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ISOFORM-SPECIFIC REGULATION OF ADENYLYL CYCLASE BY G alpha-s

by

ANYA CARMEN HARRY

A dissertation submitted to the Graduate Faculty in Biomedical Sciences in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York

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ABSTRACT

Isoform-Specific Regulation of Adenylyl Cyclase by G alpha$_s$

by

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Heterotrimeric guanine nucleotide binding proteins (G proteins) function as signal transducers for receptors for many hormones, neurotransmitters, autocrine and paracrine factors. The G protein links the receptor that receives the extracellular signal to a distinct intracellular effector. Effectors in G protein signaling pathways are often enzymes regulating second messenger production. The cAMP pathway has long been studied as a prototype of a G protein signaling system. The molecular multiplicity of receptors, G proteins and adenylyl cyclases have now been established. Nine distinct adenylyl cyclase (AC) isoforms have been cloned and grouped into distinct families. In this study, we determined if adenylyl cyclases from different families exhibited differential responses to $G\alpha_s$. For this, we used AC1, a neuronal isoform stimulated by Ca^{2+}/CaM; AC2, largely expressed in the brain and stimulated by protein kinase C and $G\beta\gamma$ subunits, and the ubiquitously expressed AC6.

Stimulation of AC1, 2 and 6 by varying concentrations of mutant (Q227L) activated $G\alpha_s$ ($G\alpha_s$*) in the presence and absence of forskolin was studied. $G\alpha_s$* stimulation of AC1 exhibited simple kinetics. The apparent K_{act} for $G\alpha_s$* was 0.9 nM, in the presence of forskolin, it measured 0.2 nM. $G\alpha_s$* stimulation of AC2 was more complex, the $G\alpha_s$* concentration-effect curve best fit a two site model. The "high affinity" site was 0.7-0.9 nM and the "low affinity" site was 7-15 nM. In the presence of forskolin, $G\alpha_s$* stimulation was monophasic with an apparent K_{act} of 0.4 nM. Regulation of AC2 by $G\beta\gamma$ subunits appeared to require occupancy of primarily the "high affinity" $G\alpha_s$ site. Maximal fold stimulation by $G\beta\gamma$ is achieved at 2 nM $G\alpha_s$*. AC6 exhibited the most complex kinetics. $G\alpha_s$* stimulation of AC6 appeared to involve two interacting sites with apparent K_{act}'s of 0.2-0.8 nM and 8-19 nM. In the presence of forskolin, stimulation was monophasic with an apparent K_{act} of 0.2 nM. Thus, different adenylyl cyclase isoforms have different patterns of responses to $G\alpha_s$. These data indicate that the amplitude and sensitivity of the intracellular cAMP response to hormones and neurotransmitters will be determined by which adenylyl cyclase isoforms are present in individual cells and tissues. Thus, to obtain a more complete understanding of transmembrane signaling, it is not only necessary to know

which receptor subtypes are present but also to know the identity of the G protein subunits as well as the effector isoforms.

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DEDICATION

This dissertation is dedicated to my parents, Norman and Luz Marina Harry, my brother Dwain Harry and sister Denise Harry-Harris.

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CHAPTER 1

INTRODUCTION

The organization of eukaryotic cells into functionally discrete tissues and organs is maintained through intercellular signal transduction processes. The components necessary for localized cell to cell signaling often include a receptor, an intermediary guanine nucleotide binding protein (G protein) and an effector. These signaling components are also utilized in the integrated regulation between organ systems necessary to maintain overall homeostasis within the organism. Because they are required for the transfer of information between cells, they are associated with the plasma membrane and are specialized to respond over variable distances, time constraints and selective signal requirements.

Early models attempted to explain the mechanism by which information is transferred across the plasma membrane by means of a single membrane-associated protein. Such a protein was hypothesized to serve a dual role as both signal receiver and intracellular converter. Today, this model represents the signaling process for some neurotransmitter systems, however a large number of known hormones, neurotransmitters, neuromodulators, autocrine and paracrine factors control cellular activity by a

pathway utilizing a heterotrimeric G protein. The G protein molecule links the exposed extracellular receptor to a separate intracellular effector. A role for the G protein component was first introduced to the field of signal transduction by Rodbell, Birnbaumer and colleagues as they attempted to dissect the signaling pathway of glucagon in the late sixties and early seventies (Rodbell et al., 1971).

G proteins have been demonstrated in the activities required for normal maintenance of the homeostatic condition such as regulation of metabolic activities of organs, transcriptional regulation and cellular differentiation and proliferation. In addition, covalently modified or mutated forms of G proteins have also been implicated in abnormal signal transduction events. Specifically, they account for the pathogenicity of many human diseases such as cholera, whooping cough, pseudohypoparathyroidism, acromegaly and the McCune-Albright syndrome (Gilman, 1987, Levine et al., 1988, Spada et al., 1990, Lyons et al., 1990 and Landis et al., 1990). For the advancement and evolution of a principle so pervasive in biomedical research today, Rodbell along with Gilman were awarded the Nobel Prize in Medicine or Physiology for 1994.

BACKGROUND

A cell targeted to respond to a signal is distinguished from all others by the presence of a selective receptor. The receptor is capable of specifically recognizing the corresponding signal and can be localized either at the cell surface or in the cytosol. In the case of receptors embedded in the membrane, the message is transferred across the lipid bilayer by ligand binding to the receptor leading to activation. The activated receptor then initiates a series of protein-protein interactions to transfer the message to the interior of the cell.

The first hormone regulated second messenger pathway to be discovered, adenylyl cyclase, remains the paradigm from which many principles of signal transduction mechanisms have been derived (Iyengar, 1993). First, is the use of an intermediary protein functioning as a signal regulator. Adenylyl cyclase activation is regulated by a heterotrimeric G protein composed of three subunits; α, β and γ (Gilman, 1987 and Neer, 1995). The G protein based signal transduction system mediates the cellular response to a variety of signals including sensory stimuli, biogenic amines, neuropeptides, polypeptide hormones and fatty acid derivatives.

Generation of a second messenger to propagate the signal intracellularly is the second principle found in diverse signaling pathways. Second messengers vary in chemical structure from cyclic nucleotides to

membrane associated lipids (i.e. 1,2-diacyl glycerol). Stimulation of adenylyl cyclase turns on the enzymatic activity catalyzing the synthesis of second messenger, cAMP (cyclic adenosine 3′, 5′ monophosphate) from the substrate, ATP/Mg^{2+}. cAMP proceeds to mediate the transfer of information to the cell interior for hormones triggering cellular metabolism, growth and differentiation.

Third, a signaling cascade of protein kinases and phosphatases is a mechanism widely used in pathways requiring controlled flexibility brought about by on/off switches and signal amplification. The balance in activity of protein kinases and phosphatases determines the switch-like cellular response, while amplification is achieved through the cascade arrangement of protein-protein interactions. In the adenylyl cyclase pathway, the second messenger cascade proceeds by cAMP activating protein kinase A which in turn, phosphorylates either enzymes, channels or transcription factors. Overall, the physiological advantage of a cascade arrangement include: (1) numerous regulatory points, (2) rapid termination and (3) amplification of the signal.

Receptor Recognition:

The defining features of all receptors are their functions to specifically recognize signals and trigger a biological response. The current working

model of the physical dynamics of receptor activation has evolved mainly through studies using mutagenesis and proteolytic treatments. A binding pocket within the receptor allows for the binding of selected ligands leaving all other ligands ineffective at the cell periphery. Generally, the ligand binds within the region of the receptor associated with the lipid bilayer causing a conformational change which converts the receptor into an active state (Dixon et al., 1987 and Dohlman et al., 1988). The activated receptor can bind to the trimeric form of a G protein. Those receptors utilizing G proteins to transduce their extracellular signals comprise the G-protein coupled receptor family.

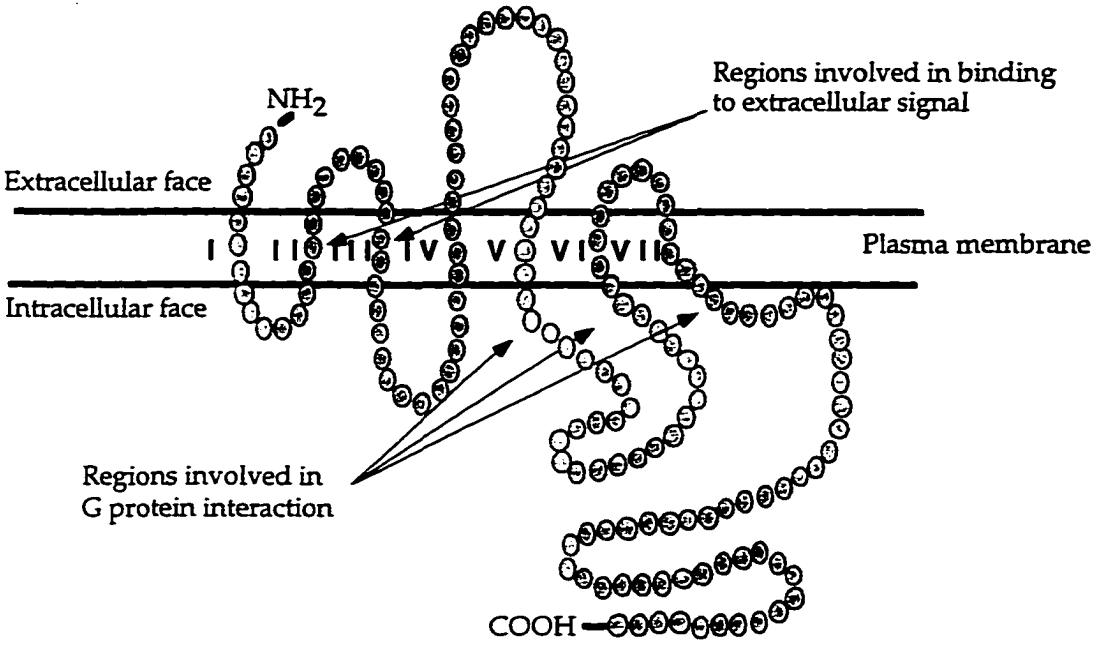
cDNA's for more than a hundred G protein-coupled receptors have been cloned. Even though they share only approximately 30% amino acid homology (mainly in the transmembrane segments), the predicted secondary structure of the G protein coupled-receptor class is remarkably conserved among species and receptor types (Bockaert, 1991 and Gudermann et al., 1995). The structural model is derived from the hydropathy pattern which depicts a single polypeptide chain with seven hydrophobic regions. These regions are thought to span the membrane as alpha helices with the amino terminus oriented towards the outside of the cell and the carboxy terminus towards the cytosol. This structure is based on a low resolution x-ray structure for bacterial rhodopsin (Henderson, 1975). Recent studies have shown that

receptor activation is associated with movement of the membrane helices. A schematic diagram is shown in Fig 1-1.

Figure 1-1 Model of prototypical G protein-coupled receptor

Schematic representation of the topology of a prototypical seven transmembrane spanning receptor. The transmembrane hydrophobic clusters are labeled in Roman numerals. Arrows point out the segments demonstrated to be involved in G protein interaction and extracellular signal binding.

Figure 1-1 Model of prototypical G protein-coupled receptor



Due to the homologous structure of G protein-coupled receptors, exchanging functionally relevant domains has provided evidence for regions involved in receptor-G protein coupling. Chimeras constructed from transmembrane segments V and VI with the intervening third cytoplasmic loop of α2-AR (adrenergic receptor) with β2-AR cDNA's have shown that the third cytoplasmic loop is critical in determining the specificity of interactions with G proteins (Kobilka et al., 1988). Thus, the primary structure of the receptor encodes a bi-directional specificity; signal recognition and the capability to transmit the signal to a specific intracellular pathway.

G-protein mediated signaling:

The G protein complex mediates the transformation of extracellular signals into second messengers produced by intracellular enzymes. The fundamental role for this transducer protein appears to be as sensor allowing for either amplification and/or diffusion of the message. In addition to regulation of signal magnitude, the G protein also imparts the specificity for the effector pathway leading to the signal-specific cellular response. To understand how G proteins couple signal specific receptors to effectors, it is necessary to understand the functional and structural features of G proteins.

(i) Functional features: the GTPase cycle

Heterotrimeric G proteins are members of the multigene GTPase superfamily. As such, its functional state oscillates between active and inactive depending on the guanine nucleotide bound (Bourne et al., 1991). In the inactive state, the three subunits, α, β and γ are associated as a single complex with guanosine diphosphate (GDP) bound to the α subunit. The activated receptor in combination with intracellular Mg^{2+} catalyzes the release of GDP forming a transient ligand-receptor-G protein high affinity ternary complex (Cassel and Selinger, 1978). See figure 1-2.

Although the affinity of Gα for both GDP and guanosine triphosphate (GTP) are comparable, the concentration of intracellular GTP is ten times greater (Gilman, 1987). Therefore, GTP binds to the empty pocket of the α subunit causing separation of the Gα subunit from the tightly associated Gβγ heterodimer. Gα-GTP assumes its activated conformation. Both Gα-GTP and Gβγ can independently propagate the signal further downstream by stimulating their respective effectors. The target effectors for the Gα-GTP and Gβγ are summarized in Table 1-1.

Table 1 Categorization of G protein subunit members

Class	Members	% Homologous	Effectors
$G\alpha_s$	α_{s1-4}*	100	AC, Ca^{2+}, K-Ca^{2+} channels
	α_{olf}	88	AC
$G\alpha_i$	α_{i1}	100	K^+ channels, AC
	α_{i2}	88	K^+ channels, AC
	α_{i3}	94	K^+ channels, AC
	α_{oA}	73	Ca^{2+} channels
	α_{oB}	73	Ca^{2+} channels
	α_{tc}	68	cGMP PDE
	α_{tr}	68	cGMP PDE
	α_{gust}	67	PDE
	α_z	60	AC
$G\alpha_q$	α_q	100	PLC (β1, 2, 3)
	α_{11}	88	PLC (β1, 2, 3)
	α_{14}	79	PLC (β1, 2, 3)
	α_{15}	57	PLC (β1, 2, 3)
	α_{16}	58	PLC (β1, 2, 3)
$G\alpha_{12}$	α_{12}	100	Na^+/K^+ exchanger
	α_{13}	67	
Gβ	β1-5	50-90	PLCβ-2, 3, K^+ channels, AC1, 2, 4, β-ARK, phosphoinositide 3 kinase
Gγ	γ1-11	27-75	

Toxins emitted during bacterial infection, namely cholera and pertussis toxins covalently modify the Gα subunits. Pertussis toxin (PTX) catalyzes the ADP ribosylation of a conserved cysteine residue in the fourth position from the carboxy terminus of Gα. This covalent modification abolishes hormonal inhibition of adenylyl cyclase (Katada and Ui, 1982). Cholera toxin (CTX) modification of $G\alpha_s$ eliminates the requirement for activation by the ligand-bound receptor and abolishes the hydrolysis activity of $G\alpha_s$. The modified $G\alpha_s$ is rendered constitutively active (Gill and Meren, 1978, Cassel and Pfeuffer, 1978 and Moss and Vaughan, 1978).

Control points for termination of the signal transduction process exist at both sides of the plasma membrane. At the external surface, there is dissociation of the agonist from the receptor and at the intracellular surface, the intrinsic GTPase activity of Gα abolishes the signal process. The catalytic rate of the GTPase can be independent of the receptor, Gβγ or effector and thus may serve as a clock defining the temporal window during which propagation of the signal may occur. More recently, "RGS" (Regulator of G protein Signaling) proteins that regulate GTPase activity of some Gα subunits have been identified (Berman et al., 1996 and Iyengar, 1997). These proteins increase the Kcat of GTPase and speed up signal transmission.

In one G protein system, Gq/phospholipase C-β the catalytic rate of the GTPase is stimulated by the effector (Ross, 1996). This allows the G protein to "hold" the signal until it has been transmitted to the effector. Hydrolysis of the gamma phosphate of the bound GTP to GDP causes the Gα subunit to dissociate from the effector and return to the inactive conformation bound to Gβγ. The GTPase cycle consistent with the present working model is illustrated in Fig 1-2.

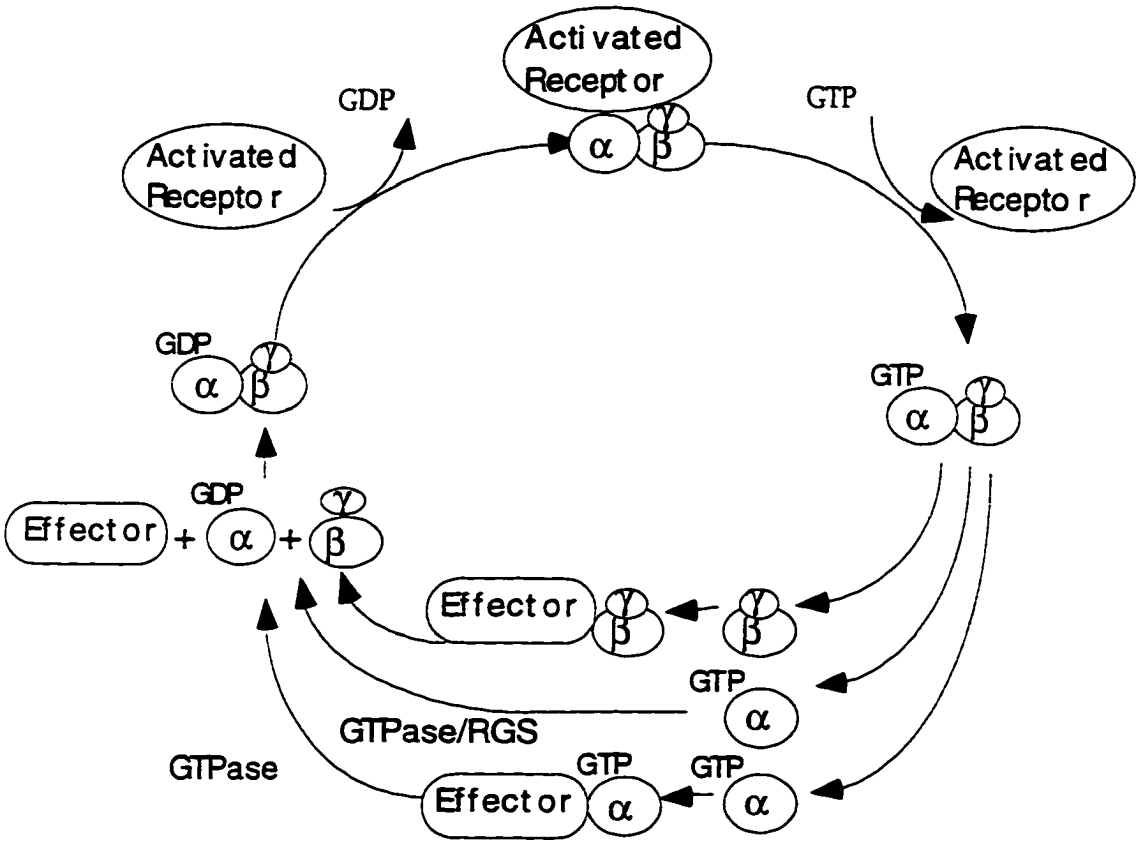
In the basal state, GDP is bound to the $G\alpha_s$ subunit and all three subunits are tightly associated. Receptor binds a selective ligand, stabilizing an activated form of the receptor. Upon receptor activation, Mg^{2+} and Gβγ seem to have opposing effects. While Gβγ increases the affinity of $G\alpha_s$ for GDP, Mg^{2+} decreases it so GDP dissociates more rapidly due to receptor activation in the presence of Mg^{2+}. Subsequently GTP binds to the empty pocket. This form is now the activated state where Gβγ and $G\alpha_s$ dissociate and are able to independently propagate signals by interacting with downstream effectors. Termination of the signal occurs when GTP bound to $G\alpha_s$ is hydrolyzed to GDP and the basal heterotrimeric form is resumed. GTP hydrolysis can be stimulated by RGS proteins. This occurs when the free Gα-GTP subunit interacts with the Ras proteins. One effector (PLC-β) can also

stimulate the GTPase activity of its interacting Gα (Gq family members) subunit.

Figure 1-2 Schematic of the Activity States involved in the GTPase Cycle

Activated receptor mediates the oscillation from basal GDP-bound heterotrimeric G protein to activated GTP-bound Gα which dissociates from Gβγ. Both GTP-Gα and Gβγ can activate effectors. The signal is terminated by the intrinsic GTPase which may be accelerated by RGS proteins. GDP-bound Gα reassociates with Gβγ and assumes the inactive heterotrimeric conformation.

Figure 1-2 Schematic of the Activity States involved in the GTPase Cycle



(ii) Structural features of Gα subunits

The first experimental evidence suggesting a distinct GTP-sensitive regulatory protein in the AC signaling pathway was provided by Pfeuffer (1977). A 42 kDa protein was partially resolved by affinity chromatography over a GTP-sepharose column. The flow through fractions exhibited no adenylyl cyclase activity, however stimulation was measured when those fractions eluted by GppNHp or GTP were added to the assay. In 1978, the requirement for GTP in visual signal transduction was discovered (Bitensky et al., 1978 and Yee and Liebman, 1978). Shortly thereafter, the protein now identified as transducin was purified (Fung et al., 1981, Godchaux and Zimmerman, 1979 and Kuhn et al., 1981).

Reconstitution studies were conducted by Gilman's group using a genetic variant of the murine S49 lymphoma cell line (Ross and Gilman, 1977 and Ross et al., 1978). No adenylyl cyclase activity could be detected from these cells in response to GppNHp or fluoride ions. It was therefore thought to be deficient in the adenylyl cyclase protein and as such, labeled Cyclase- (Cyc-) cells. Stimulation was obtained when a detergent extract from heat-inactivated wild type S49 cells was added to Cyc- membranes and assayed. It was concluded that the heat labile AC was present in Cyc- cells. However it is

stimulated by a separate component present in the wild type cells but absent in the mutant line, this component was $G\alpha_s$.

Two such $G\alpha_s$ polypeptides were isolated from rabbit liver. Analysis of the partially purified polypeptides in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) revealed two bands of apparent molecular weights, 45 and 52 kDa. Both were susceptible to covalent modification by [^{32}P]NAD and cholera toxin (Northup et al., 1980). Since the initial identification and purification of G_s, many more $G\alpha$ subunits have been identified (Hepler and Gilman, 1992 and Neer, 1995).

Currently, 20 different G protein alpha subunits have been identified and categorized into four classes (Table 1-1). These include: (i) α_s family, containing four isoforms derived from alternate splicing of a single gene, and α_{olf}; (ii) a_i family containing three forms of α_i, two forms of α_o and two forms of α_t (one expressed in rod cells and the other in cone cells) and one form of α_z; (iii) α_q family containing five members and (iv) α_{12} family containing two members.

The crystal structures for $G_t\alpha$-GTPγS, $G\alpha_{i1}$-GTPγS and $G\alpha_{i1}$-GDP-AlF4- have provided an understanding of the structural elements in the $G\alpha$ subunit

that play a functional role in the GTPase cycle (Lambright et al., 1996). The $G_t\alpha$ subunit has three domains: (i) a GTPase domain consisting of a six stranded β-sheet surrounded by six helices (α1- α5, αG). The GTPase domain is conserved among all GTPases. (ii) An alpha helical domain unique to heterotrimeric G proteins (αA) surrounded by five smaller helices (αB-αF). And (iii) a helix at the N terminus (αN) . Comparison of the crystal structures of the functionally homologous GTPases, p21ras and elongation factor Tu (EF-Tu) confirmed that the three dimensional folds of the GTPase domain are the same although the two proteins share only 17% homology. Guanine nucleotide binding is localized to a deep cleft in between the GTPase and αA domains.

Crystallography studies carried out by Hamm and Sigler's groups have determined the regions of the G protein subunits that undergo conformational changes upon activation (Lambright et al., 1996). Activation by nucleotide exchange promotes changes in the tertiary structure of $G_t\alpha$-GTPγS limited to the interfaces at the three "switch regions" within the protein. By superimposing the structures of free $G_t\alpha$-GDP and $G_t\alpha$-GTPγS onto that of the heterotrimeric complex of $G_t\alpha$-GDP, the conformational changes between heterotrimeric complexed $G_t\alpha$-GDP and the free, activated form are seen mainly in those residues that specifically interact with $G_t\beta\gamma$.

These include the Gly 199 residue which triggers conformational changes and Gln 200 which stabilizes the transition state for GTP hydrolysis, both of which are conserved among members of the heterotrimeric G protein family. Switch I and II of $G_t\alpha$-GTPγS are induced by hydrogen bonding to the γ phosphate of GTP. These structural changes can be detected in p21ras and EF-Tu. The conformational changes seen in a region unique to heterotrimeric G proteins, switch III is not induced by direct contact with guanine nucleotides, but as a conformational change induced by switch II. Overall, the structural changes induced by guanine nucleotide exchange do not affect the orientation of the α helical domain and its orientation with respect to the GTPase domain.

The regions for receptor and effector activation have been mapped out based on data from biochemical and mutational approaches. The model contains the following structural features: the tail end of the C terminus (dependent upon the α subtype) and a stretch of amino acids in the α_N region of the N terminus are postulated to contain the structural determinants required for receptor binding. The regions of $G\alpha_s$ containing the structural determinants for binding to the effector AC include the three loops, α2-β4, α3-β5 and α4-β6, and for $\beta\gamma$ interaction and the first twenty-five amino acids are necessary for contact (Berlot and Bourne, 1992).

(iii) Structural and functional features of Gβγ subunits

Initially, it was thought that the Gα subunit was the only component of the heterotrimeric complex involved in signal transduction. Currently, the Gβγ complex has been demonstrated to regulate as many effectors as there are for Gα. This dual regulation of effector pathways reflects the potential diversity in cellular responses initiated by only a single G protein complex.

The Gβγ dimer has several functions. For example, it is required for the Gα subunit to interact with the receptor (Florio and Sternweis, 1985). In addition, Gβγ has been demonstrated to activate K^+ channels (Logothetis et al., 1987 and Ito et al., 1992), PLCβ isoforms (Camps et al., 1992, Blank et al., 1992 and Smrcka and Sternweis, 1993), activate and inhibit several AC's (Tang et al., 1991, Tang and Gilman, 1991 and Gao and Gilman, 1991) and to inhibit both Ca^{2+} channels and agonist induced receptor states through phosphorylation (Herlitze et al., 1996, Haga and Haga, 1992, Pitcher et al., 1992). See Table 1-1.

The Gβ and Gγ subunits are noncovalently associated but do not separate under non-denaturing conditions (Gilman, 1987). They do not

demonstrate activity when separated. The Gβ subunit has a molecular mass of 35-36 kDa and shows considerable heterogeneity. Five mammalian Gβ subunits have been cloned sharing 50-90% sequence homology (Simon et al., 1991 and Watson et al., 1994). The Gγ subunit has a molecular mass of 7.3-8.5 kDa and may be the determining unit for Gβγ function. The Gγ subunit of the dimer is thought to serve as the plasma membrane anchor for the inactive heterotrimeric complex. Eleven Gγ subunits have been cloned exhibiting greater diversity with only 27-75% homology (Hepler and Gilman, 1992, Clapham and Neer, 1993, Ray et al., 1995). The Gβγ's can be functionally exchanged with $G\alpha_i$, $G\alpha_o$ or $G\alpha_s$. Refer to Table 1-1.

In association with Gα, Gβγ increases the affinity of Gα for GDP approximately 100 fold, stabilizing the inactive species (Higashijima et al., 1987). One working model of the structural changes that occur suggests that most of the conformational changes are limited to two of the three "switch regions" depending on the nucleotide occupying the Gα subunit. The span of amino acids from Gly 198 to Ser 202 of $G_t\alpha$ interact directly with $G_t\beta$ adopting a more flexible conformation that $G_t\beta$ can bind to. The subsequent adjustments stabilize the otherwise flexible switch I and switch II regions causing an increase in the affinity of GDP by reducing the rate of release (Lambright et al., 1996).

The specificity of dimerization between different Gβ and Gγ subtypes was identified based on the associated combinations found in cytosolic fractions (Simonds et al., 1991, Muntz et al., 1992 and Ray et al., 1995). From these studies and others, it was concluded that Gγ1 binds Gβ1 but not Gβ2 or Gβ3, Gγ 4 and Gγ 5 can both interact with Gβ1 and Gβ2 but not Gβ3.

The post translational modification occurs at the C terminus of the Gγ subunit. Different lipids are attached to different Gγ subtypes. For example, the Gγ1 and Gγ11 are farnesylated whereas the Gγ2 and other subunits are geranyl-geranylated (Kalman et al., 1995 and Ray et al., 1995). This lipid modification of the Gγ subunit is thought to anchor the heterotrimeric G protein to the membrane (Fukada et al., 1990, Lai et al., 1990, Mumby et al., 1990, Yamane et al., 1990, Maltese and Robishaw, 1990, Maltese, 1990 and Spiegel et al., 1991). Membrane anchorage of Gγ is thought to promote high local concentration and proper orientation of the components near the receptor and effector (Chabre et al., 1989).

The Gβ subunit contains seven tandem repeats of approximately 40 amino acids, called WD40 repeats (Simon et al., 1991). This repeating motif is found in a family of unrelated proteins whose functions are as diverse as

signal transduction, gene regulation and development, cell division, RNA processing, vesicular traffic, and cytosolic assembly (Neer et al., 1994). It is hypothesized that the WD40 repeats may play a role in determining the specificity of the Gγ subunit for interaction with a particular Gβ (Pronin and Gautam, 1992). The predicted structure for Gβ includes an amphipathic alpha helix with the WD40 repeats in sequence (Simon et al., 1991).

The Gβγ associated dimer displays a regulatory function at the level of the agonist activated receptor. Phosphorylation of G protein coupled receptors such as, β-AR, muscarinic acetylcholinergic receptor and rhodopsin by β-adrenergic receptor kinase (βARK) is stimulated by Gβγ (Muller et al., 1993, Richardson et al., 1993, Koch et al., 1993, Haga and Haga, 1993 and Pitcher et al., 1992). Gβγ is thought to mediate the translocation of βARK from the cytosol to the membrane. At this location βARK is in close proximity to phosphorylate the receptor. As a result the receptor is uncoupled from its G protein leading to a decrease in the cellular response, also referred to as desensitization (Pitcher et al., 1992, Haga and Haga, 1993 and Inglese et al., 1992). Muller et al., (1993) investigated the specificity of this regulation by measuring the effects of different combinations of Gβ and Gγ and found differential regulation by different combinations of Gβ and Gγ which was dependent on the receptor. For rhodopsin, β2γ2 was the most potent

combination in stimulating phosphorylation. For β2-AR, the heterodimer, β1γ2 was demonstrated to be the most potent.

G Protein Regulated Effectors and Second Messenger Production:

Signals recognized at the receptor and transduced by a G protein are sorted in accordance with the effector type. The effectors in the signal transduction pathway can be enzymes of the type that catalyze production of intracellular second messengers or ion channels which regulate the cell's membrane potential. Additional effector types remain to be identified.

G protein stimulation of effector molecules is the necessary turn on switch imparting the effector with the property to convert the primary extracellular signal into an intracellular chemical form. The production of second messengers in the form of 3',5' cyclic adenosine monophosphate (cAMP), inositol 1,4,5 triphosphate (IP_3), diacylglycerol (DAG), Ca^{2+} or 3',5' cyclic guanosine monophosphate (cGMP) leads to a biochemical or physiological response by the cell. Typical examples of G protein regulated second messenger producing enzymes are adenylyl cyclase and phospholipase C (PLC). The inward rectifying potassium channel is an example of an ion channel directly regulated by G proteins. Unique to the visual system, the G protein effector is the cGMP phosphodiesterase (cGMP-PDE) which degrades

the intracellular messenger cGMP to 5' GMP. As of yet, such a system has not been found in tissues other than sensory organs.

In contrast to the conserved structures found among receptor subtypes and G protein subtypes, the structure of the different G protein coupled effectors is very diverse. Hence, it is convenient to consider each effector separately. The structure and regulation of adenylyl cyclase will be discussed in the following section.

A. cGMP-Phosphodiesterase

The mechanism underlying the transduction events in the perception of light has been carefully worked out and has provided a paradigm from which many of the fundamental concepts demonstrated in other transduction pathways were derived. The pigmented receptors of light are the rod and cone cells of the retina. As the most peripheral sensors along the optic apparatus they connect with horizontal and bipolar cells, the latter synapsing with ganglion cells that form the optic nerve. While the components for the visual system have counterparts in other transduction pathways, the scheme differs in that without a signal, the pathway is active and in the presence of a visual target it is shut down.

The visual receptor called rhodopsin is found in the rod and cone cells of the retina. Light absorption by rhodopsin triggers replacement of GDP by GTP on transducin ($G_t\alpha$) (Wheeler et al., 1977). Subsequently, $G\beta\gamma$ is released and $G_t\alpha$-GTP now activates cGMP-PDE which in turn catalyzes the hydrolysis of cGMP into 5'GMP. In the rod cell cytoplasm, cGMP functions to maintain Na^+ channels in the open state. Therefore, a drop in cGMP levels causes the channel to close. Intracellular Na^+ will accumulate and the cell will subsequently hyperpolarize.

In contrast to the structure of adenylyl cyclase and PLC isoforms, the cGMP phosphodiesterase protein is a tetramer consisting of three subunits α, β, $\gamma 2$ (Baehr et al., 1979). Activation by transducin causes the release of the γ subunits (also called inhibitor, I subunits) producing a catalytically active $\alpha\beta$ complex. The phosphodiesterase is a soluble protein that can be attached to the membrane through the G protein subunits.

B. Phospholipase C

The renin-angiotensin system is one of several mechanisms that the kidney utilizes to regulate blood pressure. The specialized kidney cells of the juxtaglomerular apparatus synthesizes and secretes renin which catalyzes the production of angiotensin (AT) I from angiotensinogen. ATI is cleaved by

angiotensin converting enzyme to yield ATII, a potent vasoconstrictor. The signaling mediated by ATII is transmitted to the cell interior by activation of the catalytic property of PLC. The first reported data demonstrating the regulation of PLC by G protein subunits were described by Litocsh (1985) and Cockcroft and Gomperts (1985). Activation by G protein subunits causes PLC to catalyze the hydrolysis of phosphatidyl inositol-4, 5-bisphosphate (PIP_2) into inositol-1, 4, 5-trisphosphate (IP_3) and 1,2 diacylglycerol (DAG), both of which are able to carry the signal further down two interlinked pathways.

At least three subtypes of PLC's are involved in distinct cellular responses, PLC-β, PLC-γ and PLC-δ (Rhee et al., 1989, Rhee and Choi, 1992 and Cockroft and Thomas, 1992). Each is a product of distinct genes. In the AC system, $G\alpha_s$ can regulate all cyclase isoforms however, with the PLC subtypes there is no overlap in the regulatory molecules. For example, G protein subunits regulate only PLC-β while PLC-γ is not activated by G proteins but instead, it is stimulated by receptor tyrosine kinases.

To date, four mammalian forms of PLC-β have been cloned and characterized, PLC-β 1, PLC-β 2, PLC -β 3 and PLC-β 4 (Taylor et al., 1991, Suh et al., 1988, Carrozi, 1992, Jhon, et al., 1993, Ferreira, et al., 1993). The members of the G_q family: α_q, α11, α14, α15 and α16 have been demonstrated to regulate the PLC-β isozymes, PLC-β 1, PLC-β 2, PLC-β3 and PLC-β 4 (Smrcka et al., 1991,

Taylor et al., 1991, Waldo et al., 1991, Blank et al., 1991, Wu et al., 1992, Lee, C.W. et al., 1993, Lee S.B., et al., 1993, Smrcka and Sternweis, 1993, Jhon et al., 1993 and Hepler et al., 1993). The heterogeneity of PLC-β and α_q may play a role in selective coupling between different receptors and PLC's (Wu, 1993).

Structurally, the PLC-β isoforms are single chain polypeptides that are not intrinsic membrane proteins like adenylyl cyclase. Because the substrate for PLC is a component of the lipid membrane, PLC must translocate from the cytosol to the membrane. A clear understanding of the means of translocation of PLC has not yet been achieved. However, it appears that activation of the G_q pathway mediates the recruitment of PLC-β to the plasma membrane where it can catalyze the production of IP_3 and DAG.

The water-soluble, IP_3 binds to a specific endoplasmic reticulum receptor inducing an open conformation of the Ca^{2+} channel. This leads to mobilization of a high, localized concentration of Ca^{2+} into the cytosol. The release of intracellular Ca^{2+} from IP_3 sensitive stores regulates a variety of activities either through calmodulin or indirectly through the calmodulin-stimulated protein kinase (CaM-kinase). Along a parallel pathway, the membrane bound DAG activates protein kinase C (PKC). In addition, PKC can be activated by Ca^{2+} ions. PKC in general, mediates many growth and metabolism mechanisms through protein phosphorylation. For example, both normal physiological effects such as inhibition of glycogen synthesis and

pathological effects such as transformation into malignant cells are cellular responses evoked by PKC activation.

C. Ion Channels

One mechanism which accounts for the decrease in heart rate when cholinergic vagal fibers to nodal tissue are stimulated involves G protein activated inward rectifying K^+ channels (I_{KACh}) (Kubo et al., 1993 and Kofuji et al., 1995). The acetylcholine (ACh) liberated at the nerve endings binds to muscarinic receptors (m2) coupled to pertussis toxin-sensitive G proteins which directly activate I_{KAChs}. Cloning techniques utilizing cardiac and brain cDNA libraries have identified three cDNA's encoding for three isoforms of the putative I_{KACh} termed G protein inwardly rectifying K^+ channels (GIRKs); GIRK1, GIRK2 and GIRK3 (Kofuji et al.,1995, Kubo et al., 1993, Lesage et al., 1994). The channel responsible for I_{KACh} is a heteromultimer of GIRK1 and GIRK2 and this complex is known to be directly regulated by $G\beta\gamma$ subunits (Krapivinsky et al., 1995 and Kofuji et al., 1995).

Properties of the Adenylyl Cyclase Isoforms:

The cAMP-mediated signaling events regulate many aspects of cellular homeostasis. These include metabolic pathways directly initiated by cAMP

such as glycogen breakdown, fat lypolysis and the resorption of calcium from bone. In addition, the other signaling processes such as cellular proliferation, differentiation of embryonic cells and modulation of neuronal synaptic transmission have been demonstrated to be cAMP mediated (for review see, Iyengar, 1997). Due to the ubiquitous nature of this intracellular messenger a great deal of interest has been focused on characterizing the properties of the synthesizing enzyme, adenylyl cyclase.

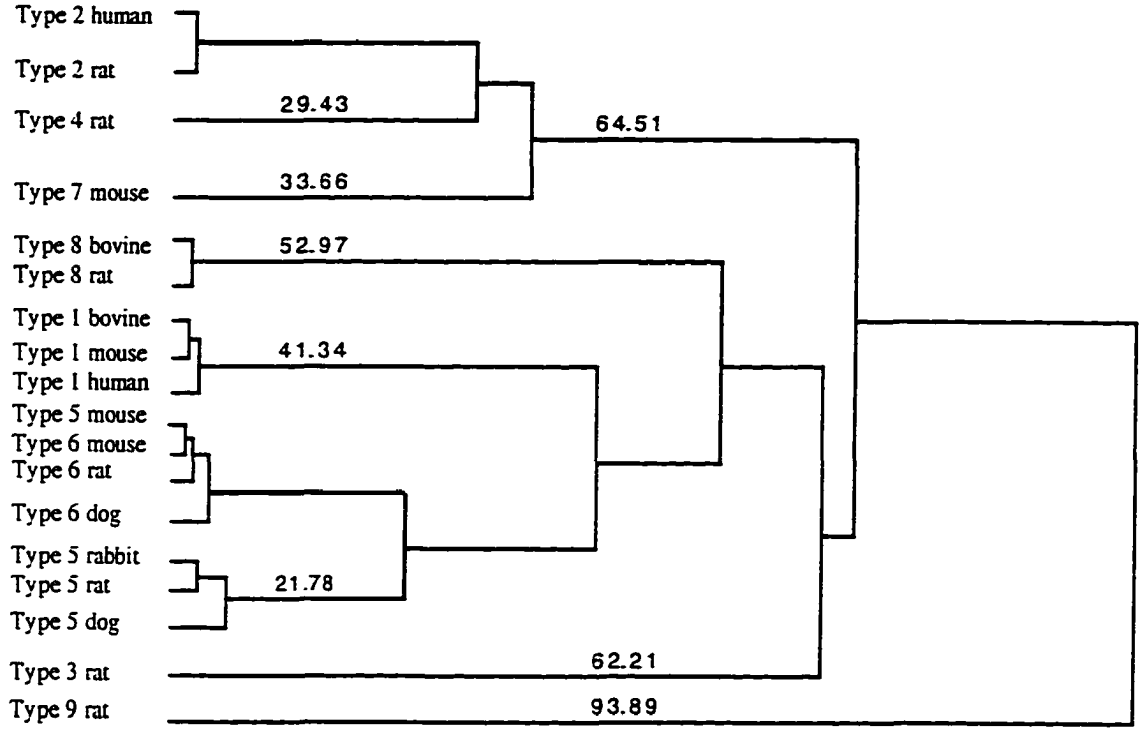
A) Structural Features and Molecular Heterogeneity

Investigations carried out by several independent laboratories have revealed considerable molecular heterogeneity in mammalian adenylyl cyclases (Fig 1-3). Nine distinct adenylyl cyclase isoforms have been cloned and characterized (Iyengar, 1993 and Premont et al., 1996). All are single polypeptides intrinsic to the membrane. However, certain bacterial isoforms and an isoform localized to the testis are cytosolic (Iyengar, 1993). Although interpretation of the hydropathy analysis suggests a structural organization resembling that of a transporter or ion channel, to date, no such transporter roles have been demonstrated to support this topographical model (Krupinski et al., 1989).

Figure 1-3 Phylogenetic Tree of Adenylyl Cyclase Isoforms

Using the GCG Growth Tree/UPGMA software from the Genetics Computer Group the homology among amino acid sequences for mammalian adenylyl cyclase isoforms were compared. A phylogenetic tree was reconstructed based on the UPGMA analysis of multiple sequence alignments. The method utilizes a distance matrix "Distances" to determine the relative distance (# in figure) between clusters of related sequences.

Figure 1-3 Phylogenetic Tree of Adenylyl Cyclase Isoforms



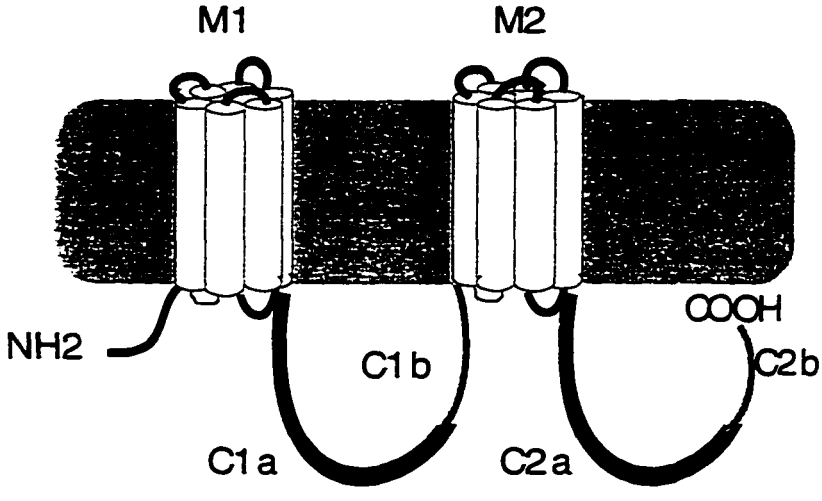
Much of our current understanding of the topographical arrangement of AC in the membrane is based on the hydropathy analysis of the amino acid sequences (see figure 1-4). There are two hydrophobic regions designated M1 and M2 each containing six transmembrane spans. Between these two regions is a large hydrophilic domain of 350 amino acids and at the C-terminal is a second cytoplasmic loop of 250-300 amino acids. These cytoplasmic loops are designated as C1 and C2 respectively. A schematic representation of the proposed topographical arrangement in the plasma membrane is depicted in figure 1-4.

Several residues within the cytoplasmic domains are conserved among populations of both adenylyl and guanylyl cyclases. Comparison of the profile of these two domains with proteins contained in the National Biomedical Research Foundation Protein Identification Resource, show the greatest scores among nucleotide-binding proteins (Krupinski et al., 1989). Early experimental evidence such as the loss of cyclase activity after a point mutation in the C1 domain of the Drosophila type 1 in combination with the sequence homology of this region with a single domain of guanylyl cyclase suggested this region as the site for catalytic activity (Livingstone et al., 1984).

Fig. 1-4 Schematic representation of proposed topographical arrangement of AC

Hydropathy analysis deduced a secondary structure as shown here. Two homologous (55%) hydrophobic domains, each believed to contain six membrane spanning regions (M1 and M2) separated by a large intermediary cytoplasmic loop (C1) and a long carboxy tail (C2). Several residues within the two cytoplasmic loops are homologous to guanylyl cyclases and thus suggested to be the site for catalytic activity.

Fig. 1-4 Schematic representation of proposed topographical arrangement of AC



While the specific residues involved in ATP/Mg^{2+} binding have not been identified, molecular biological techniques have provided methods to develop constructs of functional domains. A soluble form of AC was constructed by ligating the sequences that encode for C1a from AC type 1 and C2a from type 2 (Tang and Gilman, 1995). Constructs were coexpressed with $G\alpha_s$* or wild type G_s into an *E coli* strain deficient in adenylyl cyclase. Subsequently, activity was restored and could be measured by maltose utilization by this strain and growth on McConkey agar. While constructs of half molecules of AC expressed in Sf9 cells have not shown activity, half molecules are present in cardiac muscle (Katsushika et al., 1992). It is therefore of great interest to determine the mechanism by which $G\alpha_s$ stimulates AC and how this active conformation can convert ATP/Mg^{2+} into cAMP and PP_i.

B) Functional regulation of adenylyl cyclase isoforms:

Purification of the catalytic moiety proved difficult due to the inherent thermal instability of adenylyl cyclase and the low concentration of enzyme present in the plasma membrane. Although a soluble bacterial form of the enzyme with a specific activity of 30 mmol/min/mg was isolated in 1973 (Takai et al., 1974), the mammalian counterpart was not purified to satisfactory enzymatic activity and stability until 1982 (Pfeuffer and Metzger,

1982). Characterization of the regulatory patterns of the AC isoforms was therefore possible only after methods were developed to isolate a functional form of the enzyme.

Based on prior knowledge that the cardioactive diterpene, forskolin directly stimulated AC with high affinity (for review see Seamon and Daly, 1986), a forskolin-bound Sepharose matrix was developed and used to affinity purify AC from a rabbit myocardial preparation (Pfeuffer and Metzger, 1982). This isoform can be stimulated by G_s and Ca^{2+}/calmodulin (CaM) and is found to have an apparent molecular weight ranging from 130 to 150 kDa and a specific activity of 15 μmol/min/mg in response to forskolin (100 μM). [^{32}P] labeled G_s was crosslinked to the catalytic subunit to confirm adenylyl cyclase identity. The instability encountered in earlier studies was overcome by preactivation with GTP analogues (Pfeuffer et al., 1982, 1983, 1985a).

Because the AC species purified over the forskolin-sepharose column was stabilized by preactivation, this persistently active preparation was rendered impractical for reconstitution studies. In order to further characterize the molecular interaction with regulatory components, Smigel (1986) developed a method to resolve the catalytic moiety from G_s. Enzymatic activity in Smigel's detergent extracts from bovine brain was measured in the presence of $G\alpha_s$, forskolin and Ca^{2+}/CaM.

In most mammalian systems AC is stimulated by the $G\alpha_s$ subunit however, G_s insensitive forms are found in bacteria and yeast (Braun and Dods, 1975). Microorganisms such as bacteria and yeast, express adenylyl cyclases that are constitutive enzymes and therefore not regulated by extracellular signals. However, they contain regulated adenylyl cyclases, even though G_s is absent.

The distinguishing features used to categorize the different adenylyl cyclase forms are their sensitivity to stimulatory or inhibitory effects of Ca^{2+}, functional regulation by the $G\beta\gamma$ complex (stimulatory/inhibitory) in the presence of activated $G\alpha_s$ and susceptibility to protein kinases. Some adenylyl cyclases, especially those found in neuronal tissues, are stimulated by Ca^{2+} through the Ca^{2+} binding protein, CaM (Tang et al., 1991 and Cali et al., 1994). Other adenylyl cyclases are extensively stimulated by $G\beta\gamma$ subunits in the presence of $G\alpha_s$ (Tang and Gilman, 1991 and Taussig et al., 1993). Some adenylyl cyclase isoforms on the other hand, were found to be inhibited by Ca^{2+} or $G\beta\gamma$ subunits (Katada et al., 1987, Chiono et al., 1995, Yoshimura and Cooper, 1992). Thus, depending on which adenylyl cyclase isoform is present in the cell, they function to raise or lower cAMP levels in response to a variety of signals.

(i). Regulation by Gβγ subunits:

Type-specific regulation of adenylyl cyclase by Gβγ subunits has been demonstrated (Tang et al., 1991 and Cali et al., 1994). AC type 1 was directly inhibited by Gβγ subunits at concentrations in the range of 1-50 nM (Katada et al., 1987). Regulation of type 2 and 4 is dependent upon the conditions. As the sole regulatory factor, Gβγ has a small stimulatory effect, however in combination with activated $G\alpha_s$, Gβγ extensively stimulates adenylyl cyclase types 2 and 4 (Gao and Gilman, 1991 and Tang and Gilman, 1991). Because this effect is mediated by a nanomolar range of Gβγ, it is likely to be a relevant regulatory pathway in the intact cell. Types 3, 5, 6 and 9 are not sensitive to regulation by Gβγ (Premont et al., 1992, Tang and Gilman 1991, Premont et al., 1996).

(ii). Regulation by Ca^{2+}/CaM:

Assays measuring type 1 enzyme activity in Sf9 cells show direct stimulation (up to 60-80% of maximum) in response to Ca^{2+}/CaM (Tang, Krupinski and Gilman, 1991). While type 3 is also regulated by Ca^{2+}/CaM, the pattern of stimulation is very different from that exhibited by type 1. The EC_{50} for type 1 is 0.05 μM and is 5 μM for type 3. Also, stimulation of type 3 by Ca^{2+}

is conditional to prior activation by forskolin or activated G protein subunits and is ten fold less sensitive to CaM. Keeping in mind that the cytosolic concentration of free Ca^{2+} can range from 0.1 to 10 μM and the membrane surface of neurons may reach as high as 100 μM during an action potential (Smith and Augustine, 1988) an interesting role for type 3 is proposed. Because stimulation of type 3 requires high concentrations of Ca^{2+} and prior activation by other effector molecules, it may serve a role as a signal amplifier in the activated cAMP pathway.

Stimulation by Ca^{2+}/CaM has also been measured for type 8 (Parma et al., 1991, Cali et al., 1994 and Defer, 1994). This isoform can be expressed as either of three splice variants: 8A, 8B or 8C (Cali, 1996). Dependent upon which exon is excised, the resulting variant may be N-glycosylated or may be differentially regulated by Ca^{2+}/CaM. For example, type 8B is not N-glycosylated because the target site for N-glycosidase F is spliced out of the message. All three types are stimulated by Ca^{2+}/CaM, however, their EC_{50} values differ which may result from altered conformation or a change in activity corresponding to a regional deletion. The EC_{50} values generated from concentration-response curves are as follows: 140 ± 21 nM, 116 ± 12 nM and 30 ± 6 nM for 8A, 8B and 8C respectively.

To date, types 2, 4, 5 and 6 have not been shown to be stimulated by Ca^{2+}/CaM however, low concentrations of free Ca^{2+} (μM) appear to cause inhibition of AC type 5 and 6 (Yoshimura and Cooper, 1992 and Chiono et al., 1995). The recently cloned type 9 is also insensitive to Ca^{2+}/CaM (Premont et al., 1996).

(iii). Regulation by P-site ligands:

All adenylyl cyclases except for the bovine sperm isoform have been shown to be directly inhibited by purine nucleotides (Desaubry et al., 1996 and see references within). The inhibitory domain is called "P site" because the inhibition is demonstrated only with purine analogues. For example, 2',5'-dd-ATP and 2',5'-dd-ADP are potent inhibitors of rat membrane adenylyl cyclase with IC_{50}'s measuring 40 and 100 nM respectively. The parent ligand, adenosine also displays inhibitory effects albeit with a lower potency, the measured IC_{50} for rat brain adenylyl cyclase is 82 μM. The activated form of adenylyl cyclase is most susceptible to inhibition by adenosine derivatives. Both G_s or Mn^{2+} stimulation increased the sensitivity to inhibition (Desaubry et al., 1996 and Johnson et al., 1989).

Although no data has been offered to delineate the location of the P site, the inhibitory mechanism is thought to occur by direct interaction with domains distinct from the catalytic site and possibly some regions within it.

Lys 923 Ala mutated AC1 demonstrated greater than 90% inhibition of basal, G_s and calmodulin activity (Tang et al., 1995). In the presence of forskolin and Mn^{2+}, the mutated AC1 showed 40% of the activity of the wild type, however, there was a 100 fold decrease in sensitivity to inhibition by P site ligands. This suggests that Lys 923 is critical for both catalytic activity (in the presence of cation complexed-ATP) and for P-site ligands. Recently, Johnson et al., (1997) have presented data describing the differential sensitivity of adenylyl cyclase isoforms to inhibition by P-site ligands. AC1 was most sensitive, AC6 and AC8 followed and were comparable and AC2 was the least sensitive.

C) Tissue distribution of multiple AC forms

The tissue selective distribution of the multiple AC isoforms enables the cell to respond to extracellular signals depending on their immediate environment and the presence of an integrated network for the maintenance of homeostasis. In addition, the differing ratios for the distinct enzyme types within a single cell serves as a basis for specialized regulation.

Northern analysis reveals a limited distribution of the type 1 message in the nervous system (Xia et al., 1993). Specifically, type 1 is localized to the cortex, hippocampus and cerebellum, regions associated with learning and memory. Types 7 and 8 were found in the retinal pigment epithelium and cortex, respectively (Volkel et al., 1996 and Hellevuo et al., 1996). Type 2

message is localized to the brain and lung (Feinstein et al., 1991 and Premont et al., 1992). Type 3, once thought to be limited to olfactory sensory neurons is also expressed in adrenals, atria, lung and retina (Xia et al., 1992). Type 4, 5 and 6 are widely distributed with high levels in the brain and heart (Iyengar, 1993). The mRNA levels of the recently cloned and characterized AC9 were widely distributed with high levels detected in skeletal muscle and brain (Premont et al., 1996).

Immunohistochemical methods revealed a selective subcellular localization in neurons, the most dense signals were observed at the postsynaptic regions of dendritic spine heads as well as in presynaptic axon terminals (Mons et al., 1995). Since an antibody common to all ACs was used it was not possible to determine which ACs are present in the postsynaptic densities. Such specific subcellular localization implicates AC in neurotransmitter release and a close association between cAMP and Ca^{2+} signaling pathways.

D) Regulation of Adenylyl Cyclase by Protein Kinases

Upon activation of the signal transduction pathway, the extracellular signal is converted into an intracellular second messenger. The machinery within the cell senses the change in second messenger levels and initiates a cellular response. The intracellular molecules that function as sensors are

protein kinases. Protein kinases are enzymes that can propagate a signal by covalent modification of target proteins through phosphorylation of specific amino acids. In mammalian systems the amino acid residues targeted for phosphorylation are serine, threonine or tyrosine. Different protein kinases specifically respond to different second messengers, for example protein kinase A responds to cAMP, protein kinase C to diacylglycerol and the Ca^{2+}/CaM kinase to Ca^{2+} and hence to IP_3.

Protein kinases also exhibit substrate specificity and are thus able to regulate the activity of target proteins selectively. There is considerable diversity among protein kinases in the manner in which a response is evoked. In some cases the protein kinase directly modifies the target protein activity through phosphorylation. In other cases, the protein kinase sets off a cascade system by phosphorylating another protein kinase downstream which will in turn induce the sequential phosphorylation of several protein kinases. At the end of the cascade the target protein whose activity results in the physiological response evoked by the signals is covalently modified.

Targets for phosphorylation include metabolic enzymes, transcription factors and ion channels. Once phosphorylated, these targets exhibit a change in activity. Such changes can be either stimulatory or inhibitory. For example, regulation of glucose production in the hepatic cell is brought about by both stimulatory and inhibitory signals. Activation of protein kinase A

activates phosphorylase-kinase which in turn activates phosphorylase B leading to the production of glucose from glycogen. Simultaneously, protein kinase A phosphorylates glycogen synthetase turning off its capability to synthesize glycogen from glucose. Thus, protein kinase A, via two pathways both stimulates the production of glucose from stores and simultaneously blocks the storage of free glucose thereby raising the availability of free glucose for metabolic purposes. Using this mechanism, hormones such as epinephrine are able to mobilize glucose rapidly when needed.

CHAPTER 2

STATEMENT of PROBLEM and SPECIFIC AIMS

cAMP is an ubiquitous intracellular second messenger mediating the effects of many hormones, neurotransmitters, autocrine and paracrine factors. These include circulating hormones such as adrenocorticotrophic hormone, calcitonin, catecholamines, follicle stimulating hormones, and leutenizing hormone, neurotransmitters such as dopamine and serotonin and autocrine factors such as prostaglandin and prostacyclin to name a few. Although this second messenger is so widespread in regulating cellular activity, regulation of the enzyme that synthesizes cAMP, adenylyl cyclase by various entities is only now beginning to be understood. Nine adenylyl cyclases have been cloned and expressed. The different adenylyl cyclases are differentially regulated by $G\beta\gamma$, $G\alpha_i$ and protein kinases . All of the mammalian adenylyl cyclases are stimulated by $G\alpha_s$. Also, the diterpine, forskolin directly activates all mammalian adenylyl cyclase isoforms, albeit to varying degrees but does not activate an isoform from *Dictyostelium*, ACA even though both share the same overall topography as the other isoforms (Pitt et al., 1992). Forskolin and $G\alpha_s$ are known to stimulate adenylyl cyclases in a synergistic fashion (Neer, 1995 and Clark et al., 1982). However, it is not known if the mechanisms by which $G\alpha_s$ or forskolin stimulate the different mammalian

adenylyl cyclases is conserved throughout the isoforms. The goal of this research project is to determine if the mechanism of $G\alpha_s$ and the chemical regulator, forskolin stimulation are conserved amongst the various adenylyl cyclases. For this purpose I will:

1. characterize the regulation of adenylyl cyclases 1, 2 and 6 by varying concentrations of mutant (Q227L) activated $G\alpha_s$.

2. determine the effects of forskolin on the stimulation of adenylyl cyclases 1, 2 and 6 by varying concentrations of mutant activated $G\alpha_s$.

Mutant (Q227L) activated $G\alpha_s$ expressed *in vitro* in rabbit reticulocyte lysates was used since this method of expression produces high potency recombinant $G\alpha_s$ in contrast to $G\alpha_s$ expressed in bacteria or in Sf9 cells. Adenylyl Cyclases 1, 2 and 6 were individually expressed in Sf9 cells. These adenylyl cyclases were chosen as representative members of three different families of adenylyl cyclases. Adenylyl cyclase 1 is expressed in neuronal cells and is stimulated by Ca^{2+}/CaM in addition to $G\alpha_s$ and may function largely as a Ca^{2+} sensor in neuronal cells. Adenylyl cyclase 2 represents the prototype of an enzyme that is largely subject to stimulatory regulation from many sources and may function as a positive co-incident detector. Adenylyl cyclase 6 by contrast is stimulated by $G\alpha_s$ and inhibited by Ca^{2+}, $G\alpha_i$ and protein kinase A

and may function as a negative co-incident detector. Since these three isoforms serve different biological functions it is of interest to know if they are differentially regulated by $G\alpha_s$.

CHAPTER 3

MATERIALS and METHODS:

I. MATERIALS

- Chemicals and Reagents

Molecular Biology:

GeneClean II Kit was purchased from Bio101, (La Jolla, CA). Chemicals including: D-glucose, select peptone 140 (bactotryptone), formaldehyde-37%, agarose, formamide and the resin, Sepharose CL-4B were all purchased from Sigma Chemical Co. (St. Louis, MO). HB101 cells were purchased from Bio-Rad, (Richmond, CA). Epicurian Coli XL1-Blue supercompetent cells was from Stratagene, (LaJolla, CA). MOPS 3-(N-morpholino) propane-sulfonic acid and trichloroacetic acid were from Fisher. Reagents necessary for transcription such as: ribonucleotides, nuclease free water, RNAse inhibitor were purchased from Promega (Madison, WI). Sequenase Version 2.0 DNA Sequencing Kit was purchased from Amersham Life Science (Arlington Heights, IL).

Protein Synthesis and Analysis:

N,N'-methylene-bis-acrylamide and Tween 20 were purchased from Bio-Rad, (Richmond, CA). The brand of nonfat dry milk used for Western blotting was Carnation, (Glendale, CA). Both high molecular weight standards prestained and unstained were purchased from Gibco BRL (Gaithersburg, MD). CHAPS and Acrylamide (2X) were from Serva (Paramus, NJ). n-dodecyl β-D maltoside, L-methionine, β-mercapto-ethanol, glycine, H_2O_2-30%, EDTA, ovalbumin, lauryl sulfate, Lubrol, isobutylmethylxanthine, creatine phosphate, cAMP, ATP, phenylmethylsulfonyl fluoride, 1,10 phenanthroline, leupeptin, aprotinin, soy bean trypsin inhibitor, trizma base, HEPES, imidazole were all purchased from Sigma Chemical Co. (St. Louis, MO). The in vitro translation kits TnT and Flexi rabbit reticulocyte lysate were purchased from Promega (Madison, WI). Methanol, acetone, 2-propanol, 2 and 10N NaOH were from Fisher. Whatman microfibre filter GF/A, Hybond C extra nitrocellulose, ECL Western blotting kit were all from Amersham Life Science (Arlington Heights, IL).

Cell Culture:

Gentamycin 500X (50 mg/ml) was purchased from Boehringer Mannheim (Indianapolis, IN). Donor horse serum was from Gemini Bio-products (Calabasas, CA). All cell culture media such as: Sf-900 media, Graces

supplemented media, DMEM media, penicillin and streptomycin, fetal calf serum were purchased from Gibco BRL (Gaithersburg, MD).

- Enzymes

Restriction enzymes, T4 DNA ligase, were purchased from New England Biolab (Beverly, MA) through the Brookdale Center for Molecular Biology at Mount Sinai School of Medicine. T7 RNA polymerase was from Boehringer Mannheim, (Indianapolis, IN). Lysozyme, RNase A, creatine phosphokinase, myokinase were all from Sigma Chemical Co. (St. Louis, MO). *Pyrococcus furiosus* (Pfu) DNA polymerase was from Stratagene, (LaJolla, CA).

- Radiochemicals

[α^{32}P]-ATP, 2-,8-[^{3}H]-cAMP, [α ^{35}S]-dATP, [α ^{32}P]-dCTP were purchased from DuPont NEN (Boston, MA), translational grade [^{35}S]-L-methionine was purchased from ICN, (Irvine, CA)

- Antibodies

Antiserum labeled, AC common was prepared by Richard Premont. Rabbits were boosted with a peptide sequence NH2-IGARKPQYDIWGNT-

COOH common to adenylyl cyclases 1-8. The antibody against $G\alpha_s$, RM/1 was a gift from Allen Spiegel at the NIH.

II. METHODS

A. Cell culture

- Insect cell culture

The insect cell lines used which support AcMNPV replication, synthesis of recombinant protein and post-translational modifications are the Sf9 Fall Armyworm Ovary, Spodoptera frugiperda (Sf9) and High Five ovarian cells, *Trichoplusia ni*. The Sf9 cells were obtained from the Mount Sinai Baculovirus Expression Core Facility and American Type Culture Collection, Rockville, Maryland. The High Five cells were a generous gift from Dr. Peter Graves (Mount Sinai School of Medicine) and originally obtained from Stratagene. Monolayer cultures are incubated at 28°C in serum-free insect culture medium for Sf9 cells and Graces-supplemented with L-glutamine, yeastolate and lactalbumin with 10% fetal bovine serum and 10 µg/ml gentamycin for High Five cells. Cultures were not used beyond 25 to 30 passages. Cells in the log phase stage of the growth curve are infected with a multiplicity of infection between 5 and 10. Insect cell viability was

greater than 97% as determined by Trypan Blue exclusion. Cells were harvested 48 hours post infection.

- S49 cyc-mouse lymphoma cell culture

S49 cyc- cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) plus 10% horse serum inactivated at 56°C for 30 minutes as a suspension culture in flasks. Maintenance of exponential growth is dependent on the pH optimum of 7.4 and cell density no greater than $2x10^6$ cells/ml and no lower than $1x10^5$ cells/ml. Conditions beyond these limits result in toxicity and subsequent death. Cells were incubated at 37°C with 95:5/%O:%CO2. S49 cyc- cells were obtained from the University of California, San Francisco Cell Culture Facility.

B. Membrane preparation

To prepare membranes, viral-infected cells were pelleted at 4°C and washed with lysis buffer: 20 mM HEPES (pH 8), 5 mM EDTA, 1 mM EGTA, 150 mM NaCl, 2 mM DTT, and a cocktail of protease inhibitors including: 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM 1, 10 phenanthroline, 3.2 µg/ml leupeptin, 3.2 µg/ml soy bean trypsin inhibitor (SBTI), 2 µg/ml aprotinin. Cells were lysed by nitrogen cavitation in a Parr bomb at 600 psi for 30 minutes on ice and centrifuged at 1000xg for eight minutes at 2°C with no

break applied to pellet debris. The supernatant was spun at 100,000xg for forty-five minutes at 2°C to pellet crude membrane. The pellet was resuspended in 20 mM NaHepes (pH 8), 1 mM EDTA, 200 mM sucrose, 2 mM DTT and a cocktail of protease inhibitors including: 1 mM PMSF, 1, 10 phenanthroline, 3.2 μg/ml leupeptin, 3.2 μg/ml SBTI, 2 μg/ml aprotinin. Aliquots were frozen in a dry ice-acetone bath and stored at -80°C. Protein concentration was determined by either Lowry method or Bicinchoninic Acid (BCA) Protein Assay detection system using bovine serum albumin as the standard.

Solubilized AC6 protein was prepared by extraction in 20 mM HEPES pH 8, 150 mM NaCl, 2 mM DTT, 1 mM EGTA, 1mM EDTA, 20% glycerol, 0.8% dodecyl maltoside, 1 mM PMSF, 1, 10 phenanthroline, 3.2 μg/ml leupetin, 3.2 μg/ml SBTI, 2 μg/ml aprotinin. Preparation was rocked gently in 4°C cold room for one hour, spun down 100,000xg for 45 minutes at 4°C and the supernatant was assayed immediately.

C. Nucleic Acids, vectors and viruses

- Expression of AC2 and AC6

The cDNA's encoding adenylyl cyclase types 2 and 6 were subcloned by other members of the laboratory. AC2 and AC6 cDNA's were subcloned into plasmid vector, pVL1392 and pVL1393 respectively replacing the coding region of the polyhedron gene of the baculovirus Autographa californica. The recombinant plasmid was cotransfected with BaculoGold DNA into Sf9 cells. Recombinant viral particles were isolated and purified. The cotransfection of plasmid, infection with baculovirus and selection of clones were carried out by the Baculovirus Core Facility of Mount Sinai School of Medicine. Large scale infection of Sf9 or High Five cells was carried out with recombinant baculovirus and TPO-XS at a multiplicity of infection between 5 and 10. Sf9 or High Five cells were grown in appropriate medium at 28°C and harvested 48 hours post infection. High titer virus stock was prepared using roller flasks maintained at room temperature.

The cDNA for AC2 was the kind gift of Dr. R. Reed from Johns Hopkins School of Medicine, cDNA's for types 1 and 6 were isolated by our laboratory. The plasmid vectors, pVL1392 and pVL1393 were the kind gifts of Dr. Max Summers of Texas A&M University.

D. Adenylyl cyclase assays

The assay was carried out following the conditions of Johnson and Salomon (1991). Sf9 or High 5 cell membrane preparations were assayed in the presence of 0.1 mM [α^{32}P]-ATP (~ 1000 cpm/pmol), 1 mM [^{3}H]-cAMP (~10,000 cpm), 1 mM EDTA, 25 mM NaHepes (pH 8.0), 0.5 mM isobutylmethyl-xanthine (IBMX), ATP regenerating system (2 units/ml myokinase, 20 mM creatine phosphate and 2 units/ml phosphocreatine kinase), 10 mM $MgCl_2$. Typically, 5 µg of crude membrane protein was assayed per sample in a total volume of 30-50 µl at 32°C for 15 minutes. [^{32}P]-cAMP formed was separated from [α^{32}P]-ATP by affinity chromatography with serial DOWEX and alumina columns. 3 ml of Aquasol was added to the alumina eluent, vortexed and counted after 1 hour using Beckman LS 5000TD scintillation counter.

E. SDS-Page, Coomasie Blue staining and immunoblotting

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) separates proteins based on molecular weights (Laemmeli, 1970). Linear slab gels were prepared using 5% Acrylamide for stacking and 7% Acrylamide for separating gel for electrophoresis of adenylyl cyclase preparations or 12%

Acrylamide for electrophoresis of G protein subunits. The Acrylamide:bis ratio was 30:0.04. Gels were run at 150V constant voltage and either stained in 0.1% Coomassie blue or proteins were transferred to Hybond C nitrocellulose membrane.

The immunoblot technique was carried out according to Towbin et al., (1979). The gel containing electrophoresed proteins was incubated in transfer buffer for 15 minutes. Proteins were transferred onto Hybond-C extra nitrocellulose membrane using 80 V for one and a half hours at room temperature or 35 V overnight at 4°C in a wet blotting tank. Transfer buffer contained 25 mM Tris-HCl, 192 mM glycine and 15% methanol. Nitrocellulose membrane was blocked in phosphate buffered saline containing 5% dry nonfat milk, 1% ovalbumin, 5% fetal calf serum, 1M glycine for 30 minutes at room temperature or overnight at 4°C. The membrane was washed 3 times, 5 minutes each in phosphate buffered saline containing 0.1% dry nonfat milk, 0.1% ovalbumin, 1% fetal calf serum, 0.1% Tween 20 before being exposed to antiserum either for 3 hours at room temperature or overnight at 4°C. Prior to incubation with secondary horse-radish peroxidase-conjugated goat anti-rabbit antibody, the membrane was washed vigorously 3 times for 5 minutes in wash buffer and a final time in phosphate buffered saline containing 0.05% Tween 20 (T-PBS). The secondary antibody was diluted 3,000 fold in T-PBS and incubated with membrane for 1 hour at room temperature and washed vigorously 3 times in T-PBS buffer.

The immunodetection system was an ECL kit. The reagent was incubated with the membrane for 1 minute and immediately exposed to Hyperfilm for the necessary time to visualize the signal.

F. Dideoxy method of sequencing

The approach utilized to sequence DNA is an enzymatic-based method. A primer sequence anneals to a single stranded template DNA and elongation is catalyzed by a DNA polymerase (Sequenase). The chain elongation is terminated by incorporation of 2′,3′-dideoxy-nucleoside 5′-triphosphates (ddNTP's). Because it lacks the 3′-hydroxyl residue, it prevents the formation of a phosphodiester bond resulting in chain termination. Four different ddNTP's (ddATP, ddGTP, ddCTP, ddTTP) are included in separate reactions. A radiolabeled nucleotide is included. The resulting oligonucleotides of various lengths can be visualized through autoradiography after resolution on a polyacrylamide gel.

The sequencing protocol was provided by Sequenase v2.0 kit. Double stranded DNA template (3-5 μg) is first denatured. Next, primer is allowed to anneal as the tube is warmed to 65°C for 2 minutes and cooled slowly at room temperature over a 30 minute period. Afterwards, the tube is placed on ice. The labeling reaction includes the annealed template primer, DTT, labeled nucleotide and diluted Sequenase. All are mixed and incubated for 2-5

minutes at room temperature. The reaction is terminated by adding labeled samples to prewarmed tubes containing either ddNTP while incubating at 37°C for 3-5 minutes and finally, addition of Stop Solution to each tube. Prior to loading onto 8% polyacrylamide sequencing gels, samples are heated to 75-80°C for 2 minutes and immediately loaded.

G. Calculation of viral titer

Cells were collected, counted and diluted to 10,000 cells/100 µl. Using a Costar pipette, 200 µl of cells were dispensed into all wells except column 1 of a microtiter plate. 110 µl of media was added to all wells except A1 where 122 µl of virus was added. Serial dilution was performed as follows: 12 µl of virus was taken from A1 and added to B1, 12 µl was taken from B1 and added into C1 and so on. Then, 10 µl of diluted virus transferred from H1 into H2, from H1 into H3 and so on. No virus was added to column 12, this served as control. Proceed with row G, F, etc. Finally, 200 µl of diluted cells were added to all wells of column 1.

The plate was inserted into a plastic heat-sealable pouch (Kapak/Scotchpak™) along with several moist paper towels to maintain a humid environment. The pouch was sealed and placed in an incubator at

28°C for at least 10 days. Afterwards, plates were scored and titer was calculated by determining the 50% end point dilution. Titers typically ranged from 10^7-10^8 pfu/ml.

III. Data Analysis

Computer programs: Kaleidagraph 3.5, Microsoft Excel, Prophet (from NIH, distributed by BBN of Massachusetts). The concentration response data was analyzed using the curve fitting programs of Prophet. Equations for enzyme kinetics representing single site, two site noninteracting and two site interacting models were used to fit the data. The model best fitting the data was determined by statistical analysis (F test) of the parameter estimates. Data from concentration response curves were also transformed according to the Hofstee linear transformation method. For graphical analysis of the linearly transformed data, v/s was plotted on the abscissa and v on the ordinate (v = velocity and s = substrate concentration). Analysis of Westerns was carried out with a Hoeffer GS-300 Scanning Densitometer.

CHAPTER 4

Part A: Synthesis of $G\alpha_s$

In order to investigate the regulation of ACs by $G\alpha_s$, a mammalian expression system was selected to synthesize $G\alpha_s$. This expression system was selected because $G\alpha_s$ expressed in reticulocyte lysate functions with high potency as compared to bacterial $G\alpha_s$. The rabbit reticulocyte lysate *in vitro* translation system was utilized for its capacity to synthesize foreign proteins with high fidelity particularly posttranslational modifications, ease in optimizing translation efficiency and rapid expression time. The cDNA's encoding the wild type $G\alpha_s$ short form plus serine (wt-$G\alpha_{s+}$) and a mutant form of the same splice variant, $G\alpha_{s\ q227l}$ ($G\alpha_s$*) were the kind gifts of Dr. Juan Codina of Baylor School of Medicine.

A total of four $G\alpha_s$ isoforms have been cloned and sequenced (Bray et al., 1986). The four isoforms arise from alternative splicing of a single gene product (Kozasa et al., 1988). $G\alpha_{s\,1}$ and $G\alpha_{s\,2}$ are longer forms identical in sequence except for an additional serine residue in $G\alpha_{s\,2}$. $G\alpha_{s\,3}$ and $G\alpha_{s\,4}$ are the short forms lacking a 15 amino acid stretch due to the splicing of exon 3.

$G\alpha_{s3}$ and $G\alpha_{s4}$ are identical except for the presence of an additional serine residue near the splice junction in $G\alpha_{s4}$. All four forms have very similar functional properties.

$G\alpha_s$* is functionally similar to the cholera toxin-modified $G\alpha_s$ in that the mutation causes the $G\alpha_s$* molecule to be constituitively active. Glutamine 227 is conserved throughout the GTPase superfamily and resides within the GTP binding pocket. The point mutation of this amino acid results in a phenotype with a significantly reduced K_{cat} for GTP hydrolysis (Graziano and Gilman, 1989, Masters et al., 1989 and Landis et al., 1989). The $G\alpha_s$* form was expressed in rabbit reticulocyte lysates and used for subsequent experiments characterizing the regulation of AC isoforms by $G\alpha_s$.

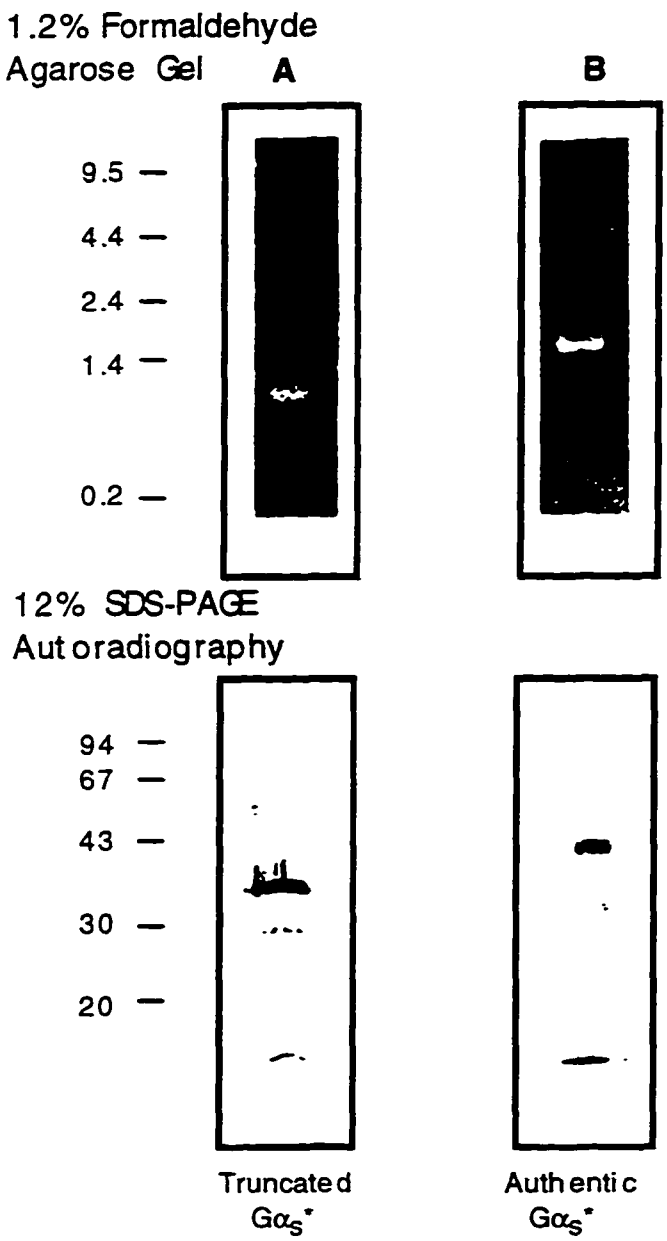
Epicurian Coli XL1-Blue cells were used for expansion of $G\alpha_s$* DNA primarily because a previously used competent cell line, HB101 produced a truncated form of the DNA. The expansion of $G\alpha_s$*-pGEM in competent HB101 bacterial cells resulted in an abnormally truncated DNA sequence and subsequent transcription of the product resulted in an mRNA species of approximately 0.9 kb (see fig. 4-1). When expanded in competent Epicurian Coli XL1-Blue cells and transcribed, the mRNA co-migrated with the 1.4 kb ladder. The translation product exhibited an apparent molecular weight of 45

kDa on a 7% SDS-PAGE, consistent with that published in the literature (Hepler and Gilman, 1992). The protein was recognized by rabbit anti- $G\alpha_s$ antisera, RM/1 in Western analysis. The apparent discrepancy in size was due to an endonuclease system present in many bacterial cells such as HB101, but mutated in XL1 Blue. The mutation (hsdR17) in XL1 Blue cells prevents the cleavage of cloned DNA by the EcoK endonuclease system. Therefore, transformation of these cells ensures expansion of full length, authentic DNA.

Figure 4-1 Comparison of $G\alpha_s$* cDNA in (A) HB101 and (B) XL1 Blue competent cells.

Expansion of $G\alpha_s$*-pGEM in (A) HB101 competent cells results in truncated transcription and translation products. The mRNA synthesized from the $G\alpha_s$*-pGEM construct was electrophoresed on a 1.2% formaldehyde agarose gel. It migrated to the 0.9 kb position. Correspondingly, the [^{35}S]-L-methionine-labeled translation product was electrophoresed on a 12% SDS-PAGE. Visualized by autoradiography, it had an apparent molecular weight of approximately 35 kDa. $G\alpha_s$*-pGEM expanded in XL1-Blue™ competent cells (B) did not result in truncated transcription or translation products. The mRNA co-migrated with the 1.4 bp marker and the protein product exhibited an apparent molecular weight of 45 kDa.

Figure 4-1 Comparison of $G\alpha_s$* cDNA in (A) HB101 and (B) XL1 Blue competent cells.



Transformation of E. Coli XL1-Blue cells was carried out by a heat shock method. 50 µl of cells in a 15 milliliter Falcon 2059 tube, resistant to degradation by β-mercaptoethanol (β-ME) was mixed with a final concentration of 25 mM β-ME. β-ME was utilized to increase transformation efficiency. 1 µl of 0.05 ng/µl $G\alpha_s^*$-pGEM in TE (10 mM Tris-HCl and 1 mM EDTA) buffer pH 8.0 was added to cells and swirled gently, placed on ice for 30 minutes, heat pulsed for 45 seconds in a 42°C water-bath and placed on ice for two minutes. Thereafter, preheated (42°C) Luria Bertani (LB) medium was added and the mixture was incubated at 37°C with 1 hour shaking at 225 rpm. 25 µl was plated on ampicillin containing, 60 µg/ml Petri plates and incubated at 37°C overnight. Several colonies were transferred to 5 ml of LB media containing 60 µg/ml ampicillin in a 50 ml polypropylene tube. The bacteria were grown overnight to late log phase. 500 ml of media containing 60 µg/ml ampicillin in a 2 liter flask was then inoculated. The culture was incubated at 37°C with vigorous shaking at 300 cycles/minute overnight.

Harvest and lysis of 500 ml culture of bacteria was carried out using 250 ml bottles centrifuged at 1000 x g for 15 minutes at 4°C (Sorvall GS3 rotor). The supernatant was discarded. The pellet was resuspended in 100 ml of STE (0.1 M NaCl, 10 mM Tris-HCl pH 8.0, 1mM EDTA pH 8). The suspension was pelleted by centrifugation at 1000 x g and resuspended in 10 ml of 50 mM

glucose, 25 mM Tris-HCl pH 8, 10 mM EDTA pH 8.0 plus 1 ml of a fresh solution of lysozyme (10 mg/ml in 10 mM Tris-HCl pH 8.0). Thereafter, 20 ml of 0.2N NaOH and 1% sodium dodecyl sulfate (SDS) was added and the mixture was gently inverted several times and incubated at room temperature for 5-10 minutes. 15 ml of ice-cold solution containing a final concentration of 3 M K+ ions and 5M OAc- ions (potassium acetate and glacial acetic acid) was centrifuged 1000 x g for 15 minutes at 4°C, allowing the rotor to stop without use of the break. The supernatant was filtered through 4 layers of cheese cloth and 0.6 volume of isopropanol was added, mixed and stored at room temperature for 10 minutes.

Nucleic acids were recovered by centrifugation at 5000 rpm for 15 minutes at room temperature. The supernatant was carefully decanted and the bottle containing the pellet was inverted and allowed to drain. The pellet was rinsed with 70% ethanol, dried at room temperature and resuspended in 3 ml of RNAse (20 mg/ml) and incubated at room temperature for 1 hour. The mixture was deproteinized by extraction with an equal volume of phenol/chloroform until no protein which segregates to the white flocculent interphase was evident. A final extraction with an equal volume of sevage (chloroform: isoamyl alcohol 24:1) was carried out. 300 mM NaOAc and 2.5 volumes of ethanol were added. The nucleic acids were precipitated at -70°C for 1 hour, spun down at 4°C for 30 minutes, rinsed with 70% ethanol, spun for 30 minutes and resuspended in 1 ml of water.

In order to separate the DNA from the RNA, the sample was placed over a column packed with 10 ml of Sepharose™ CL-4B. Gel buffer (10 mM Tris-HCl pH 8, 1 mM EDTA pH 8, 0.1% Sarkosyl, 600 mM NaCl) was added and 200 µl samples were collected. The absorbance at OD 260 was measured. Peak aliquots were run on a 1.2% agarose gel. The pooled DNA fractions were analyzed by restriction digestion (Nco I, Xho I).

Transcription of the $G\alpha_s$* message from the pGEM vector was carried out as follows: All components were brought to room temperature to avoid precipitation of DNA which occurs in the presence of spermidine. 12 µg of $G\alpha_s$*-pGEM linearized by digestion with Xho I was reacted with 320 units of T7 polymerase in the presence of 0.1 mM rNTP's, 40 units of RNAse inhibitor and transcription buffer (40 mM Tris-HCl pH 8, 6 mM $MgCl_2$, 10 mM DTT and 2 mM spermidine). Spermidine is included in the transcription buffer to stimulate T7 RNA polymerase.

Depending on the flanking regions of the expression vector, the translation of different proteins is sensitive to variations in salt concentrations (Jagus, 1987). The most critical component of the reaction is the MgOAc concentration. Translation efficiency was measured by the capability of the $G\alpha_s$ to stimulate AC.

The translation reactions were incubated at 30°C for 90 minutes with 20 μM amino acids (including methionine), translational grade of [^{35}S]-L-methionine, RNAse inhibitor (40 units), 110 mM KCl and 60% lysate in nuclease free water. 2 μg of $G\alpha_s$* mRNA was added per 100 μl of translation reaction mix. The translation product was then washed in 10 mM Tris-HCl pH 8 and 1 mM EDTA. The wash/buffer exchange was carried out using centrifugation in Centricon™-10 tubes three times for one hour each at 10,000 rpm, 4°C. This removed endogenous cofactors such as GTP (0.1 mM), MgOAc (variable) and ATP (0.5 mM) present in the lysate. After the third spin, the retentate was diluted in 25 mM HEPES, 1 mM EDTA and 20 mM β-ME, frozen in a dry-ice acetone bath and stored at -80°C until assayed.

The percentage of labeled methionine incorporated into the translation product was determined by trichloroacetic acid (TCA) precipitation and used to calculate the concentration of $G\alpha_s$* protein formed. To determine the percent incorporation of [^{35}S]-L-methionine, a 2 μl aliquot from the reactions with and without mRNA was added to 98 μl of a solution of 1 N NaOH and 2% H_2O_2 and incubated at 37°C for 10 minutes. 900 μl of ice-cold solution of 25% TCA and 2% casamino acids was added and incubated on ice for 30 minutes. Whatman™ glass fiber filters (GF/A) were soaked with 5% TCA on a

Millipore™ 12-well filtration apparatus. 500 µl of the precipitated protein was collected over vacuum filtration and rinsed 2 times with 1.5 ml of ice-cold 5% TCA. 3 ml of Aquasol™ scintillation fluid was added to a scintillation vial containing a filter, mixed and counted. For total counts, 5 µl of the TCA reaction mix was directly spotted onto the filter. Calculation of the concentration of the $G\alpha_s$* translation product was calculated from the specific activity of methionine and the percent incorporation assuming that all seven methionines in $G\alpha_s$ are equivalently labeled.

Determination of the salt concentrations necessary for optimal translation were based on utilization of the adenylyl cyclase assay. The concentrations of MgOAc and KCl which provided $G\alpha_s$ that gave the greatest levels of AC activity, as measured by [^{32}P]-cAMP formation, were selected for all subsequent translation reactions. Cyc- membranes lacking $G\alpha_s$ were used to measure AC stimulation by the various $G\alpha_s$* translation products. For each batch of lysate, the endogenous levels of MgOAc varied and therefore the optimal MgOAc levels were considered to be the concentration added to the translation reaction plus the endogenous level present in the lysate according to the technical data sheet. The concentration was varied from the indicated endogenous level, usually 1.85 to 4.45 mM. The final concentration of 2.45 mM MgOAc resulted in the greatest fold stimulation (see fig. 4-3).

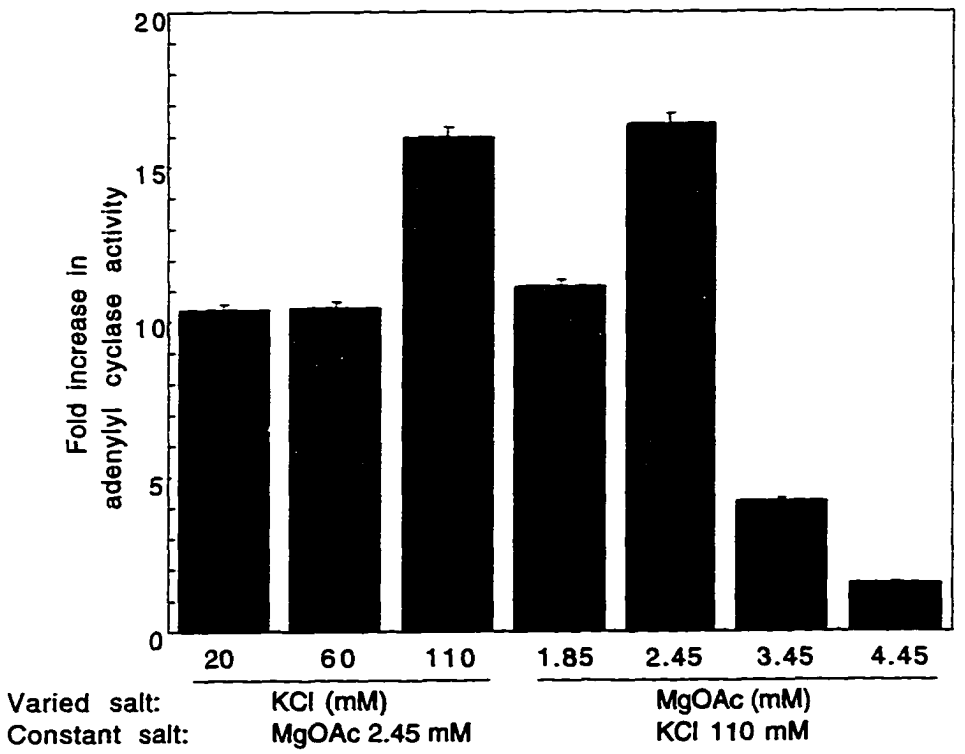
2.45 mM MgOAc resulted in a 16 fold increase in cyclase activity compared to basal. The final concentration of 4.45 mM MgOAc resulted in only a 1 fold increase in activity compared to basal.

The KCl concentration was also varied and the $G\alpha_s$ -stimulated AC activity in Cyc- membranes was measured. Originally, KOAc was utilized instead of KCl as Cl- ions were shown to have an inhibitory effect on the initiation of translation (Jackson and Hunt., 1983). However, in later studies, KCl was shown to enhance translational efficiency (Beckler, 1995). The KCl concentration was varied from 20 mM to 110 mM. The optimal efficiency of translation was obtained at a KCl concentration of 110 mM which is consistent with the translational efficiency of other constructs containing a 5' untranslated region such as that from the alfalfa mosaic virus included in the vector, pGEM.

Figure 4-2 Effect of varing MgOAc and KCl concentrations for optimal expression and activity of $G\alpha_s$*.

10 µg of Cyc- membranes in each tube were assayed with $G\alpha_s$* expressed in the presence of indicated salt concentrations. The endogenous level of MgOAc varies in the lysate mixture. The technical data sheet included with each batch of rabbit reticulocyte lysate provides the concentration of MgOAc for each batch of lysate. The level of MgOAc and KCl providing the greatest efficiency in translation is 2.45 mM and 110 mM respectively.

Figure 4-2 Effect of varing MgOAc and KCl concentrations for optimal expression and activity of $G\alpha_s^*$.

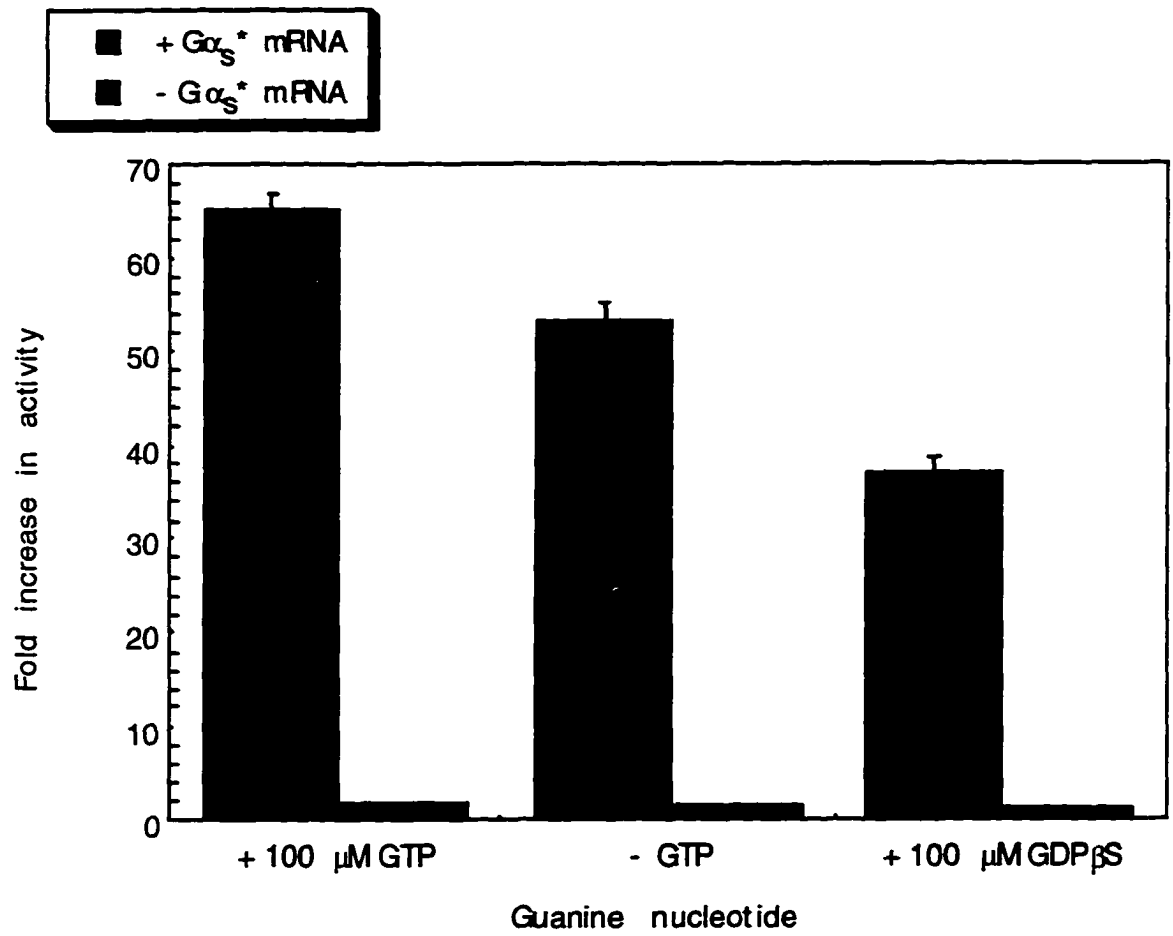


Consistent with the phenotype of $G\alpha_s$* found in pituitary tumors (Landis et al., 1989), the $G\alpha_s$* prepared in rabbit reticulocyte lysate is constituitively active. The translation product is presumably preactivated during synthesis in the presence of GTP in the lysate translation mix. The translation product was washed at first five times and in subsequent experiments three times over a Centricon™-10 to remove the endogenous GTP, ATP and MgOAc so as not to contribute to artifactual stimulation of $G\alpha_s$*. The extensive washing did not deactivate $G\alpha_s$* (see fig. 4-4). Activation of AC in Cyc- membranes by 1 nM $G\alpha_s$* resulted in a 53± 2.0 fold increase in activity over basal without the addition of guanine nucleotides (see fig. 4-4). Addition of 100 μM GTP resulted in a 64.3 ±1.59 fold increase in activity which was partially reversed by 100 μM GDPβS.

Figure. 4-3 Stimulation of Cyc- membrane adenylyl cyclases by $G\alpha_s$* in the presence and absence of guanine nucleotide

The $G\alpha_s$* translation product was diluted 25 fold and washed over a centricon-10 three times to minimize the amount of endogenous GTP, ATP and MgOAc. With each wash the $G\alpha_s$* was diluted 25 fold and regulatory factors necessary for translation were collected in the flow through filtrate. Activation of AC in Cyc- membranes (10 μg) by 1 nM $G\alpha_s$*. $G\alpha_s$* stimulation resulted in a 53 fold increase in activity without additional GTP added. In the presence of 100 μM GTP, the fold increase in activity was 65 and that in the presence of GDPβS was reduced to 36. Values are means ± SD (standard deviation) of triplicate determinations and calculated as ratios of activity in the absence and presence of reticulocyte lysates. This data suggests the extensive washing did not deactivate $G\alpha_s$* significantly. All subsequent assays included 100 μM GTP in the assay mixture.

Figure. 4-3 Stimulation of Cyc- membrane adenylyl cyclases by $G\alpha_s$* in the presence and absence of guanine nucleotide

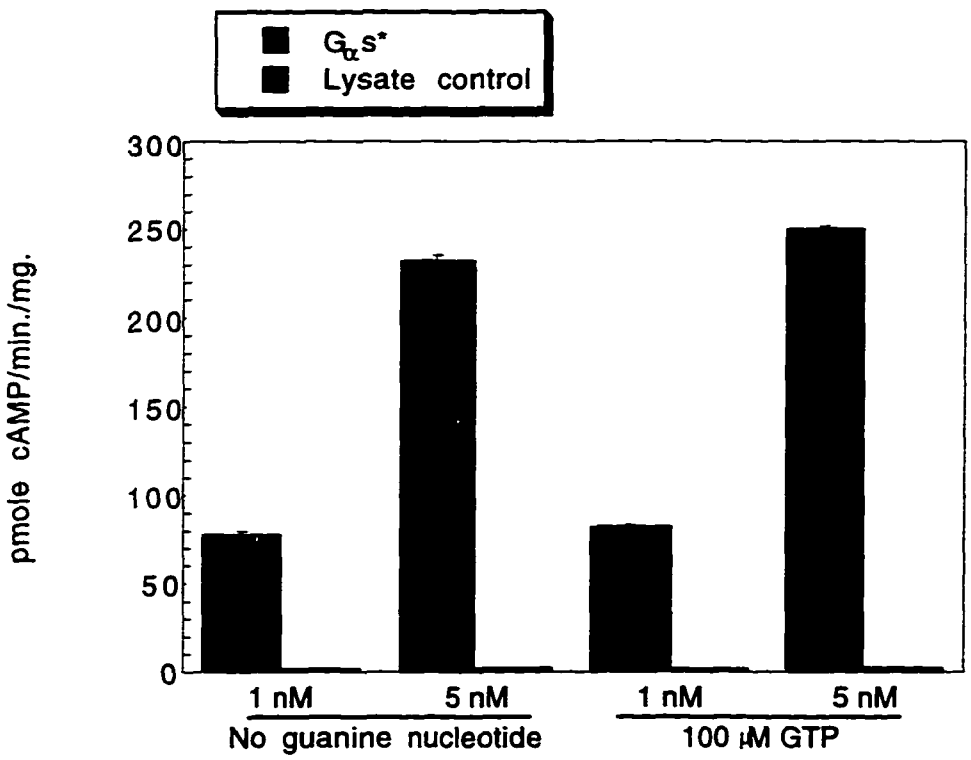


The establishment of the optimal conditions for the synthesis of $G\alpha_s$* allowed for the investigation of the concentration-dependent nature by which AC is activated by $G\alpha_s$*. Stimulation of 10 µg Cyc- membranes was assayed again in the presence and absence of 100 µM GTP with 1 and 5 nM $G\alpha_s$* (see fig. 4-5). The degree of stimulation was dependent upon the concentration of $G\alpha_s$* added. At 1 nM $G\alpha_s$*, the specific activity was measured as 78 pmol cAMP/min/mg. At 5 nM $G\alpha_s$* the specific activity was 240 pmol cAMP/min/mg. The control condition was lysate prepared in the same manner however, no $G\alpha_s$* mRNA was added. No significant stimulation was observed.

Figure 4-4 Adenylyl cyclase activity in Cyc- membranes in the presence of 1 or 5 nM $G\alpha_s$* with and without 100 μM GTP

Adenylyl cyclase activity in Cyc- membranes was measured in the presence of 1 or 5 nM $G\alpha_s$* with and without 100 μM GTP. The dark hatched bars represents adenylyl cyclase activation by $G\alpha_s$* in the absence of GTP added to the assay, the light hatched bars represents activation by $G\alpha_s$* in the presence of 100 μM GTP. The dark solid bars represents activation by the lysate control where no $G\alpha_s$* mRNA was added to the translation reaction.

Figure 4-4 Adenylyl cyclase activity in Cyc- membranes in the presence of 1 or 5 nM $G\alpha_s$* with and without 100 μM GTP

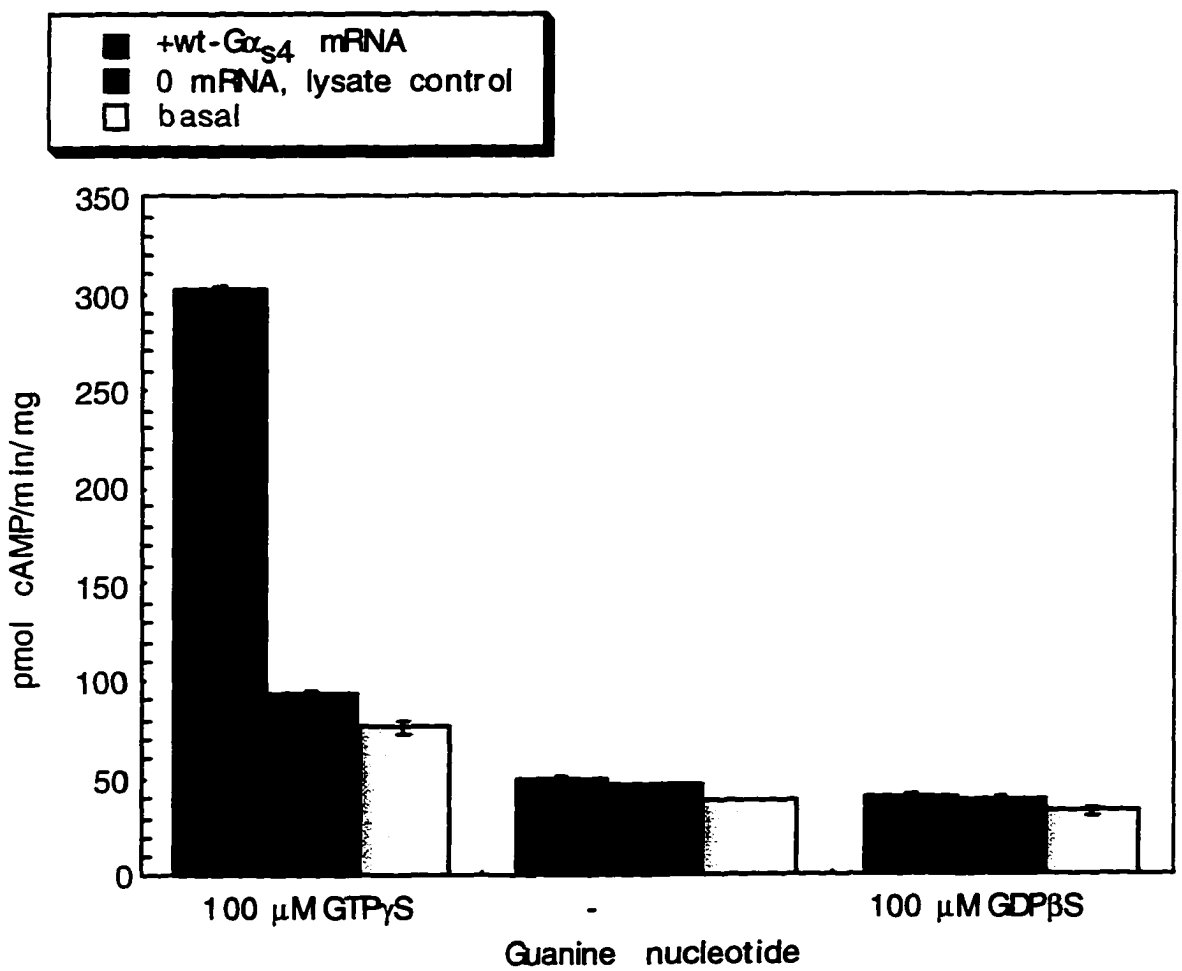


For several experiments carried out in this project, wt-$G\alpha_{s4}$ was utilized to stimulate AC. wt-$G\alpha_{s4}$ was expressed under the same conditions as that for $G\alpha_s$*. Instead of Cyc- membranes, AC2 membranes were assayed for stimulation to confirm the expression of active protein. AC 2 membranes were prepared from pVL1392-AC2 baculovirus infected insect cells and assayed for stimulation by wt-$G\alpha_{s4}$ in the presence and absence of either GTPγS or GDPβS. In the case of regulation by wt-$G\alpha_{s4}$, a nonhydrolyzable guanine nucleotide was necessary to see stimulation. Without the addition of nonhydrolyzable guanine nucleotide analogues, the level of stimulation of AC2 (5 μg) by 3 nM wt-$G\alpha_{s4}$ was measured at 50.6 ±1.43 pmol cAMP/min/mg (see fig. 4-6). In the presence of 100 μM GTPγS stimulation was measured at 302 ±1.46 pmol cAMP/min/mg and in the presence of 100 μM GDPβS, wt-$G\alpha_{s4}$ stimulation of AC2 was measured at 40.28 ± 0.76 pmol cAMP/min/mg.

Figure 4-5 Regulation of AC type 2 by wt-$G\alpha_{s4}$ expressed in rabbit reticulocyte lysate.

5 μg AC2 membranes were assayed for stimulation by 3 nM wt-$G\alpha_{s4}$ and the indicated guanine nucleotide in the assay. The dark hatched bars represents stimulation by wt-$G\alpha_{s4}$, the solid bars represents lysate control and the light hatched bars represents basal activity.

Figure 4-5 Regulation of AC type 2 by wt-$G\alpha_{s4}$ expressed in rabbit reticulocyte lysate.



Part B: **Subcloning & expression of Adenylyl Cyclase type 1**

Construction of pVL1392-AC1-FLAG

AC1 was cloned from bovine brain cDNA library into pBluescript II (KS) phagemid by Richard Premont in our laboratory. The nucleic acid sequence in the 5′ region was changed to reduce the number of GC's without changing the amino acid sequence by Ofer Jacobowitz in our laboratory. A Flag marker 8 amino acid peptide, N-Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys-C (Kodak International Biotechnologies™, Inc.) which includes the M1 antibody binding site and enterokinase cleavage site was incorporated into the 5' region for affinity purification if necessary.

A 5' 120 base pair primer (OJ050) of AC1 and Flag epitope was designed containing Hind III and Bgl II restriction sites and an internal 3′ antisense primer (OJO51), containing the Eco RI restriction site. Both primers were incorporated into the 5' region of pBS(KS)-5′AC1 by PCR mutagenesis (see fig. 4-7). The template 5′AC1 DNA (30 ng) was denatured at 94°C, 7 minutes followed by 30 cycles of 94°C for 30 seconds, 66°C for 5 minutes, 72°C for 3 minutes in a reaction volume of 100 µl containing 600 ng of each primer and 10 mM dNTP, PFU #1 buffer and PFU polymerase (Stratagene™). The 173 base pair PCR product was purified from 2% low melting agarose (Biogel™)

using Gene Clean II™ with TBE reducing the pH to approximately 6.5 which enables small DNA to bind better to glass milk. 5'AC1 was excised from pBS(KS)-5'AC1 with Hind III and XbaI. Both PCR product and excised 5'AC1 were cut with Eco RI and Hind III and ligated with 2 units of T4 DNA ligase at 4°C overnight.

The recombinant pBS(KS)-5'AC1-FLAG DNA was used to transform XL1 Blue cells with 25 mM β-ME by heat pulse to 42°C for 45 seconds. Colonies were screened and positive clones were sequenced by the dideoxy method. AC1-Flag construct was excised using Bgl II and Xba I restriction enzymes and ligated into those sites in the pVL1392 transfer vector. Transformation was repeated, colonies were screened and first 200 bp were sequenced.

Oligomers:

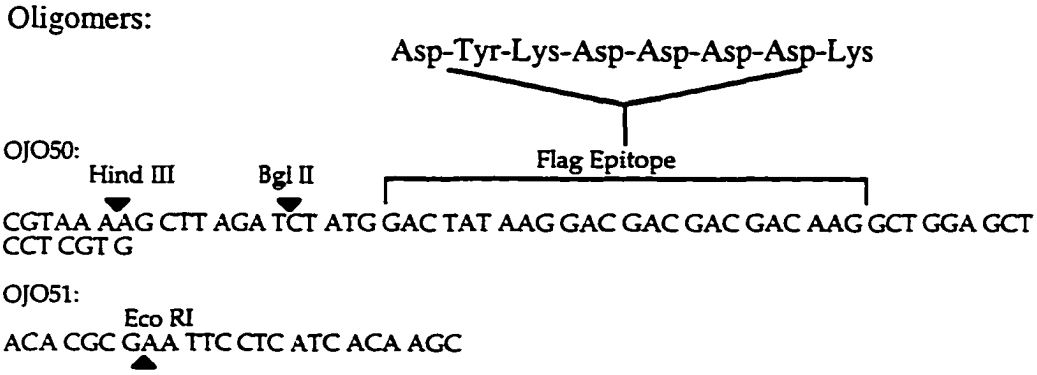
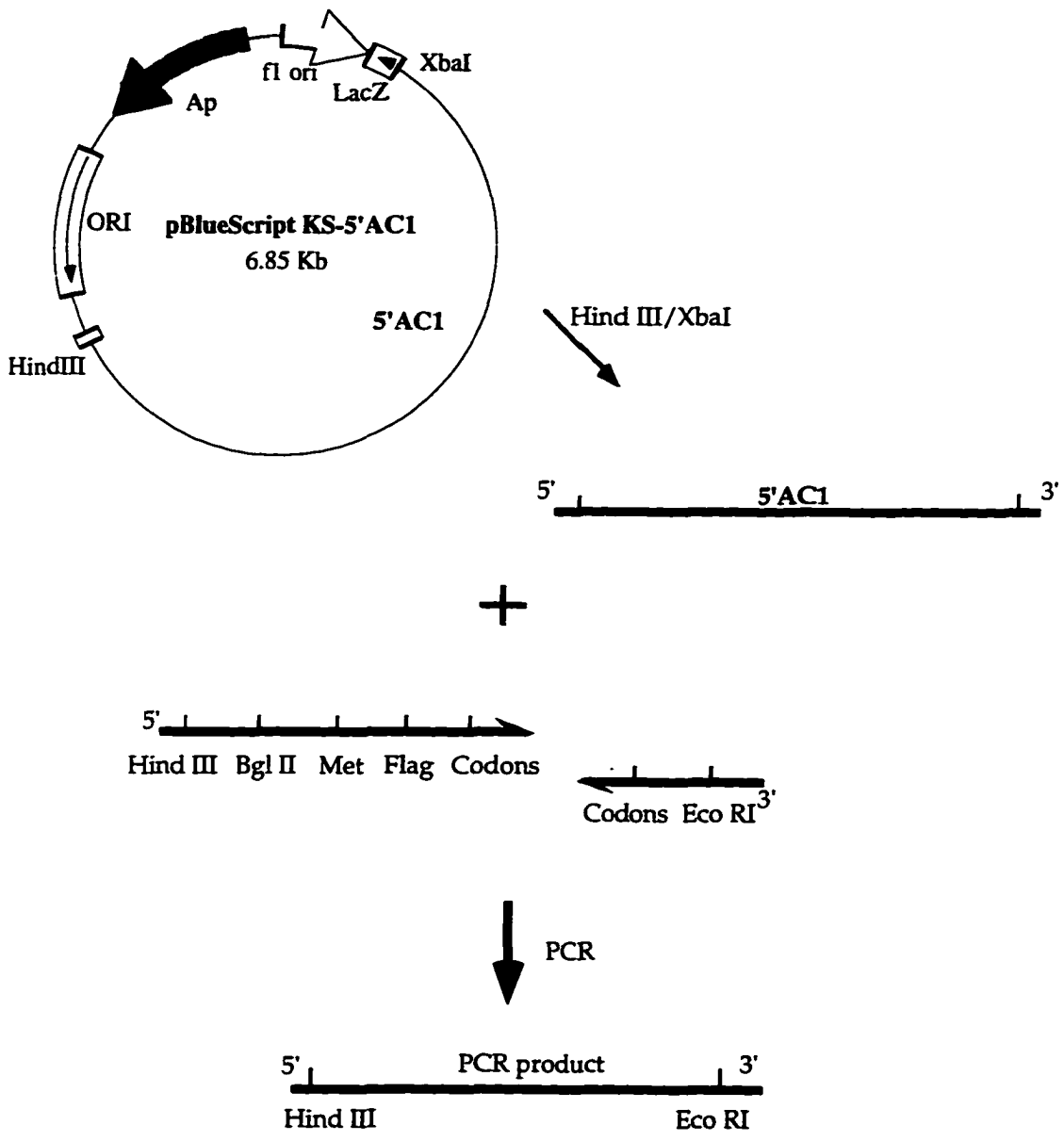


Figure 4-6 A schematic of the AC1 subcloning strategy

Flow chart of the stages in construction of recombinant pVL1392-5'AC1-Flag baculovirus expression vector.

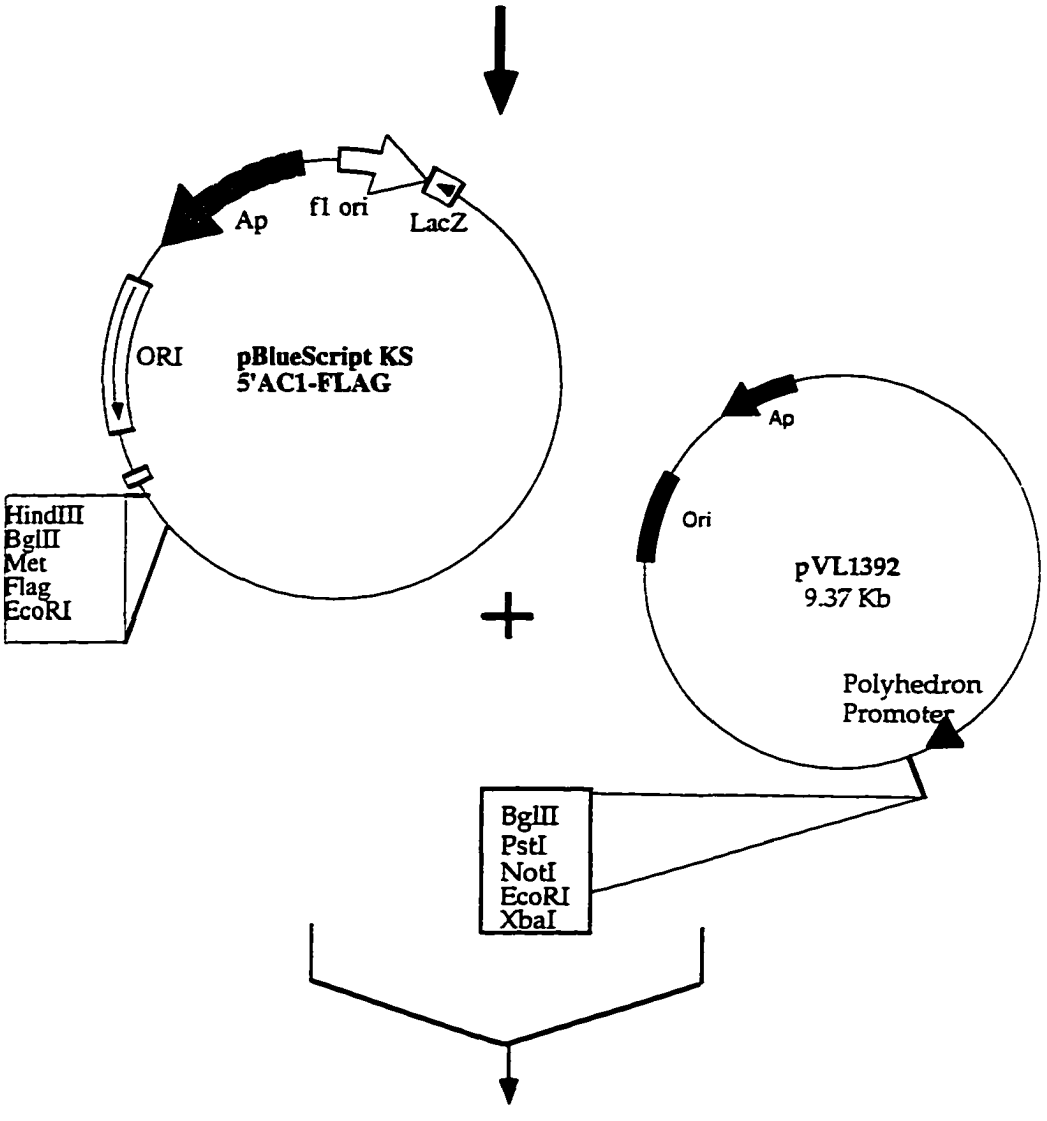
Figure 4-6 A schematic of the AC1 subcloning strategy

Step 1: Excise 5'AC1 from pBS(KS)-5'AC1, then amplify 5' 120 bp of AC1 with Flag epitope primers by PCR.



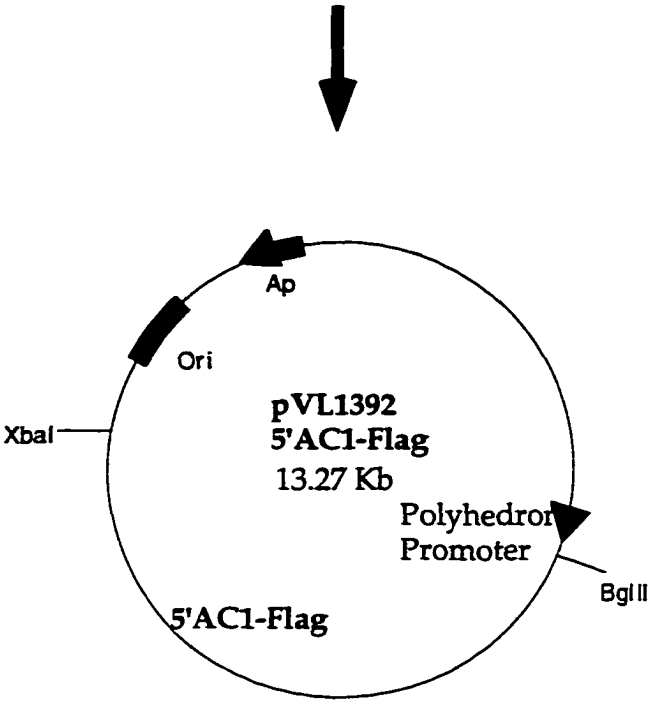
Step 2: Cut PCR product and pBS(KS)-5'AC1 using Hind III and Eco RI restriction enzymes.

Step 3: Ligate linear digest product, pBS(KS)-5'AC1 and PCR product, transform XL1 Blue cells, screen and sequence first 200 bp.



Step 4: Cut recombinant pBS(KS)-5'AC1-Flag and baculovirus transfer vector, pVL1392 with Bgl II and Xba I restriction enzymes.

Step 5: Ligate pBS(KS)-5'AC1-Flag and pVL1392, transform, screen and sequence.

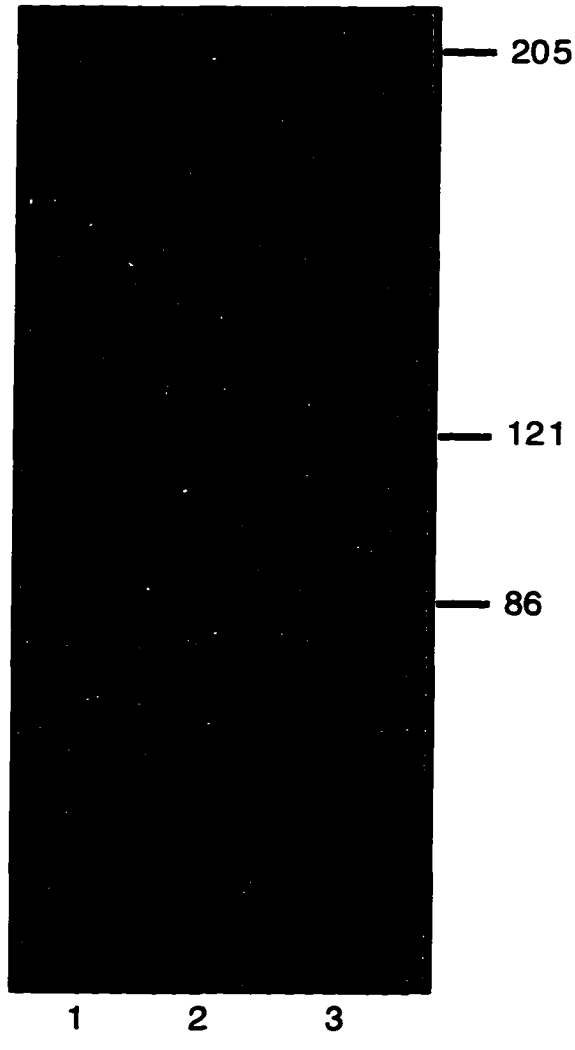


The BaculoGold™ DNA (baculovirus, AcMNPV) is designed with a lethal deletion in the neighborhood of the polyhedrin locus such that only cotransfection with a helper plasmid such as pVL1392 which complements this sequence can restore virulence. Recombinant pVL1392-5'-AC1-Flag is cotransfected with BaculoGold™ DNA into Sf9 insect cells. 3 - 4 days later viral plaques (Occ^-) are visible. Recombinant viral plaques are selected and used to infect Sf9 cells. 48 hours post infection, the cells are harvested and crude membranes were prepared. Western analysis using the AC comm antisera resulted in a signal in the lane containing membranes of recombinant pVL1392-AC1 baculovirus-infected insect cells and not in the TPO control. The AC comm antisera was generated from rabbits immunized with the peptide NH_2-IGARKPQYDIWGNT-COOH-linked keyhole hemocyanin, a sequence at the carboxy terminus common to AC types 1-8. Optimal detection was achieved at a 1:1000 dilution of the antisera (see fig 4-7). All subsequent membrane preparations containing recombinant Flag-AC1 were prepared from insect cells infected with this recombinant Flag-AC1 baculovirus. The membrane preparations were used for characterization of the regulatory properties by $G\alpha_s$.

Figure 4-7 Immunoblotting of Sf9 cell membranes infected with AC1-recombinant baculovirus

Analysis of recombinant AC1 expressed in insect cells. 8 μg of protein was loaded into each lane. AC comm antiserum (1:1000) recognized the AC1 band (lane 1) but did not recognize protein prepared from recombinant TPO baculovirus infected insect cells (lane 2). The AC comm peptide was incubated with antiserum blocking the signal in lane 3.

Figure 4-7 Immunoblotting of Sf9 cell membranes infected with AC1-recombinant baculovirus



CHAPTER 5

Adenylyl Cyclase type 6 was expressed in recombinant baculovirus-infected insect cells and crude membranes were prepared. I characterized the regulatory effects of varying concentrations of $G\alpha_s$* on AC6. The assays carried out measured the [^{32}P]-cAMP formed from [α-^{32}P]-ATP both in the absence and presence of an additional regulatory molecule, forskolin.

5.1 Construction of recombinant baculovirus vector for insect cell infection

The AC6 isoform was expressed in insect Sf9 cells. AC6 cDNA was ligated into the appropriate restriction sites in the pVL1393 transfer vector by Jianqiang Chen in our laboratory. The pVL1393-AC6 and the baculovirus Autographa californica nuclear polyhedrosis virus (AcMNPV) DNA (BaculoGold™ DNA, Invitrogen) were cotransfected into Sf9 cells. Recombinant viral plaques were selected and screened for the presence of AC6 DNA by hybridization by the Baculovirus Core Facility at the Mount Sinai School of Medicine.

The advantage of baculoviruses as expression vectors is the presence of the strong polyhedrin which directs the expression of high levels of a foreign gene. The AcMNPV DNA was engineered with a lethal deletion flanking the polyhedrin promoter locus. Therefore, a helper plasmid such as pVL1393 containing the intact promoter sequence is necessary for recombinant gene expression. In addition, insertion of a foreign gene, in this case AC6 cDNA, into the polyhedrin promoter (polh) region of pVL1393 causes the formation of recombinant viruses with an occlusion minus (occ-) phenotype under the control of the late polh promoter. The resulting construct, pVL1393-AC6 when cotransfected with AcMNPV DNA restores the virulence of AcMNPV

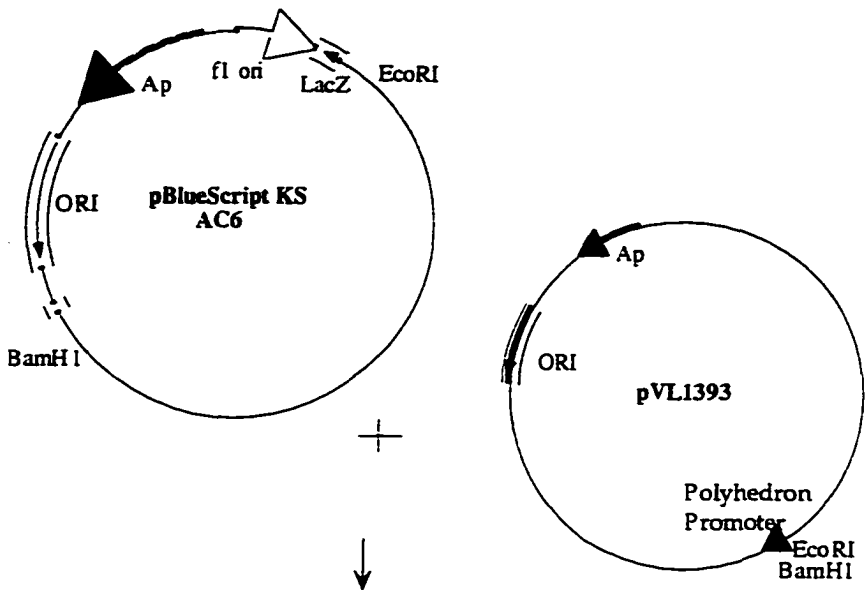
in insect cells and at the same time allows for visual selection of the recombinant viral plaques expressed as occ- forms.

The construction of pVL1393-AC6 is shown in Fig. 5-1. AC6 was excised from pBSII-AC6 and ligated into the multiple cloning site of pVL1393. Recombinant vector was digested with restriction enzymes and limited sequencing was carried out for confirmation of proper construct and orientation. The Baculovirus Core Facility performed the cotransfection with AcMNPV into Sf9 cells. Viral plaques were selected, checked for AC6 insert, plaque purified a minimum of two rounds and used to infect Sf9 cells.

Figure 5-1 Construction of pVL1393-AC6

Flow chart of stages in the construction of recombinant pVL1393-AC6 baculovirus expression vector.

Figure 5-1. Construction of pVL1393-AC6



Clone AC6 cDNA into pVL1393 Transfer Plasmid

Prepare pVL1393-AC6 Plasmid DNA

Baculovirus Core Facility:

Cotransfect AcMNPV & pVL1393-AC6

Screen for Recombinant Viruses

Plaque Purify Selected Recombinants

Amplify Selected Recombinants

Confirm Identity of Recombinant

5.2 Expression of Adenylyl Cyclases in Sf9 Cells

The Baculovirus Expression Vector System was selected for the expression of the adenylyl cyclase isoforms for its ability to produce high levels of heterologous nonfusion foreign proteins in a eukaryotic environment. Investigators have utilized this method successfully for the expression of other mammalian intrinsic membrane proteins. Sf9 cells can be cultured easily at temperatures between 25-30°C without additional CO_2 and can be grown as large scale suspension cultures.

Sf9 cells were grown in serum free insect media to log phase at which point, they were infected with recombinant baculovirus at a multiplicity of 5-10. The budded form of the virus enters the insect cells through either adsorptive endocytosis or fusion. Inside the cells, the nucleocapsids gain entry into the nucleus by interaction with nuclear pores. Capsid uncoating occurs once in the nucleus, initiating the early phase of infection characterized by cytoskeletal arrangements. The late phase is characterized by viral DNA replication, late gene expression and production of the budded form of the virus. Finally, the very late phase also known as the occlusion-specific phase is characterized by *de novo* membrane synthesis with subsequent enveloping of nucleocapsids and packaging into occlusion bodies. Accumulation of occlusion bodies in the nucleus eventually leads to cell lysis.

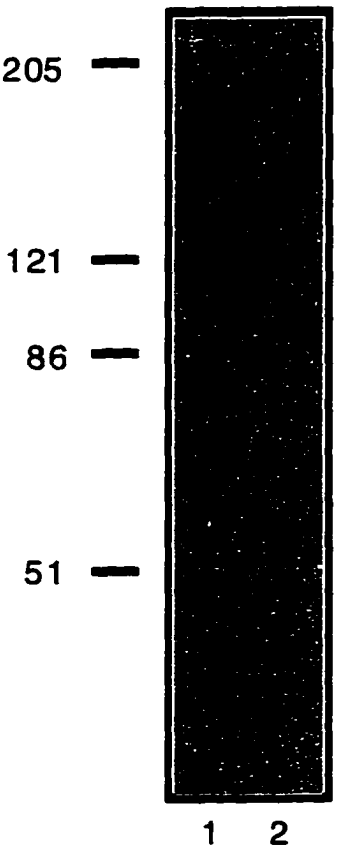
The recombinant baculovirus-infected cells were harvested 48 hours post infection and crude membranes were prepared. Western analysis was used to confirm the identity of AC6 using antiserum specific for a conserved carboxy terminus sequence common to the adenylyl cyclase isoforms (Fig. 5-2). A band was observed in the lane containing membranes prepared from recombinant AC6 baculovirus infected insect cells and not in the lane loaded with membranes prepared from the control, TPO baculovirus infected insect cells.

Preliminary studies indicated that AC6 activity was not linear beyond 15-20 minutes, subsequently all assays were carried out under 15 minutes. The original membrane preparation included cell lysis by homogenization using a Teflon pestle tissue homogenizer with 20 strokes in ice cold membrane buffer. All other conditions were the same as described in Methods. However, the responsiveness to increasing concentrations of magnesium was not concentration dependent but rather, decreased with increasing concentrations of magnesium (Fig. 5-3). Baculovirus-infected cells were instead lysed by nitrogen cavitation using a Parr bomb with all other buffers and conditions the same as described in Methods. This change resulted in a membrane preparation in which the adenylyl cyclase activity was responsive to the concentration dependent effects of magnesium (Fig. 5-4). This method for cell lysis was used in all subsequent enzyme assays.

Figure 5-2 Immunoblotting of Sf9 cell membranes infected with AC6 - recombinant baculovirus

Analysis of recombinant AC6 expressed in insect cells. 2.5 μg of protein were electrophoresed through a 7% SDS-PAGE, transferred to nitrocellulose and probed with AC comm antiserum diluted at 1:1000. Lane 1, membranes prepared from recombinant AC6 baculovirus infected insect cells. Lane 2, membranes prepared from recombinant TPO baculovirus infected insect cells.

Figure 5-2 Immunoblotting of Sf9 cell membranes infected with AC6 - recombinant baculovirus



In further experiments, the activities of AC6 and AC2 expressed in Sf9 cells were compared using membranes prepared after cell lysis in a glass/Teflon homogenizer see Fig. 5-3.

Since this method of membrane preparation did not demonstrate the expected adenylyl cyclase activity proportional to added Mg^{2+}, I tested another method of membrane preparation. In this method, the cells were lysed by nitrogen cavitation in a Parr bomb (Fig. 5-4).

Figure 5-3 Magnesium responsiveness of AC2 and AC6 membranes prepared from cells lysed by homogenization

Data are from a single experiment. Membranes were prepared from AC2 and AC6-baculovirus infected Sf9 insect cells The method for cell lysis was homogenization using a Teflon pestle tissue homogenizer with 20 strokes in ice cold membrane buffer. All other conditions were the same as described in Methods.

Figure 5-3 Magnesium responsiveness of AC2 and AC6 membranes prepared from cells lysed by homogenization

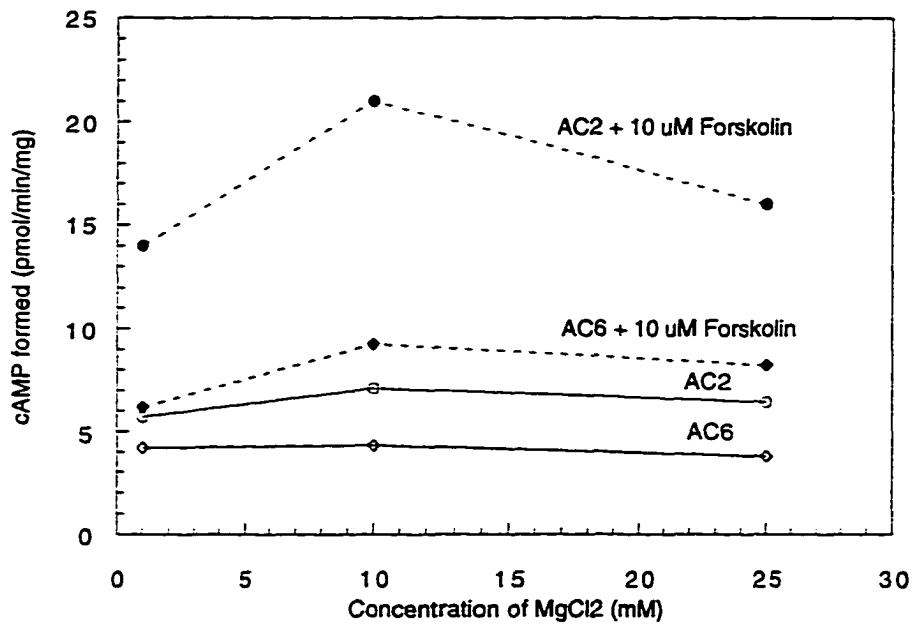
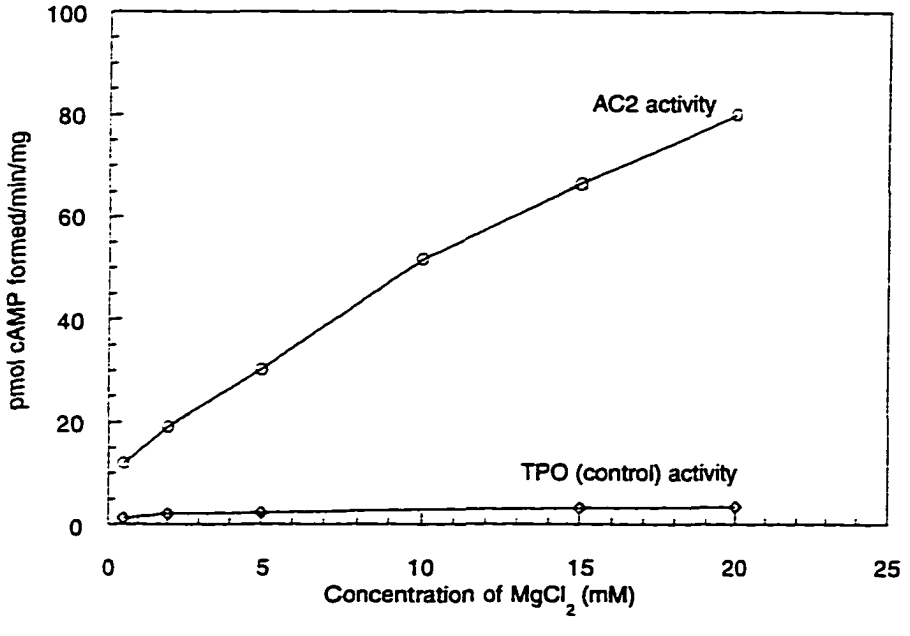


Figure 5-4 Magnesium responsiveness of AC2 membranes prepared from cells lysed by Parr bomb nitrogen cavitation

Data are from one experiment. Recombinant AC2 baculovirus-infected cells were lysed by nitrogen cavitation using a Parr bomb with all buffers and conditions the same as described in Methods. Concentration dependent effects to magnesium were observed. This method of cell lysis was used for all adenylyl cyclase isoforms prepared in subsequent experiments.

Figure 5-4 Magnesium responsiveness of AC2 membranes prepared from cells lysed by Parr bomb nitrogen cavitation



Under appropriate assay conditions, basal activity of AC6 was 18.45 ± 4.57 pmol cAMP/min/mg (n = 10). Basal activity of control membranes with recombinant thyroid peroxidase (TPO) was 6.40 ± 1.46 pmol cAMP/min/mg (n = 10). Diagnostic assays confirming a functionally intact and responsive AC6 enzyme were carried out in the presence or absence of forskolin. Stimulation by this regulatory molecule was significant and reproducible. The intrinsic activity of 100 μM forskolin-stimulated AC6 averaged 73.42 ± 21.06 pmol cAMP/min/mg (n = 6). The intrinsic activity of 100 μM forskolin stimulated control, TPO averaged 15.6 ± 5.86 pmol cAMP/min/mg (n = 6).

5.3 General Properties of the Response to $G\alpha_s$*

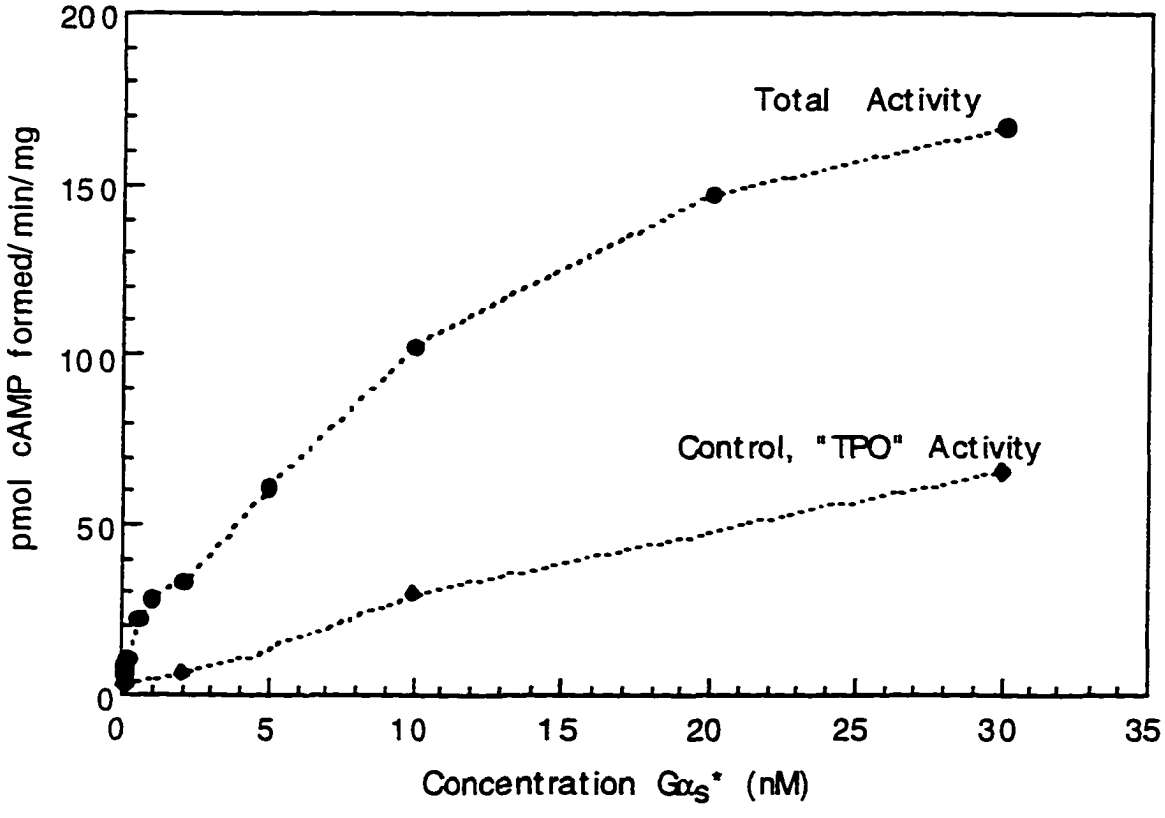
Optimization of the expression of mutant $G\alpha_s$* Q227L was carried out *in vitro* in the Flexi Rabbit Reticulocyte Lysate system. Assays measuring the $G\alpha_s$* stimulation of AC6 included samples with membranes from control thyroid peroxidase baculovirus (TPO) infected cells. Because the basal activity of AC6 was low relative to the other isoforms tested, the endogenous cyclase activity in insect cells accounted for 20-35% of the measured activity. The observed $G\alpha_s$* regulated AC6 activity was taken as the difference between the

activity measured from AC6 infected membranes and the activity measured from TPO-infected membranes (Fig. 5-5).

Figure 5-5 Effect of varying concentrations of $G\alpha_s$* on adenylyl cyclase activity in membranes from control (TPO) virus infected and AC6 virus infected cells

The concentration range of $G\alpha_s$* was 0.05 nM to 30 nM. Membranes infected with pVL1393-AC6 recombinant baculovirus and control, TPO recombinant baculovirus were prepared at the same time under the same conditions as described in Methods. For each $G\alpha_s$* concentration point, AC6 (total) membrane and TPO (control) membrane activity were measured. The endogenous adenylyl cyclase activity, measured as TPO membrane activity averaged 20-35% of total cyclase activity. The observed AC6 activity was taken as the difference between AC6 membrane (total activity) and TPO (control) membrane activity.

Figure 5-5 Effect of varying concentrations of $G\alpha_s$* on adenylyl cyclase activity in membranes from control (TPO) virus infected and AC6 virus infected cells

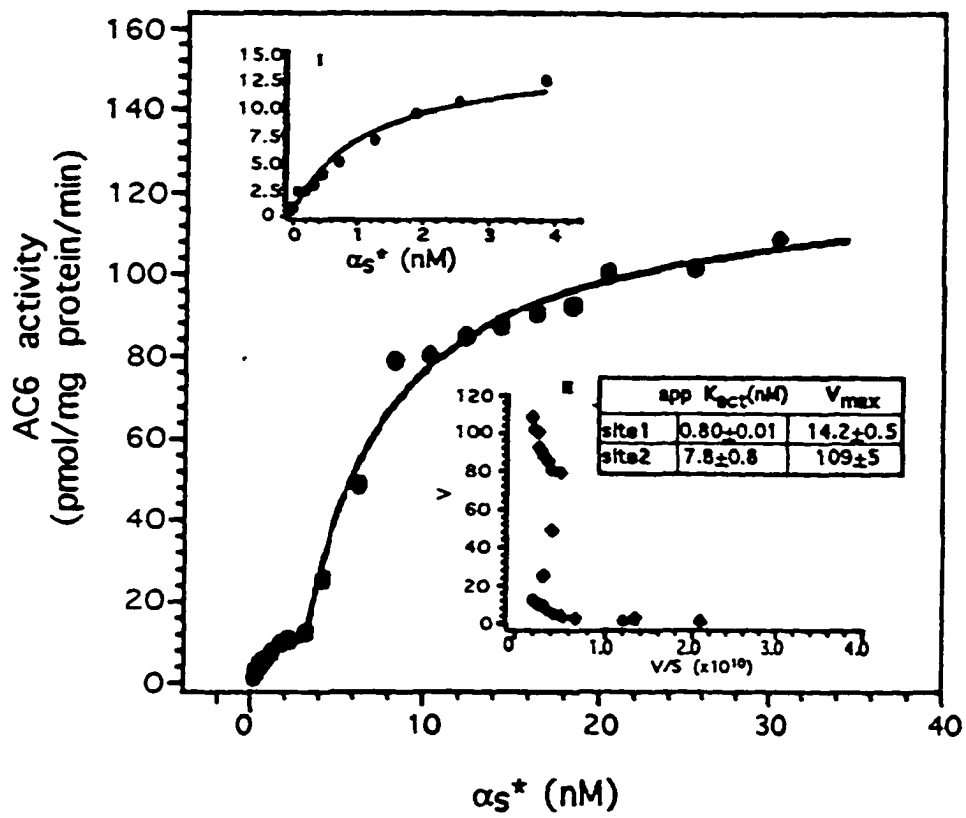


A detailed analysis with at least 24 data points of $G\alpha_s$* stimulation of membranes from recombinant AC6 and TPO baculovirus-infected insect cells was then performed. The resulting data was fit using the Prophet program. This is shown in Figure 5-6

Figure 5-6 The effect of varying concentrations of G α_s* on AC6 activity

The data represents one experiment. Profile of AC6 stimulation by varying concentrations of $G\alpha_s$*. 5 µg of protein was assayed in each tube. $G\alpha_s$* concentration typically ranged from 0.025 nM to 35 nM. Each point represents the "observed AC6" activity obtained by subtracting control activity from total activity. The line through the points was obtained by curve fitting with Prophet. The Prophet program provided the following apparent parameter estimates: high affinity site apparent $K_{act} = 0.8 \pm 0.01$ nM, low affinity site $K_{act} = 7.8 \pm 0.80$ nM, high affinity $V_{max} = 14.2 \pm 0.5$ pmol cAMP/min/mg protein and low affinity $V_{max} = 109 \pm 5$ pmol cAMP/min/mg protein. Inset I is a magnified plot of the data at lower concentrations of $G\alpha_s$*. Inset II shows the Hofstee linear transformation of the data.

Figure 5-6 Stimulation of adenylyl cyclase type 6 activity by varying concentrations of $G\alpha_s$*



$G\alpha_s$* stimulation of AC6 was concentration dependent. Assays measuring the stimulation of AC6 by $G\alpha_s$* generated a distinctly biphasic concentration-effect curve (Fig. 5-6). On average, the first component of the curve approached a plateau at $G\alpha_s$* concentrations between 3-8 nM and on average represented 13-24% (n = 4) of the maximal response. A magnified plot of the first component of the curve is shown in inset I. Higher concentrations of $G\alpha_s$* elicited a further increase in the rate of [^{32}P]-cAMP formed.

Hofstee linear transformation of the data yielded a curvilinear profile (Fig. 5-6 inset II). Linear transformations of data that characterize *[S]/v* vs. *[S]* and *v/[S]* vs. *v*, weight points at high and low *v* or high and low *[S]* more equally than the *1/v* vs. *1/[S]* plots and are therefore more useful in detecting the presence of complex enzyme kinetics (*S* = substrate concentration, *v* = velocity) (Segel, 1975). The data was best fit to a modified two site model. At low concentrations of $G\alpha_s$*, the data were fit to the function represented in equation 1 since only one (the "high affinity") site would be effective at low concentrations of $G\alpha_s$*. The second site was observable only at concentrations of $G\alpha_s$* above a certain threshold value. In the experiment shown in Fig. 5-6, the threshold value was equal to 3 nM. AC6 activities above 3 nM $G\alpha_s$* are

the summation of interactions at both sites. The data points above the threshold concentration were then fit to a modified two site model described by the function represented in equation 1 for $0 \leq A \leq A_{th}$ and to equation 2 for $A \geq A_{th}$ where A_{th} represents the threshold concentration for $G\alpha_s^*$ and $V_{max2} = {}_{Vmax}(obs)-V_{max1}$. The threshold concentration was estimated by initial visual inspection of the $G\alpha_s^*$ concentration response curve followed by an iterative fitting process to obtain a threshold value that generated a curve that best fit the data points. The apparent K_{act} for the "high affinity" site ranged 0.2 - 0.8 nM (n = 4). The apparent K_{act} for the "low affinity" site ranged 15 - 25 nM (n = 4). Maximal stimulation by $G\alpha_s^*$ at the "high affinity" site ranged 14 - 25 pmol cAMP/min/mg protein (n = 4) and at the "low affinity" site ranged 109 - 127 pmol cAMP/min/mg protein (n = 4).

Equation 1:

$$v = \frac{V_{max1} * A}{K_{act1} + A}$$

Equation 2:

$$v = \frac{V_{max1} * A}{K_{act1} + A} + \frac{V_{max2} * (A - A_{th})}{K_{act2} + (A - A_{th})}$$

The basic kinetic parameters are: v = velocity, V_{max} = maximum velocity, K_{act} = the concentration of the activator at half V_{max} and A represents the concentration of the activator. In order to distinguish between proposed

models for fitting the observed data it was necessary to obtain a quantitative estimate of goodness of fit. The best statistical test to provide an objective assessment for testing the quality of fit is the F-test shown in equation 3 (Crabbe, 1985 and references within).

Equation 3:

$$F = \frac{(R_2 - R_1)(n - p_1)}{R_1(p_1 - p_2)}$$

R_1 and p_1 are the sum of squares of the residuals and the number of parameters associated with the more complex model, R_2 and p_2 are the parameters for the simpler model, and n is the total number of data points. Two additional parameters were used to evaluate the model that best fit the data, these included p values and residual mean squares (rms). The statistical parameters showing the improvement of fit by the two site interacting model over the two site noninteracting or single site models are provided in table 5-1. Plots of the data points and the fitted curves were generated by Prophet. The printed plots were exported to the Canvas program in a Mac 8100 by use of an optical scanner. The plots were labeled and assembled within Canvas. The final plots as shown were printed as Canvas files.

These data led to the hypothesis that $G\alpha_s$* regulation of AC6 involved multiple sites of interactions. I was therefore interested in ascertaining if

these sites could be subjected to concomitant regulation or if they were interconvertible.

Stimulation of Acs by forskolin and $G\alpha_s$ is known to occur in a synergistic fashion. Hence, the regulation of AC6 by $G\alpha_s$* was measured in the presence of forskolin. An experiment characterizing the stimulation of AC6 by $G\alpha_s$* both in the absence (Fig. 5-7 top panel) and in the presence (Fig. 5-7 bottom panel) of 100 μM forskolin carried out at the same time is shown. The addition of forskolin generated a steep concentration response curve. Hofstee linear transformation of the data yielded a straight line indicating that the data could be fit to a one site Michaelis-Menten model. The fit for the data in the absence of forskolin provided the following apparent parameter estimates: high affinity site apparent $K_{act} = 0.8 \pm 0.01$ nM, low affinity site $K_{act} = 21.8 \pm 6.4$ nM, high affinity $V_{max} = 25.1 \pm 2.0$ pmol cAMP/min/mg protein and low affinity $V_{max} = 127 \pm 19$ pmol cAMP/min/mg protein. In the presence of 100 μM forskolin, a left shift in the curve is observed and analysis of the data using a one site model generated estimates for the apparent $K_{act} = 0.2 \pm 0.03$ nM and for the Vmax = 130 ± 4 pmol cAMP/min/mg protein. Comparison of the statistical factors showing the improvement of fit by the two site model over the one site model is presented in table 5-1.

Table 5 Parameter estimates and statistical values for the $G\alpha_s$* regulation of AC6

Model	app Kact (nM)	Vmax (pmol/min/mg)	F statistic	rms	r2
2 Site* "hi"	0.8±0.01 (0.001)	14.2±0.49 (0.001)	1377	5	0.996
"lo"	7.8±0.78 (0.001)	109±5.3 (0.001)			
2 Site** "hi"	0.6±0.01 (0.001)	1.0±5.3 (0.853)	275	17.8	0.98
"lo"	15±6.7 (0.037)	85±11.1 (0.001)			
1 Site	14.3±3.1 (0.002)	169.1±17.64 (0.001)	647	61	0.98

2 Site*: interacting model

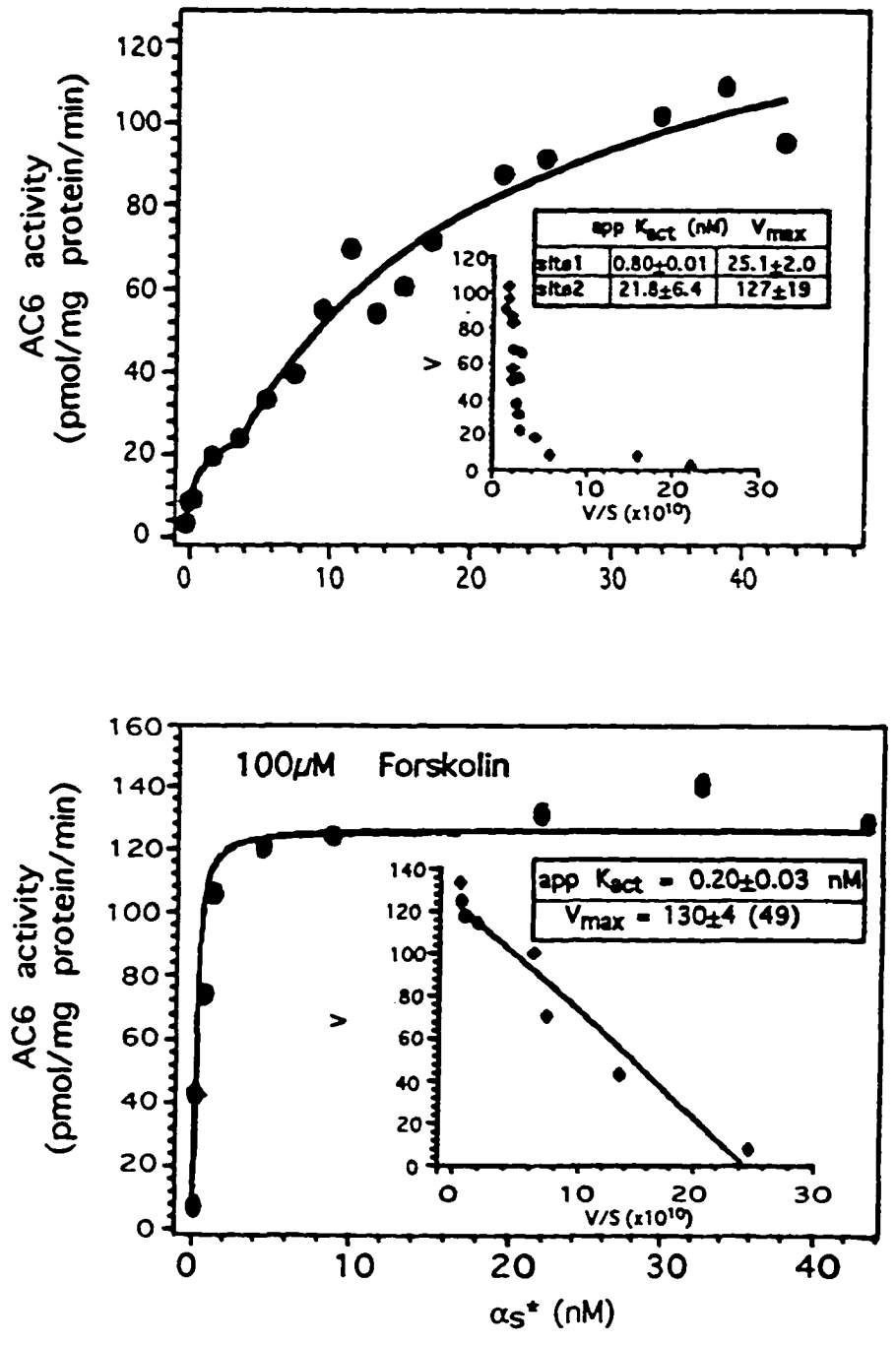
2 Site**: noninteracting model

The data is from one experiment repeated five times. Values are parameter estimates ± SD generated by computer fit, as explained above. Values within parentheses are p values. Statistical analysis was carried out utilizing the F test comparing goodness of fit for different models.

Figure 5-7 Stimulation of adenylyl cyclase type 6 activity by G α_s* in the presence of forskolin

The $G\alpha_s$* stimulation was measured in the presence of 100 μM forskolin. 5 μg of AC6 membrane was assayed in each tube. The concentration range of $G\alpha_s$* was 0.025-44 nM. The fit provided the following parameter estimates: apparent K_{act} = 0.2 nM and V_{max} = 130 pmol cAMP/min/mg. The p value constants were less than 0.01. A steep left shift in the concentration response curve is observed. This experiment is representative of a single experiment repeated five times.

Figure 5-7 Stimulation of adenylyl cyclase type 6 activity by G α_s* in the presence of forskolin

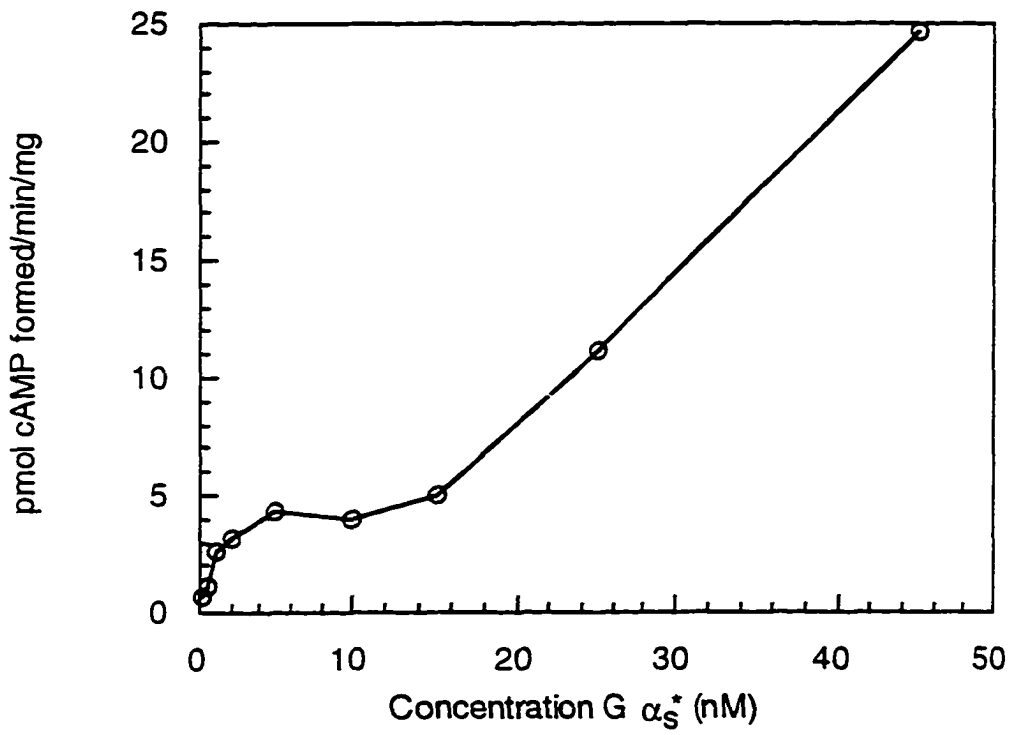


Although adenylyl cyclases are trace proteins even when expressed in Sf9 cells, the baculovirus driven level of expression in Sf9 cell is 10 - 50 fold higher than in native tissues. This could result in a biologically irrelevant association of adenylyl cyclase molecules resulting in the observed complex kinetics. To test if the observed $G\alpha_s$* response profile was due to a "membrane associated" phenomenon, I solubilized the recombinant AC6-containing membranes with dodecyl maltoside. The AC6 was obtained in the 100,000xg supernatant and the effects of varying concentrations of $G\alpha_s$* on the solubilized AC6 was determined (Fig. 5-8).

Figure 5-8 The effect of varying concentrations of G α_s* on solubilized AC6

The data is representative of a single experiment repeated twice. Solubilized membrane extracts of recombinant AC6 baculovirus infected insect cells were prepared as described in the Methods section. Each assay tube contained 5 μg protein concentration as measured by the BCA assay (Methods). The assay was carried out for 15 minutes. The range of Gα_s* was 0.05 - 45 nM.

Figure 5-8 The effect of varying concentrations of G α_s* on solubilized AC6



Assays utilizing the detergent solubilized AC6 indicate that the biphasic nature of $G\alpha_s^*$ concentration-effect curve is not restricted to the membrane bound form of the enzyme but is observed in dilute solutions as well. These observations indicate that while the formation of multimers of adenylyl cyclase is a possibility, it is not due to overexpression in the membranes.

CHAPTER 6

The varied regulatory properties and differential tissue distribution among the AC isoforms are thought to be the means by which the generation of cAMP can mediate the selective activities induced by diverse signaling molecules. One of the goals of this study was to examine whether the nature of regulation of AC6 by $G\alpha_s$* is conserved among other AC isoforms. AC types 1 and 2 were selected for comparison with AC type 6 because all three represent different adenylyl cyclase families based on differential regulation by either Ca^{2+}/CaM or $G\beta\gamma$.

6-1 Construction of AC1 and AC2

The cDNA of AC1 in pBSII was prepared in our laboratory, the cDNA for AC2 in pBSII was a kind gift from Dr. Randy Reed. The cDNA for both forms were excised from the Bluescript vector and inserted into the appropriate restriction sites in pVL1392 for both AC2 and AC1 (Eco RI/Eco RI and HindIII/XbaI, respectively). Each was cotransfected with AcMNPV and viral recombinant plaques were purified by the Baculovirus Core Facility. Membranes of insect cells infected with either AC1 or AC2 recombinant baculovirus were prepared and the protein concentration was determined using the BCA protocol as described in Methods. The assay included 5 μg of membrane in each test tube containing 30 μl of total reaction mixture. The assay was carried out under steady state conditions for 15 minutes at 30°C. Stimulation of both AC1 and AC2 isoforms was concentration dependent. The range of concentrations used to characterize the regulation was typically between 0.025 - 40 nM. The levels of $G\alpha_s$* stimulated enzyme activity due to endogenous adenylyl cyclase in insect membrane represented less than 5% of activity due to AC 1 or AC 2.

6-2 General Properties of $G\alpha_s$* Regulation of AC1

Diagnostic assays carried out in duplicates, measured basal stimulation of AC1 as 75.7 ± 15.3 pmol cAMP/min/mg (n = 7). Stimulation by 100 μM forskolin averaged 218 ± 73.4 pmol cAMP/min/mg (n = 4). I then studied the effect of varying concentrations of $G\alpha_s$* on AC1 activity. The fitted values ± SD, p value, F statistic and rms. are presented in Table 6-1. The velocity curve generated for AC1 was a normal hyperbolic function (Fig 6-1). Hofstee linear transformation of the data yielded a straight line suggestive of simple enzyme kinetics (Fig 6-1 inset). The data was best fit to a simple Michaelis-Menten one site model according to equation 1 (Chapter 5). The apparent K_{act} value for AC1 is 0.9 nM ± 0.01 nM and the V_{max} is 434 ± 11 pmol cAMP/min/mg (table 6-1). The addition of forskolin caused a left shift of the velocity curve, K_{act} = 0.157 nM ± 0.011 nM and a maximal activity, V_{max} = 428.2 ± 12.26 (Fig. 6-2). The results were transformed according to the Eadie-Hofstee method of linearization and plotted (inset Fig. 6-2). Linearization of the transformed data supports the simple enzyme kinetics.

Table 6-1. Parameter estimates and statistical values for $G\alpha_s$* regulation of AC1

Parameter	Fitted Value	SD	P value	F statistic	rms	r2
V_{max}	434	11	0.0001	802	241	.987
K_{act}	$0.9x10^{-9}$ (M)	$1.60x10^{-11}$ (M)	0.0001			
V_{max}+FSK	428.2	12.26	0.0001	610	2571	.984
K_{act} +FSK	$1.57x10^{-10}$ (M)	$1.10x10^{-11}$ (M)	0.0001			

The data is from one experiment repeated four times. Values are parameter estimates ± SD generated by computer fit, as explained in Methods. Statistical analysis was carried out utilizing the F test comparing goodness of fit for different models.

Figure 6-1 The effect of varying concentrations of G α_s* on AC1

High 5 membranes (5 µg) containing AC1 activity were assayed in the presence of varying concentrations of $G\alpha_s$*. Inset shows Hofstee transformations of the data. The Prophet program was used to analyze the data and obtain the fitted curves. The apparent K_{act} and V_{max} parameter estimates were 0.9 nM ± $6.7x10^{-13}$ M and 433.5 ± 10.8 pmol cAMP/min/mg respectively.

Figure 6-1 The effect of varying concentrations of G α_s* on AC1

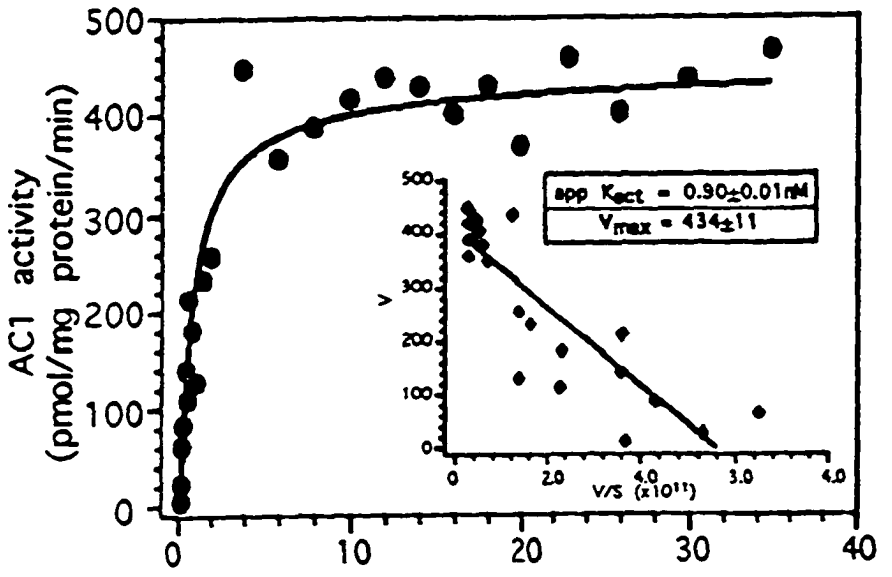
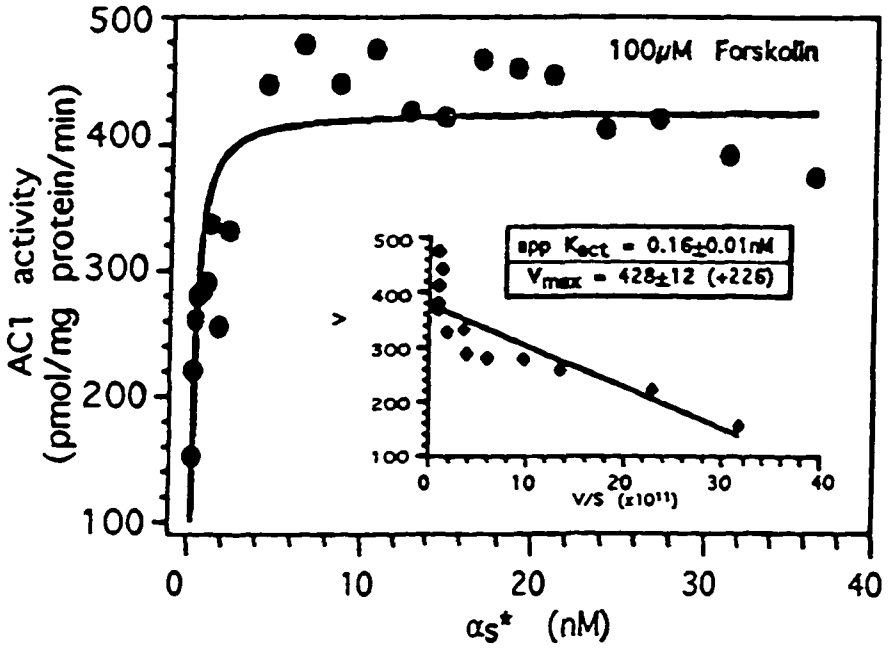


Figure 6-2 The effect of varying concentrations of G α_s* on AC1 in the presence of 100 μM forskolin

Membranes (5 μg) obtained from High 5 insect cells infected with recombinant AC1 were assayed in the presence of 100 μM forskolin and varying concentrations of Gα_s*. Inset shows Hofstee transformations of the data. The Prophet program was used to analyze the data and obtain the fitted curves. The apparent K_{act} and V_{max} parameter estimates were 0.157 nM ± 0.011 nM and 428.2 ± 12.26 pmol cAMP/min/mg respectively.

Figure 6-2 The effect of varying concentrations of G α_s* on AC1 in the presence of 100 μM forskolin



6-3 General Properties of $G\alpha_s$* Regulation of AC2

Diagnostic assays carried out in duplicates, measured basal stimulation of AC2 as 91.3 ± 10.4 (n = 9). Stimulation by 100 μM forskolin averaged 276.8 ± 65.9 (n = 5). I then studied the effect of varying concentrations of $G\alpha_s$* on AC2 (Fig. 6-3). This concentration-effect curve best fit a two site Michaelis-Menten function according to equation 4.

Equation 4:

$$v = \frac{V_{max_1} * S}{K_{act_1} + S} + \frac{V_{max_2} * S}{K_{act_2} + S}$$

The parameter estimates generated from the curve fitting program for K_{act1}, V_{max1}, K_{act2} and V_{max2} is 0.9 ± 0.01 nM, 106 ± 30 pmol cAMP/min/mg, 14.9 ± 3.8 nM and 713 ± 47. Linear transformation of the data generated a curvilinear function suggestive of complex enzyme kinetics. The addition of forskolin generated a steep rectangular velocity curve (Fig. 6-4). The data was best fit to equation 1 which generated the following parameter estimates: K_{act} equal to 0.39 nM and a V_{max2} equal to 621 ± 9 pmol cAMP/min/mg. A linear profile of the transformed data was obtained.

Figure 6-3 The effect of varying concentrations of G α_s* on AC2

Membranes (5 µg) obtained from Sf9 insect cells infected with recombinant AC2 baculovirus activity was assayed in the presence of varying concentrations of Gα_s*. Inset shows Hofstee transformations of the data. The Prophet program was used to analyze the data and obtain the fitted curves. The apparent K_{act1}, V_{max1} , K_{act2} and V_{max2} parameter estimates were 0.09 ± 0.01 nM, 106 ± 30 pmol cAMP/min/mg, 14.9 ± 3.8 nM and 713 ± 47 pmol cAMP/min/mg respectively.

Figure 6-3 The effect of varying concentrations of G α_s* on AC2

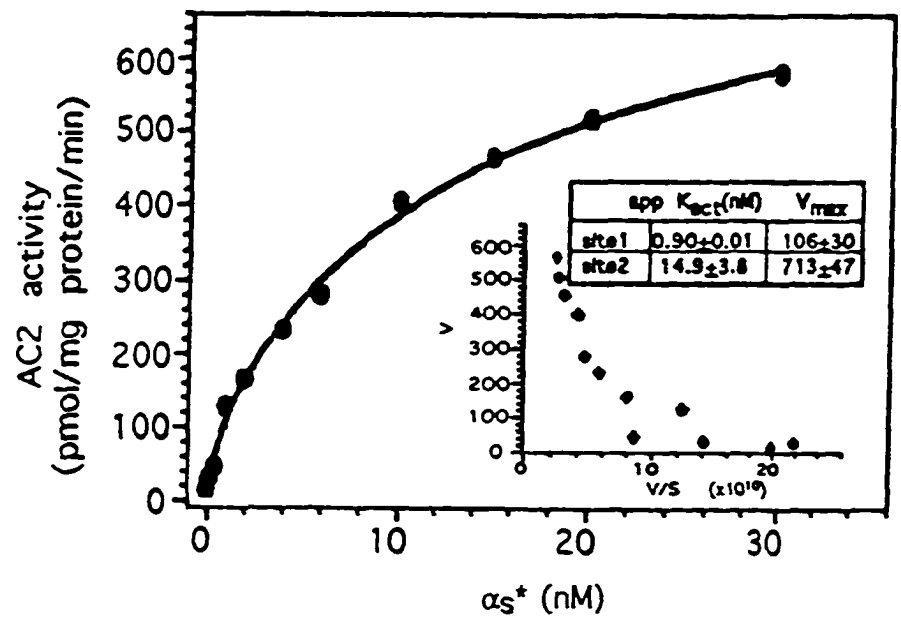


Figure 6-4 The effect of varying concentrations of G α_s* on AC2 in the presence of 100 μM forskolin

The data is representative of one experiment repeated at least five times. Membranes (5 μg) obtained from Sf9 insect cells infected with recombinant AC2 were assayed in the presence of 100 μM forskolin and varying concentrations of $G\alpha_s$*. Inset shows Hofstee transformations of the data. The Prophet program was used to analyze the data and obtain the fitted curves. The apparent K_{act} and V_{max} parameter estimates were 0.39 ± 0.02 nM and 621 ± 9 pmol cAMP/min/mg respectively.

Figure 6-4 The effect of varying concentrations of G α_s* on AC2 in the presence of 100 μM forskolin

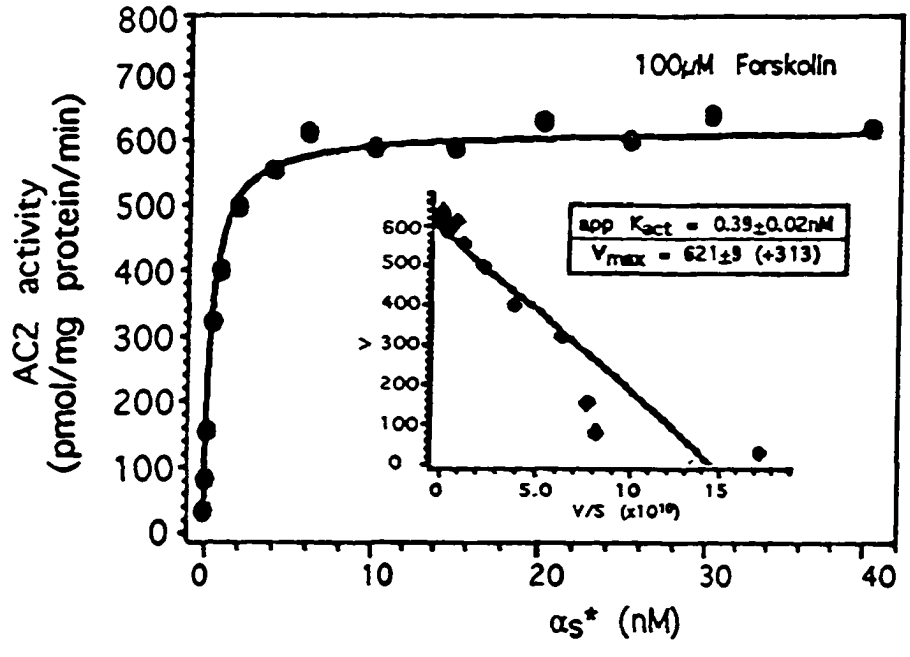


Table 6-2. Stimulation of AC2 by G α_s* with and without FSK

Model	app K_{act} (nM)	V_{max} (pmol/min/mg)	F statistic	rms	r2
2 Site "hi"	0.9±0.01 (0.0001)	105.9±30.3 (0.007)	1380	206	0.998
"lo"	14.9±3.8 (0.004)	713±47 (0.0001)			
1 Site	8.56+1.17	741+41	1298	436	0.996

The data is from one experiment repeated five times. Values are parameter estimates ± SD generated by computer fit, as explained in Methods. Values within parentheses are p values. Statistical analysis was carried out utilizing the F test comparing goodness of fit for one and two site models.

6-4 The requirement of Gα_s* for G$\beta\gamma$ stimulation of AC2

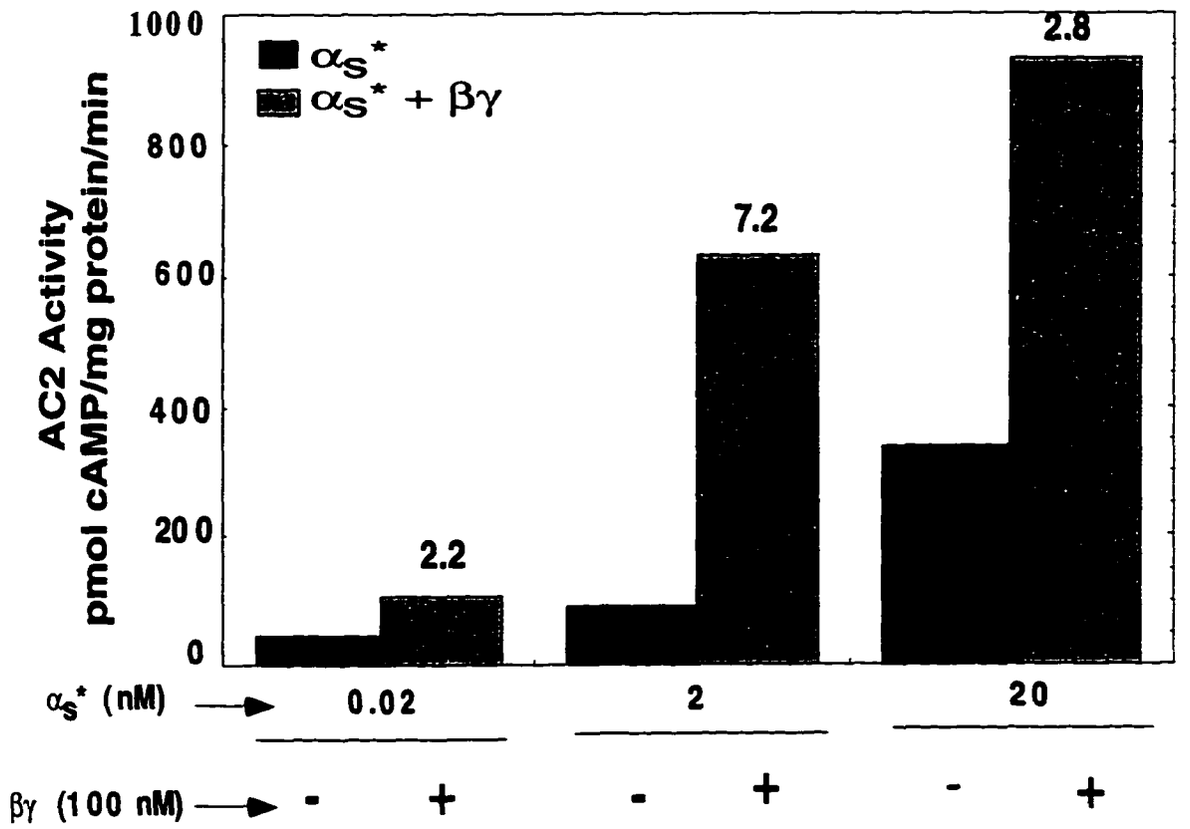
Of the adenylyl cyclase isoforms characterized to date, AC2, 4, 7 and 9 are regulated by G$\beta\gamma$ subunits in the presence of Gα_s. AC1 is inhibited by G$\beta\gamma$ and AC3, 5 and 6 are not responsive whether in the presence or absence of Gα_s (Tang and Gilman, 1991, Gao and Gilman, 1991, Premont et al., 1992 and 1996). A region in AC2 critical for G$\beta\gamma$ regulation was mapped to the carboxy tail of the second cytoplasmic loop (C2) including residues 956-982 by Jianqiang Chen in our laboratory (Chen et al., 1995).

It was of interest to determine whether both the low and high affinity sites or only one of the sites was required for Gβγ stimulation. The stimulation by different concentrations of $G\alpha_s$* was tested in the presence of Gβγ. Adenylyl cyclase activity was stimulated by Gβγ approximately 2 fold in the presence of 0.02 nM $G\alpha_s$*, this was increased to 7 fold in the presence of 2 nM $G\alpha_s$*. However, as the concentration of $G\alpha_s$* was increased to 20 nM the fold increase in activity only reached a level of 2.8 (Fig. 6-5). For AC2, occupancy of the "high affinity" $G\alpha_s$* site appears to be sufficient for Gβγ stimulation, since maximal fold stimulation by Gβγ is observed by 2 nM $G\alpha_s$*.

Figure 6-5 The effect of varying concentrations of $G\alpha_z$* on the stimulation of AC2 by 100 nM Gβγ subunits

The data is representative of a single experiment repeated three times. The Gβγ subunits were purified from bovine brain, 100 nM was used for assay. Values are mean of triplicate determinations. Coefficient of variance was less than 5%.

Figure 6-5 The effect of varying concentrations of $G\alpha_s^*$ on the stimulation of AC2 by 100 nM Gβγ subunits



CHAPTER 7

DISCUSSION

Great interest has been focused on understanding the functional and structural properties of the adenylyl cyclase isoforms primarily because they synthesize one of the most ubiquitous second messengers, cAMP. This large family of enzymes represents a challenge in our understanding of the evolutionary development of species. Not only is it found in a wide range of organisms such as eukaryotes, prokaryotes and protists but also the functions of the varied cyclase isoforms are diverse. Adenylyl cyclase in *Bacillus anthracis* is the edema factor which comprises one of the three components of anthrax. In the mammalian nervous system, the AC 1 and AC8 are involved in the complex integrative tasks of learning and memory.

In multicellular organisms, the adenylyl cyclase pathway is one of the more important signal transduction systems that mediates the conversion of extracellular signals to cellular activities. The mammalian isoforms exhibit varied tissue distribution, chromosomal mapping and regulatory properties. An understanding of these regulatory properties and functional domains of the mammalian adenylyl cyclases will contribute to the developing field of cellular communication and the integration of messages.

This study has focused on $G\alpha_s$ regulation of adenylyl cyclases 6, 2 and 1. The results obtained indicate that the kinetic mechanisms by which $G\alpha_s$ stimulates the different adenylyl cyclase are very different and may be indicative of differences in the oligomeric organization of the different $G\alpha_s$ - adenylyl cyclase complexes.

Adenylyl Cyclase 6

$G\alpha_s$ regulation of adenylyl cyclase type 6 appears to involve complex kinetic mechanism as indicated by the biphasic concentration-effect curve. Hofstee transformation of the $G\alpha_s$ concentration-effect curve yielded a curved line indicative of multiple sites of interactions. The model used to fit the AC6 data required an iterative two site fitting procedure indicative of interacting $G\alpha_s$* sites. At low concentrations of $G\alpha_s$, the data showed a single site interaction profile. The second site was observable only at concentrations of $G\alpha_s$ above a certain threshold value. AC6 activities above the threshold concentration of $G\alpha_s$ are the summation of interactions at both sites. Hence, a modified two-site model was used for activities above the threshold $G\alpha_s$* concentration, the activity due to the second site is the difference between the observed activity and the maximal activity due to occupancy of the first site. This method of fitting is suggestive of interaction between the sites and may

imply that the "high affinity site" needs to be fully occupied to permit occupancy of the "low affinity site". The estimated V_{max} of the high affinity site had to be subtracted from the observed activity at higher $G\alpha_s$* concentrations to fit the second site and obtain an overall best fit.

One hypothesis which is consistent with this behavior is that there exists at least two distinct regions in adenylyl cyclase 6 that are involved in high and low affinity interactions with $G\alpha_s$*. These "two regions" may be within a single AC6 molecule, and may become available for $G\alpha_s$ interactions with differing affinities by the dimerization of two separate AC6 molecules in the membrane, Alternatively, the observations could also be consistent with two different affinity states attained by a single site on AC6 when AC6 is part of a dimer.

Forskolin appears to have a dual regulatory effect on $G\alpha_s$* stimulation of AC6. Forskolin both increases the affinity of $G\alpha_s$* for AC6 and converts the concentration response curve from biphasic to a monophasic kinetic profile. The single $G\alpha_s$ site in the presence of forskolin as well as the detection of a single band with apparent molecular weight of 131 kDa with Western analysis of AC6 membranes indicate that the two sites displayed by AC6 are not due to two separate populations of enzymes when expressed in Sf9 cells. However, this data does not preclude the possibility that the conversion of a high and

low affinity site to two equal, high affinity sites within a single molecule can be induced by forskolin. It is also possible that in the presence of forskolin and $G\alpha_s$ AC6 exists as active monomers. These possibilities have to be resolved by further experiments.

Adenylyl Cyclase 1

The regulatory profile of AC1 stimulation by increasing concentrations of $G\alpha_s$* is the simplest of the three isoforms studied here (Chapter 6). Concentration dependent regulation of AC1 by $G\alpha_s$* is monotonic and the data are best fit to a one site model. Forskolin increases the affinity of $G\alpha_s$* for AC1 without altering the profile of the $G\alpha_s$ concentration- curve. It is noteworthy that the apparent k_{act} for $G\alpha_s$* is similar to the K_i for $G\beta\gamma$ (Tang et al., 1991). Stimulation by $G\alpha_s$ would be inhibited by $G\beta\gamma$ and thus for all practical purposes G_s coupled receptors would be unable to activate AC1 as has been recently demonstrated by Storm and co-workers (Nielsen et al., 1996).

Adenylyl cyclase 2

The concentration dependent regulation of AC2 by $G\alpha_s$* is somewhat similar to the complex kinetic phenomena observed with AC6. Hofstee transformation of the $G\alpha_s$ concentration-effect curve for AC2 yield a curvilinear profile. The data are best be explained by a two site model. The

kinetic constants can are obtained by resolution of the curve into its linear components. The regulation of AC2 by $G\alpha_s$ in the presence of forskolin not only shifts the concentration response curve to the left, but also renders stimulation monotonic indicating that both the low and high affinity $G\alpha_s$ sites can be converted to a higher single affinity site in the presence of forskolin. Hofstee transformation of the $G\alpha_s$ concentration-effect curve in the presence of forskolin yielded a straight line.

Though $G\alpha_s^*$ stimulation of both AC2 and AC6 best fit a two site model, the kinetic analysis presented here indicate that there may be crucial mechanistic differences in $G\alpha_s^*$ stimulation of the two isoforms. The $G\alpha_s$ stimulation of AC2 data can be best described by a model that uses a two site Michaelis-Menten type function. These conclusions are based on the curve-fitting procedures with statistical analysis measuring the improvement in fit by the two site model as compared to a single site model. In contrast to the analysis used for AC6 constants, both the "high" and "low" affinity sites are obtained by resolution of the curvilinear Hofstee into two linear components. This model is compatible with the existence of two "non-interactive sites". Thus, in contrast to AC6, for AC2 the analysis does not indicate that the high affinity site needs to be occupied to obtain the low affinity stimulation.

For AC2, occupancy of the "high affinity" $G\alpha_s$* site appears to be sufficient for $G\beta\gamma$ stimulation. While $G\alpha_s$* stimulation of AC2 in the presence of $G\beta\gamma$ is concentration dependent, this is so only at low concentrations of $G\alpha_s$*. The maximal fold stimulation by $G\beta\gamma$ is observed by 2 nM $G\alpha_s$*. $G\beta\gamma$ stimulation remains constant with further increases in the concentration of $G\alpha_s$. However, the fold stimulation in AC2 activity due to $G\beta\gamma$ drops with increasing concentrations of $G\alpha_s$* beyond 2 nM. These observations that high affinity $G\alpha_s$ interactions with AC2 is sufficient for $G\beta\gamma$ stimulation may indicate that some of the determinants for $G\alpha_s$* binding are in the cytoplasmic tail. The amino acid sequence between residues 956 and 982 of the carboxy tail of the C2 cytoplasmic loop appears to be the region in AC2 critical for $G\beta\gamma$ regulation (Chen et al., 1995). Point mutations in the cytoplasmic tail in the 950-1050 region of AC1 results in abolishment of $G\alpha_s$ binding. Interaction of $G\alpha_s$* with these determinants could open up one of the regions involved in $G\beta\gamma$ binding. Such a model would provide a molecular explanation for why $G\beta\gamma$ only stimulates AC2 activated by $G\alpha_s$*.

Forskolin modulation of $G\alpha_s$ regulation

Forskolin is a lipophilic molecule that directly stimulates most AC isoforms, including the soluble enzyme derived from the cytosolic domains

of AC1 and AC2 (Seamon and Daly, 1986, Tang and Gilman, 1995 and Laurenza et al., 1987). Forskolin stimulation does not require $G\alpha_s$. However stimulation by $G\alpha_s$ and forskolin are synergistic (Clark et al., 1982 and Neer, 1995). This synergism is best seen at sub-saturating concentrations of $G\alpha_s$ and forskolin presumably due to the increase in potency with which $G\alpha_s$ stimulates adenylyl cyclase in the forskolin and vice versa.

The close interactions between $G\alpha_s$ and forskolin in regulating the stimulation of adenylyl cyclase suggest that the $G\alpha_s$ and forskolin sites on adenylyl cyclases could be close to each other. The C1 cytoplasmic loop with 350 amino acids and the C2 with 250-300 amino acids contain the catalytic core of the enzyme. This was initially predicted on the basis of sequence homology with cloned guanylyl cyclases (Chinkers, et al., 1991). Recent experiments with soluble constructs that utilize the C1 domain of AC1 and the C2 domain of AC2 indicate that $G\alpha_s$ and forskolin stimulated adenylyl cyclase activity is contained within these regions (Tang and Gilman, 1995, Whisnant et al., 1996 and Yan et al., 1996).

The crystal structure of the truncated cytoplasmic tail of AC2 complexed with forskolin has been solved (Zhang et al., 1997). Both in solution (Whisnant et al., 1996) as well as in crystal, the cytoplasmic tail exists as a dimer. The dimer has a wreath like structure with a cleft in the center.

Each dimeric structure contains two molecules of forskolin. Several residues that line the center of the ventral cavity have been shown by site-directed mutagenesis to bind either ATP or ATP analogs. Hence, the ventral cavity may contain the ATP binding site. Forskolin binds to the two ends of the ventral cavity. As expected the forskolin binding pockets are highly hydrophobic. This dimer of the truncated C2 region has been proposed as a model for the interaction between the central cytoplasmic loop and the C-terminal tail in the formation of an active enzyme. Further studies are required to determine the validity of this proposal. However it is note worthy that the forskolin binding site is very close to the residues on AC1 that is involved in $G\alpha_s$ interactions. Now, crystal structures of the C1-C2 complex with $G\alpha_s$ and forskolin are needed to obtain a rigorous mechanistic understanding of this synergistic stimulation.

One vs. two $G\alpha_s$ sites per adenylyl cyclase molecule

The kinetic descriptions of $G\alpha_s$* interactions with the various adenylyl cyclases provided here should not be construed as an indication that each molecule of AC2 or AC6 contains two $G\alpha_s$* binding sites. This is one possible explanation. In such a case, AC1 would either have only one $G\alpha_s$* site or two sites with very similar affinities. Studies by Neer and co-workers on the hydrodynamic properties of the $G\alpha_s$-adenylyl cyclase from bovine caudate which yield molecular weights for $G\alpha_s$-adenylyl cyclase complex in the

presence of forskolin of 197 to 225 kDa (Bender et al., 1984) may support the stoichiometry of two $G\alpha_s$ per adenylyl cyclase. This possibility needs to be explored further in detail for the different adenylyl cyclases.

There also exists data that do not support two $G\alpha_s$ sites per adenylyl cyclase model. Crosslinking of $G\alpha_s$ to the olfactory adenylyl cyclase(s) suggest a 1:1 stoichiometry (Pfeuffer et al., 1989). If each AC2 or AC6 molecule contains one $G\alpha_s$ site, then our data may be indicative of adenylyl cyclases to dimerize in the membrane environment. If differing adenylyl cyclases have differing capabilities to form homodimers, then our data would suggest that AC1 had a very low capability to form homodimers, while AC2 and AC6 are more likely to dimerize.

Future studies incorporating crosslinking protocols are required to determine the stoichiometry of $G\alpha_s$-adenylyl cyclase interactions. Further investigations into the regulatory properties of the naturally occurring half AC molecules in heart may also provide a greater picture of the relationship between the structure and enzymatic activity of AC. In addition, crystallography studies of the adenylyl cyclase- $G\alpha_s$ complex AC should useful in obtaining a clear description of the mechanistic details of how $G\alpha_s$ activates adenylyl cyclase .

Irrespective of the details that emerge from these studies it has become abundantly clear from the data presented here, that each adenylyl cyclase can respond differently to $G\alpha_s$ and that such differential responses could be crucial factors in determining why different tissues and organs show specialized capability to regulate cAMP elevation.

CHAPTER 8

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