

Gai	267	F L N K K D	321	T H F T C A T
Gao	268	F L N K K D	321	C H M T C A T
Gas	290	F L N K Q D	361	P H F T C A V
Gat	263	F L N K K D	317	S H M T C A T
Gα2	270	F L N K S D	323	S H I T C A T
Gaz	268	F L N K K D	322	S H F T C A T
Gaq	266	F L N K K D	320	S H F T C A T

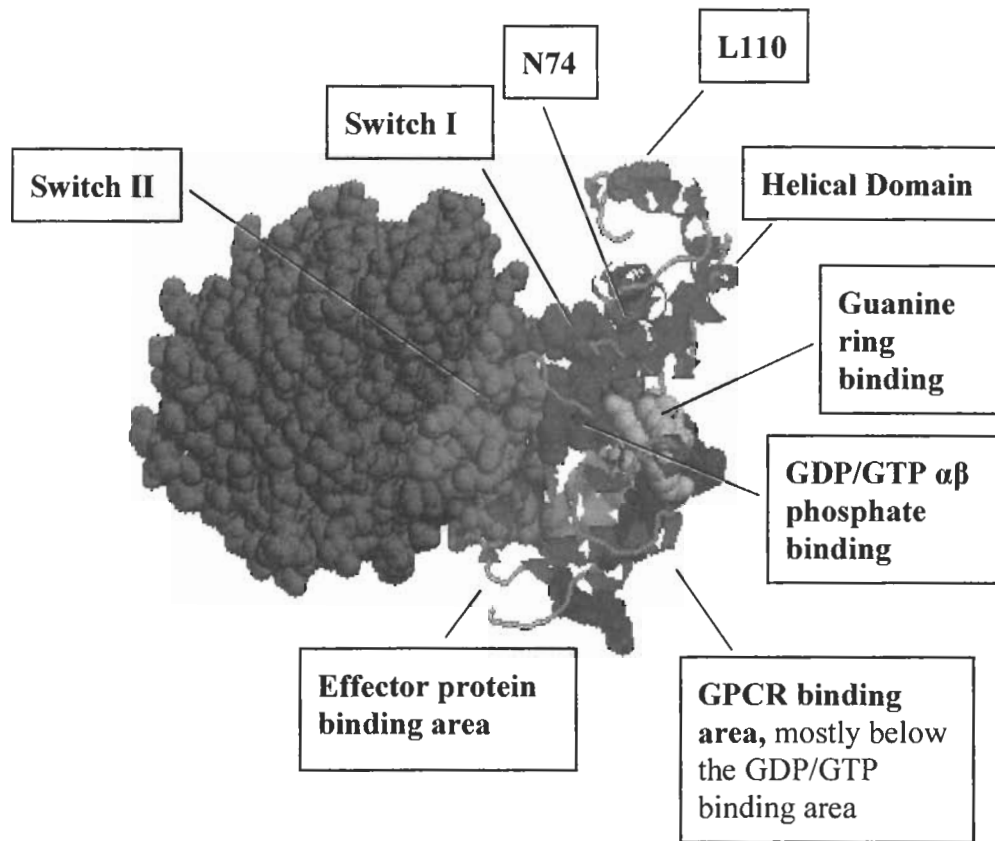


Figure 4. The Helical Domain. This orientation is looking down from above the G-protein with the plasma membrane lying flat beneath. The location of the helical domain and the N74 amino acid between switch I and the GPCR binding area is clearly seen in this view. Note the location near the surface of the $G\alpha$ subunit of the guanine nucleotide ring binding area. This is opposite the switch regions and the interface with the $\beta\gamma$ subunit. It may be that the GDP/GTP exchange occurs on this surface as this side is exposed before the $\beta\gamma$ subunit disassociates and GTP binding with resulting reorientation of switch II logically must be occurring at the moment of $\beta\gamma$ dissociation. The covering of the switch regions by the $\beta\gamma$ subunits (in gray spacefill) is well demonstrated in this orientation. Effector proteins bind at both the switch regions and the indicated face of the $G\alpha$ subunit.

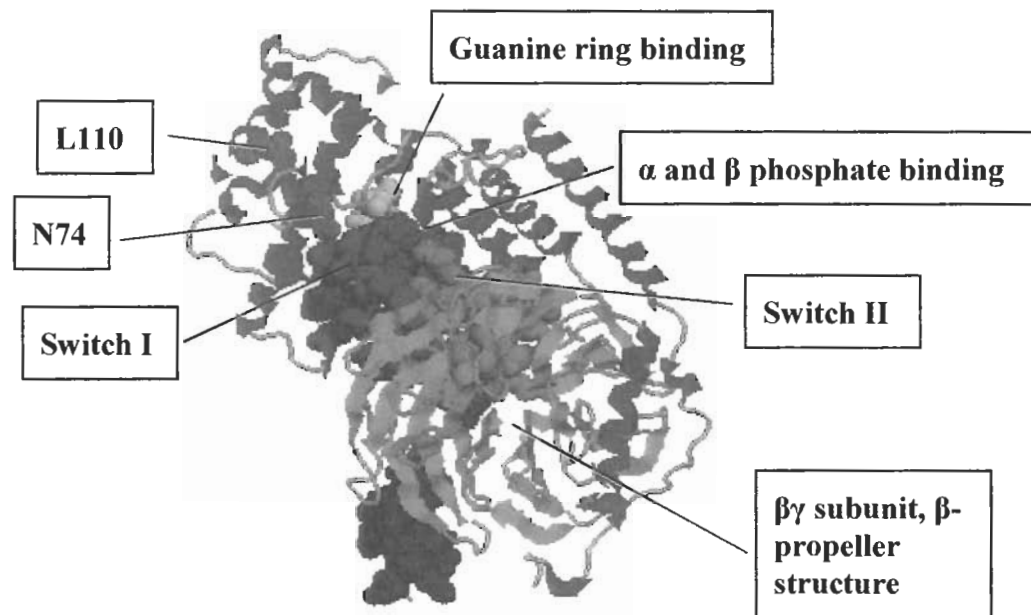


Figure 5. The GDP/GTP binding pocket. The GTP γ phosphate is bound between switches I and II in front and the guanine ring is bound toward the back. The $\beta\gamma$ subunit is in the foreground. Its β -propeller structure in the dark yellow ribbon is well seen from this angle. Note the point of apparent contact between the N74 amino acid and switch I.

switch I (R182 in $G\alpha_2$) (Figures 6 and 7). This arginine is the site of ADP-ribosylation, catalyzed by cholera toxin, which allows GTP binding but eliminates GTPase activity of the subunit. The resulting G-protein is constitutively active. RGS proteins bind to the switch I and II loops and have a higher affinity for the GDP- AlF_4^- complex, thought to represent the enzymatic transitional state of GTP hydrolysis (Privé *et al.*, 1992), than for the GTP γ S complex (Berman *et al.*, 1996). So in stabilizing the transitional state, the rate of hydrolysis is increased. The last two loops are involved in the selective binding of the guanine ring. The conserved sequence, NKXD, in the $\beta 5/\alpha G$ loop along with the conserved residues, CA, in the $\beta 6/\alpha 5$ loop sterically favor the guanine ring structure over adenine.

The heterotrimeric G-protein $G\alpha$ subunits have other important structural areas not found on monomeric G-proteins (reviewed in Sprang, 1997). The N-terminus is required for association with the $\beta\gamma$ subunits and the GPCR (Taylor *et al.*, 1994) and is lipid modified for membrane attachment in some $G\alpha$ subunits. Between the $\alpha 1$ helix and switch I is the helical domain mentioned earlier. Structurally this domain forms a cleft with the G domain where the guanine nucleotide binding occurs (Figure 4). The conformational change between GDP and GTP bound forms also involves a slight opening of this cleft (Benjamin *et al.*, 1995). The role of the helical domain has been hypothesized but not defined (see Helical Domain section). The $\beta 4$ strand connects switch II to another flexible loop, termed switch III. Effector proteins have been shown to bind at all three of these switch regions. More detail of this subject follows later. Between the $\beta 5$ strand and $\alpha 4$ helix, the $G\alpha$ subunits have an extra helical element, termed the αG loop. Finally the C-terminus of the $G\alpha$ subunits, especially the last 7 amino acids, are required for receptor interaction (Martin *et al.*, 1996).

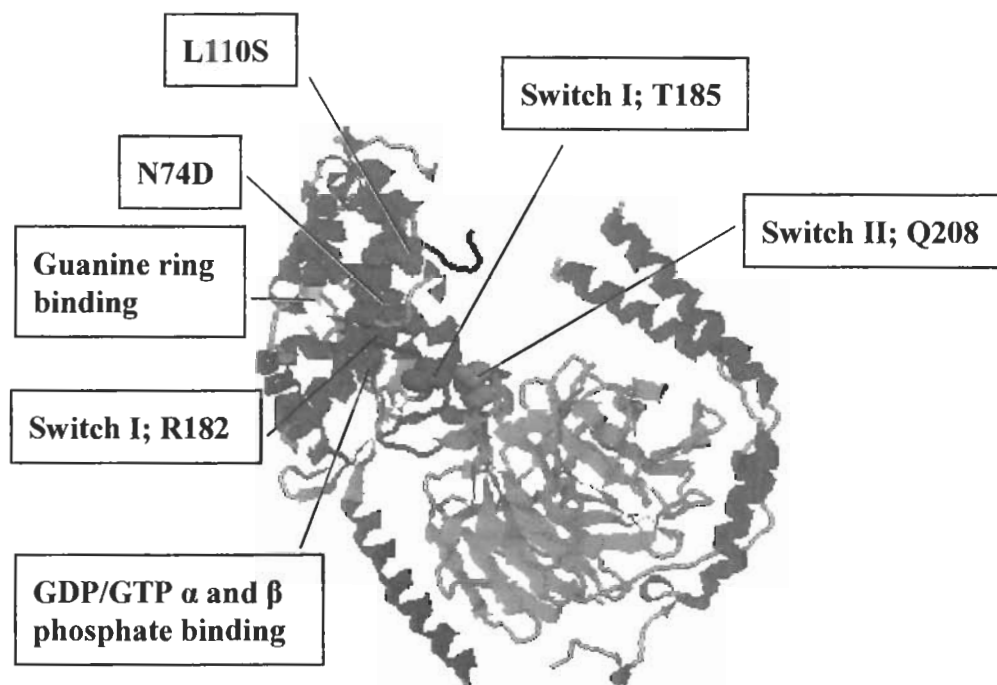


Figure 6. The N74D and L110S mutants. This figure highlights the locations of critical switch I and switch II amino acids and their relation to the mutant amino acids. All color schemes are the same as in Figure 2, however only the discussed amino acids are in spacefill. The N74D mutation (wild type amino acid shown) lies adjacent to the switch I amino acid R182, involved in GTP γ phosphate hydrolysis. The switch I amino acid, T185, which binds the γ phosphate of GTP, is in red spacefill. The switch II amino acid, Q208, is in gold spacefill. This glutamine is involved in γ phosphate hydrolysis and is directly next to the DXXG motif (not in spacefill) which coordinates with a Mg^{++} ion in binding the GTP γ phosphate. Compare with Figure 7 which provides a clearer view of the γ phosphate binding pocket, with slight rotation of the protein.

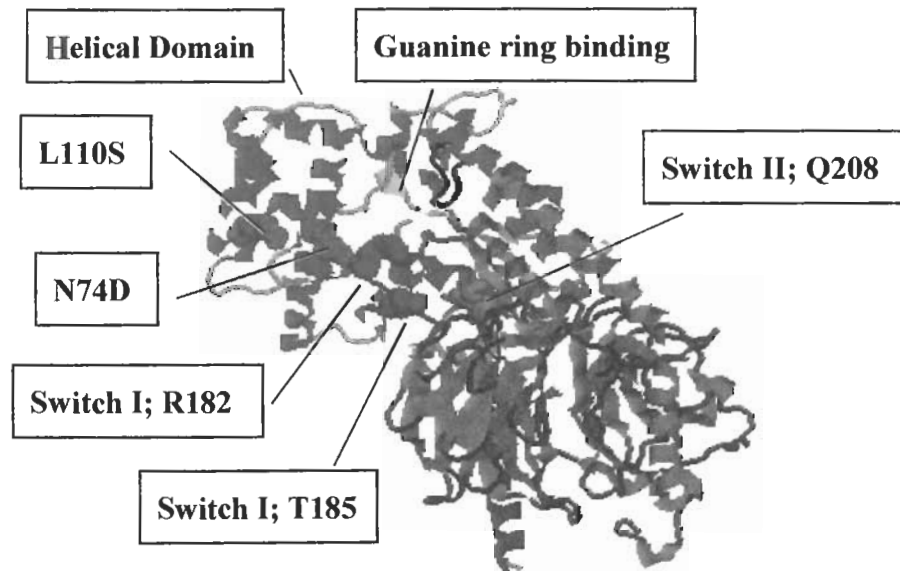


Figure 7. The GTP γ phosphate binding pocket. This view is Figure 6 rotated slightly and with the $\beta\gamma$ subunit in gray. The spacial inter-relationship between the mutations and critical switch I and switch II amino acids is clearly demonstrated in this view. Note both mutations are in the helical domain of the $G\alpha$ subunit and the N74D residue is adjacent to the R182 residue in switch I.

The structure of the β subunit consists of seven blades aligned in a radial fashion as in a propeller. Each 'blade' is formed by four antiparallel β strands, with each strand running roughly parallel to the central axis of the entire β -propeller structure (see Figures 5 and 6). Seven 40-amino acid WD repeats constitute the blades (Sondek *et al.*, 1996; Lambright *et al.*, 1996; Wall *et al.*, 1995). The γ subunit consists of two helical segments joined by a loop and has no tertiary structure by itself. The γ subunit N-terminal helix forms a coiled coil with the N-terminal helix of the β subunit and the γ C-terminal helix lies against two of the β subunit blades. Therefore, a portion of the receptor, the N-terminus of the $G\alpha$ subunit, a portion of the β subunit, and the C-terminus of the γ subunit all lie in close proximity to each other and the cell membrane (Figure 2). The C-terminus of the γ subunit has been shown to provide some of the specificity between the various heterotrimeric G protein complexes and receptors (Kisselev *et al.*, 1995; Yasuda *et al.*, 1996).

In the model system *Dictyostelium discoideum*, the $G\alpha_2$ subunit is critical for an aggregation response and will be discussed later. Several mutations causing aggregation negative phenotype were analyzed. Two of these are in the $G\alpha_2$ helical domain. One of these two lies directly adjacent to the conserved arginine involved in GTP γ phosphate hydrolysis while the other lies near the surface of the helical domain (see Figure 7).

1.3 *Dictyostelium discoideum* as a Model System

1.3.1 *Dictyostelium discoideum* Development

Dictyostelium discoideum is a primitive eukaryotic organism with several characteristics which make it useful for laboratory study (reviewed in Kessin, 2001). It is a haploid cellular slime mold whose natural habitat is the soil of the forest floor where it feeds on bacteria. The earliest strains used for modern studies were isolated from decaying leaves in forests near Asheville, North Carolina (Raper, 1935). During the feeding vegetative stage, individual amoeba undergo chemotaxis toward their food source up chemical gradients supplied as by-products of bacterial metabolism, predominantly folate. During times of starvation, certain cells begin emitting extracellular pulses of cAMP. This further stimulates other cells to do the same, thus relaying the signal. This chemical signaling induces a transition from the unicellular vegetative stage through aggregation to the multicellular stage which, under other regulators, progresses from mounds to the formation of a mobile slug moving toward light and heat, and culminating in a fruiting body (Figure 8). This final developmental stage has a pocket of spores attached atop a stalk. The spores are heat and dessication resistant and, in the wild, the stalk extends out into spaces in the soil such that the spores can be passively moved to other locations by attachment to mobile organisms present in the soil habitat. Strains of *Dictyostelium* that can live on a nutrient broth mixture instead of bacteria (axenic strains) have been developed (Watts and Ashworth, 1970). The starvation change can be induced abruptly by a change to a non-nutrient buffered medium and the resulting biochemical events then studied. *In vivo*, the series of biochemical events

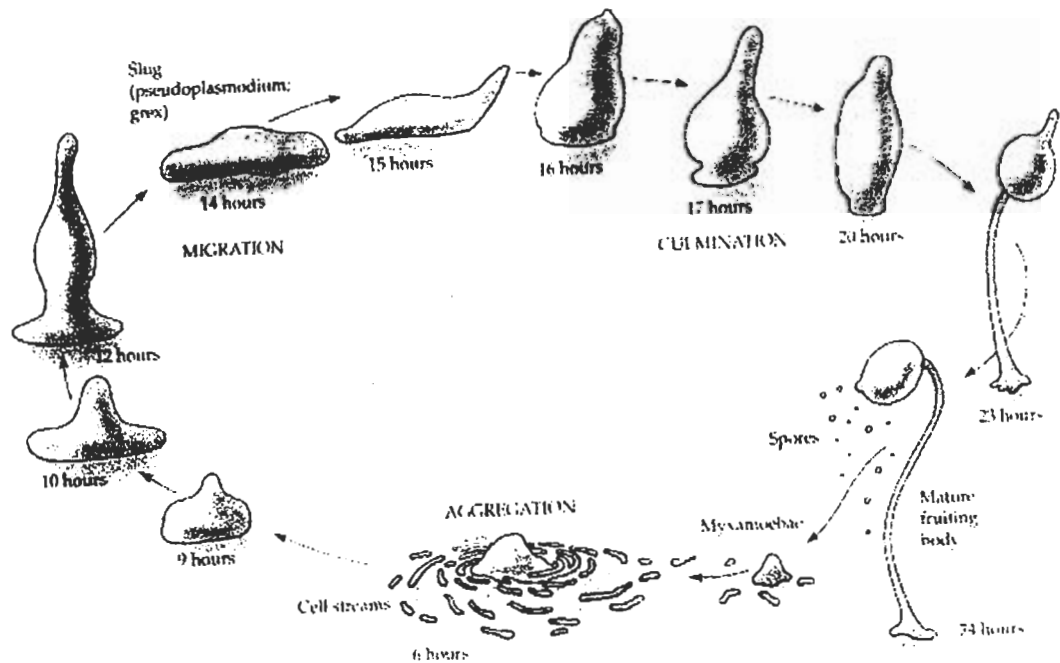


Figure 8. *Dictyostelium discoideum* lifecycle. The transition from unicellular amoeba to the aggregation stage is easily induced in the lab and specifically requires the Gα2 subunit.

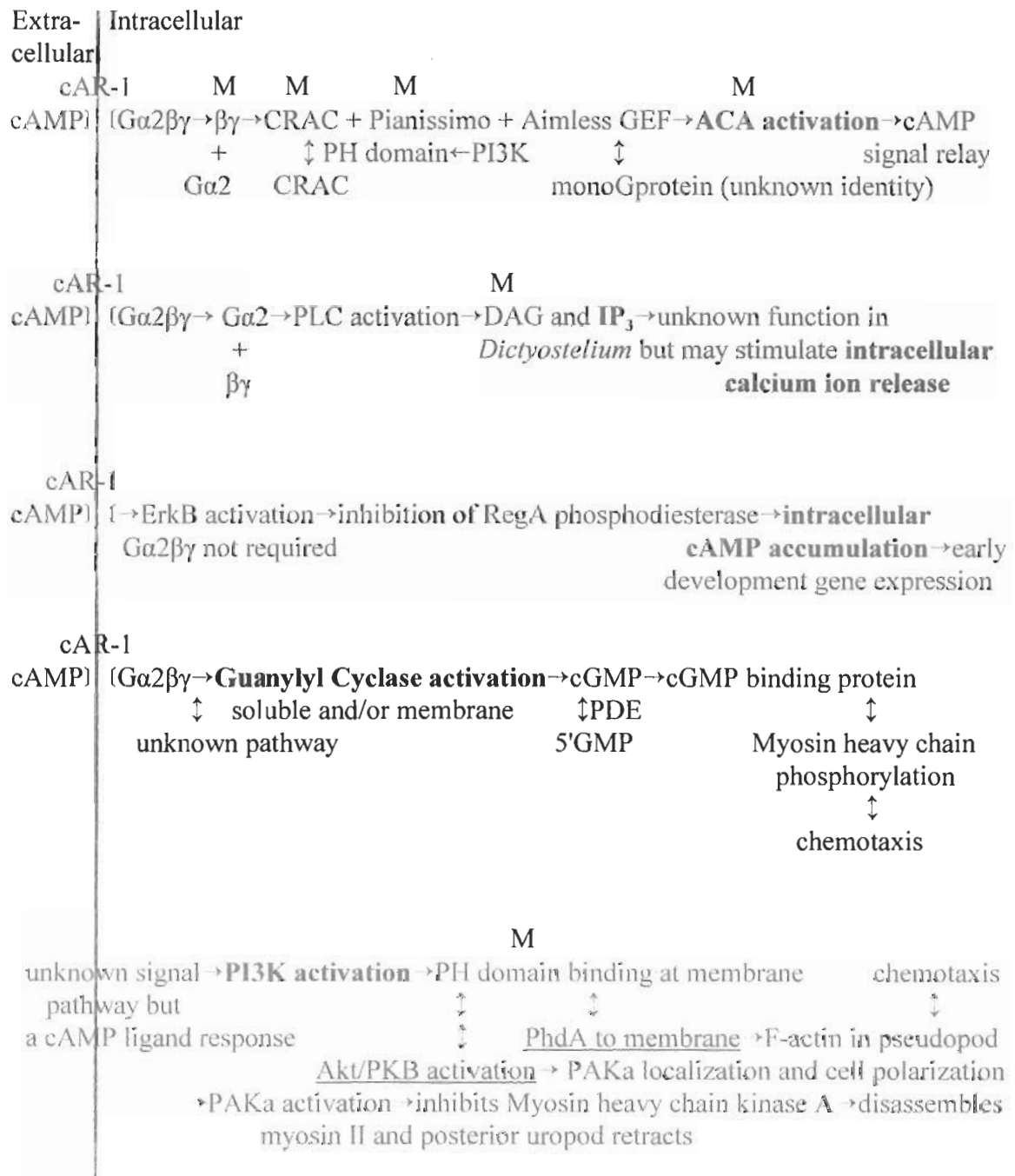
that lead to chemotaxis up a cAMP gradient driving toward aggregation are completely dependent on the extracellular signaling progressing through heterotrimeric G-protein signaling and specifically requiring the Ga2 subunit (Coukell *et al.*, 1983; Kesbeke *et al.*, 1988). In *Dictyostelium*, there is only one β subunit gene (Lilly *et al.*, 1993). This lends itself to study of mutations of the Ga2 subunit and the resulting effects on interactions of this protein with the other proteins involved in signaling at this early cascade branch point.

1.3.2 The *Dictyostelium discoideum* Ga2 subunit

In *Dictyostelium*, eight Ga subunits have been cloned to date. Others are presumed to exist based on sequence comparison within database sequences (Parent and Devreotes, 1996). Between them there is a 30-35% amino acid identity and a similar amount of identity exists between *Dictyostelium* Ga subunits and mammalian ones in general. The major mammalian Ga subunit families, Gai, Gas, Gaq (Simon *et al.*, 1991), do not correlate directly with any of the *Dictyostelium* Ga subunits, however numerous amino acids in important areas of these proteins are identical.

Much work has been done on the interactions between cAR-1 (the GPCR specific for Ga2), the Ga2 subunit, the $\beta\gamma$ subunits, and several of the immediate downstream effector proteins (reviewed in Kessin, 2001). A summary of these pathways is found in Figure 9. The downstream effects from extracellular cAMP binding to cAR-1 include induction of early development genes, activation of the membrane enzyme adenylyl cyclase A (ACA), activation of guanylyl cyclase (GC) activities (there are two GC's in *Dictyostelium* (Roelofs J, Snippe H *et al.*, 2001; Roelofs J, Keima M *et al.*, 2001)), activation of phospholipase C

Figure 9. Known Pathways involving the cAR-1 receptor and the $G\alpha 2\beta\gamma$ subunit. The overall responses in each pathway to extracellular stimulation of the cAR-1 GPCR by cAMP are highlighted in bold type. The vertical line represents the plasma membrane with the cAR-1 receptor traversing the membrane. Biochemical events requiring membrane localization have the capital letter 'M' over them (data from Kessin, 2000).



(PLC), and an increase in intracellular calcium ion concentration. The pathway leading to ACA activation has been extensively defined (reviewed in Kessin, 2001). It involves the $\beta\gamma$ subunits and includes membrane localization of Cytosolic Regulator of Adenylyl Cyclase (CRAC), using a pleckstrin homology (PH) domain as a mechanistic component. A cytosolic protein, Pianissimo, is required as is the *ras* guanine nucleotide exchange factor, Aimless. The cAMP produced by ACA is mostly released extracellularly as part of the signal relay during aggregation. Mutations of the β subunit have been developed which do not activate ACA yet are functional in other heterotrimeric G-protein activities, including chemotaxis toward cAMP (Jin, Amzel *et al.*, 1998). The pathway leading to induction of early development and chemotaxis protein genes involves intracellular cAMP as a second messenger, produced by a different AC than ACA. In this pathway, the activated cAR-1 receptor itself, independent of the heterotrimeric G-protein complex, leads to phosphorylation and thus activation of ErkB (Gaskins *et al.*, 1996; Segall *et al.*, 1995; Wang *et al.*, 1998). ErkB is a mitogen activated protein kinase (MAK) whose phosphorylation is required for chemotaxis and is involved with other proteins in regulating intracellular second-messenger cAMP levels. Intracellular cAMP levels regulate cAMP-dependent protein kinase (PKA), a critical protein throughout the aggregation and development stages in *Dictyostelium* (Wu *et al.*, 1995). The $\beta\gamma$ subunits have been shown to be essential for expression of genes involved in aggregation but not later development (Jin, Soede *et al.*, 1998). Figure 10 shows the timing of the expression of various proteins required during the aggregation stage of the *Dictyostelium* lifecycle.

Figure 10. Gene expression during aggregation. All of these proteins are required for the aggregation response to extracellular cAMP in *Dictyostelium* {from Parent and Devreotes, 1996}. There is only one G β subunit gene in *Dictyostelium* and it is constitutively expressed. Although GCA is expressed during chemotaxis, it's level of expression decreases during this time.

Time from starvation onset{hours}:	Stage of development					
	<u>amoeba</u>	<u>chemotaxis</u>	<u>mound</u>	<u>slug</u>	<u>fruiting body</u>	
	0	5	10	15	20	25
Expressed protein						
cAR-1		*****				
G α 2		*****				
CRAC		*****				
Aimless		*****				
ACA		*****				
G β	*****	*****	*****	*****	*****	*****
sGC		*****				
GCA	*****			*****	*****	*****

cAR-1 → the G-protein coupled receptor stimulated by cAMP and associate with the G α 2 subunit

G α 2 → the G α 2 subunit

CRAC → Cytosolic Regulator of Adenylyl Cyclase; in the pathway leading to ACA activation and cAMP production

Aimless → a protein in the pathway leading to ACA activation

ACA → Adenylyl Cyclase A; a membrane bound enzyme which produces the cAMP secreted extracellularly, termed signal relay, producing cell aggregation

G β → the G β subunit

sGC → soluble Guanylyl Cyclase; in the pathway leading to chemotaxis

GCA → membrane bound Guanylyl Cyclase; possibly not in the pathway leading to chemotaxis

The chemotaxis and aggregation responses to extracellular cAMP require the presence of the Gα₂ subunit *in vivo*, but several of the biochemical events can be bypassed *in vitro* (reviewed in Parent and Devreotes, 1996). Beyond this observation, the role of the Gα₂ subunit in this process has not been well defined. Also unclear, but with more data, is the role of cGMP as a second intracellular messenger. Activation of guanylyl cyclase activity with cGMP production occurs rapidly after cAMP binding and cGMP is detected in 15 seconds (Table 1). Actin polymerization into F-actin and possibly both production of inositol phosphate (IP₃) and activation of phosphoinositol 3' kinase occur within 5 seconds, by unknown pathway, and are the first detectable effects on cAMP binding to the receptor (reviewed in Kessin, 2000). Intracellular cAMP reaches a peak 60 seconds from binding. Altered metabolism of cGMP has long been known to influence chemotaxis in *Dictyostelium*. Streamer F mutants demonstrate a prolonged chemotactic response, although initially delayed and slower throughout, which correlates with an abnormally prolonged cGMP rise (reviewed in Newell and Liu, 1992). This is caused by a defective cGMP-phosphodiesterase. Concurrent and presumably cGMP influenced events include a prolonged calcium ion influx and increased myosin II light chain phosphorylation and myosin association with the cytoskeleton. Interestingly, F-actin production in streamer mutants is unaffected. In contrast to streamer mutants, the K-10 mutant, which does not mount a cGMP response to cAMP, does not induce myosin II association with the cytoskeleton (Kuwayama *et al.*, 1993). Its protein defect has not been characterized. Recently, the two guanylyl cyclase genes identified in *Dictyostelium* and their enzyme products have been characterized (Roelofs J, Snippe H *et al.*, 2001; Roelofs J, Keima M *et al.*, 2001).

Table 1. Secondary Events after stimulation of the cAR-1 receptor (from Kessin, 2000).
Figure 9 shows the known pathway proteins involved in these events.

Time (seconds)	Biochemical Event
0	Extracellular cAMP stimulation of the cAR-1 receptor
5	Initial cytoskeletal actin accumulation; PI3K activation (?)
10	cGMP production peaks; CRAC translocation to the membrane peaks
20	Intracellular Ca^{++} reaching its peak; PI3K activation (?); secondary cytoskeletal actin accumulation
30	Cytoskeletal myosin II accumulation peaks
60	Initial cAMP production response peaks

cAR-1 → the GPCR for cAMP and the $\text{G}\alpha 2\beta\gamma$ G-protein

PI3K → phosphatidylinositol 3' kinase

CRAC → Cytosolic Regulator of Adenylyl Cyclase

Although GC activity clearly modulates the chemotactic response as noted, the deletion of both GC genes, and thus all resulting cGMP production, only partly affects but does not eliminate chemotaxis to cAMP (Roelofs and van Haastert, 2002). These two GC's appear to be more closely related to mammalian adenylyl cyclases. Guanylyl cyclase A (GCA) is a twelve-transmembrane protein with two cyclase domains, with the active cyclase domain appearing to be opposite it's mammalian AC counterpart. The other GC, soluble guanylyl cyclase (sGC), demonstrated significant identity with the recently discovered human soluble AC. Elimination of sGC produced the greatest chemotactic defect, although only a moderate one. This was demonstrated by the inability of sGC-null cells to aggregate at a density of 3×10^4 cells/cm² which did not prevent wild type aggregation. Also, a 20-fold increase in cAMP concentration was required to induce a chemotactic response in sGC-null cells (Roelofs, Meima, *et al.*, 2001). Eliminating both genes did not further reduce this defect. Interestingly, the gene expression of the membrane bound GCA temporarily decreases during aggregation while sGC expression temporarily increases. During other stages, GCA expression predominates (Figure 10). A very recent report introduces two novel cGMP binding proteins, GbpC and GbpD, into the pathway leading toward effective chemotaxis. These proteins are homologous to one another and contain Ras, MAPKKK and Ras-GEF domains (Bosgraff *et al.*, 2002). Their elimination is reported to produce a severe chemotactic deficit. In another recent experiment, the membrane bound guanylyl cyclase, GCA, was mutated in it's purine recognition site to recognize ATP instead. This revealed an unexpected observation that, *in vitro*, GTP γ S activates GCA in mutant clones where both the G α 2 and G α 4 (the latter required for chemotaxis to folate during the vegetative stage) genes

have been eliminated. This suggests another G-protein, such as the novel monomeric G-protein, is involved as part of the pathway leading from $G\alpha\beta\gamma$ activation to cGMP production (Roelofs, Looovers *et al.*, 2001). Defining the role of cGMP in chemotaxis has been hampered by the lack of success, to date, of purifying the protein or cloning the gene of both cGMP phosphodiesterase and a cGMP-dependent protein kinase. Evidence of the former's existence is clear (streamer F mutants) however evidence of the latter's existence is only speculative. Further investigation into the newly discovered GbpC and GbpD proteins likely will yield more detail regarding chemotaxis initiation.

As mentioned earlier, F-actin formation is detectable by 5 seconds after cAMP stimulation in wild type cells. This occurs at leading edges, termed pseudopods, in chemotactic movement (Funamoto *et al.*, 2001). The protein, PhD is required for this F-actin formation in the pseudopod. Activation of phosphoinositol-3 kinase (PI3K) also plays a role in chemotaxis. The monomeric G-protein Ras is a known activator of PI3K. Activity of PI3K creates membrane sites for localization of signaling proteins having PH domains. The protein, PhD, is localized by this mechanism and requires PI3K activation to localize (Funamoto *et al.*, 2001). This implies early activation of PI3K, possibly involving a *ras*-like activator. Note that myosin II accumulation, requiring the production of cGMP, occurs at the trailing edge of chemotactic movement.

The direct interactions between the heterotrimeric G-protein complex and these and/or other proteins, including cGMP phosphodiesterase and monomeric G-proteins, leading to the chemotactic response have not been defined. These experiments do suggest however that intermediate proteins are involved, either in a cascading manner or as part of

a complex. It is also not clear whether the G α 2 subunit or the $\beta\gamma$ subunits are the critical conductor of the signal leading toward chemotaxis. As there is only one β subunit gene in *Dictyostelium*, its deletion attenuates all heterotrimeric G-protein activity in this organism. Some of the β subunit *synag* mutants are normally chemotactic and some are not (Jin, Amzel *et al.*, 1998) and nothing can be concluded about the $\beta\gamma$ subunits role in chemotaxis from these mutants. Also, although the G α 2 subunit is absolutely required *in vivo* for chemotaxis to cAMP, it's interaction with any downstream effector in an *in vivo* system requires the single and invariant β subunit. Therefore, nothing can be concluded from the 'absolute requirement' observation regarding the G α 2 subunit's chemotactic signaling role either.

1.4 $G\alpha$ subunit: Critical Amino Acids

The members of the heterotrimeric G-protein superfamily show similar general structure, similar conformational changes effected by GDP/GTP exchange and hydrolysis, and similar functions. In addition to the separation of the $G\alpha$ and $\beta\gamma$ subunits, all $G\alpha$ subunits undergo a conformational change in the switch I, II, and III regions between GDP and GTP bound forms (Noel *et al.*, 1993; Lambright *et al.*, 1994; Coleman *et al.*, 1994; Mixon *et al.*, 1995). The most dramatic of these involves switch II. The conformational changes affect the mutual orientations of the switch regions with various other surfaces, importantly the $\alpha 3$ helix, the $\alpha 3$ - $\beta 5$ loop, and the $\alpha 4$ - $\beta 6$ loop, even though these latter areas do not themselves change conformation. The $\alpha 3$ helix and the $\alpha 3$ - $\beta 5$ loop, along with the $\alpha 2$ helix containing switch II, lie on or near the same face of the α subunit. The $\alpha 4$ - $\beta 6$ loop is on an adjacent face and could easily be visualized as interacting with an effector protein which wraps around these two adjacent faces (see Figures 4 and 11). Effector proteins appear to interact with varying sets of these switch and effector binding areas rather than with a single region.

The $G\alpha$ subunit/effector protein interactions of $G\alpha_i$ and $G\alpha_s$ with adenylyl cyclase (Grishina and Berlot, 1997) and of $G\alpha_t$ with cGMP phosphodiesterase (Natochin *et al.*, 1998) are the best studied interactions to date. Solvent exposed amino acids in the switch regions, the $\alpha 3$ helix and the $\alpha 3$ - $\beta 5$ and $\alpha 4$ - $\beta 6$ loops have been mutated individually and in clusters and the resulting effects studied regarding ability to activate effector proteins (Berlot and Bourne, 1992; Rarik *et al.*, 1992; Spickofsky *et al.*, 1994; Faurobert *et al.*, 1993; Mittal *et al.*, 1996; Medina *et al.*, 1996; Itoh *et al.*, 1991; Artemyev *et al.*, 1992; Skiba *et al.*, 1996;

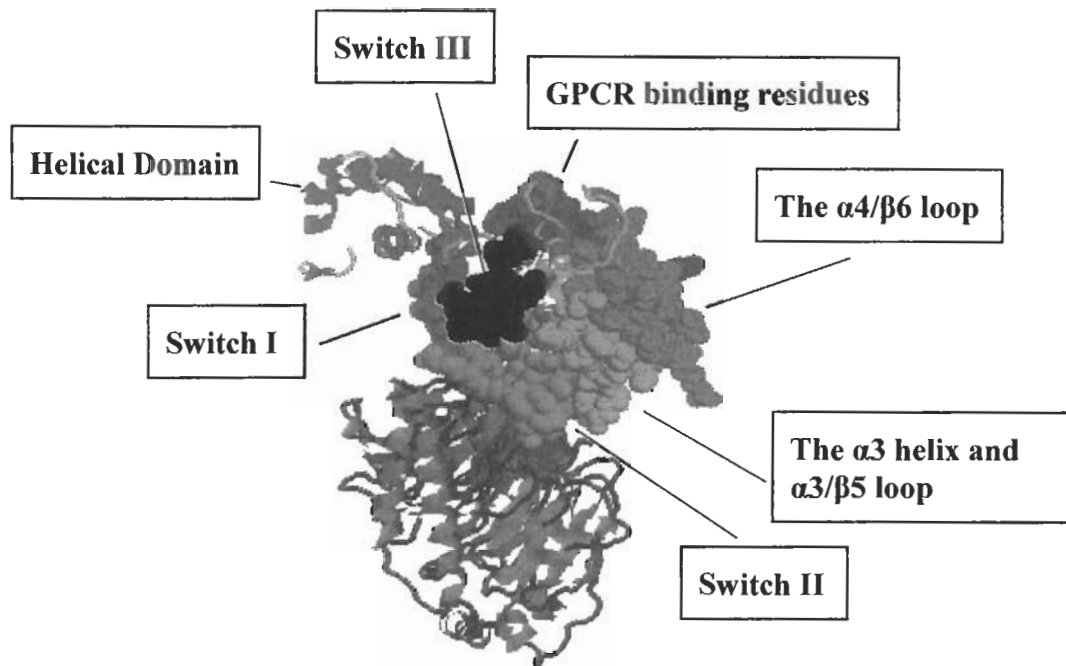


Figure 11. Effector protein binding areas. In this figure, the orientation is looking down from above the G-protein with the plasma membrane lying flat beneath. Effector interacting amino acids in the $\alpha 3$ helix and $\alpha 3/\beta 5$ loop are highlighted in violet and are on the same face of the $G\alpha$ subunit as switch II in gold spacefill. The effector interacting residues on the $\alpha 4/\beta 6$ loop are colored light green and are on a different face. There is a slight ridge between the two effector interacting faces of the $G\alpha$ subunit. Both faces would be easily available to a single effector protein upon disassociation of the $\beta\gamma$ subunit colored in gray. This view also highlights the positioning of the helical domain between the receptor binding area in blue spacefill and switch I in red spacefill.

Tesmer *et al.*, 1997; Sunahara *et al.*, 1997). The emerging general picture is one where certain specific amino acids from some but not necessarily all of these areas combine together to activate the effector enzyme. Amino acids in the switch I and II regions provide selective affinity of the GTP-bound, active form of the $G\alpha$ subunit for the effector enzyme. Specificity of $G\alpha$ subunit to effector protein is not exclusively provided by any single one of these areas but rather on differing combinations depending on the particular $G\alpha$ subunit/effector protein combination. In this manner, for example, the same effector enzyme, adenylyl cyclase, can bind to different sets of binding sites on G_{ai} and G_{as} . This also involves different locations on adenylyl cyclase so the enzyme can be inhibited versus activated.

In the G_{at} subunit switch II region, neighboring amino acids to effector interacting amino acids have been shown to interact with RGS proteins. Space filling models suggest that differing faces of the G_{at} subunit are involved with the RGS protein versus the effector enzyme cGMP phosphodiesterase and use separate sets of amino acids (Tesmer *et al.*, 1997). This implies the possibility of a complex forming involving the $G\alpha$ subunit, the regulatory protein, and the effector enzyme. The effector enzyme may be activated, but does not remain activated for what might be too long in regards to other downstream signaling events.

Figure 12 shows the alignments in effector areas between various $G\alpha$ subunits as well as the wild type amino acids whose mutation caused the aggregation phenotype and were studied in our research. Note the high conservation of an asparagine residue at position 74 in the $G_{\alpha 2}$ subunit and a branched hydrophobic chain at position 110. Space filling models show this asparagine is in a position to interact with the arginine in switch I, also highly

Figure 12. Alignments between $G\alpha$ subunits. Switch II shows the highest level of conservation reflecting its more general role of involvement in activating the $G\alpha$ subunit and, by its changed orientation, representing this activated state to effector proteins. The $\alpha 3$ helix and $\alpha 3$ - $\beta 5$ loop are just C-terminal from switch III, are effector protein binding sites, and have either minimal or no conformational change on $G\alpha$ subunit activation. The $\alpha 4$ - $\beta 6$ loop is an effector protein binding area and does not change conformation. These latter two regions have less ($\alpha 3$ helix and $\alpha 3$ - $\beta 5$ loop) or minimal ($\alpha 4$ - $\beta 6$ loop) amino acid conservation suggesting their more significant role of imparting specificity to $G\alpha$ subunit/effector protein interaction. The N74D and L110S mutations are in locations of high conservation of amino acids. The N74D conservation is specific and total among these subunits and the L110S conservation is that of a hydrophobic side chain. The amino acids highlighted in bold in possible effector binding areas (the $\alpha 3$ helix, $\alpha 3$ - $\beta 5$ loop and the $\alpha 4/\beta 6$ loop) are the four mutations resulting in aggregation negative phenotype which will be studied further in Dr. Gundersen's laboratory in future experiments.

Switch II.

Gai2	(200-221)	F D V G G Q R S E R K K W I H C F E G V T A
Gas	(222-243)	F D V G G Q R D E R R K W I Q C F N D V T A
Gat	(195-216)	F D V G G Q R S E R K K W I H C F E G V T C
Ga2	(203-224)	V D V G G Q R S E R K K W L S C F D D V T A

$\alpha 3$ helix and $\alpha 3$ - $\beta 5$ loop

Gai1	(247-261)	M K L F D S I C N N K W F T D
Gas	(267-281)	L N L F K S I W N N R W L R T
Gat	(243-257)	L H L F N S I C N H R Y F A T
Ga2	(251-264)	L R V F S D V C N S - W F V N

$\alpha 4$ - $\beta 6$ loop

Gai2	(300-321)	A A S Y I Q S K F E D L N K R K D T K E I Y
Gas	(337-358)	A K Y F I R D E F L R I S T A S G D G R H Y
Gat	(295-316)	A G N Y I K V Q F L E L N M R R D V K E I Y
Ga2	(302-322)	A S N Y I K E R F W Q I N - K T E Q K A I Y

Conservation at the mutation sites (see discussion)

	<u>N74D</u>	<u>L110S</u>
Gai	76 N	110 L
Gao	76 N	110 V
Gas	98 N	133 L
Gat	72 N	106 M
Ga2	74 N	110 L
Gaz	76 N	110 L
Gaq	76 N	110 V

conserved (Figure 7). The insertion of the negatively charged aspartic acid instead of its amide, asparagine, is likely to affect the function of this arginine in the $G\alpha$ subunit (see Discussion). At position 110, the branched hydrophobic side chain on leucine may be important in local folding. Insertion of a smaller side chain with a polar -OH would affect hydrophobic interaction. The location of L110 in the helical domain is best shown in Figure 7. These mutations are in an area of the helical domain that is away from the cytoplasmic portion of the receptor (Figure 4) so would not be expected to affect receptor binding. They may however have some influence on the role of the helical domain.

1.5 $G\alpha$ subunit: Helical Domain

The helical domain of the $G\alpha$ subunit is so named as it consists of six helices of varying length connected by short loops. This domain is the N-terminal half of the $G\alpha$ subunit and is an independently folded, autonomous rigid domain which experimentally has been separately produced. When produced and added separately, it imparts $G\alpha$ subunit activity to a related G-domain (Markby *et al.*, 1993; Benjamin *et al.*, 1995), so may best be considered as an attached subunit. This characteristic and its location immediately adjacent to the GDP/GTP binding site has led to its hypothesized role as a lid, opened by the receptor, to facilitate GDP/GTP exchange (Noel *et al.*, 1993). The addition of the helical domain to the G-domain is associated with GTPase activation of the latter (Markby *et al.*, 1993). This observation along with other observations of the GPCR acting as a GEF (Franke *et al.*, 1992; Ernst *et al.*, 1995; Acharya *et al.*, 1996) leads to the hypothesis that the helical domain is an attached GEF/GAP regulated by the receptor and possibly by other RGS proteins. The third

cytoplasmic loop of the GPCR contains a short conserved E(D)RY sequence required for the receptor to effect GDP release (Acharya *et al.*, 1996). Specifically the arginine residue is critical for this function. Interestingly, the third cytoplasmic loop also is involved with specificity of $G\alpha$ subunit binding whereas the second cytoplasmic loop plays more of a general role of inducing conformational change in the $G\alpha$ subunit (Yamashita *et al.*, 2000). With these observations in mind, a proposed mechanism for GDP/GTP exchange would be the GPCR binding part of the $G\alpha$ subunit and sterically interacting through the helical domain of the $G\alpha$ subunit to transmit the receptor's conformational change into the $G\alpha$ subunit such that an empty state can be maintained while the GDP molecule exits and is replaced by a GTP molecule. The addition of this higher energy molecule and its extra charge would further complete the GDP/GTP exchange by creating $G\alpha$ subunit conformations in the switch regions which have low affinity for both the $\beta\gamma$ subunits and the receptor, promoting dissociation. The final, but essentially simultaneous, event is the receptor being left in a low affinity state for its ligand. The binding of GTP must be 'registered' with the receptor in some manner to alter its ligand affinity. This may be done by release of the $\beta\gamma$ subunits, release of the $G\alpha$ subunit and/or $G\alpha_2$ subunit conformational change from the binding of the GTP molecule itself. The N74D mutant and L110S mutants provide some data and insight in support of the proposed role, mentioned above, of the helical domain.

1.6 Rationale

The role of the G-domain of the $G\alpha$ subunit has been well studied. Critical amino acids have been identified both by site-directed mutagenesis, as in alanine scanning, as well

as characterization of mutants. In the *Dictyostelium* Ga2 subunit, critical mutations are most easily screened for by their phenotypic appearance as aggregation negative mutants. The helical domain of the Ga subunit is less well characterized than the G-domain but is an integral part of all heterotrimeric G-proteins. This observation leads to the hypothesis that the helical domain is used for a function that is conserved throughout G-protein signaling. The *Dictyostelium* Ga2 subunit provides a useful way to investigate this hypothesis. If it is performing a conserved function, a helical domain mutation which disrupts that function should produce a defective Ga2 subunit that results in an aggregation negative mutant. Screening a library of randomly generated mutations in the *ga2* gene would focus mutations in just the Ga2 subunit and may identify mutations located only in the helical domain but producing non-functional Ga2 subunits. As the crystal structure of the Ga β γ complex is known, the location of specific mutations may further suggest which mutants to study in more detail.

Taking this approach, a library of *ga2* mutant plasmids was screened for aggregation minus phenotype when introduced into *ga2*-null *Dictyostelium*. This library was previously created by Jian-xin You in Dr. Gundersen's lab by the technique of PCR amplification of the Ga2 gene during conditions of unbalanced nucleotide concentrations (You, 1996). Further evaluation led to the identification of two aggregation negative mutations located in the helical domain of the Ga subunit near switch I. Study of one of the mutants, the N74D mutant, suggests an important role for the helical domain in GDP/GTP exchange.

2. MATERIALS AND METHODS

2.1 Cell Culture

Dictyostelium discoideum cell lines used in this project were: Ax-3, an axenic wild-type cell line; MYC-2, a *ga2*-null cell line; Ga2 Wild Type, MYC-2 with a plasmid containing the un-mutated Ga2 subunit gene cloned into the *Dictyostelium* extrachromosomal expression vector pJK1; and SN5A2, a specific clone of the *synag 5* cell line containing a G β mutation plasmid (Jin, Amzel *et al.*, 1998) transformed into the *g β* -null cell line LW-6. Untransformed cells were grown axenically in HL-5 medium in shaking culture (Watts and Ashworth, 1970) or on plastic petri dishes. Transformed *Dictyostelium* lines were grown in HL-5 with the antibiotic G418 at 20 μ g/ml.

2.2 Ga2 Random Mutation Library

A PCR generated DNA library containing random mutations in the Ga2 gene, cloned into the pJK1 plasmid for use either in *E. coli* or *Dictyostelium* (Jian-xin You Thesis, 1996), was utilized to focus on the effects of Ga2 subunit mutations. This library had been transformed into electroporation-competent JS4 *E. coli* and stored in 16% glycerol/LB at -70°C. Frozen samples were grown under ampicillin selection (50 μ l/ml) with plasmid extracted utilizing plasmid mini prep protocol and kits (Qiagen).

2.3 Expression of the Mutant Library in *Dictyostelium*

The Ga2 random mutation library plasmid DNA was transformed into MYC-2 cells by electroporation using a Bio-Rad Gene pulser (Howard *et al.*, 1988) with the settings of 1.2 kv, 200 Ω and 3 μ FD. Heat killed *Klebsiella aerogenes* bacteria (1×10^{10} /ml stock concentration) were added to HL-5 medium at 1% v/v to improve effectiveness of transformation (Joly *et al.*, 1993). After 24 hours, the broth was changed to HL-5 supplemented with G418 and the transformants divided equally into two 24-well plates per transformation, resulting in partial separation of transformants.

2.4 Phenotype Screen

Transformants were screened for aggregation negative phenotype by placing approximately 50 cells in 300 μ l Development Buffer (DB; 5 mM Na₂HPO₄, 5 mM KH₂PO₄, 2 mM MgSO₄, 0.2 mM CaCl₂) containing live *Klebsiella* in suspension, then spread on SM/5 plates and incubated at 22°C. The individual cells grow into plaques on the bacterial lawn. In the nutrient poor centers of the plaques, cells either enter into multicellular development or remain unicellular (aggregation negative). On plates displaying entirely or nearly total aggregation negative phenotype, cells from an individual plaque were picked and regrown in 24-well plates under G418 antibiotic selection.

2.5 Development Phenotype Observation

The aggregation negative phenotype of selected clones was re-confirmed by plating 5×10^7 cells on 150mm plates made with Development Buffer and agar at 1.5% w/v. The harvested cells were first washed in 30 ml DB then plated. Observations of development were made at 24 and 48 hours.

2.6 Western Blot/Immunoblotting

The aggregation negative clones were evaluated for Gα2 protein expression. A sample of 2×10^6 cells was solubilized in SDS-PAGE sample buffer and components separated by 10% SDS-polyacrylamide gel electrophoresis (Laemmli, 1970). Transfer of separated components from the gel to a nitrocellulose membrane (0.45μm) was done using a Hoefer transfer box filled with Transfer Buffer using a current of 200mA for 45 minutes at 4°C. The membrane was stained with Ponceau S (Sigma) to identify molecular weight markers which were then marked with clay pencil. Blocking of the membrane was performed in 5% non-fat dried milk in Tris Buffered Saline (0.02 M Tris, pH 8; 0.14 M NaCl) with 0.1% Tween 20 (TBS/Tween) added and incubated at 4°C overnight. The primary antibody (rabbit) directed against the 20 amino acid Gα2 peptide sequence H₂N-CASSMEGEKTNNTDINLSIEK-COOH, which begins at amino acid #4 of the N-terminus (Kumagai *et al.*, 1989), was diluted 1:5000 in TBS/Tween and incubated with the membrane for 1 hour at room temperature. The blot was rinsed, washed 3 times in TBS/Tween for 10 to 15 minutes each wash. The blots were incubated with secondary antibody (horseradish peroxidase-coupled goat anti-rabbit IgG from Sigma, diluted 1:5000 in TBS/Tween) for 1

hour. The blots were washed as above except the final wash was in 0.1 M Tris, pH 8.5. Reactive bands were detected by enhanced chemiluminescence (Root *et al.*, 1999) and the Gα2 bands qualitatively identified.

Immunoblots of cAR-1 receptor expression were done in a similar manner as above except lysis was done osmotically. Cells were placed in cold 98% saturated ammonium sulfate for 5 minutes. They were micro-centrifuged at 5000 rpm for 5 minutes at 0°C with the supernatant quickly aspirated off. ERB buffer (Root *et al.*, 1999) was added with vortexing. The lysate was centrifuged down at 16,000 rpm for 10 minutes. The pellet was processed for immunoblot as above. Primary antibody was against the cAR-1 receptor (antibody a gift of P.N. Devreotes).

2.7 Plasmid Recovery from *Dictyostelium*

For recovery of plasmid from *Dictyostelium*, 5×10^7 cells were harvested, washed in DB buffer once and resuspended in 0.5ml 'Real Lysis' buffer (0.32 M sucrose; 10 mM Tris, pH 7.5; 5 mM MgCl₂; 1% v/v Triton X-100). The nuclear pellet containing the plasmid was separated by centrifugation in an Eppendorf tube (16,000×g for 10 min.). The pellet was resuspended in 200 µl Buffer A (10 mM Tris, pH 7.5; 10 mM EDTA) and 220 µl of Buffer B (10 mM Tris, pH 7.5; 0.7% v/v SDS). Residual RNA was removed by adding 30 µl RNase A (10mg/ml) and incubating at 65°C for 40 minutes. Subsequently, digestion with Proteinase K was done by adding 30µl of a 20 mg/ml concentration and incubating at 65°C for 50 minutes. DNA was isolated by phenol/chloroform extraction, then ethanol/sodium acetate precipitation. This DNA was transformed into CaCl₂-competent MC1061 *E. coli* and plasmid

transformation was identified using ampicillin selection. The rescued plasmids were biologically amplified using the bacteria and the plasmids extracted using the Qiagen plasmid mini prep protocol.

2.8 DNA Sequence Analysis

Sequencing of the mutated *ga2* DNA was done by the University of Maine DNA Sequencing Facility. The *Ga2* internal primers G2-2S and G2-3A were used. The *ga2* gene is 1174bps total. These two 17mer primers are sense/antisense in orientation, producing a 195bp overlapping sequence in the central area of the gene. The primers' central location and the usual 6-700bp sequencing run allows for accurate and complete sequencing of the *ga2* gene using the equipment and programming at the University of Maine (University of Maine DNA Sequencing Facility, Patricia Singer, Director. Equipment: ABI 373 Stretch DNA Sequencer with XL Upgrade. Software: ABI Sequence Navigator DNA and Protein Sequence Comparison Software. Reagents: ABI Prism BigDye Terminator V 3.0 Cycle Sequencing Ready Reaction Kit). Sequences were compared with the wild type *ga2* sequence and clones containing one or two amino acid changes were selected for further study.

2.9 Site Directed Mutagenesis with PCR Amplification

Two mutants, each containing a single amino acid change, were used directly in further experiments. Selected mutants containing two amino acid changes were evaluated for possible re-introduction of single mutations by site-directed mutagenesis.

The primers used to create the individual mutants were obtained commercially (Operon) and are listed in Table 2.

2.10 Chemotaxis Assay

Chemotaxis to exogenously supplied cAMP was performed by a modification of the protocol described by Dr. Jared Rifkin (Queens University, personal communication). A total of 35×10^6 Dictyostelium cells were harvested, washed once in DB buffer and resuspended in 5 ml of DB at a 7×10^6 cells/ml concentration. These were placed in a thoroughly rinsed 125 ml Erlenmeyer flask rotating at 100 rpm for 4 to 5 hours. Chemotaxis plates were 150mm petri dishes filled with 20 ml DB and 2.3% non-nutrient agar. Wells were made in the agar using 3 mm hollow skin biopsy forceps attached to vacuum. Drops (1 μ l) of the pre-starved cell line of interest were placed 4 mm from the edge of the well in 3 or 4 locations. The well was filled with 20 μ l of 10 μ M cAMP and the plate was incubated at 22°C. Observations were made 3 to 5 hours later and chemotaxis was judged positive if cells had migrated specifically to the well side of the drop in all of the three drops (See Figures 13 and 14). Chemotaxis was judged negative if all three of the drops showed both uniform distribution in the drop and the cells had been judged healthy at the time of drop placement by observing clear pseudopod formation/polarity development in at least three quarter of a 10 μ l sample of cells under the microscope. Negative chemotaxis assessment also required a positive control on each plate using Ga2 Wild Type cells where Ga2 expression is by transformed plasmid as in the experimental groups.

Table 2. Primers for site directed mutagenesis. These mutations all cluster in possible effector interacting areas ($\alpha 3$ helix through $\alpha 3/\beta 5$ loop and $\alpha 4/\beta 6$ loop) on the G $\alpha 2$ subunit and will be used in future experiments. See Table 3 for more details on the locations of these mutations.

V257G

s: GTTTACGTGTATTCAGTGATGGTTGCAATAGTTGGTTTGTAATAC

a: GTATTTACAAACCAACTATTGCAACCATCACTGAATACACGTAAAC

I306V

s: CGAAAGAGCCTCAAACCTATGTCAAAGAACGTTTCTGGCAAATC

a: GATTTGCCAGAAACGTTCTTTGACATAGTTTGAGGCTCTTTTCG

F310S

s: CAAACTATATCAAAGAACGTTCTTGGCAAATCAATAAAACCGAAC

a: GTTCGGTTTTATTGATTTGCCAGGAACGTTCTTTGATATAGTTTG

I320V

s: CAATAAAACCGAACAAAAAGCAGTCTATTCTCATATCACTTGTGC

a: GCACAAGTGATATGAGAATAGACTGCTTTTTGTTTCGGTTTTATTG

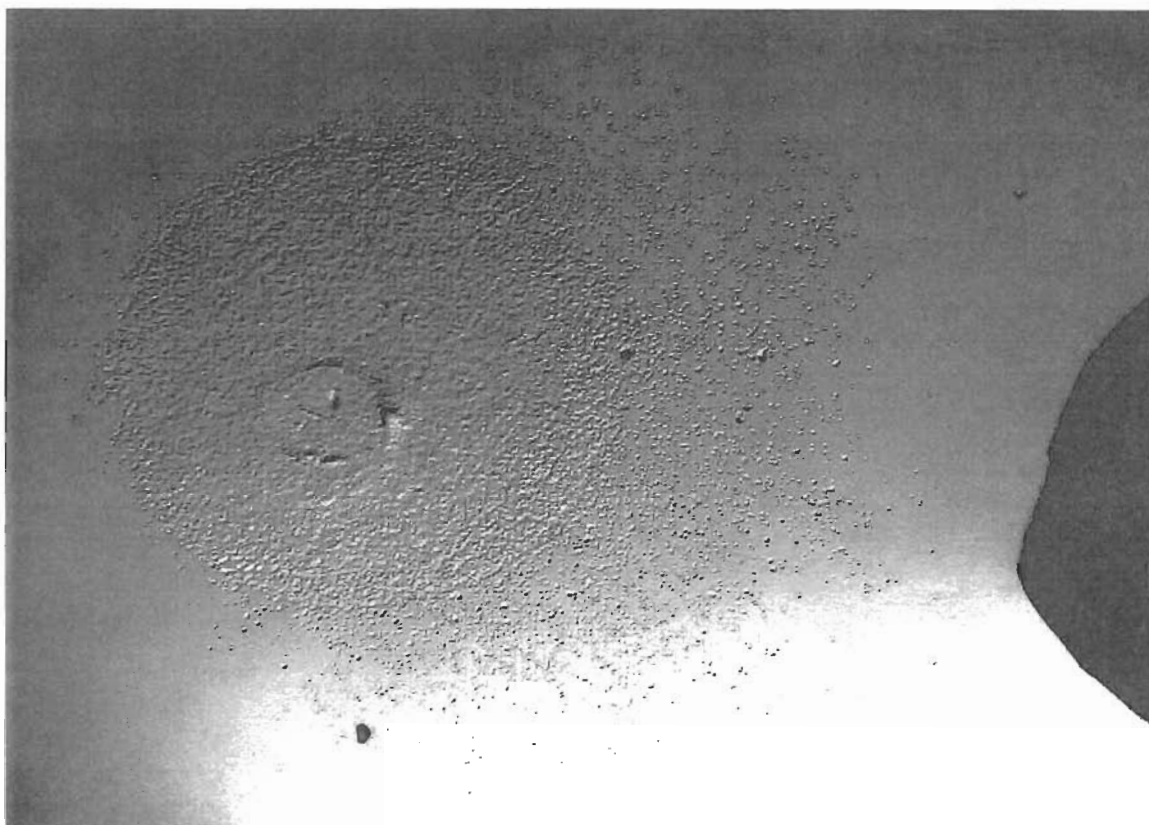


Figure 13. Positive chemotaxis. This is the wild type pattern of chemotaxis using a cell line which produces the $G\alpha$ subunit protein from a plasmid, as in the mutants. The well containing cAMP is the dark region on the right. Cells were starved at 7×10^6 cells/ml without cAMP pulsing for 4 to 5 hours. Drops ($1\mu\text{l}$) were placed on DB agar plates approximately 4mm from a well filled with $10\mu\text{M}$ cAMP. Observations were made 3 to 5 hours later.

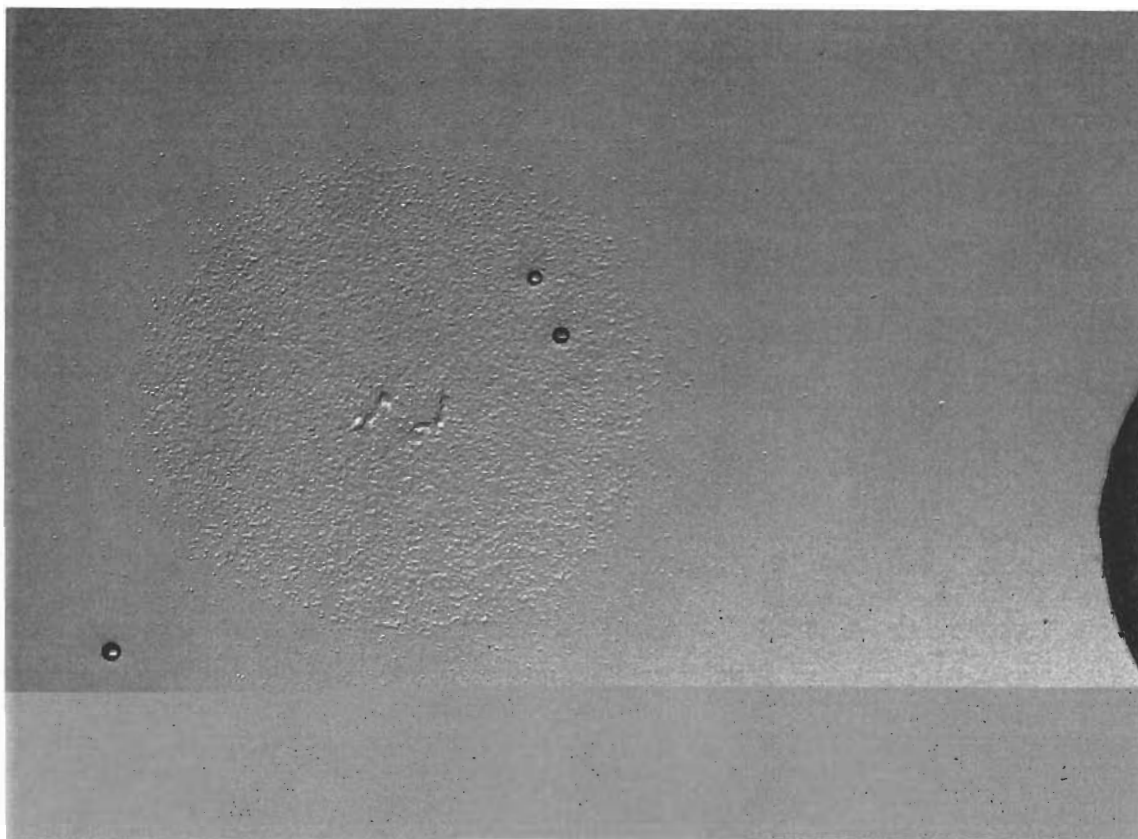


Figure 14. Negative chemotaxis. This is the pattern of chemotaxis displayed by all mutants. The well containing cAMP is on the right. Although some cell movement occurs, it is non-directional as the cell is not sensing any chemical gradient from an outside source. The amount of non-directional movement varied between mutants but none demonstrated any directionality. Cells were starved at 7×10^6 cells/ml without cAMP pulsing for 4 to 5 hours. Drops (1 μ l) were placed on DB agar plates approximately 4mm from a well filled with 10 μ M cAMP. Observations were made 3 to 5 hours later.

2.11 GTP γ S Inhibition of cAMP Binding Assay

The influence of GTP γ S (a non-hydrolyzable form of GTP) on receptor affinity for cAMP was evaluated as previously described (Van Haastert, 1984). *Dictyostelium* cells were harvested, washed and suspended in DB for starvation at 2×10^7 cells/ml, with slow shaking at 100rpm. After the first hour, cAMP pulses every six minutes at 50nM concentration per pulse were started. At 5 hours of pulsing, cells were diluted to 2×10^6 cells/ml with rapid shaking at 200 rpm for $\frac{1}{2}$ hour. Cells were washed in DB, then washed in cold AC⁻ buffer (40mM HEPES, pH 7.4 and 0.5 mM EDTA). They were resuspended at 10^8 cells/ml in ice cold AC⁺ buffer (AC⁻ buffer plus 250mM sucrose). One ml of cells were lysed by rapid straining through a 5 μ m millipore filter and centrifuged at $16,000 \times g$ for 10 min. at 0°C. The pellet containing cell membrane was washed in Phosphate Buffer (PB; 5mM Na₂HPO₄ and 5 mM KH₂PO₄, pH 6.2) and resuspended in one ml PB for an equivalent of 10^8 cells/ml. Receptor binding of cAMP was evaluated by incubating 80 μ l of the membrane preparation and (³H)cAMP (5nM final concentration) with either water (no GTP stimulation), GTP γ S (100 μ M final concentration), or unlabeled cAMP (10 μ M final concentration to reveal non-specific, non-receptor background binding). After 5 minutes incubation on ice and 3 minutes centrifugation at $16,000 \times g$, 4°C, the supernatant containing unbound (³H)cAMP was aspirated off. Bound (³H)cAMP was measured by scintillation counting. Data for the Scatchard plot of receptor affinity of the N74D mutant was obtained by a similar protocol as above. GTP γ S stimulation was not done and increasing concentrations of (³H)cAMP were used. Less than 10% of total counts were bound by N74D membrane receptors using

80 μ l of membrane preparation (data not shown). Therefore counts made on equal quantity of (3 H)cAMP without membrane added were used to represent unbound cAMP (B_o).

2.12 *In vivo* cAMP and cGMP Production Assay

For both assays, *Dictyostelium* cells were harvested and starved with cAMP-pulsing as described for the GTP γ S inhibition assay. After pulsing, cells were diluted tenfold with DB and incubated at room temperature for 30 minutes with rapid shaking (200rpm). The cells were resuspended in DB at 5×10^7 cells/ml. One ml of these cells was placed in an assay well with rapid shaking for 15 minutes. For the cAMP assay, cells were stimulated with 5 μ M 2'-deoxy cAMP. Cells (100 μ l) were sampled after stimulation at the time points noted in the cAMP graph and placed in 100 μ l of 3.5% perchloric acid. Lysates were neutralized with 35 μ l of 50% saturated potassium bicarbonate. After centrifugation at 16,000 \times g at 4°C, aliquots of 100 μ l were assayed for cAMP using the protocols in the cAMP radioassay kit supplied by (Amersham).

For the cGMP assay, cells were stimulated with 1 μ M cAMP. Cells were sampled and processed as in the cAMP assay except using different time points given the more rapid production of cGMP after stimulation.

3. RESULTS

3.1 Transformation and Screening of the Ga2 Mutant Library

Using DNA from the random Ga2 mutant library created by Jian-xin You (You, 1996), transformation into MYC2 yielded 152 successful transformants. These were screened for aggregation phenotype by plating on *Klebsiella* bacterial lawns. With growth on the lawn, the center of a plaque becomes a nutrient poor area and induces aggregation in competent cell lines. The phenotype of 108 transformants was aggregation positive, indicating either non-critical or no mutation, and these were discarded. The remaining 44 were re-screened under starvation conditions on DB plates and were confirmed as aggregation negative.

3.2 Ga2 Protein Expression by Aggregation Negative Mutants

The 44 aggregation negative mutants were screened for Ga2 subunit expression using the N-terminal antibody as described. Immunoblots on crude cell lysates performed soon after transformation revealed generally poor expression of the Ga2 protein. After the mutants had been allowed to grow for several weeks, much improved protein expression was noted. Expression was compared to equal quantities of wild type and MYC2 cells as positive and negative controls. Strength of expression was by qualitative evaluation of the Ga2 subunit band produced by equal numbers of cells ranging between MYC2 (*ga2*-null) and Wild Type. Sixteen mutants expressing protein levels close to wild type were evaluated further. Of these, 6 were now found to be aggregation positive on DB plates. The remaining 10 were reconfirmed as continuing to express the Ga2 subunit and their plasmid DNA was extracted.

The Gα2 protein expression of the N74D and L110S mutants, with controls is shown in Figures 15 and 16.

3.3 Chemotaxis Assay

The 10 aggregation negative mutants expressing the Gα2 subunit were evaluated for chemotaxis. Repeated trials under various conditions produced consistently chemotactic negative results, with the transformed Wild Type Gα2 cell line serving as a positive control. See Figures 13 and 14 for the positive Wild Type result and a representative negative result, which all mutants displayed. If any of these mutants indeed had only partial impairment of chemotaxis, this was below the sensitivity of the assay available to us in the lab.

3.4 DNA Sequencing of Selected Mutants

The pJK-1 plasmid of the 10 aggregation negative mutants expressing the Gα2 subunit was extracted and sequenced by the University of Maine DNA Sequencing Facility. Incomplete sequence was obtained from some of the mutant clones. However all had sufficient sequence of the *ga2* gene to identify the usefulness of the mutant in terms of studying specific parts of the protein. Table 3 lists all the mutations that were sequenced. Two of the mutants each had single amino acid mutations and both of these were in the helical domain of the Gα2 subunit, whose function in signaling is unclear. These are the N74D and L110S mutants discussed later. One mutant had taken up two different plasmids at transformation, resulting in a total of 11 sequences.

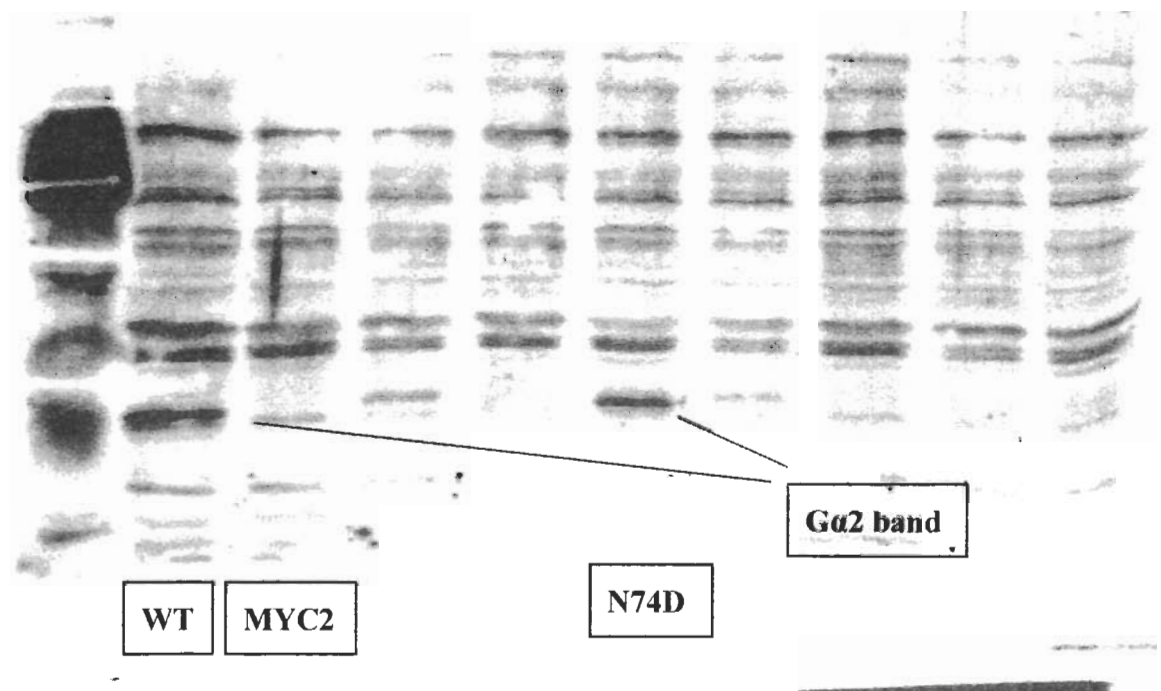


Figure 15. Expression of Ga2 protein by the N74D mutant. Mutant cell lines were maintained for 2 months before protein expression was tested. This allowed for full adaptation of the MYC2 cell line to the introduced plasmid with much improved protein expression by all transformed cell lines. Each lane represents a separate mutant with Wild Type and MYC2 (*ga2*-null) serving as positive and negative controls respectively. On the far left is a low molecular weight marker lane. The N74D mutant was tested twice after the two month period, showing strong expression on both occasions, and was therefore studied further. Equal numbers of cells (2×10^6) were lysed and the whole cell lysate subjected to SDS-PAGE. Protein was transferred to nitrocellulose membranes for immunoblotting. Primary antibody was to an N-terminal fragment of the Ga2 subunit. Development was by enhanced ECL.

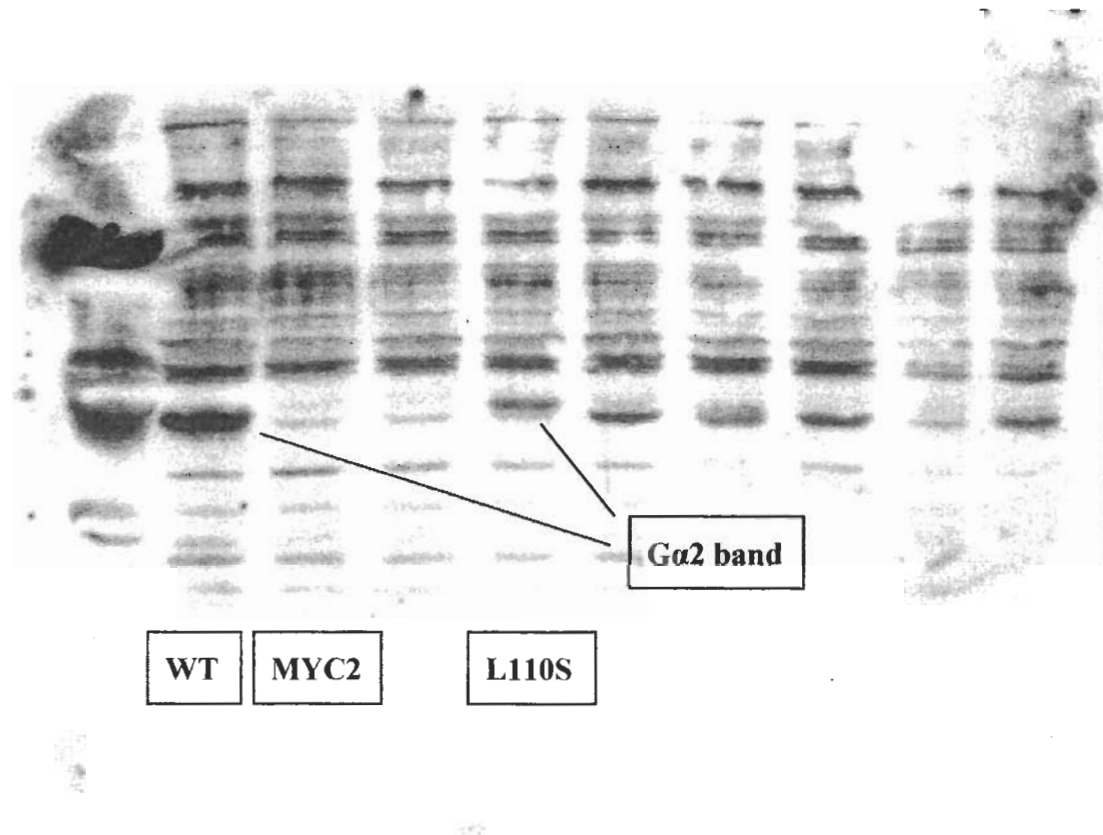


Figure 16. Expression of the Ga2 subunit by the L110S mutant. Mutant cell lines were maintained for 2 months as in Figure 15. The L110S mutant was tested once after the two month adaptation period, showing strong protein expression when compared to Wild Type. The L110S mutant also began to occasionally produce small fruiting bodies with limited and delayed aggregation. This suggested incomplete impairment of Ga2 subunit function. Equal numbers of cells (2×10^6) were lysed and the whole cell lysate subjected to SDS-PAGE. Protein was transferred to nitrocellulose membranes for immunoblotting. Primary antibody was to an N-terminal fragment of the Ga2 subunit. Development was by enhanced ECL.

Table 3. Sequence Changes of aggregation negative mutants. Mutants 1 and 2 (L110S and N74D) were selected for further study. Mutants 3 and 4 contain mutations located in possible effector protein binding areas. Mutants 5 and 6 contain mutations in previously characterized regions . The last five were discarded due to the high number or type of mutations.

<u>Mutant</u>	<u># of Sequence Changes</u>		<u>Amino Acid</u>	<u>Location on Gα Subunit</u>
	<u>Nucleotide</u>	<u>Amino Acid</u>	<u>Mutation</u>	
				<u>Helical Domain</u>
1)	4	1	L110S	within the α B helix
2)	3	1	N74D	within the α A helix
				<u>G Domain</u>
3)	2	2	V257G I306V	within the α 3 helix within the α 4 helix
4)	7	2	F310S I320V	junction of α 4 helix and α 4/ β 6 loop within the α 4/ β 6 loop
5)	2	2	E190G D220V	switch I switch II
6)	2	2	G206S I334T	in the DXXG Mg ⁺⁺ binding motif within the α 5 helix
	<u>Discarded</u>			
7)	wrong sized plasmid			
8)	6	6		
9)	reading frame shift			
10)	8	8		
11)	4	4		

The helical domain mutants were studied further to gain insight about the function of the helical domain. One of the changed amino acids is highly conserved and is directly adjacent to a critical and conserved amino acid in switch I of the G domain of the G α subunit. Asparagine-74 is adjacent to arginine-182 respectively. Note that threonine-185 is the residue in switch I which binds to the γ phosphate of GTP and thus orients switch I into a different conformation from that when bound to the receptor. Arginine-182 is the residue which is ADP-ribosylated by the cholera toxin resulting in GTP binding without GTP hydrolysis. This switch I residue is involved along with Glu-208 of switch II in hydrolyzing the γ phosphate of GTP. Switch II is bound to the γ phosphate of GTP by the DXXG motif immediately adjacent to Glu-208. The L110S mutation is in the α B helix of the helical domain, structurally close to the N74D mutation within the α A helix.

The four mutations in the α 4/ β 6 loop of G α 2 (V257G, I306V, F310S, I320V), although interesting in terms of possible identification of G α 2 subunit effectors and best studied as a group, will be evaluated in a future project in Dr. Gundersen's laboratory.

3.5 GTP γ S Inhibition of cAMP Binding by the cAR-1 Receptor

In Wild Type *Dictyostelium*, activation of the cAR-1 GPCR by cAMP results in both activation of the G α 2 β γ heterotrimer and a lowering of receptor affinity for cAMP from a K_D of 25nM to 230nM (Kessin, 2001). GTP γ S is a non-hydrolyzable form of GTP which allows the comparison of receptor affinity for cAMP between the activated and inactivated state of the G-protein. It also identifies if a mutant G-protein is able to associate with the receptor, thus producing high affinity data for cAMP by the receptor. Figure 17 shows stimulation of

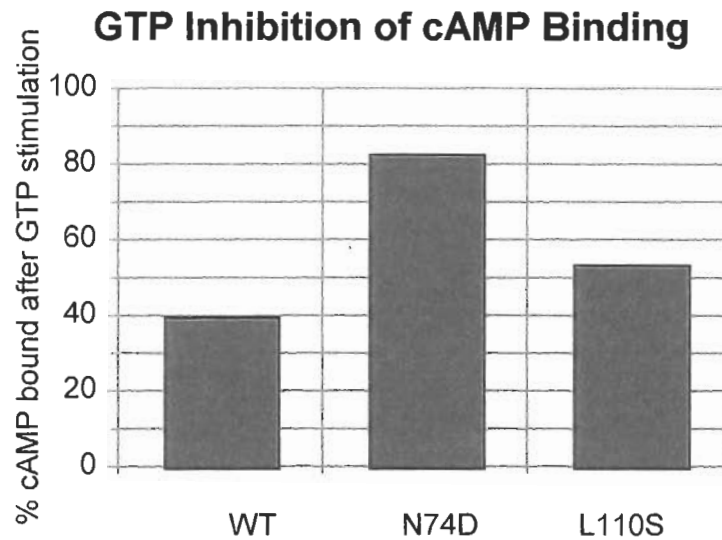


Figure 17. GTP γ S inhibition of cAMP binding by the cAR-1 G-protein coupled receptor. Cells were starved at 2×10^7 cells/ml for 6 hours with cAMP pulsing after the first hour. Membrane preparations were made from equal numbers of cells (1×10^8 cells). The binding of [3 H]-cAMP was measured in unstimulated (water only added) and GTP γ S stimulated membranes. Non-specific binding was measured by adding high concentration unlabeled cAMP and was subtracted from experimental results. Adequate receptor expression was judged by total [3 H]-cAMP binding being 5 to 10 times non-specific binding levels. The graph represents the averaged results of four separate experiments, each having good receptor expression. Compared to Wild Type, the N74D mutant shows little change in affinity for cAMP after exposure to GTP γ S stimulation.

Numerical data:

Wild Type 39% \pm 10%

N74D 82% \pm 14%

L110S 53% \pm 13%

Wild Type membrane preparations with GTP γ S results in significant lowering of receptor affinity with only 39% ($\pm 10\%$) of labeled cAMP remaining bound compared to unstimulated membrane binding of labeled cAMP. This is consistent with previous results (Gundersen, personal communication). The L110S mutant shows a commonly found condition (Gundersen, personal communication) of an intermediate reduction in affinity with 53% ($\pm 13\%$) remaining bound. The N74D mutant however shows 82% ($\pm 14\%$) of labeled cAMP remains bound after stimulation. This suggests the receptor does interact with the N74D mutant heterotrimer to produce the high affinity state of the receptor and the affinity remains high after stimulation with cAMP. To look further at this, an immunoblot of the cAR-1 receptor was done, using the same membrane preparations that were used in the affinity assays and comparing Wild Type with N74D levels of receptor protein expression. Receptor expression by equal numbers of cells revealed less expression by the N74D mutant (see Figure 18). This is consistent with high receptor affinity as the scintillation counts from N74D binding were generally about half the counts of wild type binding (10,000 for WT versus 6000 for N74D). In order to obtain false positive high affinity results for cAMP binding by the N74D receptor, the mutant would need to be found expressing higher levels of receptor than Wild Type such that low affinity binding of cAMP by the N74D receptor (at cAMP concentrations equal to those of wild type high affinity binding) would yield scintillation counts of sufficient number to mis-lead the investigator.

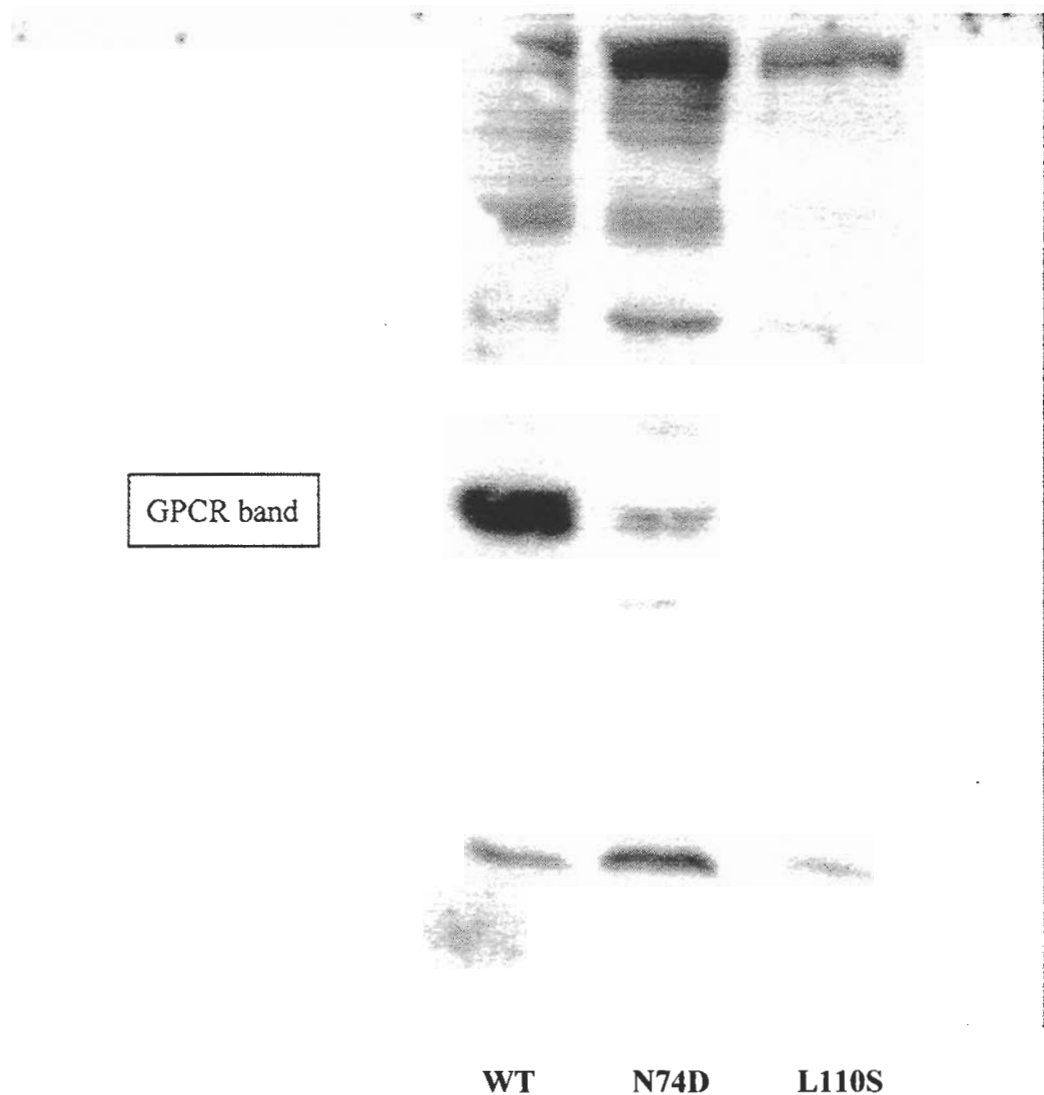


Figure 18. cAR-1 receptor immunoblot. Expression of the N74D cAR-1 receptor is present and at much less the expression of wild type. L110S receptor expression is present however only at trace amount. Cells were starved at 2×10^7 cells/ml for 6 hours with cAMP pulsing after the first hour. Equal numbers of cells were lysed osmotically using 98% saturated ammonium sulfate and the resulting lysates subjected to SDS-PAGE. After transfer to a nitrocellulose membrane, immunoblot was done with the primary antibody directed against the cAR-1 receptor (cAR-1 antibody a gift from P.N. Devreotes). Development was by enhanced ECL.

3.6 Scatchard Plot of the N74D Mutant

A Scatchard analysis was performed to examine receptor affinity more directly. A Scatchard plot of receptor binding will identify the number of affinity states. Under conditions of bound ligand being less than 10% of total ligand, accurate evaluation can be obtained using increasing amounts of labeled ligand (Bennett, 1978). The 10% limit allows the assumption that unbound cAMP (difficult to identify as separate from bound) is equal to total cAMP in the assay (easily obtained from counting an equal aliquot of labeled cAMP separately from the membrane bound labeled cAMP assay). Labeled cAMP binding by membrane preparations similar to those of the GTP γ S stimulation assay was measured. First, an experiment was performed using differing quantities (20, 50, and 80 μ l) of N74D membrane preparation to evaluate that the proportion of the total labeled cAMP bound to the receptor under the conditions of the assay (without GTP γ S stimulation) was indeed less than 10% of total cAMP label in controls containing no membrane preparation. The 80 μ l aliquot, which is the usual quantity used in the GTP γ S inhibition of receptor affinity assay, bound 2% of the total cAMP, so this volume could be used in a Scatchard analysis of the N74D mutant. Increasing concentrations of labeled cAMP from 1nM to 200nM were added to the membrane preparations. Plotting bound ligand divided by free ligand (total ligand under the conditions mentioned) versus bound ligand will yield differing slopes if concentrations of ligand progress up through different affinity states of the receptor for the ligand. The graph in Figure 19 is a representative example of three separate receptor binding assays. It demonstrates the two slope graph which is similar to that seen in Wild Type assays of the cAR-1 receptor

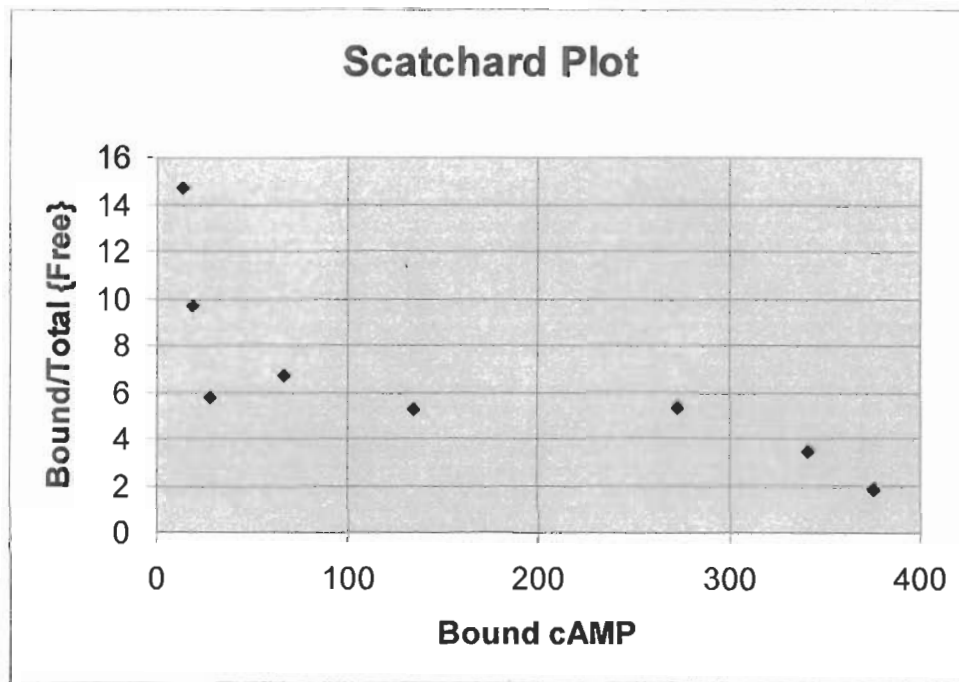


Figure19. Scatchard Plot of cAR-1 affinity for cAMP in the N74D mutant. Cells were starved at $2 \cdot 10^7$ cells/ml for 6 hours with cAMP pulsing after the first hour. Membrane preparations were made from equal numbers of cells ($1 \cdot 10^8$ cells). Equal quantity of membrane preparation was incubated with increasing concentrations of [3 H]-cAMP from 1 to 200 nM. At each of these concentrations the amount of bound cAMP was measured after centrifugation, removal of unbound cAMP in the supernatant, then scintillation counting. Total [3 H]-cAMP added in blank vials was considered equal to free cAMP as, in a preparatory experiment, less than 10% of the total cAMP added was bound by the amount of membrane preparation used in the Scatchard analysis (see text).

Estimation of K_D of high affinity and low affinity receptor binding. The slope at the low concentrations of cAMP on the left (1, 2 and 5nM cAMP) versus the slope at higher cAMP concentrations on the right (25, 50, 100, 200 nM cAMP) represent differing affinity binding sites on the cAR-1 receptor, the higher affinity sites being saturated first at lower cAMP concentrations followed by lower affinity binding as cAMP concentration increases. The 10nM cAMP point was included in both slope calculations. The graph is representative of three separate experiments yielding similar data. From a Scatchard plot, the equilibrium dissociation constant of the ligand for the receptor, K_D , can be estimated as the negative slope of the best fit linear regression line through selected data points corresponding to a single affinity state. From this graph, the K_D at the higher affinity binding is estimated as 9.6nM (Wild Type known value = 25nM). The K_D at the lower affinity binding is estimated as 79.0nM (Wild Type known value = 230nM).

(Gundersen, 1997). Receptors with only a low affinity state do not show the change in slope as the bound ligand concentration decreases to low levels.

3.7 *In vivo* Adenylyl Cyclase Activity Assay

This assay determines the cAMP production by intact *Dictyostelium* cell lines in response to cAR-1 receptor stimulation. The stimulating ligand is 2'-deoxy cAMP which is not detected in the assay. In cells expressing the wild type, cAMP production peaks at 60 seconds and returns to baseline by 10 minutes, as seen in Figure 20. The N74D mutant did not produce any detectable rise in cAMP indicating that downstream activation of adenylyl cyclase A by the $\beta\gamma$ subunits (in *Dictyostelium*) did not occur. The L110S mutant also did not produce cAMP after stimulation.

3.8 *In vivo* Guanylyl Cyclase Activity Assay

Like the cAMP assay, the cGMP assay reflects cGMP production resulting from guanylyl cyclase activation, known to occur through the cAR-1 receptor and $G\alpha 2\beta\gamma$ subunit in intact cells. Production of cGMP peaks in 20-30 seconds and returns to baseline by 60 seconds, as demonstrated in the Wild Type cell line (Figure 21). The N74D mutant did not produce any detectable rise in cGMP production indicating lack of activation of the $G\alpha 2\beta\gamma$ subunit by the cAR-1 receptor. An interesting result in the L110S mutant was an intermediate production of cGMP suggesting activation of at least some of the $G\alpha 2\beta\gamma$ subunits. This finding and the occasional production of small fruiting bodies by this mutant suggests a less severe disruption of helical domain function by the L110S mutation.

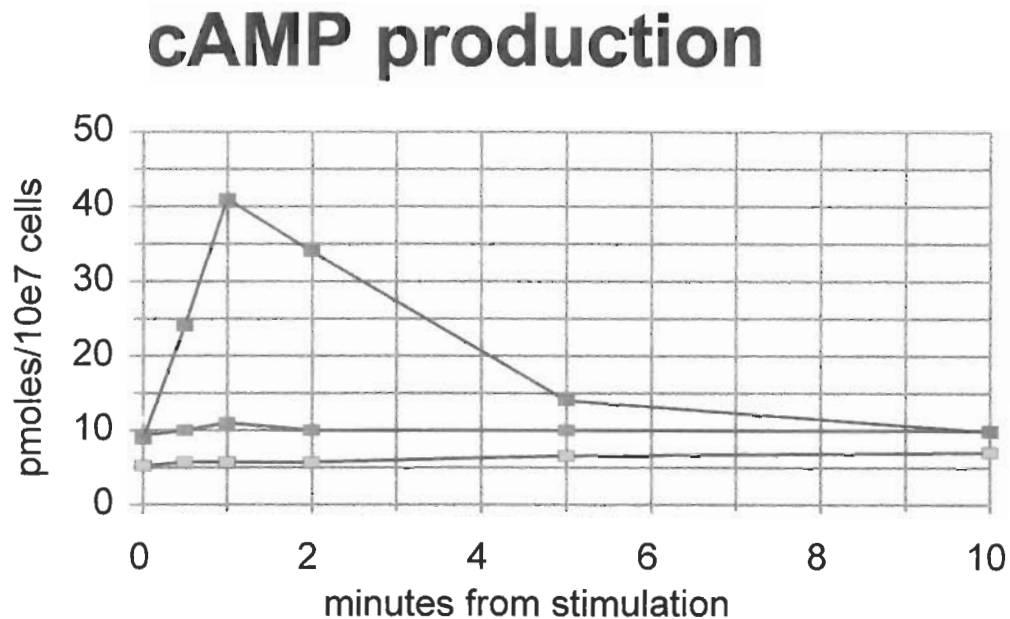


Figure 20. *In vivo* Adenylyl Cyclase Activity. Wild type response is in green, N74D is in blue, and L110S is in red. The wild type response is typical. The mutants show no response indicating inability of the receptor to activate the G-protein. Cells were starved at 2×10^7 cells/ml for 6 hours with cAMP pulsing after the first hour. After pulsing, cells were diluted tenfold and incubated at room temperature with rapid shaking for 30 minutes to complete degradation of extracellular cAMP. Cells were then concentrated to 5×10^7 cells/ml and stimulated with $5 \mu\text{M}$ 2'-deoxy cAMP, which is not detected by the cAMP assay used. Cells were sampled at the times points indicated in the graph and the activity of the cells proteins stopped abruptly by placing in cold 3.5% perchloric acid. Total cAMP levels in the resulting lysate were measure using the protocols of the cAMP Assay Kit (Amersham).

cGMP production

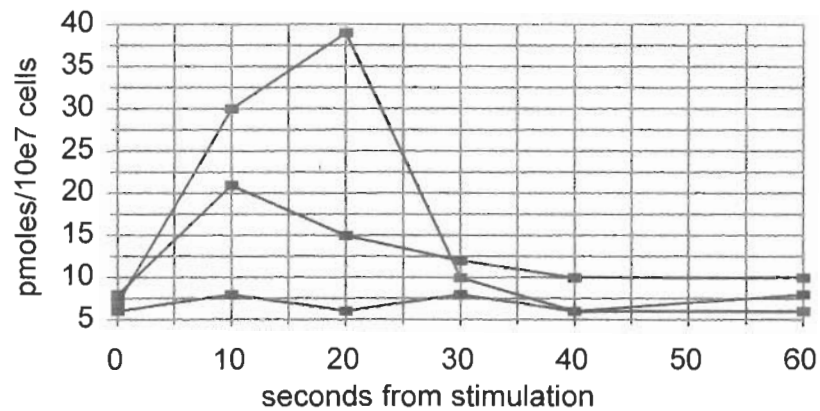


Figure 21. *In vivo* Guanylyl Cyclase Activity. Wild Type is in red, the N74D mutant is in green, the L110S mutant is in blue. The wild type response is typical. The N74D mutant shows no response indicating inability of the receptor to activate the G-protein. Interestingly, the L110S mutant shows a partial response. Cells were starved at 2×10^7 cells/ml for 6 hours with cAMP pulsing after the first hour. After pulsing, cells were diluted tenfold and incubated at room temperature with rapid shaking for 30 minutes to complete degradation of extracellular cAMP. Cells were then concentrated to 5×10^7 cells/ml and stimulated with $1 \mu\text{M}$ cAMP, which is not detected by the cGMP assay used. Cells were sampled at the times points indicated in the graph and the activity of the cells proteins stopped abruptly by placing in cold 3.5% perchloric acid. Total cGMP levels in the resulting lysate were measure using the protocols of the cGMP Assay Kit (Amersham).

4. DISCUSSION

Biochemical signaling involving G-protein coupled receptors (GPCR) and heterotrimeric G-proteins encompasses a truly vast array of information transfer in eukaryotic systems. There are on the order of 1000 GPCR genes, representing one of the largest gene families (Bourne, 1997). The large variety of information these receptors recognize is funneled across the plasma membrane into a relatively small number of genes encoding the heterotrimeric subunits $G\alpha$ (16 known), β (5 known), and γ (11 known). From this intracellular point a very complex network of interacting proteins from many types of signaling pathways integrate to produce a viable organism. There are, however, points of significant conservation in this complexity which represent the core mechanisms of signal transfer rather than recognition of environmental input or effector output.

In the GPCR/heterotrimeric G-protein families the common mechanistic functions to all members are 1) receptor conformational change being communicated to a heterotrimeric G-protein, 2) the heterotrimer receiving this communication of receptor activation such that a molecule of GDP is ejected from the $G\alpha$ subunit and replaced by a separate molecule of GTP, 3) the $G\alpha$ subunit undergoing a conformational change effected by the now present γ phosphate of GTP, 4) the heterotrimer receiving receptor communication, or having GTP induced conformational changes, such that the $\beta\gamma$ subunits separate from both the $G\alpha$ subunit and from the receptor, 5) the $G\alpha$ subunit separating from the GPCR with receptor affinity for its extracellular ligand being lowered, and 6) the hydrolysis of the γ phosphate of GTP. These events occur in all GPCR/heterotrimeric G-

protein combinations and do not provide any specificity as to the content of the signal message. This has, of course, led to our ability to discover them and study them in different organisms. In all heterotrimeric G-protein $G\alpha$ subunits, there is a helical domain which is not present in the related families of the monomeric G-proteins. The fact that the helical domain is highly conserved implies an equally conserved function in all $G\alpha$ subunits.

The sites of protein-protein interactions that are involved in these events are becoming better defined. All GPCRs have seven transmembrane domains with intracellular cytoplasmic loops (Figure 22) which communicate the receptor conformational change to the G-protein. Receptor/ $G\alpha$ subunit interaction is known to involve the N- and C-terminals and $\alpha 4/\beta 6$ loop of the $G\alpha$ subunit. Receptor/ $\beta\gamma$ subunits interaction is known to involve the C-terminal portion of the γ subunit and the $\beta\gamma$ subunits do not change conformation during activation of the G-protein (Wall *et al.*, 1995; Lambright *et al.*, 1996). Interactions of the $G\alpha$ and $\beta\gamma$ subunits primarily involve the switch II region of the $G\alpha$ subunit with some contact also at the $G\alpha$ subunit N-terminus, the latter being the only area where receptor, $G\alpha$ subunit, and $\beta\gamma$ subunits are in close proximity to each other (Wall *et al.*, 1995; Lambright *et al.*, 1996). All G-proteins have a G-domain where GDP/GTP exchange and hydrolysis activity occur. The motifs which carry out the enzymatic activity are present in the G-domain itself and are highly conserved. However, amino acid interactions within the body of the $G\alpha$ subunit which are involved in inducing the conformational change in this protein are not well defined.

With the activation process being so rapid, the time sequence of these events is a significant source of speculation. One assumes a simultaneous activation event but this is

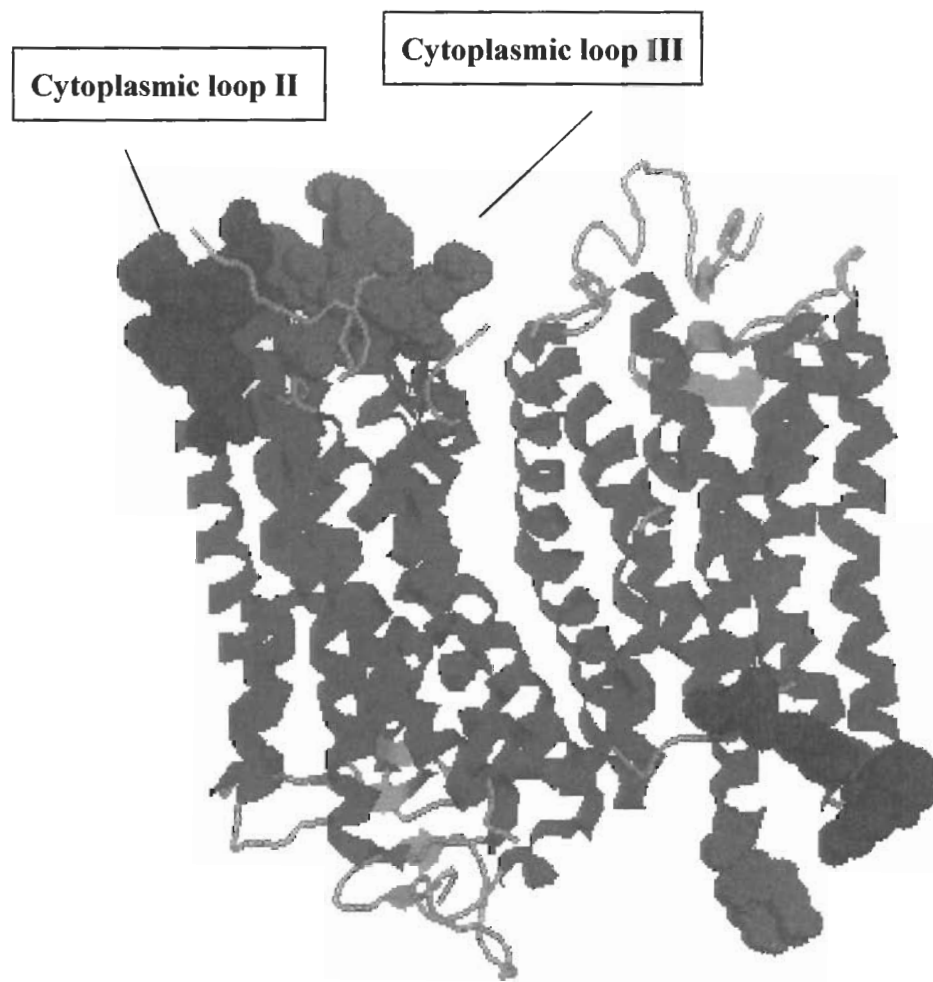


Figure 22. Rhodopsin, the GPCR for *Gat* (Palczewski *et al*, 2000). There are two molecules of rhodopsin in this image. The helices in dark pink traverse the plasma membrane. The second (blue spacefill) and third (green spacefill) cytoplasmic loops are the known sites of interaction with the G-protein heterotrimer, as indicated in blue on the heterotrimer figures throughout the paper.

intuitively not required, nor likely. In the step of GDP/GTP exchange, the guanine nucleotide molecule almost traverses the center of the $G\alpha$ subunit so could enter/exit from either of two faces of the subunit (Figure 4). One face, involving the switch regions of the $G\alpha$ subunit and the phosphate end of GDP/GTP, is covered by the $\beta\gamma$ subunits, either by direct binding or by space filling steric hindrance. If $\beta\gamma$ subunits separation occurred before GDP/GTP exchange, the switch II region would be exposed and GTP could enter from this face. This is unlikely, as this scenario would imply that the $\beta\gamma$ subunits' downstream effectors could be activated in the absence of GTP, by receptor activation alone, a finding never seen in G-protein experiments. The face of the $G\alpha$ subunit opposite the switch regions, which is also adjacent to the receptor binding area, is exposed (Figure 4). This may actually be the side where GDP/GTP exchange occurs. GPCR's are guanine nucleotide exchange factors (GEFs; reviewed in Bourne, 1997) for $G\alpha$ subunits and may act to stabilize the empty state of the $G\alpha$ subunit during the moment of nucleotide exchange. This presumes some interaction with the $G\alpha$ subunit such that the activated conformation of the receptor induces or temporarily stabilizes an empty state where the $G\alpha$ subunit has no nucleotide bound. For obvious steric reasons the receptor or the $\beta\gamma$ subunits cannot block the presumed cleft where GDP/GTP exchange occurs. While receptor/ $G\alpha$ subunit and receptor/ γ subunit binding amino acids are known, the crystal structure of a GPCR/G-protein complex has not been determined. The $G\alpha$ subunit N- and C-terminals and some residues in the $\alpha 4/\beta 6$ loop are known areas of receptor interactions (Lambright *et al.*, 1996). Recent studies suggest that the third cytoplasmic loop of the GPCR may be involved in specificity of interaction with the C-terminal amino acids of the $G\alpha$ subunit while the second cytoplasmic loop of the receptor may supply the

conformational change needed to activate the heterotrimer (Terakita *et al.*, 2002; Yamashita *et al.*, 2000). This 'push/pull' action would occur at the point of binding between the receptor and $G\alpha$ subunit which is located immediately adjacent to the GDP/GTP binding pocket. Therefore, sterically the GDP/GTP exchange is favored to occur on the receptor binding face opposite the switch regions on the $G\alpha$ subunit. Functioning as a GEF, the receptor may act by physically holding open the guanine binding pocket and/or by creating a more favorable environment for GTP to bind versus GDP. For the latter to occur, receptor conformational changes would need to influence the orientations of the switch I and II regions. The helical domain is the structural connection between the receptor binding area and switch I so this domain would be expected to be involved in transmitting conformational changes between these two areas.

Using the argument that the $\beta\gamma$ subunits are never seen causing downstream activation in the absence of GTP, a molecule of GTP is in place and contributing to the conformational changes in the $G\alpha$ subunit causing $\beta\gamma$ subunits separation. Crystal structure data clearly show a marked conformational change occurring, mostly in the switch II region and less in switch I, between the GDP bound heterotrimeric form and the GTP γ S bound monomeric $G\alpha$ subunit form present after receptor activation. Recalling that switch I and II are flexible loops, their conformations are determined by hydrogen and ionic binding with the GTP γ phosphate, a Mg^{++} ion, and other structural elements of the protein. For switch I, the structural element is the helical domain; for switch II, it is the $\beta\gamma$ subunits. With these structural elements in mind, the data provided by studies of the N74D mutant become very interesting.

The N74D mutant demonstrates a phenotype suggesting $G\alpha 2\beta\gamma$ complex formation with normal binding to the receptor. The Scatchard analysis of the N74D mutant (Figure 19) suggests that the mutated $G\alpha 2$ subunit is able to associate with the receptor such that the receptor can assume a high affinity state for the cAMP ligand, the normal resting or 'off' state of the GPCR/G-protein complex. However, the GTP γ S induced inhibition of cAMP binding assay suggests that cAMP binding by the receptor does not change the affinity state of the receptor for cAMP in this mutant. By using the $G\alpha 2$ mutant library, the mutation in this set of proteins is in the $G\alpha 2$ subunit and the receptor is expected to be functioning normally. The N74D mutation is at a location in the $G\alpha$ subunit which spatially connects the helical domain to switch I of the G-domain. The helical domain and to a lesser extent switch I are not physically in close proximity with the $\beta\gamma$ subunits and there are no areas of direct contact. The switch II region, the main area of significant $G\alpha$ subunit conformational change on GTP binding, is closely in contact with the $\beta\gamma$ subunit. Therefore, when the GTP γ phosphate reorders these two switches, only switch II would be able to participate in the disassociation of the $\beta\gamma$ subunit. However, it follows that switch I must be structurally intact to act as the other half of the binding pocket (see Figure 7). Without this bridging phosphate, these switch regions are known to become disordered with respect to one another. A mutation in the area of the helical domain near switch I would be expected to affect the role of the helical domain and its interaction with switch I, if any. The effects of such a mutation on switch II conformational change and $\beta\gamma$ subunits separation would be indirect via switch I disruption. The N74D mutation is in the helical domain at a place directly adjacent to arginine-182, a specific conserved amino acid residue in switch I known to be involved in

hydrolyzing the γ phosphate of GTP. Threonine-185 is the specific switch I amino acid involved in binding the γ phosphate, and therefore, reorienting switch I. Threonine-185 may be significantly influenced by any repositioning of arginine-182 in the N74D mutant.

Therefore, the receptor conformational change is occurring in the N74D mutant but does not result in G-protein activation. The interrupted step must be either 1) an inability for GTP to bind or 2) GTP exchange occurring, but without induction of the conformational changes in the switch areas of the $G\alpha 2$ subunit which would normally lead to the $\beta\gamma$ subunits separation and lowered receptor affinity for its extracellular ligand. At the present stage of this experiment, one cannot differentiate between these two possibilities. We will come back to this after reviewing the structural detail of the mutation.

In the wild type $G\alpha$ subunit, crystal structural data, as interpreted using the Rasmol software, show the side chain of asparagine-74 in the helical domain reaching the backbone carbon of arginine-182 in switch I of the G-domain (see Figure 7). The same crystal structure data, when interpreted by the MolProbity software which uses inter-molecular distances to predict hydrogen bonding and other atom/atom interactions (available for unrestricted use on www.kinemage.com), demonstrate hydrogen bonding occurring between the asparagine-74 side chain and the protein backbone of arginine-182. As mentioned, this is the arginine involved in hydrolysis of the γ phosphate of GTP and is near threonine-185 involved in binding the γ phosphate. Asparagine (N) is the amide of aspartic acid (D) and otherwise is the same. The amine portion of the amide of asparagine-74 hydrogen bonds with the peptide backbone α -carbonyl group of arginine-182 while the carbonyl oxygen of the amide of asparagine-74 hydrogen bonds with the nitrogen of arginine-182 involved in the peptide

bond (Figure 23). This would orient the arginine-182 side group towards the opposite side of the peptide backbone from the asparagine-74 amide as is seen in the crystal structure. Mutation of asparagine-74 to aspartic acid would dramatically change the local ionic environment. The hydrogen bonding predicted in the wild type protein to occur between the asparagine-74 amide and the peptide backbone portion of arginine-182 would be significantly disrupted in the mutated protein by replacing one of the two hydrogen bonds with a repelling interaction between oxygen atoms (Figure 23). The carboxyl side chain group of aspartic acid is not protonated at physiologic pH. Indeed the negative ionic charge may result in an attractive interaction with the positively charged arginine side group. Either way, the spatial orientations of amino acids located in the $G\alpha 2$ subunit helical domain on one side and the switch I area of the G-domain on the other are going to be changed by this mutation. Also, the attractive forces between the rigid peptide backbone and the non-rigid amide group of asparagine are going to either be eliminated or significantly altered when the carboxyl group of aspartic acid is interacting instead. The conformational change in switch I likely involves arginine-182 as this residue is near threonine-185 which is involved in binding the γ phosphate of GTP. Asparagine-74 hydrogen bonds with arginine-182 but this is likely disrupted in the mutant.

Reconsidering the two possibilities of disrupted signaling mentioned earlier, in the first possibility where GTP cannot bind, the hypothesis would be the helical domain is acting in conjunction with the receptor as a GEF. The receptor binds to the $G\alpha$ subunit primarily in regions of the C-terminus which are adjacent to the helical domain and not directly in contact with switch I (see Figure 4). If the receptor influences the conformation of switch I during

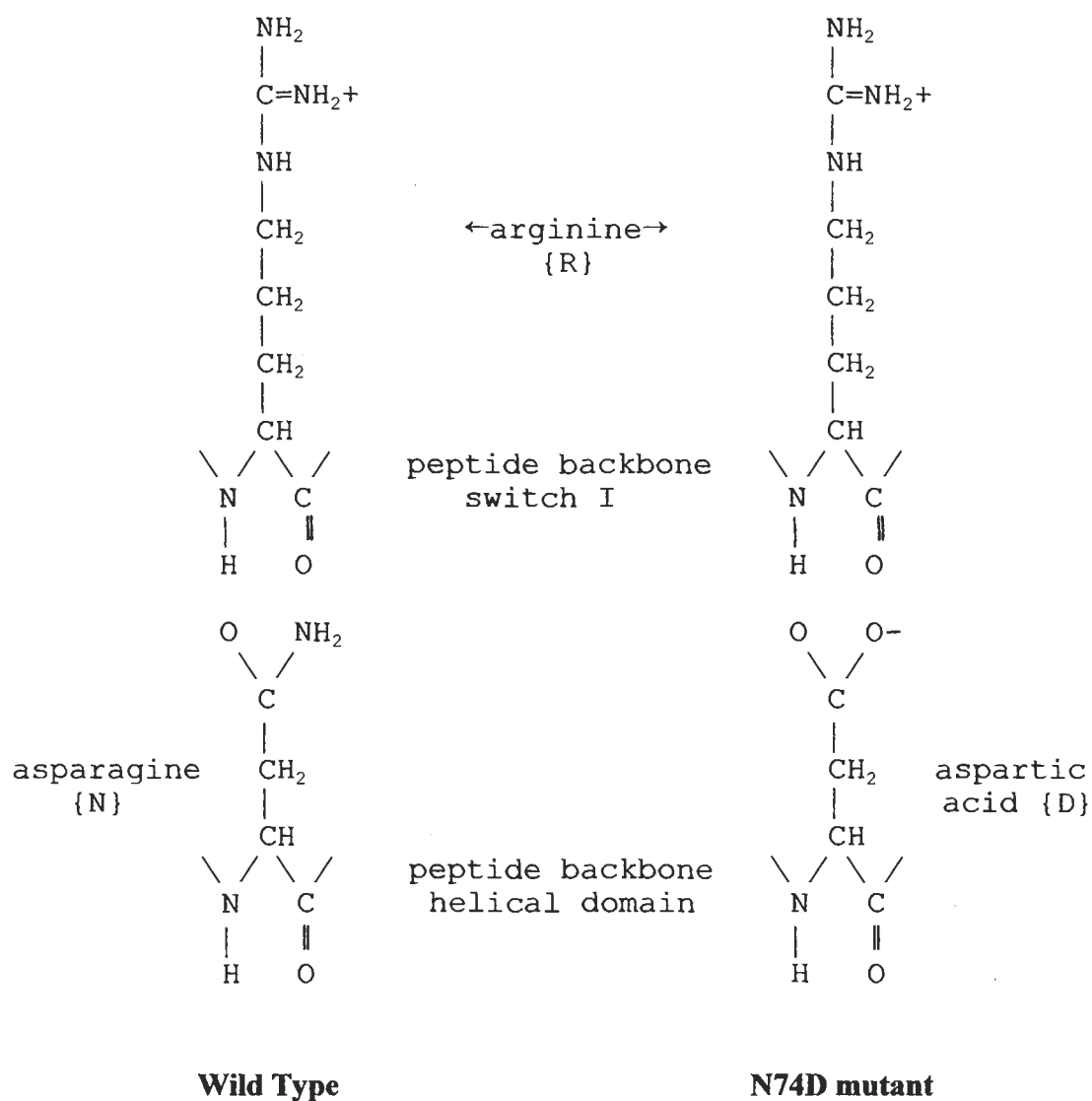


Figure 23. The hydrogen bonding between Arg-182 and Asn-74. Hydrogen bonding between the helical domain and switch I involving these two amino acids would be disrupted in the N74D mutant.

GDP/GTP exchange and the helical domain is located between the receptor binding area and switch I, the helical domain might be an area of rigidity involved in holding open the binding pocket during exchange by the receptor. The N74D mutation may disrupt the ability of the helical domain to maintain stability of the switch I part of the binding pocket during the open pocket stage of GDP/GTP exchange. In this situation either the GDP molecule is unable to exit or, perhaps more likely given the location of the mutation, a GTP molecule, as opposed to another GDP molecule, is unable to bind. The GTP γ phosphate may meet a repelling chemical environment in the mutant rather than an attractive one.

In the second possibility mentioned earlier where GTP exchange is hypothesized to occur without effect, the stability of the switch I side of the guanine binding pocket again becomes a mechanistic issue. GTP binding may not be able to induce the switch II conformational change. If switch I is improperly positioned or can move rather than be held rigid, switch II may not be repositioned by the γ phosphate of GTP as is normally done. The resulting lack of, or incomplete switch II conformational change would be the reason the $\beta\gamma$ subunits do not disassociate. In some way, the energy of the receptor conformational change (and/or the additional energy in the GTP molecule) is not effectively transmitted to the $G\alpha$ subunit to activate it.

The L110S mutant provides limited but interesting data. Its phenotype is interesting because the mutant does occasionally reach the fruiting body stage with multiple small stalks with spores at a delayed rate. At other times, cells on DB plates show limited chemotaxis (apparently below the limit of our chemotaxis assay) with mound formation and nothing more. This suggests only partial impairment of $G\alpha$ subunit

function. Cyclic-AMP binding assays and immunoblot detection of receptor expression suggest poor expression of the receptor. Effective signal transduction through the cAR-1 receptor, independent of the $G\alpha 2\beta\gamma$ complex, is involved in feedback stimulation of receptor expression during the aggregation phase. Perhaps the L110S mutant $G\alpha 2$ subunit interferes with that loop or is unable to complete other signals which must coordinate and lead to effective receptor function. It does not appear to activate cAMP production *in vivo*. It does appear to stimulate production of some cGMP, *in vivo*, but at lower levels than Wild Type. As adenylyl cyclase A activation is through the $\beta\gamma$ subunit, this subunit may not be released in the L110S mutant, or only released in a limited amount. As the pathway leading to guanylyl cyclase activation is not well defined, how this limited cGMP production comes about is unknown. However, *in vivo*, both of these pathways would be necessary to some degree for later development to occur. It may be that these functions actually do occur in the mutant but are below limits necessary and, in the case of cAMP production, lower than the limit of our assay. The findings in the L110S mutant would suggest a functional but significantly impaired $G\alpha 2$ subunit. The location of the L110S mutation in a structural α -helix within the helical domain, and the replacement of a non-polar side chain with a polar one, suggest a local breakdown of proper folding or effective rigidity. The fact that this mutant demonstrates significant disruption in phenotype does again point to the necessity of an intact helical domain.

The N74D mutant suggests the helical domain is functioning mechanistically in at least GDP/GTP exchange. The location of the mutation adjacent to arginine-182 suggests a disruption of GTPase activity would occur in this mutant as well. Arginine-182 in switch I

is the site of ADP-ribosylation catalyzed by the cholera toxin which results in GTP binding without hydrolysis. The G-protein in this situation is constitutively activated whereas the N74D mutation does not allow activation of the G protein. In the monomeric G-proteins, the G-domain is the entire protein and other proteins must associate to cause hydrolysis of GTP . The GTPase activity of the heterotrimeric $G\alpha$ subunit is intrinsic, although its rate can be affected by other regulatory proteins. The GTPase activating protein (GAP) needed by the representative monomeric G-protein, Ras, is known to interact with the switch I and II regions of Ras (reviewed in Sprang, 1997). It has been proposed that the helical domain of the $G\alpha$ subunit acts as a GAP for the G-domain (Markby *et al.*, 1993; Noel *et al.*, 1993). The helical domain's proximity to switch I is consistent with this role. To directly test the GTPase activity of the activated N74D mutant $G\alpha_2$ subunit, the $G\alpha_2$ subunit protein would need to be purified to separate it from the many other GTPases present in *Dictyostelium*. Given the significant difficulty to date in purifying the wild type $G\alpha_2$ subunit protein (Gundersen, personal communication), the effect of the N74D mutation on GTPase activity of the isolated $G\alpha_2$ subunit cannot be done by this method.

Future investigation of this interesting mutation would first include extraction of the mutant plasmid and re-introduction into MYC2 to ensure proper GPCR function. Then, site-directed mutagenesis of asparagine-74 to a neutral amino acid such as alanine or isoleucine would be useful as this would eliminate the amide/peptide backbone attraction but not introduce ionic disruption. Isoleucine would occupy an approximately similar space in the protein compared to asparagine but without hydrogen bonding potential. Substituting the similar, but longer, side group of glutamine would be a way

to evaluate the importance of amide to peptide backbone hydrogen bonding as a factor in altering this mutant's phenotype. Distance may be a factor confusing the results of such a trial as the longer side chain of glutamine may sterically shrink the γ phosphate binding pocket.

The findings from the N74D mutant suggest the helical domain functions in GDP/GTP exchange. As regulators of this exchange are identified and become more defined, it will be interesting to see if they bind to any areas on the helical domain. The role of the helical domain in GTPase activation certainly is suggested by earlier experiments however our data do not contribute either positively or negatively to this hypothesis. In the GDP/GTP exchange role, the helical domain may be mostly a rigid platform against which the receptor conformational change transmits energy into the $G\alpha$ subunit conformational change.

Helical domains are conserved in all heterotrimeric G-proteins and are not present in the monomeric G-proteins. The locations and roles of these two groups also differ. The monomeric G-proteins are cytosolic, interact with diffusible proteins, and integrate pathways resulting in slower processes such as the induction of cell differentiation. The heterotrimeric G-proteins are membrane localized and transmit changes in the extracellular environment into more rapid cellular responses such as chemotaxis or neuronal impulse generation. Logically GDP/GTP exchange is the first one of the many nearly simultaneous events resulting in G-protein activation. This is a required early step in G-protein signaling and if a rapid activation of the G-protein is important in subsequent cellular response, this exchange must be catalyzed rapidly. If the helical domain of the $G\alpha$ subunit is involved in the mechanism for carrying out

this exchange rapidly and reliably in the heterotrimeric G-protein after receptor activation, this would help to explain the conserved presence of the helical domain as an integral part of the $G\alpha$ subunit in heterotrimeric G-proteins.

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BIOGRAPHY OF THE AUTHOR

Steven Martin Rauch was born in Columbus, Ohio in November, 1954. He graduated from Upper Arlington High School in 1972 and was a member of the National Honor Society, the Spanish National Honor Society, and the Biology Honor Society for high school students. He attended Northwestern University in Evanston, Illinois where he graduated in 1976 with a B.A. degree in Biochemistry and Molecular Biology. He then attended Case Western Reserve University School of Medicine in Cleveland, Ohio where he received his M.D. degree in 1980.

He settled in Maine with his new family where he attended the Maine-Dartmouth Family Practice Residency. During this time he was granted a license to practice medicine in the state of Maine and completed residency in 1983. He received his Board Certification in Family Practice at that time. He moved to Waterville, Maine where he served the community until 1995 by founding the Waterville Family Practice outpatient clinic and being on the Active Staff at Mid-Maine Medical Center (now MaineGeneral Medical Center). He also served as the college physician at Thomas College from 1983 until 1995. In 1995, he moved to Fort Kent, Maine where he served the French-Acadian community there as an Emergency Room physician and was on the Active Staff at Northern Maine Medical Center in Fort Kent.

He retired from medical practice in 1999 and embarked on a new career by enrolling in the Graduate School at The University of Maine. He served as a teaching assistant and a research assistant during his time there. He is a candidate for the Master of Science degree in Biochemistry from The University of Maine in December, 2002.