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Adenylyl cyclase 6 is selectively regulated by Protein Kinase A in a region involved in Galphas stimulation

by

Yibang Chen

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

1998

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Abstract

Adenylyl cyclase 6 is selectively regulated by Protein Kinase A in a region involved in Galphas stimulation

by

Yibang Chen

Adviser: Professor Ravi Iyengar

Transmembrane signaling through the receptor-G_s-adenylyl cyclase complex has been studied for a long time as a model for signal transduction through heterotrimeric G proteins. Agonists bind to receptors triggering activation of $G\alpha_s$, activated $G\alpha_s$ then stimulates adenylyl cyclases to increase intracellular cAMP. As a second messenger, cAMP is involved in many downstream biological activities through the activation of protein kinase A. Previous studies have shown that in certain cell types that express adenylyl cyclase 6 (AC6), heterologous desensitization includes reduction of the capability of adenylyl cyclases to be stimulated. In my thesis I have studied the effect of Protein Kinase A on adenylyl cyclases. Protein Kinase A treatment of recombinant adenylyl cyclase 6 in insect cell membranes results in a selective loss of stimulation by high (>10nM) concentrations of $G\alpha_s$. Similar treatment of AC1 or AC2 did not affect $G\alpha_s$ stimulation. Conversion of Ser-674 in AC6 to an Ala blocks protein kinase A phosphorylation and Protein Kinase A mediated inhibition of $G\alpha_s$ stimulation. A peptide FLLT encoding the region 660-682 of AC6 blocks stimulation of AC6 and AC2 by high concentrations of $G\alpha_s$.

Substitution of Ser-674 to Asp in the peptide renders the peptide ineffective, indicating that the region 660-682 of AC6 is involved in regulation of signal transfer from $G\alpha_s$. This region contains a conserved motif present in most adenylyl cyclases, however the Protein Kinase A phosphorylation site is unique to members of the AC6 family. These observations suggest a mechanism of how isoform selective regulatory diversity can be obtained within conserved regions involved in signal communication.

Dedication

This dissertation is dedicated to my wife chuenlan and my parents

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Table of Contents

List of Figures

Chapter 1

Introduction

Chapter 1

1. Background

Mammalian organs and tissues are made up of different types of cells. All of the cells are surrounded by a plasma membrane that serves to separate cells from the extracellular milieu, and hence, form the different cell types. The plasma membrane which is made up of phospholipids, cholesterols, glycolipids and various kinds of proteins serves as a semi-permeable barrier. Within each eukaryotic cell type there exists: a nucleus, subcellular organelles, vesicles, ribosomes, proteins, and enzymes which are all necessary for cell survival and function. Sometimes the extracellular environment, within which all different cell types coexist can suddenly change and cause damage to the cell, and hence the tissue. To survive these drastic and sometimes lethal changes in the cellular environment cells must communicate with each other to better cope with changing conditions.

Specific organs such as the hypothalamus, pituitary, liver, adrenal gland, or pancreas secrete various kinds of signaling molecules that play an important role in cellular communication. Blood circulation, other body fluids and other mechanisms transport these signaling molecules between communicating cells. This process of cellular signaling and communication is now known as signal transduction. Signaling systems allow cells to

precisely control their function within a normal range. Malfunctions in signal transduction pathways can cause damage or disease in an organism.

Under normal conditions the body can very precisely monitor changes in living environment and can regulate cell function by releasing various kinds of hormones and factors in order to maintain synchrony with the environment. There are an enormous number of extracellular signaling agents such as hormones, neurotransmitters, auto and paracrine factors. Most lipophilic molecules (such as steroid hormones) can readily pass through the membrane into the cytosol by means of diffusion, but the majority of hydrophilic molecules do not enter cells to have their biological effects. Instead they convey their signal by binding to a special protein called a receptor, and then by a complicated mechanism, transduce the signal into the cell. We call those substances agonists, since they can bind to the receptors and are capable of evoking a biological response. This binding and trigging cellular response process is one of the most important parts of cell surface signal transduction.

2. Three major components of G protein-coupled receptor mediated signal transduction

Cell surface signaling systems are important cellular mechanisms used for information transfer from the cell surface into the cell. A large number of cell surface receptors use heterotrimeric G proteins as transducers. In G protein mediated signal transduction systems, at the plasma membrane, there are three major components that are essential for G protein-coupled signal transduction.

Fig.1-1

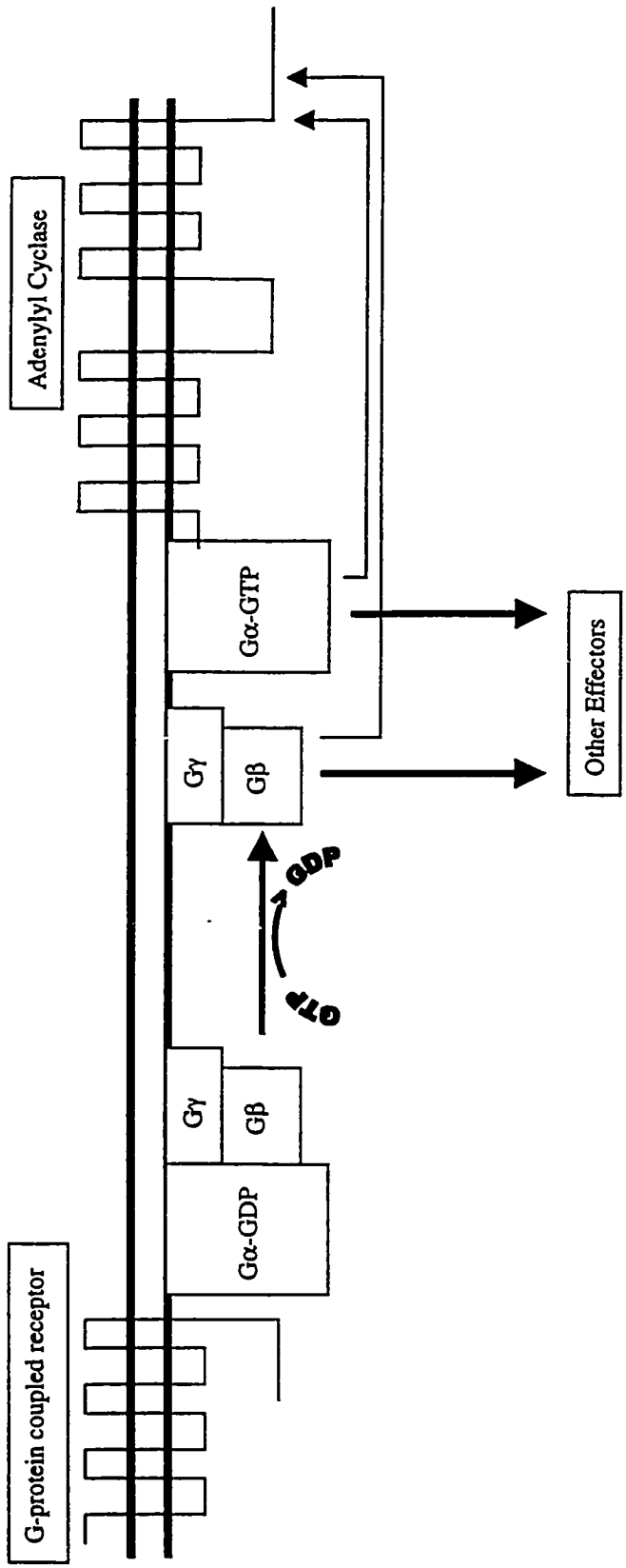


Fig. 1-1 Diagram of G protein-coupled receptor, G protein and effector

Three components are essential for receptor mediated signal transduction pathways. A large number of currently used therapeutic agents directly target either G protein-coupled receptors or G protein mediated signal transduction pathways.

(1) Receptors that bind agonists and elicit biologic activities. Receptors specifically recognize certain molecules rather than nonspecifically binding all substances.
(2) The G proteins which transmit signals from activated receptors to correspondent effectors e.g. enzymes or ion channels.
(3) Effectors (enzymes or ion channels) that generate second messengers such as cAMP, IP3, diacylglycerol or gate ion fluxes.

3. Various kind of receptors

Over a hundred receptors have been identified and cloned. Receptors are proteins that can bind physiologically active substances and convey signals from these molecules to the cells. Generally, they can be classified into five types of receptors:
(1) The ion channel receptors, such as the Na^+ and Ca^{2+} channel receptors. These receptors contain ion channel and ligand binding sites. When activated these receptors can cause ions to enter or leave the cell or intracellular compartments. Therefore these receptors are controlled either by physiologically active substances or by voltage changes.

(2) The tyrosine kinase receptors, such as the insulin receptor. They are usually transmembrane proteins. They convey their signals by phosphorylation of tyrosine residues which are located on the receptor itself. Such phosphorylation or dephosphorylation causes a cascade reaction downstream of the receptor; these pathways are closely related to cell growth and proliferation.

(3) The receptors containing intrinsic guanylyl cyclase activity, such as atrial natriuretic receptor, they coupled guanylyl cyclase pathway.

(4) Intracellular receptors, these receptors normally are not present in cell plasma membrane, but are rather in the intracellularlly located, or even in the nucleus.

Lipophilic hormones such as estrogens, progesterones and other steroid hormones, can pass through the membrane, but their functional effects need to be accomplished by intracellular receptors. Their functional sites are usually in the nucleus.

(5)The guanine nucleotide-binding regulatory protein (G protein)-coupled receptors, such as the glucagon receptor. Activating these receptor by agonists can stimulate adenylyl cyclase(AC) to increase cAMP production by acting through several different pathways to ultimately increase blood sugar. The G protein-coupled receptors represent the majority receptors of all known receptors.

4. Guanine nucleotide binding proteins

These proteins form a large family. A common feature of these proteins is that they can bind to GTP and GDP and they have an intrinsic

GTPase function which hydrolyzes GTP to GDP. There are three major classes of G proteins:

1)The small, p21 Ras-like proteins which are involved in mitogen-activated protein kinase(MAPkinase) cascades, affecing cell cycle. In this group some mutations can cause cell oncogenesis.

2) The elongation factors which play an important role in protein and polypeptide synthesis.

3)The heterotrimeric G proteins (G proteins) which are made up of three polypeptides, namely α, β, γ.

5. Heterotrimeric G proteins subunit and the GTPase cycle

Heterotrimeric G proteins are composed of three polypeptides: an α subunit, which can bind and hydrolyze GTP, a β subunit and a γ subunit. The β and the γ are made of two separate gene products but once they are expressed and form a dimer, the heterodimer does not dissociate except under denaturing condition. When GDP is bound, the α subunit is tightly associated with the βγ subunit forming the inactive heterotrimer. Only the heterotrimer interacts with the receptor. When ligand(agonist) binds to the receptor, the receptor become activated and interacts with the heterotrimeric GαGDP-βγ complex , triggering a conformational change leading to the release of GDP, creating a $G\alpha_{empty}\beta\gamma$, a very short lived transition state. In the absence of bound guanine nucleotide, $G\alpha_{empty}\beta\gamma$ has high affinity to receptors. In the cell cytosol the concentration of GTP is much higher than that of GDP, therefore the empty pocket of the nucleotide-binding site is soon occupied by GTP. After GTP binds to the receptor- G protein complex, it initiates a conformational change inducing rapid dissociation of activated receptor from

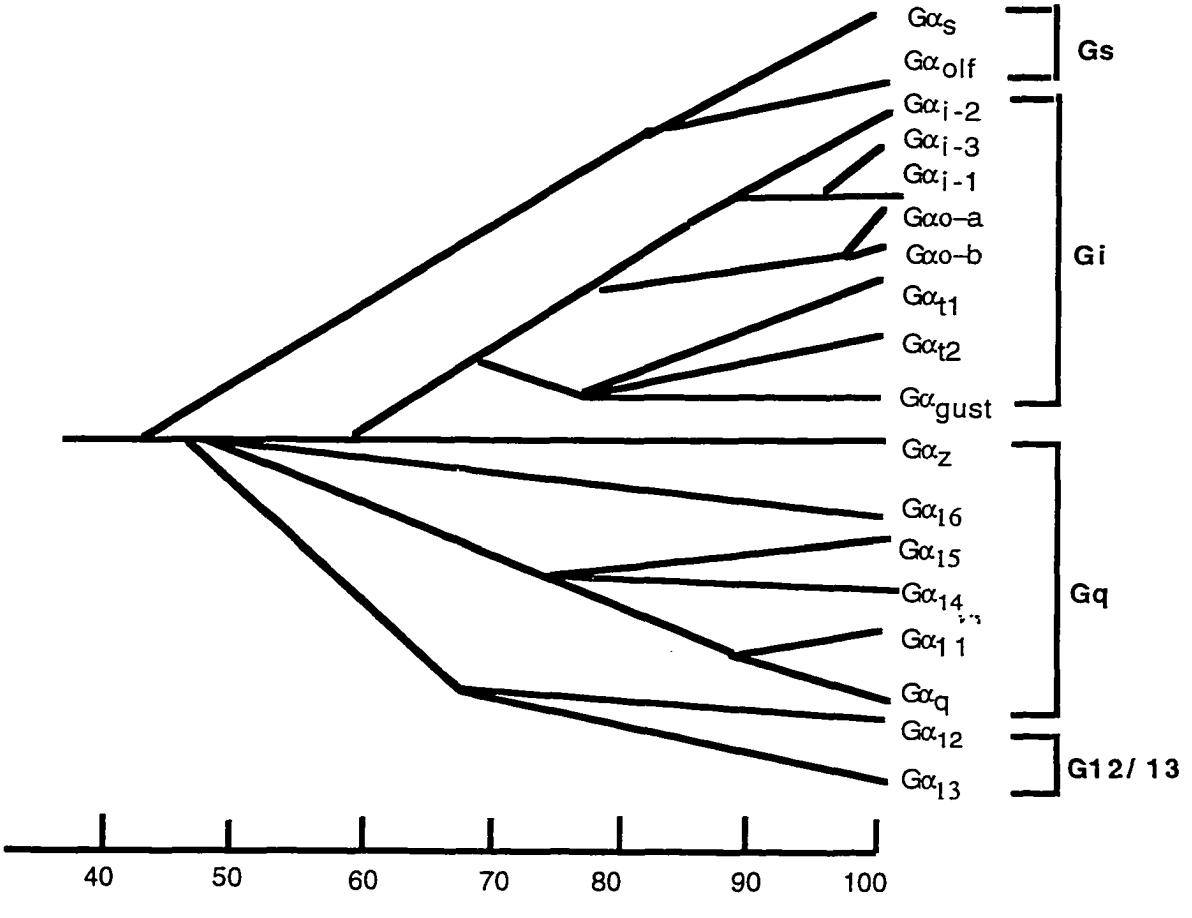
Gα GTP and the Gβγ complex. The dissociated Gα GTP and Gβγ are then able to activate their appropriate effectors. Once GTP is bound, the α subunit is activated. The activated state lasts until the GTP is hydrolyzed into GDP by Gα subunit itself by means of the Gα's intrinsic GTPase activity. As soon as GTP is cleaved into GDP, the α and the βγ subunit reassociate again. Ligand-activated receptor complexes can then interact with the heterotrimeric Gαβγ complex again. For quite a long time, it appeared that G-protein mediated signal transduction was the function of α subunit; the βγ subunit was considered to be inactive for effector modulation or a negative regulator of G protein signaling. This concept soon was challenged by the discovery that the βγ subunit itself could activate the muscarinic K^+ channel. (Logothetis et al., 1987) Since then, it has been demonstrated that Gβγ can stimulate adenylyl cyclase type 2 and 4 in the presence of $G\alpha_s$ (Tang WJ. et al., 1991; Gao, BN. et al., 1991), have inhibitory effects on Ca^{2+}/CaM stimulated AC1(Tang WJ. et al., 1991), stimulate PLCβ (Camps, M. et al., 1992a; and b), and recently it was reported that Gβγ can modulate both N-type and P/Q-type Ca^{2+} channels(Herlitze S, et al 1996, Ikeda, S. R., 1996), and MAP kinase pathways (Van et al., 1995 , Touhara et al., 1995).

6. Classification of G protein and nomenclature

Before the finding that Gβγ was directly involved in receptor mediated signal transduction pathways, Gαs were regarded as the only active part of the G proteins. The nomenclature of G protein was also influenced by this opinion. G proteins were named by using Gα function to designate whole G protein regardless of the βγ subunit. More than 20 Gα subunits have been

cloned. On the basis of their functions and the amino acid sequence relationships among Gα subunits, Gα subunit can be further subdivided into four major classes: G_s, G_i, G_q and G_{12} (Hepler, JR. and Gilman, AG. 1992; Neer, E J. 1995. ; Hamm, HE. 1996).

Fig.1-2



Mammalian G Protein α Subunit

Fig. 1-2 Diversity of G protein and the classification of G protein subclasses
The classification of G proteins is dependent on the molecular homology and functional similarity of Gα subunits. Four subclasses of G proteins can be subdivided according to their molecular structure and function relationships.

The G_s subclass includes G_s and G_{olf} ; S stands for stimulation. All members of this class can stimulate adenylyl cyclases(Graziano MP. et al., 1987). There are four splice variants of $G\alpha_s$, and $G\alpha_s$ is found widely distributed in various kinds of tissue. The G_{olf} is selectively expressed in the olfactory neuroepithelium (Jones DT. and Reed RR. 1987). In addition to stimulating adenylyl cyclase, $G\alpha_s$ also regulates Ca^{2+} channels (Mattera R. et al., 1989) and cardiac Na^+ channels (Schubert B. et al., 1989). The G_s subclass can be constitutively turned on when it is ADP-ribosylated by cholera toxin, resulting in a shut-down of the intrinsic GTPase activity of the G protein, i.e. remains in intact (Cassel D. and Pfeuffer T. 1978) and constantly stimulates adenylyl cyclase. This can cause dramatically increased intracellular cAMP concentrations. In humans infected with cholera, this causes serious diarrhea, and patients become so dehydrated that lethality results if untreated.

There is another class of G protein denoted as $G\alpha_i$ (i stands for inhibition). Originally these proteins were discovered by their ability to inhibit adenylyl cyclase. They can be ADP-ribosylated by pertussis toxin. Unlike $G\alpha_s$ which remains "on" after it is ADP-ribosylated, the G_i remains "off" since pertussis toxin mediated ADP-ribosylated prevents the G protein from being activated by agonist activated receptor complexes (Katada T. and Ui M. 1982).

According to their function the G_i subclasses can be further subdivided into three subgroups $G\alpha_i$, $G\alpha_o$ and $G\alpha_t$. The G_i subgroup contains eight members G_{i1}, G_{i2} , G_{i3} , G_o, G_{t1} ,G_{t2}, G_g and G_z. The $G\alpha_i$ subgroup includes $G\alpha_{i1}$, $G\alpha_{i2}$ and $G\alpha_{i3}$, they inhibit adenylyl cyclases (Katada T. et al., 1984), activate K^+ channels (Yatani A. et al., 1988) and attenuate phospholipase A2 (PLA 2) (Lowndes JM. et al., 1991). It was reported that $G\alpha_{i1}$, but not $G\alpha_{i2}$ or $G\alpha_{i3}$ potently inhibits an inwardly rectifying K^+ channel of the GIRK family which are induced by G $\beta_1\gamma_2$.(Schreibmayer, W. et al., 1996). Since this effect is $G\alpha_{i1}$ specific the inhibition mechanism is unlikely to be simply a G_i chelated $G\beta\gamma$ effect . The G_o subclass closes Ca^{2+} channels (Hescheler J. et al., 1987) and stimulates pertussis toxin-sensitive phospholipase C(PLC) in oocytes(Moriarty TM. et al., 1990). The G_t subclass which is expressed almost exclusively in the visual system stimulates cGMP-phosphodiesterase (cGMP-PDE) (Stryer L. 1986). G_t was the first G-protein crystalized and is the most well studied among all heterotrimeric G proteins families. The G_g subclass is specifically expressed in taste buds, and is believed to be involved in taste signal transduction(McLaughlin SK. et al., 1992). G_z is a G protein of about 40 kDa that is 41-67% identical with other known $G\alpha$ subunits (Fong, HK; 1988; Matsuoka et al., 1988; Matsuoka et al., 1990). High levels of a $G\alpha_z$-subunit were found in rat liver membranes and in brain cytosol. G_z lacks a consensus site for ADP-ribosylation by pertussis toxin but it strongly inhibits adenylyl cyclase (Kozasa and Gilman 1995).

The G_q subclass, which contains $G\alpha_q$, $G\alpha_{11}$, $G\alpha_{14}$, $G\alpha_{15}$ and $G\alpha_{16}$, all stimulate PLCβ subtypes (Smrcka AV, et al., 1991; Taylor SJ, et al., 1990 and 1991 a and b ; Wu D, et al., 1992 a and b). However the PLCβ_2 subtype is barely stimulated by G_q. $G\alpha_q$ and $G\alpha_{11}$ are highly homologous, their amino acid

identity is greater than 88%. $G\alpha_{14}$ is found in stromal and epithelial cells. It has been reported that G_q-coupled receptors are linked with GRB2 and SoS to activate the MAP kinase signaling pathway(Dikic, I. et al, 1996, Wan, Y., 1996).

Recently, it has also been reported that G_q can directly stimulated Bruton's tyrosine Kinase(btk)(Bence, K. et al., 1997). More interestingly, a group in Japan found that G_q and G_{11} can be activated by phosphorylation of a tyrosine residue (Tyr356)(Umemori, H. et al., 1997). G_q deficient mice have a tendency of increasing bleeding time because of defective platelet activation (Offermanns, S. et al., 1997).

$G\alpha_{14}$, $G\alpha_{15}$ and $G\alpha_{16}$ cDNAs encode distinct α subunits of heterotrimeric G proteins. These α subunits are structurally related to members of the G_q family and share certain sequence characteristics with $G\alpha_q$ and other member of G_q family, such as the absence of a pertussis toxin ADP-ribosylation site. $G\alpha_{11}$ and G_q are ubiquitously expressed among murine tissues, $G\alpha_{14}$ is predominantly expressed in spleen, lung, kidney, and testis, whereas $G\alpha_{15}$ is primarily restricted to hematopoietic lineages. $G\alpha_{16}$ is exclusively expressed in hematopoietc cells. Among hematopoietic cell lines, $G\alpha_{11}$ is found in all cell lines tested, $G\alpha_q$ is widely expressed but is not found in most T-cell lines, $G\alpha_{15}$ is predominantly expressed in myeloid and B-cell lineages, and $G\alpha_{14}$ is expressed in bone marrow adherent (stromal) cells, certain early myeloid cells, and progenitor B cells. $G\alpha_{16}$ is expressed in myeloid cells. It has two forms long and short forms(Tenailleau S et al., 1997). It is involved in interleukin(IL)-8 receptor mediated pathway (Knall C. et al., 1996; Xie W. et al.,1997). It activate PLC β subfamily (Kozasa T. et al., 1993). The G_q class may be involved in signal-transduction pathways that are fundamental to hematopoietic cell differentiation and function.

The $G\alpha_{12}$ subclass has only 2 members, $G\alpha_{12}$ and $G\alpha_{13}$. Both are about 44-kDa proteins and are expressed ubiquitously but at low levels. $G\alpha_{12}$ and $G\alpha_{13}$ have 67% amino acid sequence identity with each other and less than 45% identity with other Gα subunits. They are insensitive to ADP-ribosylation by pertussis toxin (Strathmann MP. and Simon MI. 1991), and $G\alpha_{12}$ is required for thrombin-stimulated gene expression and DNA synthesis (Aragay, AM. 1995). Purified $G\alpha_{12}$ and $G\alpha_{13}$ are both coupled to a recombinant thromboxane A2 (TXA2) receptor when they are reconstituted into phospholipid vesicles(Harhammer, R. et al., 1996), and both $G\alpha_{12}$ and $G\alpha_{13}$ stimulate Rho-dependent stress fiber formation and focal adhesion assembly (Buhl, AM., et al., 1995). $G\alpha_{12}$ has also been demonstrated to stimulate c-Jun NH2-terminal kinase through Ras and Rac (Collins, LR. et al., 1996). Purified $G\alpha_{12}$ has a slow rate of guanine nucleotide exchange, and $G\alpha_{12}$ does not regulate the activity of several adenylyl cyclases or phospholipases (Kozasa, T. and Gilman, A. 1995). Protein kinase C can phosphorylate $G\alpha_{12}$ and inhibit its binding to βγ (Kozasa, T. Gilman, A. 1996). $G\alpha_{13}$ can stimulate the activity of the ubiquitous Na-H exchanger (NHE1 and other isomorms) which is also regulated by a number of receptors (Voyno Yasenetskaya, T. 1994). However mutationally activated $G\alpha_{12}$ inhibit the NHE1 isoform but activate NHE2 andNHE 3 isoforms(Lin X. et al., 1996). $G\alpha_{12}$ and $G\alpha_{13}$ are also phosphorylated during platelet activation(Offermanns, S. et al., 1996). Recently, the functional data has shown $G\alpha_{12}$ and $G\alpha_{13}$ mutants to be oncogenic. The potent transforming activity of the $G\alpha_{12}$ and $G\alpha_{13}$ subunits has been identified them as a novel family of oncogenes (Xu N. et al.,1993; Xu N. et al., 1994).

7. Molecular Structure of Gα , structure similarity among Gαs and structural requirement for receptor or effector regulation

Using modern technology like x-ray crystallography, NMR, biochemical and genetic approaches, and computer modeling techniques it is possible to determine the molecular structure and define relationships between the structure and function of Gα subunits (Bourne HR, et al., 1991). The early studies of Gα structure were on the p21 Ras superfamily of proteins. These studies revealed a lot of information about the conformation of guanine nucleotide-binding proteins and the conformational events that play important roles in the regulation of cell function. Gα is predicted to be very similar to p21 Ras. From the Ras structure however, there was a lack of information about the crucial interaction between so called the switch II region and the γ phosphate of GTP that causes the structural changes in Gα and triggers deactivation. Recently, the crystal structures of $G\alpha_t$ and $G\alpha_{i1}$ have been solved by several groups(Noel JP. et al., 1993; Lambright, DG. et al., 1994; Coleman DE. et al., 1994; Sondek L. et al., 1996).

$G\alpha_t$ consists of a GTPase domain which is common to other GTPases such as Ras and Ef-Tu, and a helix domain which is unique to heterotrimeric G proteins. Since GTP is buried deep in the cleft between the two domains, there is no GDP/GTP exchange in the absence of agonist activation(Noel JP. et al., 1993). The GTPase domain has five α helices (α1-α5)surrounding a six-stranded β sheet (β1-β6) . GDP and GTP binding cause conformational changes in structure. Comparison of structural differences between the conformations of $G\alpha_t$-GDP and $G\alpha_t$ -GTPγS, show that the overall Gα conformation does not change too much in whether GTP or GDP is bound, however there are three regions with significant conformational changes, which are denoted as three

switches regions, switch I, II and III (Lambright DG, et al., 1994). Activation of Gα through direct contact with γ phosphate of GTP causes conformational changes in the switch regions, which then propagate the signal to the regions which are involved in effector interaction. Crystallographic analysis of the structural change of $G\alpha_{i1}$ induced by GTP hydrolysis at 2.2 A resolution shows that guanosine triphosphate (GTP) hydrolysis triggers conformational changes in the $G\alpha_{i1}$ subunit (Mixon, et al., 1995) and reveals information about $G\alpha_{i1}$ in more detail.

The crystal structure analysis of $G\alpha_{i1}$ (GDP)$\beta_1\gamma_2$ reveals two nonoverlapping regions of contact between α and β, an extended interface between Gβ and nearly all of Gγ, as well as a limited interaction of Gα with Gγ. The major α/β interface covers the switch II region of $G\alpha_{i1}$, and the GTP binding induced conformational change in the switch II region also results in dissociation of Gβγ from Gα (Wall MA. et al., 1995).

Heteronuclear three-dimensional NMR spectroscopy was used to determine the solution structure of a 141 residue protein containing the GTPase activating domain from $G\alpha_s$(Benjamin, D. R et al. 1995). The domain contains six alpha-helices and is stable and structured in solution despite having been excised from the intact $G\alpha_s$ protein. It reveals that the majority of the structure is remarkably similar to that observed for the cognate domains in crystal structures of the homologous proteins $G\alpha_t$ and $G\alpha_{i1}$. However, the orientations of the second helix and the subsequent interhelical loops differ markedly among the three proteins. This structural divergence, may indicate functional differences between these two sets of proteins. It is noteworthy that the NMR technique detects the molecule in solution, a condition much similar to the protein in the cell, rather than in a crystal. The majority of pharmacologists and biochemists believe the G-protein active form is GTP

bound and inactive form is GDP bound. But interestingly Dr Iyengar's early data (Iyengar R and Birnbaumer 1979, Iyengar R et al 1980) and Gilman's recently published data(Sunahara RK. et al., 1997 a) show GDP bound $G\alpha_s$ can also stimulate adenylyl cyclase to the same Emax as $G\alpha_s$ -GTP. The only difference is $G\alpha_s$ -GDP has low affinity. Thus with an increase in $G\alpha_s$ -GDP concentration the same amount of AC stimulation can be obtained as with $G\alpha_s$ -GTP. This finding is important for structural research. We know by comparing the GDP and GTP bound Gα structures that the conformations of the three switches regions are significantly different. The majority think that those changes may contribute to active and inactive states of Gα. The data of Iyengar and Gilman may indicate that the structural changes in the three switches regions of $G\alpha_s$ may only alter the affinity of $G\alpha_s$ for both receptors and effector(AC). The activation conformation may not fundamentally change since $G\alpha_s$ -GDP can also stimulate AC to the same extent as $G\alpha_s$ -GTP. One possible function of the conformational change in the three switches regions by GTP binding is to shift Gα high affinity state from receptor to effector. Obviously GDP bound Gα has high affinity for Gβγ and $G\alpha_s$-GTP has low affinity for Gβγ.

The minimal structural requirement of Gα for effector regulation has been studied. $G\alpha_s$ and $G\alpha_{i2}$ are different subclasses of G proteins whose α subunits are 65% homologous. Using the 355 amino acid $G\alpha_{i2}$ polypeptide, substitution of residues Ile 213-Lys319 with the corresponding $G\alpha_s$ region (Ile235-Arg356) generated a chimeric Gα that can activate adenylyl cyclase, indicating that the 122 a.a. residues near the C terminus of $G\alpha_s$ are sufficient to stimulate adenylyl cyclase(Masters SB, et al., 1988; Osawa S. et al., 1990). Another study with chimeric Gα subunits which uses a chimera $\alpha_q/\alpha_{i2}/\alpha_q$ containing 78 residue of α_{i2} (residue245-322) shows inhibition of AC

activity(Medina R. et al., 1996). The results imply that those 78 a.a. residues may be involved in effector specifying function. The regions of Gα that interact with effectors have been tested for $G\alpha_t$. A peptide corresponding to residues 293-314 from the COOH-terminal region of $G\alpha_t$ binds to cGMP-PDEγ and can fully mimic $G\alpha_t$ activation of PDE. This region is just adjacent to the receptor activation motif of $G\alpha_t$; thus, the α subunit of $G\alpha_t$ protein has a region for interaction with both effector and receptor near the C-terminus (Rarick HM, et al., 1992). All these studies have shown that the effector specifying region may located between residue 200 to 300 of the Gα subunit.

There is evidence indicating that the C terminal region of $G\alpha_t$ is critical for interaction with the receptor. A peptide corresponding to the C-terminal 11 residues of $G\alpha_t$ not only inhibits stimulation of $G\alpha_t$ by photorhodopsin, but also induces spectral changes in photorhodopsin, mimicking the effects of $G\alpha_t$ (Hamm HE, et al., 1988). Using several approaches such as antibodies, site-directed mutagenesis, and biochemical modification by pertussis toxin, it was found that the C terminal region was crucial for coupling to the receptor (Gutowski S, et al., 1991; Shenker A, et al., 1991; West et, al, 1991; Sullivan KA, et al., 1987). Moreover the C-terminal region is important in determining specificity of coupling to the receptor. Replacement of three residues in the C-terminal region of $G\alpha_q$ with the corresponding residues from $G\alpha_i$ results in a switch of receptor specificity from $G\alpha_q$ to $G\alpha_i$ interacting receptor(Conklin, et al., 1993). In addition to the C-terminal region, the N-terminal region also may be also involved in interactions with the receptor (Hamm HE, et al., 1988). Crosslinking experiments indicate that there is spatial proximity between the C-terminal and N-terminal regions. Additionally, another region

corresponding to a.a. 311-328 of $G\alpha_t$ may also be involved in contacts with the receptor (Hamm HE, 1991).

A chimeric Gα subunit G alpha s/i(38), containing the first 356 a.a. residues of the rat $G\alpha_s$ and the last 38 a.a. for rat $G\alpha_i$ shows more extensive stimulation of AC than Wt. $G\alpha_s$. Dynamic analysis demonstrates that the increase in AC activity may be related to an increase in the GDP dissociation rate for the chimeric Gα(Woon, et al 1989). This may indicate that the $G\alpha_s$ tail has some element that relates to GDP binding.

The N-terminal region of Gα may be important for interaction with Gβγ. Mutation or deletion of the N terminal region impairs Gα and Gβγ binding (Denker BM. et al., 1992; Graf R. et al., 1992; Journot L. et al., 1991, Neer EJ. et al., 1988). This hypothesis was further supported by experiments using monoclonal antibodies against the N-terminal region and the myristoylated N terminal peptide which attenuates Gα and Gβγ binding(Mazzoni MR. et al., 1991; Kokame K. et al., 1992).

The $G\alpha_s$ subunit contains an intrinsic GAP-like domain. When this region is deleted, the truncated protein can still bind GTP and stimulate adenylyl cyclase but it loses its GTP hydrolyzing function. If the GAP-like domain of $G\alpha_{i1}$ is added, GTP hydrolysis function is restored (Markby DW. et al., 1993).

8. Classification of G protein βγ subunits

Like Gα, Gβγ subunits also have regulatory functions. They activate PLA2 in rod outer segments, stimulate cardiac muscarinic-gated K^+ channel, stimulate adenylyl cyclase type 2 and 4 in the presence of activated $G\alpha_s$ and

inhibit Ca^{2+}/CaM stimulated AC1. Gβγ also stimulates PLCβ and modulates some types of Ca^{2+} channels (Herlitze et al., 1996, Ikeda et al.,1996). Gβγ works as a single functional unit. The two subunits are not stable nor folded correctly in a cell when expressed separately (Higgins JB, et al., 1994). To date at least five Gβ and twelve Gγ subunits have been cloned. Gβ, contains on average about 340 amino residues, with about 80% amino acid homology between the different Gβ subunits. The Gγ subunit is only about 75 amino acids long with little homology among subunits. It has been speculated that functional differences among different Gβγ may be related by Gγ (Neer EJ. 1995). The association of different types of Gβ and Gγ is selective. It has been found that Gγ1 does not associate with Gβ2 and Gβ3, whereas Gβ3 does not associate with Gγ 1 and Gγ 2 (Pronin AN. and Gautam N. 1992). Such specific association may be related to structural differences among different Gγ subunits.

Although there is no strong evidence indicating that Gβγ directly interacts with the receptor, it is certain that only the heterotrimer is recognized by receptor (Bourne H. et al., 1991). Some reports indicate that the C-terminal region of Gγ may be involved in specific interactions with the receptor (Kisselev OG. et al., 1995). Data from microinjection of antisense nucleotides into cells suggests that different Gβ and Gγ may be involved in coupling specific receptors to calcium channels (Kleuss C. et al., 1992; Kleuss C. et al., 1993). Many ion channels have been reported to be regulated by G protein α or βγ subunits.

More recently, data from our laboratory indicated that the Gβ surface is primarily involved in contact with effectors (such as AC1, AC2, and $PLC\beta_2$). A peptide from Gβ (residue 85 to 105) can inhibit Gβγ stimulated AC2 activity and simulate $PLC\beta_2$ and partially reverse βγ inhibition of AC1.

Gβγ may also be involved in the desensitization of some G protein-coupled receptors. Activation of the receptor results in dissociation of G proteins from Gα-GTP and Gβγ. Gβγ alone binds to βARK causing βARK to translocate from the cytosol to the cell membrane and phosphorylate the β2 adrenergic receptor (Pitcher JA. et al., 1992; Inglese J, et al., 1992).

9. Molecular structure of Gβγ

It was predicted that Gβ has an α-helix at the N terminal region followed by seven WD repeats (Neer EJ. 1995). Recently, the crystal structure of $G\alpha_{i1}\beta_1\gamma_2$ has shown that the repeated WD motifs in Gβ form a circularized seven fold β propeller. There are two nonoverlapping region of contact between the α and β subunit, an extended interface between Gβ and nearly the complete Gγ subunit that limits Gα and Gγ interaction (Wall MA. et al., 1995). The C-terminal tail of the Gγ, which is modified by prenylation, is believed to be attached to the membranes (Neer EJ. 1995). The crystal structure of the βγ dimer of the G protein transducin has been solved recently. Compared to the structure with $G\alpha_{i1}\beta_1\gamma_2$, this structure reveals interactions between G protein β and γ subunits and highlights regions implicated in effector modulation for the conserved family of G protein βγ dimer (Sondek J. et al., 1996). At the same time, the crystal structure of the heterotrimeric G protein ($G\alpha\beta\gamma_t$) was solved. The comparison of $G\alpha_t$ GDP with the free $G_t\beta\gamma$ structure reveals the mechanism of the nucleotide-dependent engagement of the Gα and Gβγ regulating their interaction with receptor and effector molecules. There are two distinct interfaces that are dramatically changed in the conformation of the Gα but not of the Gβγ subunits (Lambrigh DG. et al., 1996).

10. Lipid Modification of G protein α and βγ subunits

Some Gα subunits need to be modified by lipids. $G\alpha_1$, $G\alpha_z$ and $G\alpha_o$ are post-translationally myristoylated at the N-terminal glycine. Myristoylation of Gα is not only important for binding with Gβγ subunits but also for membrane attachment (Linder ME, et al., 1991). Without such modification, $G\alpha_i$ can not inhibit adenylyl cyclase (Taussig R. et al., 1993 a). However, data from our lab with a purified mutant $G\alpha_i$ * protein that is bacterially expressed and not myristoylated shows a 45% inhibition of FSK stimulated AC activity in cyc-S49 cell membranes (Dedrick et al., unpublished data). Some α subunits also have a palmitoyl group attached to cysteine in the N terminal region (cysteine 3 for $G\alpha_s$ and $G\alpha_o$, cysteine 9 and 10 for $G\alpha_q$). Changes in those cysteine residues abolishes palmitoylation and prevents their membrane attachment (Degtyarev MY. et al., 1994). Palmitoylation of the α subunit is reversible. Some evidence has shown that depalmitoylation of $G\alpha_s$ causes desensitization of $G\alpha_s$ coupled receptors, presumably from a loss of its potent activity to stimulate adenylyl cyclase (Casey PJ. et al., 1994). Many of these findings are controversial, and all of the effects of lipid modification have not been generally observed. An unidentified, possible covalent N-terminal modified $G\alpha_s$ purified from liver shows several hundred fold increase in affinity compare to $G\alpha_s$ purified from other sources such as Sf9 cell and bacteria (Kleuss C. and Gilman A 1997). More recently, it was found that palmitoylation of G_z can inhibit GTPase-accelerating activity of G_z GAP activity to 90% (Tu Y. et al., 1997).

The G_γ subunit can also be posttranslationally modified by isoprenylation. The modifications involve a cysteine residue in the carboxyl

terminal CAAX box(C, cysteine, A, aliphatic, X, any amino acid). The modification is a farnesyl group when x is serine, and a geranylgeranyl group when x is leucine. Only γ1 from G_t (transducin) is farnesylated, while all other G_γ subunits are geranylgeranylated. Farnesyltransferase, the protein which catalyzes protein farnesylation, has been crystallized and the structure has been defined(Park HW. et al., 1997). Isoprenylation may be involved in specific interactions between receptor and βγ subunits (Kisselev OG. et al., 1994). It is also required for membrane translocation (Simond WF. et al., 1991). Mutation of the cysteine residue in CAAX box of Gγ blocks membrane translocation of Gβγ, and revealed the Gβγ anable to regulate effectors (Iniguez-Lluhi J, et al., 1992).

10. Mutations of G protein and G protein related human diseases.

It was found that the mutations in G protein can cause human genetic diseases. The consequences of mutations may not be predictable. It can be either a loss or gain of function(Spiegel AM. et al, 1993). In either case the loss of regulatory function may cause disease. For example, $G\alpha_{i2}$ deficiency may cause human insulin resistance or noninsulin-dependent diabetes mellitus (NIDDM), implicating $G\alpha_{i2}$ as a positive regulator of insulin action (Moxham, et al., 1996). Gene therapy approaches for expressing $G\alpha_{i2}$ in $G\alpha_{i2}$ deficient animals may be a therapeutic modality for NIDDM.

Mutations in G proteins which cause loss of function may result in uncoupling of G proteins from either receptors or effectors. In some Albright hereditary dystrophies, a missense mutation (R385H) of $G\alpha_s$ results in an

uncoupling of $G\alpha_s$ from receptor (Miric A. et al., 1993). Mutations can also cause a G protein to gain function and may result from inhibition of GTPase activity. In some pituitary adenomas, mutations (R201 or Q227) of $G\alpha_s$ (gsp) results in GTPase-deficient proteins that constitutively stimulate adenylyl cyclase (Vallar L. et al., 1987; Landis CA. et al., 1989). Similar mutations have also been found in thyroid tumors (Lyons, J. et al., 1990). The GTPase inhibiting mutation of $G\alpha_{i2}$ (Q205L)(gip2) has been detected in ovarian and adrenal tumors (Lyons J. et al., 1990). In pseudohypoparathyroidism, type Ia patients were found to have an R231H mutation in $G\alpha_s$ (Farfel Z , at al., 1996). This mutation is located in the putative $G\alpha_s$ and $G\beta\gamma$ contact region. Even though the a.a. residue change is among basic residues, receptor mediated $G\alpha_s$ stimulation of AC activity is totally inhibited because $G\alpha_s$ can not bind to $G\beta\gamma$ and only the trimeric G- protein can be activated by agonist activated receptor.

There are some cases of mutations in G protein-regulated effectors which are linked to human genetic disease. A new form of pseudohypoparathyroidism has been found to have normal Gs function but abnormal adenylyl cyclase activity (Barrett D. et al., 1989).

Constitutively activating mutations in the $\alpha 1\beta$-adrenergic receptor can cause neoplastic transformation (Allen LF. et al., 1991). Expression of a GTPase-deficient $G\alpha_i$ mutant ($G\alpha_i$Q205L) can cause transformation of NIH 3T3 cells whereas a deactivating mutant ($G\alpha_i$ G204A) inhibits cell growth (Hermouet S. et al., 1991). $G\alpha_q$ Q205L and $G\alpha_o$ Q205L have also been shown to transform cells (De Vivo M. et al., 1992; Kroll SD. et al., 1992). Up to now point mutations of $G\alpha_q$ and $G\alpha_o$ have not been identified in human genetic diseases.

Since the G protein-signaling pathway is important in cell differentiation and proliferation, any component of the G protein pathway

may be a good candidate for a protooncogene. G proteins may also be involved in anti-neoplastic action since G proteins can increase phosphotyrosine phosphatase activity, which counteracts the action of the tyrosine kinase pathway in cell growth and malignant cell transformation (Pan MG. et al., 1992). Dr. Jianghao Chen in our lab found that co-expressed Ras and $G\alpha_s$* supressed Ras induced transformation of NIH 3T3 cell (Chen J. and Iyengar R. 1994). It also can also suppress tumor growth in Nu/Nu human breast cancer cells (Chen J. et al., 1998). Expression of $G\alpha_s$* in NIH 3T3 cells increases intracellular concentrations of cAMP activating protein kinase A, which inturn inhibits H-Ras-stimulated DNA synthesis and mitogen-activated protein kinase activity.

11. Effectors for G protein

Among all known G protein-regulated effectors, most are transmembrane proteins or membrane-associated proteins. They are either enzymes or ion channels. However, unlike G protein-coupled receptors, the G protein-regulated effectors do not share apparent common features in their structures. This has given rise to the hypothesis that different domains of a distinct subtype of G proteins may be involved in specific effector interactions. Our data however, shows that a single region of Gβ(a.a.86-105) is involved in Gβγ regulation of AC2, AC1, and PLCβ2. A peptide encoding this region can inhibit Gβγ stimulation of AC2 and reverse Gβγ inhibition of Ca^{2+}/CaM activaredAC1. It can also mimic Gβγ stimulation of PLCβ subtypes, even though the effector structures are different (Chen Yb. Weng G. et al., 1997, Ma H. et al., unpublished observation).

Phospholipase C catalyzes the hydrolysis of phosphatidylinositol-4,5-biphosphate to generate two important second messengers: diacylglycerol (DAG), which activates protein kinase C (PKC), and inositol-1,4,5-triphosphate (IP3), which mobilizes Ca^{2+} from the endoplasmic reticulum. Of the three types of PLC (β,γ and δ), only PLCβ is activated by $G\alpha_q$(Taylor SJ. et al., 1991 a and b). At least four subtypes of mammalian PLCβ have been cloned or purified and characterized (Suh PG. et al., 1988; Carrozi A. 1992; Ferreira PA. et al., 1993; John DY. et al., 1993, and 1994). PLCβ1 is highly susceptible to stimulation by $G\alpha_q$ but is only slightly stimulated by Gβγ. In contrast Gβγ is a more potent activator than $G\alpha_q$ for PLCβ2. (Carozzi A. et al., 1993; Park D, et al., 1993a; Smrcka AV and Sternweis PC, 1993; Lee CW. et al., 1993). PLC β4, which has been purified and cloned (Lee CW. et al., 1993), is stimulated by $G\alpha_q$ but not by Gβγ (Lee CW. et al., 1994). The $G\alpha_q$ binding domain in PLCβ1 has been located, but there is still no strong evidence identifying the βγ binding domain in PLCβ subclasses (Park D. et al., 1993b; Wu D. et al., 1993 b). Using an expressed polypeptide competition assay, it has been shown that the polypeptide encoding residues 435-641 in PLCβ2 can strongly inhibit βγ mediated IP3 release from Cos-7 cells in vivo. This indicates that this region may interact with βγ (Kuang Y. et al., 1996).

cGMP-PDE is the effector of transducin (G_t) in the visual signal transduction of the retina. After rhodopsin is activated by photons, it activates the heterotrimeric G protein, $G_t\alpha\beta\gamma$, causing dissociation of $G\alpha_t$-GTP from $\beta_1\gamma_1$. Gαt-GTP activates a potent cyclic GMP holo-phosphodiesterase (holo-PDE (αβγ2)) by displacing its two inhibitory γ subunits. As soon as the $G\alpha_t$-GTP-γPDE dissociates from the αβ-PDE, the αβ-PDE subunits convert cGMP into 5'-GMP. Since cGMP is an activator of the inward Na^+ channels which

are essential for cell membrane depolarization, the sudden decrease in the concentration of cGMP results in a shut down of the inward sodium channels and causes hyperpolarization of the cell membrane potential (Stryer L. 1986 a and b).

12. Adenylyl Cyclase and subclasses

Among all known G protein regulated effector systems, the adenylyl cyclases(AC) are one of most well characterized systems. Adenylyl cyclase activity is stimulated or inhibited in a hormone-dependent manner. Those stimulatory or inhibitory effects are mediated by G_s (Ross EM. and Gilman AG. 1977) or by G_i (Hildebrandt J. et al., 1983), respectively. In the past eight years, nine mammalian AC subtypes have been cloned and characterized. The specific isoforms of adenylyl cyclase are not only different in their amino acid sequences but in their signal recognition and integration as well. These diversities in the functional capabilities of adenylyl cyclase allow for a more versatile response to a wide variety of external and intracellular signals.

13. Molecular homology and functional diversity of adenylyl cyclase

Molecular cloning studies indicate diversity among the 9 forms of AC which have been cloned, isolated, and expressed. For a given AC isoform, conservation across species is typically greater than 90%(Krupinski J. et al., 1992). Adenylyl cyclase1 (AC1) was the first mammalian adenylyl cyclase to be cloned (Krupinski J. et al., 1989). This enzyme was purified from bovine brain.

The amino acid a.a sequence of the protein was identified. Using peptide fragments from this protein the DNA sequence of the fragments was deduced and oligonucleotide probes were made, a bovine brain cDNA library was screened and the AC1 cDNA was cloned. This enzyme is stimulated by Ca^{2+}/CaM, $G\alpha_s$, and FSK, and was inhibited by G_i and $G\beta\gamma$. Again, using the AC1 cDNA sequence, eight additional mammalian AC cDNA types (2 - 9) have been identified. AC2 cDNA was cloned from brain (Feinstein PG. et al., 1991) and AC4 cDNA from testes (Gao B and Gilman AG. 1991). A cDNA cloned from mouse S49 lymphoma cells encodes an adenylyl cyclase (AC7) related to AC2 (Watson PA. et al., 1994). AC7 has also been cloned from erythrolukemia cells (Hellevuo K. , et al 1993). AC3 was cloned from an olfactory neuronal epithelial cDNA library (Bakalyar HA. and Reed RR. 1990). AC5 cDNAs have been cloned from heart (Katsushika, S. et al., 1992), rat liver, and kidney(Premont RT, et al., 1992a). AC6 cDNAs have been cloned from heart (Katsushika S. et al., 1992), liver and kidney (Premont RT. et al., 1992a), S49 lymphoma cells(Premont RT. 1992b) and NCB-20 cells (Yoshimura M and Cooper DMF. 1992). AC8 has been cloned from brain libraries of several species (Cali J. et al., 1994; Defer N. et al., 1994). AC9 has been recently cloned from a mouse brain library (Premont RT. et al., 1996).

Fig. 1-3

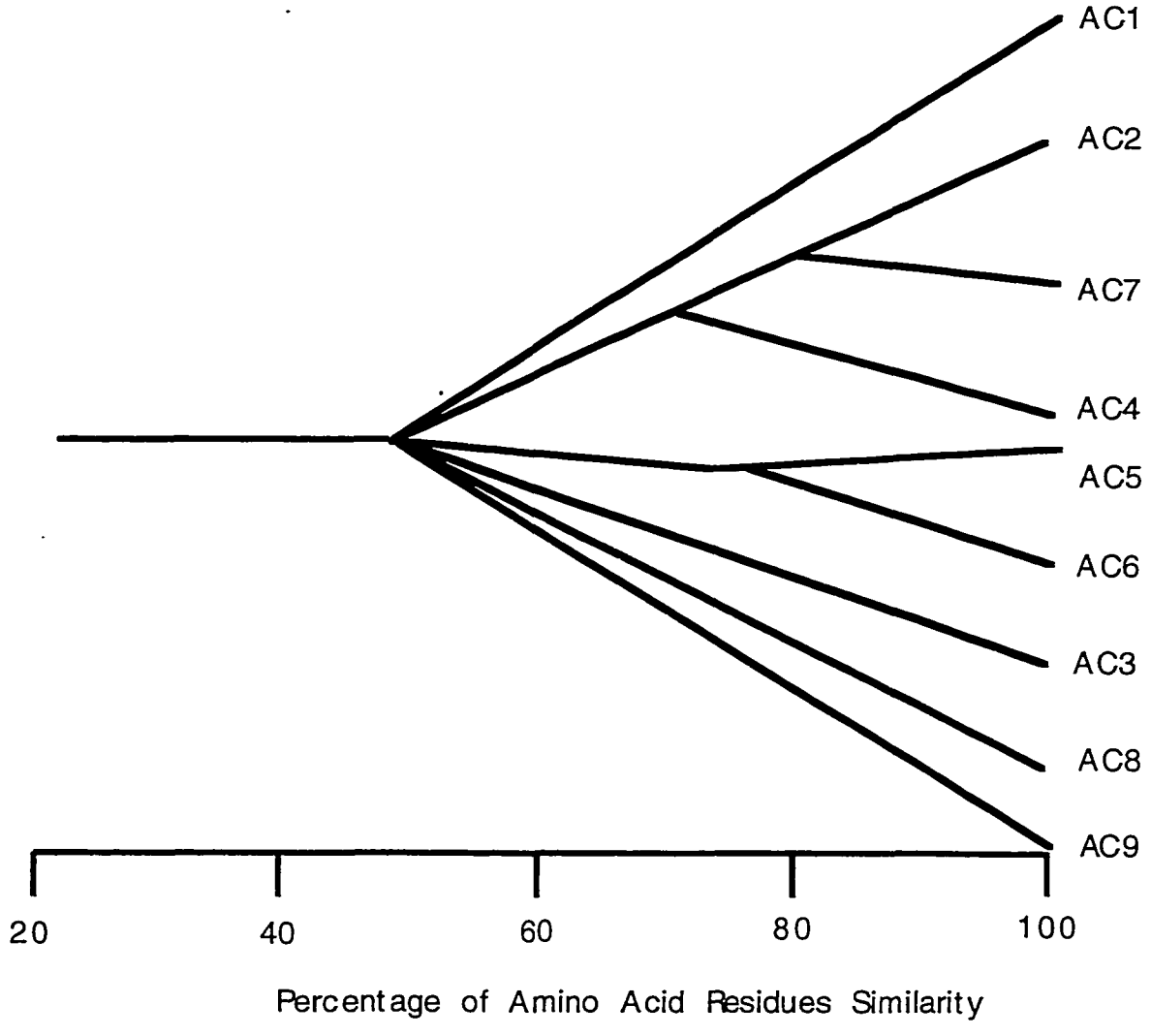


Fig. 1-3 Mammalian adenylyl cyclases nomenclature and similarity among subclasses

Mammalian adenylyl cyclases display similarity and identity of amino acids among their subclasses. The amino acid similarity among each subclasses is between 48 to 53%. The similarity in each subclasses is high, about 70% to 80%.

a) Classification of AC by molecular similarity

The overall similarity between the various mammalian AC isoforms is about 50%. Using homology analysis, mammalian ACs can be divided into at least five sub-families. Two of these families have multiple members: the AC 2 family has three members AC 2, 4, 7. Among the subfamily their similarity is more than 70%. While the AC5 family has two members AC 5, 6, their similarity is also about 70%. The other isoforms AC1, 3, 8 and 9 have a similarity of about 50%. From the view of molecular similarity, they are likely to be members of different subfamilies. The relationships between the different adenylyl cyclases are shown in fig. 1-3.

b. Classification of AC by functional capability

On the basis of similarity of function, adenylyl cyclases can be divided into five subfamilies: The AC 1 subfamily contains AC 1, 3 and 8. This group of ACs are stimulated by calcium (Ca^{2+}) and calmodulin(CaM). The second group is the AC 2 subfamily. This group has two members(AC 2, 4), which are stimulated by $\beta\gamma$ subunits in the presence of activated $G\alpha_s$. The third subfamily is the AC7 group which contains AC 2 and 7. They can be regulated by protein kinase C. They can be directly phosphorylated by PKC which results in stimulation. The next group is the AC5 subfamily which has two members (AC 5 and 6). This group can be inhibited by calcium and by PKA. The PKC effect on this group is complex; AC5 can be stimulated and AC6 can be inhibited(Lai HL. et al., 1997). The last group is the AC9 subfamily. this group has only one member. It is divergent from the other groups in terms of its structure. Functionally, AC9 also shows some differences, it insensitive to Ca^{2+}/CaM and $\beta\gamma$ but is regulated by calcineurin (Paterson JM. et al., 1995).

Within each subfamily, even though each member may function very similarly, whether they are interchangable is still a question? For instance, the functions of AC2 and AC4 are very similar, but are they interchangeable? We don't know the answer, but a targeted knock-out experiment could give us a clue. We know that even though AC1 and AC8 have only 50% sequence similarity, both have very extensive functional similarity. Both can be stimulated by Ca^{2+}/CaM. AC 1 is involved in LTP and learning. In AC 1 knock-out mice, spatial memory and LTP are damaged (Wu Z. et al., 1993). Compensatory increases in the expression of AC 8 does not recover spatial memory. This may indicate that AC1 and AC8 have distinct functional properties in vivo and are not interchangeable even though they have functional similarity in vitro.

14. Natural Evolution and Tissue Distribution

There are substantial differences between the tissue distributions of the different AC: (Iyengar R 1993 a) Different types of adenylyl cyclase are distributed distinctively in the central nervous system (CNS) and peripheral tissues. After millions years of natural evolution, more than 9 mammalian AC subtype exist and all display important characteristics and functions. They must possess profound function which are indispensable from the other enzymes.

AC 1 is localized in the neocortex, hippocampus and dentate gyrus of the brain which are thought to be involved in learning, (Xia Z. et al., 1991;). AC 2 is found in the lung and the brain. In the brain, it is abundant in hippocampus, the granular layer of the cerebellum, and the piriform cortex

(Glatt CE & Snyder SH, 1993). AC 3, originally isolated in olfactory neurons, appears to exist both in neuronal and nonneuronal tissue (Xia Z. et al., 1991). AC 4 is present at very low levels in the brain and is ubiquitous in the peripheral tissues (Gao B and Gilman AG, 1991). AC 5 and AC 6 are also widely distributed in all tissues (Premont RT, et al., 1992a; Pieroni JP. et al., 1993 a and b), but AC 6 is of low abundance in the brain. Although AC 2 and AC 7 belong to the same subfamily, their distribution in the brain and peripheral tissues is different. AC 7 is found in platelets, S49 lymphoma cells, liver and brain cells (Hellevuo K. et al., 1993). In the brain, human AC 7 is mainly localized in the granular layer of the cerebellum (Hellevuo K. et al., 1995). AC 8, like AC 1, is found only in the brain (Matsuoka I. et al., 1992). But AC 8, unlike AC 1, is expressed in hypothalamus (Cali, J. et al., 1994). The AC9 enzyme is widely distributed. The highest amounts of AC9 mRNA are found in skeletal muscle, with significant expression in other tissues(such as brain, liver, kidney, heart and lung)(Premont et al 1996).

It is noteworthy that the distribution of different types of AC is determined by mRNA expression. However, mRNA expression does not always correlate with protein expression. The development AC subtype specific antibodies may help us to further recognize the tissue distribution of AC subtypes.

15. Chromosome location of AC in human genome

Genetic studies indicate that the genes for different subtypes of mammalian adenylyl cyclases are randomly located on different chromosomes. For the human adenylyl cyclases, AC 1 is located on

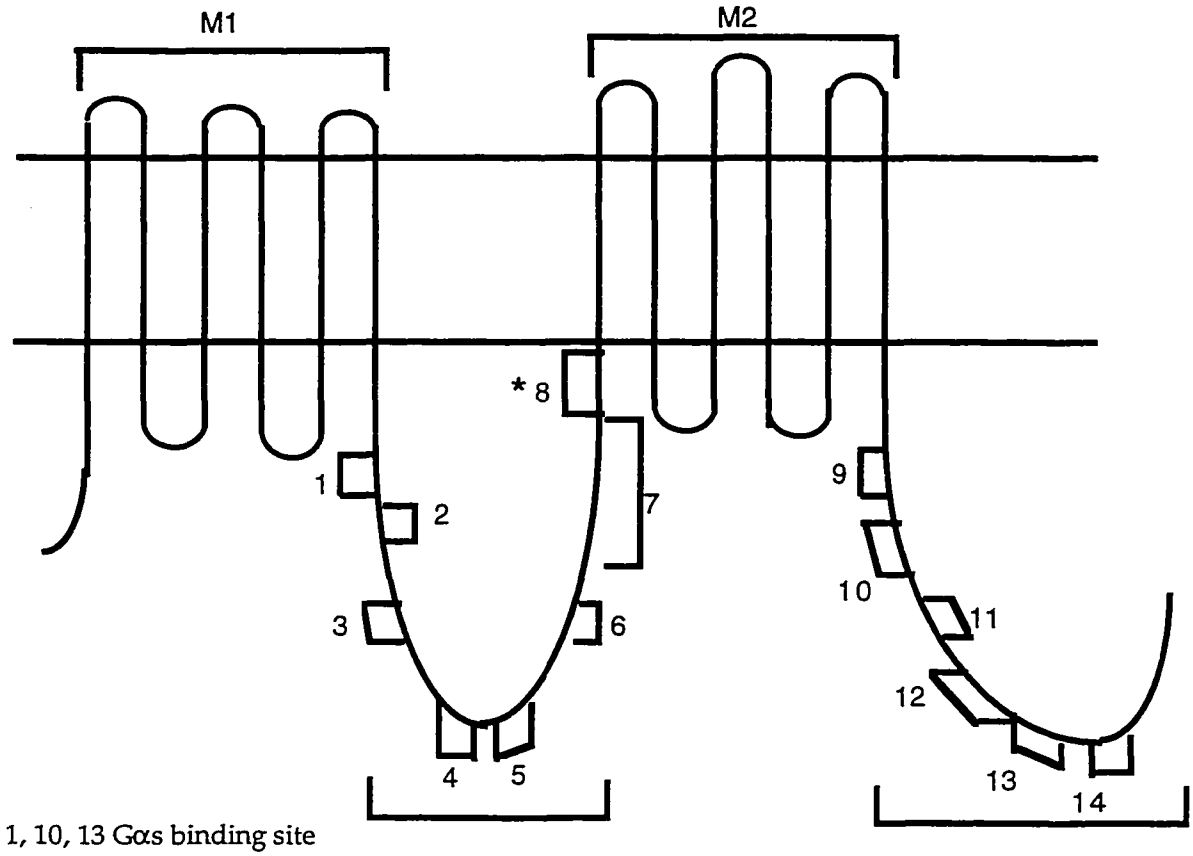
chromosome 7 at 7p12; AC 2 in chromosome 5 at 5p15.5, AC 3 on chromosome 2 at 2p22-2p24; AC 4 on chromosome 14 ; AC 5 on chromosome 3 at 3q13.2-3q21, AC6 on chromosomes 12 at 12q12-12q13, AC 7 on chromosome 16, and AC8 in chromosome sub-band 8q24.2, and AC9 in chromosome 16 at 16p13.3-13.2 (Harber et al., 1994, Gaudin C. et al., 1994; Hellevuo K. et al. 1995 b;, Premont RT. et al., 1996). In addition to different genes, the same gene may encode a single type of adenylyl cyclase but can generate variants through posttranscriptional regulation. Two splice variants of AC 5 (V-large and V-small) are coexpressed in the same species but in a tissue-specific manner (Iwami, G et al., 1996). These two variants are almost identical except in the N-terminal regions. Both can be activated by forskolin and $G\alpha_s$ even though N-terminal regions are different. In AC8, three alternatively spliced isoforms have been seen. Each of the variant's activity is stimulated by Ca^{2+}/CaM, with a distinct K_m for substrate.

16. Molecular Structural Features and Identity of Adenylyl Cyclase Family

Hydropathy analysis of a.a. sequences and structure studies have shown that the known nine cloned mammalian adenylyl cyclases share a common structural feature: they are very similar with each other and look like ion transporters or cell membrane ion channels (Krupinski J et al 1989). A short N-terminal region (N), a six hydrophobic transmembrane domain(M1) and cytoplasmic loop (C1) connected with another hydrophobic domain(M2) and a cytoplasmic tail (C2). C1 and C2 have high similarity and identity. In the two hydrophobic domains, each domain contains six transmembrane segments (Fig. 1-4). The known catalytic and regulatory

activities are in the cytoplasmic domains. The function of the transmembrane domains is not well understood.

Fig. 1-4



1, 10, 13 Gαs binding site
2, 3, 5, 9, 11 FSK binding site
2, 3 Mg2+ binding site
2 pyrophosphate binding site
2, 3, 4, 10, 14 P-site inhibitor binding site or possible ATP biding site
Ca^{2+}/CaM binding site in AC1?
7 Ca^{2+} interaction site in AC5/AC6
8 low affinity Gαs interaction site
12 βγ interaction site

Fig. 1-4 Molecular structural features of mammalian adenylyl cyclases

Mammalian adenylyl cyclases share some common features: both their N-terminal and C-terminal are cytosolic, They have 2 transmembrane domains (M1 and M2), each transmembrane domain contains six transmembrane segments. They have two cytosolic domain(C1 and C2), both share high similarity and identity. The functional interaction regions of AC with different regulators are shown within the predicted secondary structure.

The C1 and C2 regions are similar in many regions among all AC isozymes. They are also similar to the catalytic domain of guanylyl cyclase (Chinkers M, et al. 1991). C1 or C2 when expressed alone does not have catalytic activity. But, when coexpressed, activity and many regulatory functions are restored. This may indicate that the two intracellular domains are not a simple repetition. Some element may be needed for mutual compensation or interaction in order to obtain catalytic function. Newly reported, artificially engineered soluble intracellular domains show catalytic activity and regulatory characteristics. Expression of two intracellular domains, AC1CI and AC2CII, linked artificially with an a.a. residue make up a soluble chimeric AC (Tang WJ, et al., 1995 a). More recently, a non-chimeric soluble form of AC5 was constructed which contains the C1 and C2 regions from AC5(Scholich K et. al, 1997). This enzyme can be stimulated either by FSK or $G\alpha_s$. This may indicate that transmembrane regions are not essential for forskolin or $G\alpha_s$ stimulation. More recently, Tang and his collaborators expressed IC1 and IIC2 separately, then mixed them. Only when two fragments are mixed catalytic activity is obtained(Whisnant R and Gilman A 1996, Yan, SZ. et al., 1996). The difference between those soluble ACs may indicate that there are still some regulatory elements in the transmembrane domain or that the transmembrane domain may be important to maintain structural conformation of the naturally expressed AC. Also, we can not rule out the possibility that the transmembrane regions are important for effective interactions of adenylyl cyclase with the G protein-receptor complex, since G protein-coupled receptors are transmembrane proteins and G proteins are membrane-associated proteins.

Although kinetic experiments with the membrane bound enzyme suggested that there might be multiple FSK binding sites, only one FSK site was identified by equilibrium dialysis for hetero chimeric soluble for forskolinAC. Its K_d is about 0.1μM, which corresponds to EC50 for soluble AC activation(Dessauer C. et al., 1997a). This is an artificially engineered enzyme. In the C1 domain both N-terminal and C-terminal are cut. Also both the M1 and M2 transmembranes domain are absent. FSK is lipophilic, and so it may also interact with the lipophilic transmembrane domains. Moreover, the maximal FSK stimulation of full length AC is by obtained around 300 μM. There are more than three orders of magnitude difference between the EC_{50} and the concentration at which maximal stimulation is observed, indicating that there might be more than one binding or interaction site. Recently, the crystal structure of the truncated cytoplasmic C2 domain of AC2 complexed with FSK was solved(Zhang G. et al., 1997). The crystal structure of AC2 C2 forms a head to tail wreath-like homodimer, The ventral part of two molecules form a ventral cavity. Two molecules of FSK bind in hydrophobic pockets at the ends of the ventral cavity. Since this homodimer does not show good AC activity, the relevance of these forskolin binding sites have been questioned. A couple of weeks ago the structure of the soluble hetero dimer AC5C1-AC2C2 crystal was solved. Because this heterodimer shows $G\alpha_s$ and FSK regulatory AC activity, it was thought as a good model for the native enzyme. In this soluble hetero dimer there are two sets a.a. residues forming a FSK binding site. One set of a.a. residues located on C2 domain organized by Lys^{896}, Gly^{941}, and Ser^{942} form half a binding site. The other half of the binding site is formed by Phe^{394}, Try^{507},Val^{511} and Tyr^{443}, which located on C1 domain(Tesmer et al 1997).

The calmodulin binding domain in AC 1 has been localized to the region encoding amino acid residues 492-522 (the beginning part of C1b region). A peptide corresponding to 492-522 of AC 1 was shown to bind to calmodulin and inhibit Ca^{2+}/CaM stimulation of AC 1 activity(Vorherr T. et al., 1993). This binding site in AC 1 was further confirmed by point mutations of Lys^{504} and Phe^{503} (Wu Z, et al., 1993). The calmodulin binding site in AC 1 has the same structural motif found in other calmodulin-regulated proteins (Vorherr T. et al., 1993). However, other regions may also be involved. Tang et al. identified two amino acids, K350 and K923, which when modified to Ala residues resulted in lack of Ca^{2+}/CaM stimulation (Tang WJ. et al., 1995 b).

Recently, three alternatively spliced AC8 isoforms have been identified. There is no apparent sequence homology between the calmodulin-binding domain of AC1 and the corresponding sequence in AC8-A(Cali JJ. et al., 1996). If AC8-A is aligned with AC1, the calmodulin binding domain terminates 7 amino acids before the start of the 66 amino acid region missing in AC8-C. Interestingly, AC8-C has a three fold higher affinity for Ca^{2+} /CaM. These data suggest that the 66 amino acid region in AC8-A and AC8-B affects the conformational change or plays an inhibitory role in the affinity towards Ca^{2+} /CaM.

All of the regions of adenylyl cyclase involved in interactions with $G\alpha_s$ are not yet known. Anya Harry in our lab has shown that there may be two $G\alpha_s$ binding sites present in AC6; a high affinity binding site and a low affinity binding site (Harry A. et al., 1997). Investigation of the regions of AC 6 involved in $G\alpha_s$ interactions are an important part of this thesis, and I will discuss this issue in detail in the subsequent chapter. A peptide encoding the

conserved region, 425-444 of AC5, located in the beginning part of cytoplasmic loop C1a, significantly inhibits different types of adenylyl cyclase activity (Kawabe J, et al., 1994). Whether this region is a important regulatory element or catalytic element of AC5 is still not known.

17. Differential regulations of adenylyl cyclases

In spite of the overall structural similarity, there are still many differences in the amino acid sequences of various types of adenylyl cyclases. These diversities in sequence among the various types of adenylyl cyclases result in distinct functional differences among each type of adenylyl cyclase. Generally, the activity of mammalian adenylyl cyclases can be regulated by $G\alpha_s$, $G\alpha_i$, $G\beta\gamma$, Ca^{2+} PKC and PKA.

Table 1-1

Regulation of the various adenylyl cyclase subclasses

Regulatory Entity	Adenyly Cyclase types stimulatory effect	inhibitory effect
$G\alpha_s$	1-9	
$G\alpha_i$		1, 3, 5, 6
$G\beta\gamma$	2, 4, 7	1, 8
Forskolin	1-8, 9(barely)	
Ca^{2+}/CaM	1, 3, 8	
Ca^{2+}		5, 6
PKC	2, 5, 7	
PKA		5, 6
CaM kinase		1, 3

Table1-1

All of the cloned mammalian adenylyl cyclases are known to be regulated by at least two types of regulators. Since type-specific regulation of adenylyl cyclases can be stimulatory or inhibitory, the net result is difficult to predict.

$G\alpha_s$

All of the known mammalian adenylyl cyclases are stimulated by $G\alpha_s$. Kinetic analysis of the $G\alpha_s$ stimulation of the different adenylyl cyclases shows that there may be mechanistic differences in the regulation of the different adenylyl cyclases by $G\alpha_s$. Stimulation of AC1 appears to involve a single site, or two sites with similar affinity. But stimulation of AC2 and AC6 appear to involve multiple sites (Harry A. et al., 1997). For AC2 and AC6, forskolin not only increases the affinity of the enzyme for $G\alpha_s$ but also changes the profile of the curve such that stimulation by $G\alpha_s$ appears to involve a single high affinity site. Thus, it is possible that the $G\alpha_s$ and forskolin sites are proximal to each other either primary or secondary, tertiary structure. Point mutations in the cytoplasmic tail in the 950-1050 region of AC2 results in dramatically reduced $G\alpha_s$ stimulation of IC1 IIC2 hetero soluble AC activity(Zhang et al., 1997). The involvement of this region is in agreement with the recently identified forskolin binding site(Zhang G. et al., 1997). More recently, it was shown that the purified VC1 and IIC2 mixture has full FSK and $G\alpha_s$ stimulated activity. The minimum requirement of VC1 is only about a 230 a.a. residue polypeptide. More importantly, it shows $G\alpha_s$ function may simply bring the two polypeptides together to form a high-activity catalytic heterodimer and that FSK may facilitate heterodimer formation(Sunahara et al., 1997).

$G\alpha_i$

All ACs can be inhibited by $G\alpha_i$, although with different sensitivities. Inhibition of cAMP production by receptors that couple to pertussis toxin substrates has been known for the past fifteen years. However, extensive inhibition of adenylyl cyclase is only seen in some cells and tissues, while in others much more modest inhibition is observed. It was found that the different adenylyl cyclases were susceptible to differing extents of inhibition by activated $G\alpha_i$. AC6 was most extensively inhibited in whole cells (Chen J. and Iyengar R. 1993) as well as in vitro assays (Taussig R. et al., 1994). AC5, which is very similar to AC6, is also extensively inhibited by $G\alpha_i$. In contrast , of all adenylyl cyclases tested, AC2 was the least inhibitable in the intact cell

$G\beta\gamma$

Regulation of adenylyl cyclases by $G\beta\gamma$ subunits is the most complex phenomena in this system. Several type of adenylyl cyclases can be regulated by $G\beta\gamma$. AC 1 is inhibited by $G\beta\gamma$ (Katada T, et al., 1987), but AC 2 and AC 4 are stimulated or potentiated by $G\beta\gamma$ in the presence of activated $G\alpha_s$(Tang WJ and Gilman AG, 1991). The effects of $G\beta\gamma$ on purified AC 1 and AC 2 indicate that interaction between $G\beta\gamma$ and adenylyl cyclase is direct (Taussig R, et al., 1993 b). Since the concentration of $G\beta\gamma$ required to modulate adenylyl cyclase activity is much higher than that of $G\alpha_s$, it appears likely that $G\alpha_s$ and $G\beta\gamma$ bind to different sites on AC2. However less than 2 nM $G\alpha_s$ is sufficient to obtain full stimulation by $G\beta\gamma$ subunits. Moreover, since such low concentrations of $G\alpha_s$ can initiate such powerful $\beta\gamma$ stimulation of AC2, my guess is that the sites for high affinity $G\alpha_s$ interaction and $G\beta\gamma$ interaction will not be too far apart from one another, at least in three-dimensional space.

Gβγ and AC2 interaction site

A Gβγ interaction site on AC2 was identified by Chen J in our lab (Chen J et al., 1995). Peptides encoding different regions of AC2 were designed to compete Gβγ binding to AC2 to find the regions in AC2 which are important for Gβγ binding. A peptide named QEHA, encoding residue 956 to 982 in AC2, strongly inhibits Gβγ stimulation of AC2 but does not affect basal or $G\alpha_s$ stimulate AC2 activity. A functionally important motif, Gln-X-X-Glu-Arg, is also found in conserved regions of the potassium channel and β adrenergic receptor. Cross linking experiments reveal the QEHA peptide interacts with Gβ (Weng G. et al., 1996). Using the yeast two hybrid system it was found that the QEHA region of AC2 specifically interacts with a.a. residues 50-100 of Gβ(Yan K. et al., 1996), with these data and the crystal structure of Gβγ, Dr. Weng G., in our lab, used computer docking and modeling techniques to find a surface on the G protein β-subunit involved in interaction and regulation with AC2 and AC1 (Weng G. et al., 1997).

Ca^{2+} and Ca^{2+}/CaM

Almost all types of adenylyl cyclases are regulated by the PLC pathway. AC 1 is activated by Ca^{2+} /CaM in the absence of $G\alpha_s$ (Tang WJ, et al., 1991). In intact transfected cells, AC 1 can be activated by the muscarinic ACh receptor which activates the PLC pathway (Choi EJ. et al., 1992a). AC 8 is also stimulated by Ca^{2+}/CaM, although there is very low similarity of a.a. sequence between AC 8 and AC 1 (Cali J, et al., 1994).

The function of AC 1 may be involved in learning because loss AC 1 function causes defects in learning in Drosophila(Levin LR. et al., 1992). In mammalian systems, it has been demonstrated that an increase in cAMP is

critically important to LTP in the hippocampus (Chetkovich DM. et al., 1993) and LTP is important for learning.

AC 3 is also activated by Ca^{2+} /CaM in the presence of $G\alpha_s$ or forskolin activation, but a higher concentration of Ca^{2+} is needed for stimulation than for AC1 (Choi EJ. et al., 1992b).

In contrast to AC1, 3, 8, AC 5 and AC 6 are inhibited by low concentrations of Ca^{2+} (Yoshimura M & Cooper DMF, 1992). Since they are highly expressed in cardiac tissue, it has been proposed that these ACs are important in regulation of cardiac contractility (Cooper DMF and Brooker G. 1993). As cAMP causes Ca^{2+} entry, Ca^{2+} feedback inhibition of cAMP production is predicted to be the mechanism of oscillation for both the levels of cAMP and Ca^{2+} (Cooper D. et al., 1995). In AC5, the Ca^{2+} binding regions are located in the intracellular domain near second transmenbrane domain (Scholich K. et al., 1997). It may also be true for AC6 since in this region the two enzymes share high homology.

Regulation of adenylyl cyclases by P-site ligands

AC can be inhibited by purine nucleotides. These compounds are referred to as P-site inhibitors because an intact purine ring is required for inhibition (Johnson RA. et al., 1989). The most potent P-site inhibitors are the dideoxy adenosine analogs(Desaubry L et al., 1996). The stimulated forms of adenylyl cyclases, such as activity in the presence of Mn^{2+} or hormone stimulated activity, are most susceptible to inhibition by P-site compounds. The exact location of P-site inhibition has been solved by the

crystal strucuture of the hetero soluble adenylyl cyclase. The P-site is formed by α1 and β2-β3 loop β4 and α4 in both C1 and C2 domains. D^{396} D^{440} in C1 K^{938}, D^{1018}, R^{1029} in C2 are involved in contact with P-site inhibitor 2'd3'AMP(Tesmer et al., 1997). Inhibition is thought to occur by direct interactions with a domain distinct from the catalytic site, although some parts of the P-site also appear to be part of the catalytic site. Some mutations in AC1 were shown to alter ATP and P-site ligand binding to the same extent, whereas other mutations has differential effects (Tang WJ. et al., 1995 b). Mutation of Lys ^{923} to Ala in AC1 results in a greater than 90% inhibition of basal, or $G\alpha_s$ and CaM stimulated activity. The mutant enzyme shows substantial (40% greater than wild type) activity in the presence of Mn^{2+} and FSK but shows a hundred-fold decrease in sensitivity to P-site inhibition. These data indicate that Lys^{923} is important for both catalytic activity in the presence of Mg-ATP and for P-site ligands. Besides, the configuration of the catalytic site appears to be different for Mg-ATP and Mn-ATP.

PKC

Adenylyl cyclases can be regulated by PKC. Experimental data in other labs and ours have shown that AC 2 activity is greatly stimulated by phorbol ester treatment (Jacobowitz O, et al., 1993; Yoshimura and Cooper DMF., 1993; Jacobowitz O. and Iyengar R., 1994). Immunoprecipitation of Flag epitope tagged AC2 reveals direct phosphorylation of AC 2 following phorbol ester treatment or direct PKC treatment (Jacobowitz O. and Iyengar R., 1994). It was found that the region important for phorbol ester stimulation is located near the C terminal region of AC 2 (Levin LR. and Reed RR., 1995). Since AC 2 can

be stimulated by $G\alpha_s$, $G\beta\gamma$ and PKC, it serves as a good example of a G protein-regulated effector that is capable of integrating coincident signals from the G_s, G_i and G_q pathways (Lustig KD.et al., 1993). AC 7, which belongs to AC 2 subfamily, is also stimulated by pretreatment with phorbol esters (Hellevuo K, et al., 1995a). AC4, displaying almost identical regulatory properties as AC2 with respect to their responsiveness towards G protein subunits, is not stimulated by protein kinase C . No significant stimulation or inhibition is observed for AC 1 and AC 3 (Jacobowitz O, et al., 1993; Yoshimura M and Cooper D. 1993; Choi EJ, et al., 1993). Purified PKC can directly stimulate purified AC 5 by direct phosphorylation of AC 5, but for some reason, in intact cells such stimulation could not be seen (Kawabe J. et al., 1994). Lately, the same group has shown a 5 fold increase in forskolin stimulated cAMP accumulation in HEK 293 cells stably expressing AC5, with only a 60% increase of basal activity on phorbol ester activation(Kawabe J.et al., 1994). More recently, it was shown that AC5 is differentially phosphorylated and activated by PKC isoenzymes α and ζ, suggesting the AC5 is regulated by PKC in an isozyme-specific manner (Kawabe J., et al., 1996). PKC ζ can phosphorylate AC5, leading to a 20 fold increase in its catalytic activity.

18. PKA and AC regulation

PKA regulation of the adenylyl cyclase pathway will be discussed in detail in the next several chapters. AC converts ATP to cAMP. cAMP can directly activate cAMP-dependent protein kinase (PKA) and other downstream elements which contain important biological functions. The PKA pathway is important because it regulates various target proteins by phosphorylation

cascades. PKA can phosphorylate the key enzyme glycogen phosphorylase in glycogen metabolism, PKA also regulates transription factors which interact with cAMP response elements (CREs) in their promoter regions. Phosphorylation of the CRE binding protein (CREB) causes binding of CREB to CREs and regulates target gene transcription (Lalli E. and Sassone-Corsi P. 1994; Sheng M. and McFadden G.,1990). It has been demonstrated that cAMP and PKA are required for long-term potentiation (LTP)of synaptic response.

Since the identification of cAMP as an intracellular second messenger for hormone mediated cellular responsive events by Sutherland (Sutherland EW. et al., 1958; Sutherland EW. et al., 1972), many investigators have focused on downstream effectors of cAMP. This led to the discovery of a cAMP-dependent protein kinase (PKA) by Krebs and colleagues (Krebs and Fisher 1956; Walsh DA. et al., 1968). Since then, it has been demonstrated that more and more, different hormones utilize the cAMP pathway and activate PKA. PKA phosphorylates its target substrates and initiates a variety of diverse biochemical events and physiological responses (Krebs et al 1989, Taylor et al 1990).

Any protein containing the motif of amino acid(a.a.) residues BBXS(T) can be a substrate of PKA. Here, B stand for basic a.a. residues either (Arg or Lys), X represents any amino acid residue, S represent serine. If the substrate motif BB is two Arg in substrate, PKA has the greatest catalytic activity. But if XS is changed to NA, the motif XXNA, then this is a strong PKA inhibitor. PKA is a soluble enzyme. It is hard to believe that the kinase is able to rapidly and preferentially phosphorylate the correct target proteins in response to activation of specific individual hormones by freely diffusing throughout the cytoplasm and picking up the target. It is also hard to believe that in order to

activate PKA cytosolic cAMP should be keep at a high concentration level, which may also affect regular cellular activity. It might be more logical to assume that PKA is compartmentalized with its substrates, and therefore, individual hormones can preferentially activate specific pools of the kinase. Compartmentalization of PKA might then be a key regulatory event. cAMP accumulates in different cellular compartments (Adams SR. et al., 1991), and PKA subunits are detected in distinct cellular compartments (Nigg EA., et al 1985a, 1985 b). Recently, it has been shown that type II PKA holoenzyme (R2C2) can be tethered at specific subcellular locations through interaction with A-kinase Anchoring proteins (AKAPs) (Scott JD. et al., 1992).

Chapter 2

statement of problems and specific aim

Chapter 2

Statement of Problems and Specific aim

In the past eight years nine mammalian adenylyl cyclases have been cloned. Several important functions such as catalytic and regulatory properties common to all AC subtypes have been characterized. Some functions specific to the individual isoforms have been less well characterized. AC6 and AC5 were cloned in our lab. Their function and activity have been characterized by R. Premont, J.Pieroni, and A. Harry in our laboratory (Premont R. et al., 1992 a; Pieroni J., et al b., 1995, Harry A. et al., 1997), and researchers of several other groups (Katsushika, S. et al., 1992, Yoshimura M. et al., 1992, Scholich K. et al., 1997). AC5 and AC6 are widely distributed in different species and different organs. They are present in almost all important organs such as heart, liver, and brain. A thorough understanding of the function of these enzymes has as not yet been achieved and the regulatory mechanisms of these enzymes are only partially understood.

Compared to other signal transduction pathways, the G protein-coupled adenylyl cyclase pathway is among the most well studied. In recent years research in this area has made tremendous progress, but an enormous number of questions and problems still remain. It is important to further study this system in an intensive fashion.

During studies on glucagon-induced desensitization in chick hepatocytes in our laboratory, R. Premont found a component of AC desensitization that was cAMP dependent. Addition of excess purified $G\alpha_s$ to desensitized membranes did not restore full activity to membranes from cells treated either with glucagon or 8-Br-cAMP. Treatment of membranes from naive but not desensitized cells, *in vitro* with PKA also resulted in decreased AC activity (Premont R. et al., 1992 b). Studies on the kin$^-$ variant of S49 cells also indicate that PKA treatment results in decreased forskolin stimulated adenylyl cyclase activity . Since $G\alpha_s$ itself is not a target for PKA regulation (Premont R. et al, 1992 b), it appeared that ACs in these cells was the most likely target for the PKA mediated regulation. mRNA expression analysis had shown that chick hepatocytes contain both AC6 and AC5 (Premont R., et al., 1992 b). AC6 and AC7 are also expressed in S49 cells (Krupinski et al., 1992). Since both chick hepatocytes and murine S49 cells have AC6 in common, it is possible that this is the isoform of adenylyl cyclase which is inhibited by PKA. PKA has a broad specificity; any protein or peptide containing the Lys/Arg-Lys/Arg-X-Ser motif can be a candidate for PKA phosphorylation. Using this definition as a rationale, we screened the sequences of cloned ACs and found that all ACs except AC4 contain one or more consensus PKA phosphorylation sites. Although the regions of individual AC subtypes which contain the PKA phosphorylation motif are varied, AC5 and AC6 have one region in the same position near the M2 cluster transmembrane domain. My preliminary data showed that $G\alpha_s$ stimulated activity of AC6 can be regulated by PKA. At the same time, Ishikawa and co-workers showed that PKA treatment of AC5 results in decreased FSK stimulated AC5 activity as well (Iwami G. et al., 1995).

The molecular mechanism of AC6 regulation by PKA has not been elucidated. It has yet to be demonstrated whether AC6 is the substrate of PKA,

or if the PKA effect on AC6 is a direct or indirect effect. If PKA directly regulates AC6 then how does phosphorylation affect $G\alpha_s$ stimulation? Does PKA phosphorylation change the AC6 active conformation? Does PKA phosphorylation change AC6 folding? Does phosphorylation block $G\alpha_s$ binding?

During my thesis research I could not answer all the questions, rather I chose to focus on major questions.

1)Is AC6 selectively regulated by PKA?

2)What are the consequences of PKA regulation of AC6? Is the basal catalytic activity affected or only stimulation by various entities? These experiments include the study of the effects of PKA on $G\alpha_s$, forskolin and Mn^{2+} stimulated activities.

3)What is the location of the site phosphorylated by PKA? Does phosphorylation at this site regulate AC6 activity?

Chapter 3

Materials and methods

Chapter 3

Materials and Methods

I. Materials

a. Chemicals and Reagents

Molecular Biology:

Plasmid mini preparation kit and Maxi preparation kit were purchased from Qiagen. Magic Minipreps and Promega Magic Maxipreps were from Promega(Madison, WI). through the Brookdale Center. GeneClean II 3106 Kit was purchased from Bio101, (La Jolla, Ca). Original TA cloning^{R} kit was purchased from Invitrogen(San Diago, CA). Epicurian Coli XL1-Blue supercompetent cells were from Stratagene. (La Jolla, CA) Sequenase Version 2.0 DNA Sequencing Kit was purchased from Amersham Life Science (Arlington Heights, IL).

Bacterial growth medium

Bacterial broth and agar ingredient including: D-glucose, bactotryptone , yeast extracts agar were purchased from DIFCO(Detroit MI). Tetracycline and Ampicillin Agarose, Trizma base(Tris) , EDTA, glacial acetic acid, Phenol and phenol chloroform isoamyl alcohol(25:24:1) were from Sigma (St louis MO). Glassfiber was purchased from Fisher Scientific (Fair Lawn NJ) and coated with Sigmacote, which was also from Sigma.

Protein Analysis:

Acrylamide, 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 purchased from 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-mercaptoethanol, DTT, glycine, EDTA, EGTA ovalbumin, Lauryl sulfate (SDS), Lubrol, Isobutylmethylxanthine(IBMX), Creatine phosphate, cAMP, ATP, GTP, Phenylmethylsulfonyl fluoride(PMSF), 1,10 phenanthroline, Leupeptin, Aprotinin, Trizma base, HEPES, Amonium persulfate, Temed, Magnesium chloride were all purchased from Sigma(St. Louis, MO). Methanol, Acetone, 2-propanol, 2 and 10N NaOH were from Fisher. Whatman microfibre filter GF/A GF/B, Hybond C extra nitrocellulose, ECL Western blotting kit were all from Amersham Life Science (Arlington Heights, IL).

Cell Culture:

Gentamycin 500X (50mg/ml) was purchased from Boehringer Mannheim (Indianapolis, IN). Horse serum was from Gibco BRL (Gaithersburg, MD) or Hyclone (Logan Utah). All cell culture media such as: Sf-900 media, Graces supplemented media, DMEM media, Penicillin and

Streptomycin, Fetal calf serum or Fetal bovine serum were purchased from Gibco BRL (Gaithersburg, MD), or Hyclone(from Hybrydoma Center MSSM).

b) Biochemicals and proteins

cAMP dependent protein kinase catalytic subunits and cAMP dependent protein kinase inhibitors were purchased from Promega (Madison, WI), GTPγs, Okadaic acid was from Sigma(St. Louis, MO). Most $G\alpha_s$* used in these studies was in vitro expressed and prepared by Dr. Anya Harry. Some bacterial $G\alpha_s$* was purified by Dr. G. Weng. The purified βγ subunit was a gift of Dr. John Hikdebrandt. All the peptides used in these studies were made by Dr. Jingrong Li.

Enzymes

Restriction enzymes, T4 DNA ligase, T7 DNA polymerase, Alkaline Phosphatase, T4 polynucleotidase were purchased from New England Biolab (Beverly, MA). Lysozyme, DNase, RNase, creatine phosphokinase, myokinase were all from Sigma (St. Louis, MO). *Pyrococcus furiosus* (Pfu) DNA polymerase was from Stratagene (LaJolla, CA). Taq DNA polymerase was from Promega (Madison, WI).

Radiochemicals

[α^{32}P]-ATP(30 Ci/mmol, 2.0mCi/ml), [γ^{32}P]-ATP(3000ci/mmol, 10.0mCi/ml), [α^{35}S]-dATP(250μCi/25μl), were purchased from DuPont NEN (Boston, MA),

2-,8-[^{3}H]-cAMP (28 Ci/mmol, 1mCi/ml), [^{35}S L-]-methionine was purchased from ICN (Irvine, CA).

Antibodies

Antiserum labeled, AC common was prepared by Dr. Richard Premont. Rabbits were boosted with a peptide sequence NH2-IGARKPQYDIWGNT-COOH common to adenylyl cyclases 1-8(Premont R. et al., 1989). Anti-FLAG-M-2-antibody and anti-FLAG-M-2-affinity Gel purchased from Eastman Kodak(New Haven, CT).

II. Methods

A. Cell culture

1) Insect cell culture

The insect cell lines used for expression of recombinant AC were a)the Sf9 cell, derived from Armyworm Ovary, Spodoptera frugiperda (Sf9). b) Hi-5 derived from *Trichoplusia ni.* egg cell. The Sf9 cells were obtained from the Mount Sinai Baculovirus Expression Core Facility and American Type Culture Collection, Rockville, Maryland. The Hi-5 cells were a gift from Dr. Peter Graves (Mount Sinai School of Medicine) and originally obtained from Invitrogen. Monolayer cultures were incubated at 26-28$\pm$ 1°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 mg/ml gentamycin for Hi-5 cells. Cultures were not used beyond 30 passages.

Cells in the log phase stage of the growth curve (at about 80% confluent) were infected with a multiplicity of infection (MOI) between 5 and 10. Cell viability was greater than 97% as determined by Trypan Blue exclusion. Cells were harvested 48-96 hours post infection.

2)S49 cyc-mouse lymphoma cell culture

S49 cyc- cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) plus 10% horse donor serum which was heat inactivated at 56°C for 30 minutes, and 5% Fetal bovine serum(FBS) in loosely capped flasks. Maintenance of exponential growth is dependent on a pH optimum around about pH 7.4. Cells were incubated at 37°C with 95:5/% O2:CO2. S49 cyc- cells were obtained from the University of California, San Francisco Cell Culture Facility.

B. Membrane preparation

To prepare membranes from cells expressing ACs, 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 MgCl 2, 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, (optional 3.2 μg/ml soybean trypsin inhibitor (SBTI)), 2 mg/ml aprotinin. Cells were lysed by nitrogen cavitation in a Parr bomb at 600 psi for 20 - 30 minutes on ice in the

cold room, then centrifuged at 1000xg for eight minutes at 2°C with no brake applied to pellet debris that contain intact cell and nuclei. The supernatant was taken and spun at 100,000xg for forty-five minutes at 2-4°C to pellet the crude membranes. The pellet was resuspended in 20 mM NaHepes (pH8.0), 1 mM EDTA, 200 mM sucrose, 2 mM DTT and a cocktail of protease inhibitors (same as lysis buffer). Crude membranes were aliquoted and were frozen in dry ice-acetone bath or liquid nitrogen and stored in a -80°C frezer. Protein concentration was determined by either the Lowry method or Bicinchoninic Acid (BCA) Protein Assay detection system using bovine serum albumin as the standard.

Solubilized AC2 and AC6 protein was prepared by extraction with 0.8% dodecyl maltoside in 20 mM HEPES pH 8.0, 150 mM NaCl, 2 mM DTT, 1mM EGTA, 1mM EDTA, 20% glycerol, , 1mM PMSF, 1mM phenanthrolin, 3.2 μg/ml leupetin, 3.2 μg/ml SBTI, 2 mg/ml aprotinin. The mixture was first rocked gently in a 4°C cold room for 4 hours, and then spun down at 100,000xg for 45 minutes at 4°C. The supernatant was removed and the detergent concentration reduced to less than 0.05% by Centricon10. The preparation was assayed immediately or frozen and stored at -80°C.

cDNA, Vectors and Viruses

Subcloning and Expression FLAG AC6 and FLAG S674A- AC6

Construction of FLAG AC6 cDNA

The reason for adding an epitope such as FLAG to AC6 was that I might need to purify the recombinant enzyme expressed in insect cells to be able to visualize the phosphorylated enzyme and purify AC6 for further functional characterization. Additionally, monoclonal anti-FLAG-M2-antibodies are commercially available for identification of the expressed enzyme.

All oligos were synthesized from Genset(La Jolla CA)

PCR primers 1

5′ primer, named YBC 005 from 5′ to 3′ GCTCT**GGATCCT-----ATG-----**

BamHI site initiation site

GACTACAAGGACGACGATGACAAG-CCCCTGCCCGTGGCCCGATCC,

FLAG-epitope first seven amino acids

This oligonucletide(oligo) contains a BamH1 site and a methionine initiation site , FLAG and the first 7 amino acids of AC6.

PCR primer 2

3′primer, named PR 187 from 5′ to 3′ is 5′GG CGCGGCGTGGAAGGTC

This oligonucleotide was not newly synthesized. Dr. R. Premont had used it for cloning AC6. Using AC6-PVL1393 (which was subcloned by Chen J) as template, the PCR reaction was performed for 30 cycles (10 min denaturation then adding DNA polymerase pfu, 1 min at 95°C for denaturing, then 1 min at 45°C for annealing, and 2′30′′ at 72°C for elongation). A 0.6kb PCR product was obtained. The 0.6kb fragment contained FLAG and the first 185 amino

acids of AC6. This fragment was cut with BamHI/NheI, and run on a 1% agarose gel to obtain a 0.4 kb fragment which was purified with Gene Clean. A fresh maxiprep of AC6-PVL1393 was cut with BamHI/NheI and treated with Alkaline Phosphoatase and run on a 1% agarose gel. The 12.0kb fragment was separated by agarose gel electrophoresis and purified by gene clean. The 12.0kb fragment and 0.4kb fragment were ligated by T4 DNA Ligase. The ligation products were transformed into XL1Blue competent cells. Ampicilin and Tetracycline were used for selection. 12 positive colonies were picked and cultured for miniprep. The DNA was verified by restriction enzyme digestion, and the DNA of correct size FLAG-AC6-PVL1393 were selected for sequencing. After sequencing, AC6-PVL1393 cDNA were further purified and sent to the Core Facility of MSSM for production of recombinant baculovirus.

Construction of S674A-AC6 -PVL1393 cDNA

PCR primer 2

5′ primer named YBC003 from 5′ to 3′ was

5′CCAGAGGGAGGATCTCGAGAAGAAGTATGCACGGAAAGTAGACCC,

This oligo encodes AC6 cDNA from 2005 to 2049. Here the T2033 is changed into G2033. In this oligo TCA which encodes a Ser residue is mutated into GCA which encodes Ala. The 3′ primer antisense oligo from 5′ to 3′ was GCGGGCGGTAGATTCCACCTG which encodes base pair 2812 to 2792 of DNA of AC6, AC6-PVL1393 cDNA used as a template. The PCR reaction began at 95°C 5 min for denaturation, 1 min. annealing at 55°C then Taq DNA

polymerase was added and then the reaction was run for 28 cycles (1 min at 95°C for denaturation, 1 min at 55°C for annealing, and 2'30'' at 72°C for elongation). The reaction yielded a single 0.8kb PCR product which encodes the sequence of the PKA phosphorylation motif LEKKYSRKVDP, but with S now changed to A. The 0.8kb PCR product was subcloned into pCR 2.1 vector by using the TA cloning kit(Invitrogen). After sequencing the 0.8kb PCR-pCR^{R}2.1TA cloning products, the correct DNA product was cut with XhoI/BlpI and a 0.6kb fragment was obtained. Since XhoI, which was used to cut the PCR-pCR^{R}2.1TA cloning product which contains the mutated site, also cuts the vector-pVL1393, a three piece fragment ligation method was designed.

Fig3-1 Schematic diagram for the construction of FLAG S674A-AC6

1) FLAG-AC6 was cut with BamHI/XhoI, resolved on an agarose gel, and a 2kb fragment was obtained. The fragment named, fragment 1, contains a BamHI site and a XhoI site.

2) FLAG AC6 was cut with BamHI/BlpI. Here the fragment contains the pVL1393 vector and the tail of AC6. The fragment was treated with Alkaline Phosphatase to prevent self annealing. This fragment was named Fragment 2.

3) The 0.6kb fragment described in the previous section was named fragment 3.

The three fragments were ligated together by T4 DNA Ligase. After transformation the clones were selected and identified using procedures previously described as FLAG-AC6 construction.

The cDNA's encoding adenylyl cyclase types 2(AC2) and 6(AC6) were subcloned by other members of our laboratory. AC2 and AC6 cDNA's were subcloned into 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 the Mount Sinai School of Medicine. Large scale infection of Sf9 or Hi-5 cells was carried out with recombinant baculovirus and TPO-XS (as control) at a multiplicity of infection between 5 and 10. Sf9 or Hi-5 cells were grown in appropriate medium at 26- 28°C and harvested 48-96 hours post infection.

The cDNA for AC2 was the kind gift of Dr. R. Reed from Johns Hopkins School of Medicine, cDNA's for AC1 and AC6 were isolated by our laboratory. The plasmid vectors, pVL1392 and pVL1393 were the kind gifts of Dr. Max Summers of Texas A&M University.

Adenylyl cyclase assays

The assay was carried out following the conditions of Johnson and Solomon (1991). Sf9 or High 5 cell membrane preparations were assayed in the presence of 0.1 mM ATP and [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 (0.01 mg/ml myokinase, 200 mM creatine phosphate and 0.1 mg/ml phosphocreatine kinase), 2-10 mM $MgCl_2$. Typically, 2-10 µg of crude membrane protein was assayed per sample in a total volume of 50 µl at 33°C for 15 minutes. The reaction was stopped by adding 100 µl of stop buffer which contains 2 mM of ATP, 2 mM of cAMP, and 1% of SDS. Samples were mixed with 1 ml ddH_2O and applied to Dowex-50 columns. The Dowex columns were then washed with 3.0 ml water. This was followed by 4.5 ml elution from Dowex columns into alumina columns. The cAMP-containing portion (3 ml) was collected which eluted from alumina columns with application of 3.0 ml 0.1 mM imidazole (pH 7.5) after an initial wash with 1.5 ml 0.1 mM imidazole (pH 7.5). The sample was vortexed and mixed very well with scintillation cocktail. After 1 hour of sitting in the dark, the sample were counted in a Beckman LS 5000TD scintillation counter . Adenylyl cyclase activity was expressed as pmol /mg protein/min.

AC6 phosphorylation by PKA

AC6 membranes were treated with PKA in buffer solution containing 25-40mM Tris-HCl (pH7.5),10-20mM $MgCl_2$, 50-75μM PKA catalytic subunit(2500U/50ul), 0.8-1.0mM ATP, proteinase inhibitor cocktail, 0.1-0.2mM DTT, Typically either 5-10μg membrane protein was assayed in a test tube or a big batch (150-300 μg membrane) in one tube. Samples were incubated for 15-30 min. at 33- 35°C, and stopped by placing the incubation tube on ice. The sample for control was treated with same buffer, but no PKA was added. The membranes were then aliquoted for individual adenylyl cyclase assay.

SDS-PAGE, Immunoblotting

Sample treatment and solubilization

Adenylyl cyclase containing crude membranes (in some cases, treated with 1% DNase for 15 min. at room temperature) were dissociated in 1% SDS solution by adding 10% SDS solution and adjusted to a final concentration of SDS of 1%. The mixture was rocked gently at room temperature for 60 min. or 45 min. at 37°C for solublization. Boiling the proteins has to be avoided since it will cause aggregation of the membrane proteins. After one hour solubilization the samples were centrifuged at 10000g to spin down any insoluble material and the supernatant was applied to an acrylamide gel.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) separates proteins based on molecular weights (Laemmeli, 1970). 7% Acrylamide separating gel was used for electrophoresis of adenylyl cyclase preparations. The Acrylamide: Bis ratio was 29:1. Gels were run at 150V constant voltage and either stained in 0.1% Coomassie blue or proteins were transferred to Hybond C nitrocellulose membrane for immunoblotting.

Immunoblotting

The immunoblotting technique was carried out according to Towbin (Towbin H. et al., 1979). The gel containing the resolved proteins was incubated in transfer buffer which contained 25 mM Tris, 0.192M Glycine, and 15% methanol for 10 minutes. Proteins were transferred onto Hybond-C extra nitrocellulose membrane using 70 V for one hours at 4°C or 35 V overnight at 4°C in a wet blotting tank. Nitrocellulose membranes were 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 antisera either for 3 hours at room temperature or overnight at 4°C. Prior to incubation with horseradish peroxidase-conjugated goat anti-rabbit secondary 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 ECL system was used as the immunodetection system. The reagent was incubated with the membrane for 1 minute and immediately exposed to Hyperfilm for the necessary time to visualize the bands.

Autoradiography

a) AC6 phosphorylation autoradiography in crude membranes.
70µl of reaction buffer contained 40mM Tris-HCl (pH7.4),20mM $MgCl_2$, 5-10µM cold ATP, 2µM Okadaic acid, 75µM PKA catalytic subunit, proteinase inhibitor cocktail(concentration of each chemical was similar to membrane preparation) was mixed with 20µl AC6 membrane which contained about 150µg AC6 membranes protein, 15µl fresh[γ^{32}P]ATP(3000Ci/mM, 10mCi/ml), and incubated for 45 min at 35°C. After incubation 2µl of 1.5mg/ml solution of DNAse was added to the membranes and incubated for an additional 15 min. 13µl of 10% SDS solution was added to the reaction solution to dissolve the membranes at room temperature for 1 hour or 35°C for 45 min. When the solution became totally clear, the solution was ready to run on a gel.

50ul of sample were electrophoretically resolved on 6-7% SDS-PAGE. The gels were run at 150V about 3 hours The gels were fixed and dried, the

bands were visualized by exposure to reflection autoradiography film(Du Pont) for 20 sec to 2 min.

b) Purified AC6 phosphorylation autoradiography

1) ^{32}P-phosphorylation labeling

F-AC6 phosphorylationwas carried out in 40 mM Tri-HCl pH7.4, 20mM $MgCl_2$ 10μM ATP (cold), 300 μCi [γ^{32}P] ATP(3000Ci/mmol), 2μM okadaic acid. proteinase inhibitor cocktail, 75nM protein kinase A catalytic subunit, 2 mg F-AC6 or (S674A)-F-AC6 membrane protein in a final volume 150μl. When present, the concentration of WIPTIDE was 75μM. The reactions were incubated for 30 min. at 35°C. then 2μl of DNAse (1.5mg/ml) was added and incubated for 15 min.

2)AC6 solubilization

The ^{32}P labeled F-AC6 and (S674A)-F-AC6 membranes were centrifuged at 13000rpm for 30 min. at 4°C, and radioactive supernatant was discarded. 1ml modified Ripa buffer which contained 50mm Tris-HCl pH7.4, 150mM NaCl, 5mM EDTA pH8.0, 2μM okadaic acid, 10mM NaF, 10mM β-glycero-phosphate, 1mMNa_3VO_4 , 1.5% TritonX-100, 0.1%NP40, 0.2% digitonin, proteinase inhibitor cocktail was added to the pellets and the sample was rocked in the cold room 4°C for 4 hr. The sample was then spun down in the cold room at 13000 rpm for 30 min. The supernatant was used for purification of AC6.

3) Purification F-AC6 and (S674A)-F-AC6 with anti-FLAG-M2-affinity gel.

Anti-FLAG-M2-affinity gel was washed with TBS (50 mMTris-HCl, 150 mMNaCl pH7.4), followed by three 5 ml washes of 0.1M glycine-HCl pH at 3.5. The gel was then washed with 4x5ml of TBS. Finally, the gel was equilibrated with diluted Ripa buffer which contained 50mM Tris-HCl pH7.4, 150mM NaCl, 5mM EDTA pH8.0, 2μM okadaic acid, 10mM NaF, 10mM β-glycero -phosphate, 1mMNa_3VO_4, 0.75 % TritonX-100, 0.05% NP40, 0.1% digitonin, proteinase inhibitor cocktail. Generally 1ml of solubilized F-AC6 or (S674A)-F-AC6 was combined with 120 μl Anti-FLAG-M2-affinity gel and 1ml dilution buffer (Ripa without detergent). This was rocked in the cold room 4-8 hours. The gel was washed 3 times with diluted Ripa buffer and eluted with 180μl 0.1M glycine-HCl pH3.5 containing 0.05% Triton -X100 and 2μM okadaic acid .

4X sample buffer was added to eluted F-AC6 and (S674A)-F-AC6 samples and the samples were incubated at room temperature for 1 hour. The samples were then loaded on 6%SDS gels, electrophoresed, dried, and exposed to X-ray film.

Peptides

All the peptide were synthesized by Dr. Jinrong Li. The quality of the peptides was verified by Mass spectrometry and the purity was checked by both mass spectrometry and HPLC.

III. Data Analysis

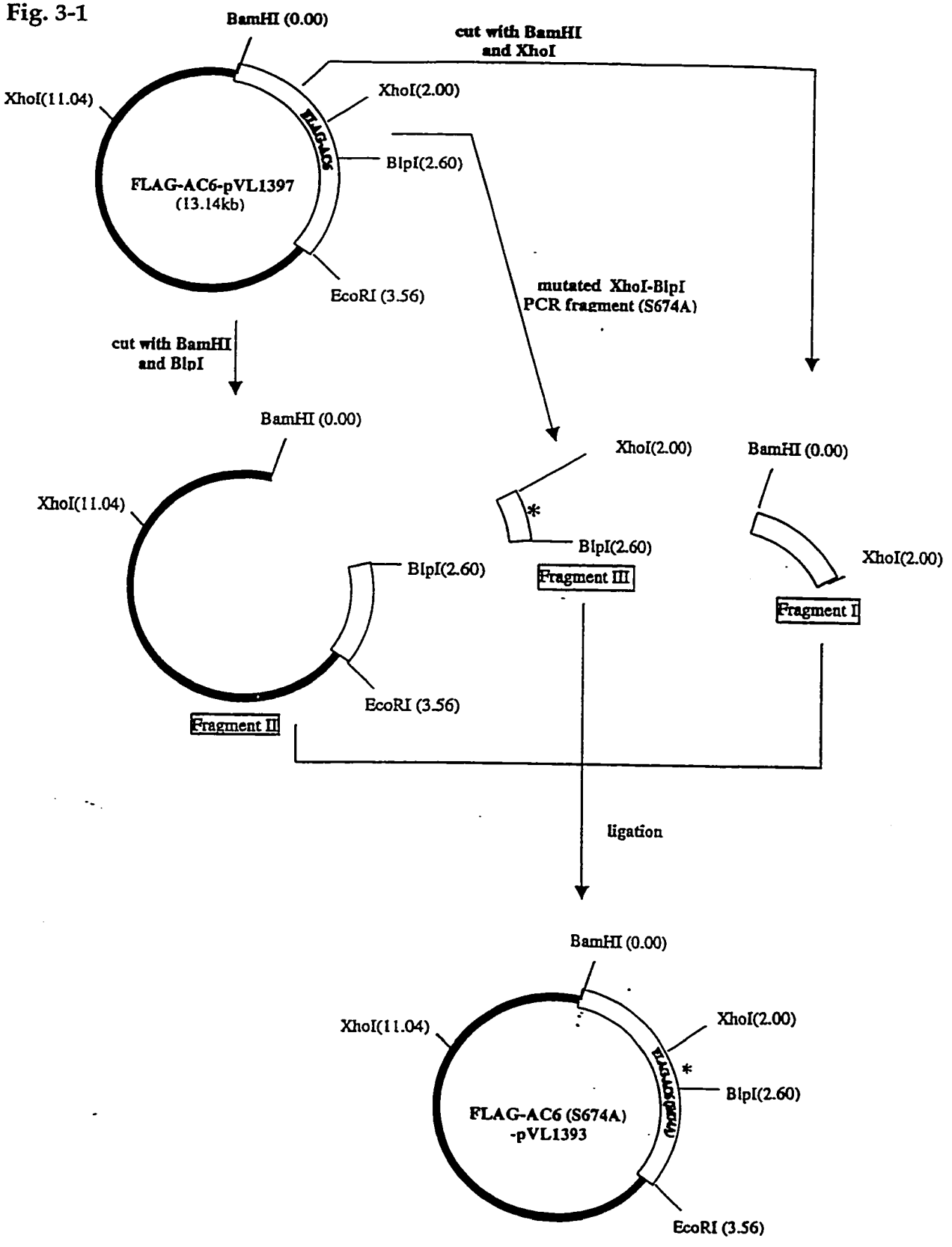
Computer programs used for Data Analysis included: Kaleidagraph 3.5, Microsoft Excel, Prophet (from NIH, distributed by BBN of Massachusetts). The dose-response data was analyzed using the curve fitting programs of Prophet. Analysis of immunoblots and autoradiography were carried out with a Scanning Densitometer.

Figure legends

Fig. 3-1 Diagram of FLAG-(S674A)-AC6-pVL1393 construction.

FLAG-AC6-pVL1393 were digested by BamHI/XhoI, and BamHI/BlpI separately. Two fragments were obtained and named fragment 1 and fragment 2. A PCR product which contained mutant site T2033G was cut with XhoI /BlpI and named fragment 3. The three fragements were ligated to obtain FLAG-(S674A)-AC6-pVL1393.

Fig. 3-1



Custruction of FLAG-(S674A)-AC6-pVL1393

Chapter 4

PKA regulation of $G\alpha_s$ stimulation of AC6

Chapter 4

PKA regulation of $G\alpha_s$ stimulation of AC6

Before I started the experiments on PKA regulation of AC6 I tested all my assay components to determine whether they were at optimal conditions. First, I determined the time course of AC6 expression. High 5 cells grown to 80% confluence were infected with AC6 recombinant baculovirus and thyroid peroxidase (TPO) baculovirus (used as a control). The concentration of virus was at a multiplicity of infection (MOI) of 5. Cells were harvested at different times between 48 to 120 hours post infection and membranes were prepared and frozen at -80°C. All these membranes were then assayed simultaneously. Fig. 4-1 shows that basal, FSK and $G\alpha_s$* stimulated activities of AC6 increase from 48 to 96 hours post infection. At 120 hours post infection AC6 membranes showed a slight increase in basal AC activity, but $G\alpha_s$* stimulated activity was decreased compared to 96 hours infection. AC6 membranes harvested at both 72 and 96 hours post infection have shown good $G\alpha_s$* stimulated activity, but 96 hours post infection has a better $G\alpha_s$* stimulated AC activity than 72 hours post infection. For control, TPO infected membranes, increases in infection time decrease basal and $G\alpha_s$* AC activity. These results are not surprising since the virus may take over the host

protein expression systems subsequently affecting the normal host cells protein expression.

I next tested the linearity of assay with respect to time of incubation. The purpose of these experiments was to test the stability of AC6 enzyme activity during incubation under assay conditions. Fig. 4-2 shows that there is no significant change in the basal activity or $G\alpha_s$* stimulated AC6 activity in 5 to 45min incubation. However, with prolonged incubation(60 to 75 min) $G\alpha_s$* stimulated AC6 activity is significantly decreased. These data indicate that PKA pretreatment of AC6 and the subsequent assay should limited to within 45 min. Thus, a 15 to 30 min preincubation followed by a 15 min incubation should yield linear assays. To determine the effect of PKA on AC6 activity a series of experiments was designed.

Membranes containing AC6 were treated with or without 50 nM purified PKA catalytic subunit (see Methods) for 15min at 35°C. The treated membranes were then assayed for the indicated activities. The experiments have been repeated several times, and the results are consistent. PKA is a well studied enzyme. There were some reports of the regulation of AC subtypes by PKA(Iwami, G. et al., 1995b), and there is strong evidence and speculation of possible regulation of AC6 by PKA(Premont R. et al., 1992b; Iyengar, R. 1993 a). However, direct evidence of PKA effect on regulation of adenylyl cyclase 6 has not been established.

PKA treatment does not affect AC6 basal activity, but inhibits $G\alpha_s$* stimulated AC6 activity up to about 40- 50 %.

PKA also moderately reduces FSK stimulated AC6 activity about 30 %(Fig. 4-3). The cAMP-dependent protein kinase (PKA) catalytic subunit is a 40 kDa protein, which is purified from bovine heart. Several batches of purified PKA which were purchased from Promega were tested and all showed consistent results, thus presumably the PKA quality was good, and it is reasonable to believe this inhibition is mediated by PKA. In these experiments $G\alpha_s$* concentration was 12nM and the FSK concentration was 50μM. Obviously a 50 % inhibition was significant but it was still necessary to further confirm that this effect was truly mediated by PKA.

In order to control for the possible effect of PKA regulation on the endogenous AC of Hi-5 and TPO infected cells, both Hi-5 cell membranes and TPO infected Hi-5 cell membranes were tested after treatment with and without PKA. Even though from Fig 4-1 we know that native Hi-5 and TPO virus infected cell membranes have low basal and $G\alpha_s$* stimulated AC activities compared to AC6 expressed membranes, it is possible that PKA treatment may alter native membrane AC activity so as to influence the effects on AC6.

Fig. 4-4 shows that treatment with and without PKA of native H-5 and TPO virus infected cell membranes show no significant change in AC activity. This may indicate that the PKA regulatory effects are not common to all AC subtypes, and it may be subtype specific. This data also shows that in both native Hi-5 and TPO infected membranes AC activities are low, thus the effects of the background are not substantial.

The cAMP-dependent protein kinase (PKA) catalytic subunit is the active subunit of PKA. In order to adjust the PKA concentration to achieve better catalytic activity or optimal phosphorylation conditions, several experimental conditions were investigated.

1) pH

2) Time course of PKA treatment

3) Concentration of PKA during treatment

If the $G\alpha_s$* stimulated AC6 activity is negatively regulated by PKA mediated phosphorylation, the more AC6 that is phosphorylated the greater the inhibition. In order to observe a maximum PKA mediated inhibition of $G\alpha_s$* stimulated AC6 activity, I first wanted to know what the optimal pH conditions for PKA phosphorylation of AC6 were. The Promega technical bulletin states that the optimal pH for PKA storage is 6.8 and for optimal catalytic activity for phosphorylation about 7.4 for in vitro experimental conditions.

The consensus PKA phosphorylation experiments were carried out at pH 7.4 in Tris-HCl buffer in presence of 10-20mM $MgCl_2$, 0.8-1.0mM ATP and 0.1-0.2mM DTT. A pH below 7.5 may increase PKA activity but could affect the substrate AC6 activity. Experiments were designed to better test conditions for PKA phosphorylation which would not affect AC6 activity in the subsequent AC assay. Three pH conditions were tested.

AC is not very sensitive to pH change in a certain pH range. Since the optimal pH conditions for the AC6 assay are from 7.5 to 8.0, pH 7.4, 7.5, 8.0

were used during PKA treatment. The reason I tested these three conditions is that pH 7.4 is the pH of normal PKA treatment, pH 8.0 is the pH we use to assay AC activity in our laboratory and pH 7.5 is the pH for AC assay in other laboratories.

Fig. 4-5 shows pretreatment of AC6 membranes at different pH. The inhibition $G\alpha_s$* stimulated AC6 activity at pH 8.0 was less than that compared to effects at pH 7.4 or 7.5. This may be because at pH 8.0, PKA is less active and, only a small portion of AC6 may be phosphorylated. There was no significant difference between pH 7.4 and pH 7.5 for $G\alpha_s$* stimulated AC6 activity. Since pH 7.5 showed a good PKA inhibition effect and also there was no any evidence that adenylyl cyclase activity was different as compared to activity measured at pH 8.0, the treatment and assay were conducted at pH 7.5

PKA treatment time course

The second series experiments I did was to test the optimal time course of PKA treatment (Fig. 4-6). AC6 membranes were treated with or without PKA(for the control Tris buffer was added) for 0, 10 ,15, and 30 min, and then assayed for AC6 activity. Fifteen and thirty min. treatment with PKA show a substantial inhibition of $G\alpha_s$* stimulated AC6 activity. Increased incubation times would increase PKA inhibition, but there were no significant differences between 15 min. and 30 min. Further, the basal activity of AC6 was not affected. The concentration of PKA in these experiments was 75 nM. The

data in Fig. 4-2 showed that there was no significant AC6 activity change up to 45 min. incubation. It is therefore possible to treat AC6 with PKA for both 15-30 min. without loss of adenylyl cyclase activity.

On the basis of the predicted consensus sequence, AC6 is not a good PKA substrate. Fig. 4-7 shows 25, 50, 75 and 100 nM PKA were used for 15 min. at 35°C pretreatment. 75 nM and 100 nM PKA produce better inhibition than 25 nM. 100 nM and 75 nM show a slight difference of inhibition. However, higher concentrations of PKA have not been used since PKA is a broad spectrum enzyme and a high concentration of enzyme might phosphorylate biologically irrelevant sites. These data show 50 to 100 nM of PKA are needed in order to get better inhibition. Higher concentrations may not be needed since 100 nM when compared to 75 nM does not show a significant difference. Again, there was no change in basal AC6 activity after PKA treatment. In these experiments the $G\alpha_s$* concentration was 15 nM. This data also indicated the phosphorylation motif LE**KKYS**RKVDPA in AC6 may not be a good substrate for PKA. High PKA concentrations for phosphorylation are needed, but 75 nM to 100 nM of PKA is not a unreasonably high concentration if compartmentization or colocalization of PKA with its substrates are considered.

Mg^{2+} effect on PKA mediated regulation of AC6 activity

Increasing concentrations of Mg^{2+} will increase AC basal activity. PKA treatment does not affect basal activity of AC6 in the presence of different concentrations of Mg^{2+}. Mg^{2+} significantly facilitates other modes of stimulation of AC activity such as FSK and $G\alpha_s$* (Fig. 4-8). Mg^{2+}-ATP is the substrate for AC, and it is speculated that Mg^{2+} stabilizes ATP in the catalytic center. Thus increasing Mg^{2+} concentration facilitates AC catalytic activity. ATP has a strong negative charge and Mg^{2+} binding statically neutralizes the negative charge of ATP. Keeping a high concentration of Mg^{2+} in the catalytic center can trap more ATP in the catalytic site. With increasing concentrations of Mg^{2+} both $G\alpha_s$* and FSK stimulated AC6 activity are increased, however the negative regulatory effect of PKA is somewhat diminished. This indicates that the PKA effect on AC6 may involve a charge change and Mg^{2+} may neutralize such change.

Verification of PKA effect on AC6

I next verified that the inhibition of $G\alpha_s$* and FSK stimulated AC6 activity is a specific PKA effect. One approach to address this question is to use a specific PKA inhibitor to antagonize the PKA effect. For this I used WIPTIDE. This WIPTIDE is a 20 a.a. peptide (Thr-Thr-Tyr-Ala-Asp-Phe-Ile-

Ala-Ser-Gly-Arg-Thr-Gly-Arg-Arg-Asn-Ala-Ile-His-Asp-NH2) derived from the Walsh inhibitor. If the PKA effect is blocked by PKA inhibitor, this indicates that the regulatory effect of AC6 is mediated by PKA; otherwise something else may be involved. AC6 membranes were treated with or without purified PKA in presence or absence of PKA inhibitor, then assayed for basal, $G\alpha_s$* and Forskolin stimulated activities (Fig. 4-9). PKA inhibitor was preincubated with AC6 membranes on ice for 10 min., then PKA was added. The data shows that the PKA mediated inhibition of $G\alpha_s$* and FSK stimulation of AC6 activity can be blocked by the PKA inhibitor. This clearly indicates PKA is involved in the inhibition of $G\alpha_s$* and FSK stimulated AC6 activity. In these experiments the PKA concentration was 75 nM and PKA inhibitor concentration was 50 μM, the $G\alpha_s$* concentration was 12 nM and the FSK concentration was10μM, $G\alpha_s$* stimulated AC6 activity in the presence of PKA inhibitor shows more activity than control AC6 membranes implying that there may be a small amount of endogenous PKA present.

Verification that the PKA effect on AC is subtype specific

In order to test if the regulatory effect of PKA is subtype specific, AC1 and AC2 together with AC6 were pretreated with PKA and their activities were tested. Basal and $G\alpha_s$* stimulated activity were measured. Fig. 4-10 shows that there was no observable effect of PKA on basal activity of either

AC1 or AC2. For $G\alpha_s$* stimulated AC activity, no inhibition was seen with AC1, and a modest (about 20%) inhibition was seen with AC2. These experimental results indicate that PKA regulation is AC subtype specific. In these experiments, the concentration of Mg^{2+} was 2 mM, PKA concentration was 75 nM and $G\alpha_s$* concentration was 12 nM. PKA treatment was 15 min. at 35°C

Mn^{2+} effect on PKA and AC6 activity

Protein kinases and many other energy consuming enzymes use ATP as a substrate. In many situations, Mn^{2+} has shown many similarities in function to Mg^{2+}. The crystal structure of the PKA catalytic domain showed Mg^{2+}-ATP and Mn^{2+}-AMP-NPN bind at almost the same position in the catalytic center. In the PKA catalyzed phosphorylation reaction, the function of Mg^{2+} and Mn^{2+} are similar, both stabilize ATP in catalytic center (Bossemeyer D et al 1993). In AC which catalyzes ATP conversion to cAMP, Mg^{2+} and Mn^{2+} show a different mechanism. First in cells, Mg^{2+}-ATP is a physiological substrate, Mn^{2+}-ATP is a nonphysiological substrate for AC (Ross EM. et al., 1978), and Mn^{2+} regulates AC activity by interaction at an allosteric site (Somkuti S. et al., 1982). Both Mg^{2+} and Mn^{2+} can stimulate AC activity but show a different efficacy and potency. Mg^{2+} produces maximally about a 2 fold increase in basal AC activity in the range of 2 to 20 mM,

however the Mn^{2+} shows about 5 fold increase in AC6 activity at same assay conditions.

Figure 4-11 shows that Mn^{2+} stimulated AC6 activity was not affected by PKA treatment. This implies that the allosteric Mn^{2+} regulated site in AC6 is the site that is not affected by PKA. That also indicates that the Mn^{2+} regulatory site is different from the $G\alpha_s$* and FSK regulatory site. They are insensitive to PKA regulation.

Effect of PKA on $G\alpha_s$* stimulation of AC6

In order to quantitatively characterize the PKA effect on $G\alpha_s$* stimulated AC6 activity, $G\alpha_s$* dose response curves have been carried out. $G\alpha_s$* dose response curve for AC6 was first characterized by Anya Harry in our laboratory (Harry A. et al., 1997). It is biphasic-like with distinct "high" and "low" affinity components. In these experiments (Fig. 4-12), the apparent K_{act} for the high affinity site is about 1.2 +/- 0.9 nM and 28 +/- 10 nM for the low affinity site. The Vmax at the high affinity site is 114+/-24 pmol/mg/min for control and 108 +/- 7 pmol/mg/min. for PKA treated. At the low affinity site the Vmax's are 620 +/- 23 pmol/mg/min for control and 179 +/- 9 pmol/mg/min for PKA treated. The dose response curve shows at $G\alpha_s$* levels less than 5 nM there is no significant difference in AC6 activity upon PKA

treatment. As $G\alpha_s$* concentration increases, AC6 control activity increases. In contrast, for PKA treated AC6 membranes further increases in $G\alpha_s$* concentration do not significantly increase AC6 activity. This indicates that PKA regulation of AC6 blocks higher concentrations of $G\alpha_s$* stimulation and does not affect low concentration $G\alpha_s$* stimulation of AC6.

Construction of (S674A)-FLAG-AC6((674A)-F-AC6)

In order to determine whether the negative regulatory effect of PKA on $G\alpha_s$* stimulated AC6 activity is a direct effect or an indirect effect. I decided to determine if AC6 was phosphorylaed by PKA, and if so the location of the phosphorylation site. I constructed a FLAG-tagged AC6 with an S -->A mutation at a.a. position 674(S674A-F-AC6). AC6 itself is a good candidate for the target of PKA because 1) $G\alpha_s$* is not the target(Premont, et al., 1992. b). 2)There is a single predicted PKA phosphorylation site at S674 in AC6. 3)both hepatocytes and S49 lymphoma cell are desensitized by a cAMP dependent process and both cell lines contain AC6.

If S674 of AC6 is the only site for PKA phosphorylation, mutating this residue will result in an AC6 enzyme which is no longer regulated by PKA.

In order to facilitate purification of the phosphorylated AC6, I introduced an epitope tag (FLAG) at the N-terminus of the protein. Previous

studies with AC2 in our laboratory had shown that phosphorylation of AC2 by protein kinase C was easily observed in the epitope tagged protein. Further, the epitope tag does not affect the adenylyl cyclase 2 activity (Jacobowitz, O. et al., 1994). FLAG tagged AC6(F-AC6) and mutant (S674A) FLAG tagged AC6((S674A)-F-AC6) were expressed in Hi-5 cells. Both of the F-AC6 and (S674A)-F-AC6 containing Hi-5 cell membranes were solublilized, resolved on SDS-PAGE, transferred to nitrocellulose membranes and immunoblotted with an anti-FLAG-M2-antibody. In each sample a protein which migrated with a expected molecular weight of 135kDa was detected(Fig. 4-13)

The F-AC6 was compared to wt. AC6 for the effects of PKA treatment as well as stimulation by $G\alpha_s$* and FSK. Addition of FLAG epitope did not affect any of the functional properties of AC6(Fig. 4-14).

I next determined if PKA directly phosphorylated F-AC6 as was the case for (S674A)-F-AC6. For this, Hi-5 membranes containing the required enzyme was treated with PKA catalytic subunit for 30 min. at 35°C. The PKA concentration was 75 nM, and fresh [γ^{32}P]ATP was used (For details see materials and experiment). In Fig.4-15 lanes 1 to 3 were F-AC6 containing membranes and lanes 4 to 6 were (S674A)-F-AC6 containing membranes. Lanes 1 and 4 were the negative control, (no PKA treatment), lanes 2 and 5 were treated with PKA, and lanes 3 and lane 6 were treated with PKA in presence of PKA inhibitor WIPTIDE. In lane 2 a major band labeled with a molecular weight approximately 135kDa was observed, indicating that PKA phosphorylated AC6 can be visualized without further purification. No

phosphorylated protein was present when PKA inhibitor was added. In contrast, the (S674A)-F-AC6 does not show any phosphorylation.

Experiments such as those in Fig 4-15 showed some background differences between PKA treatment and basal, PKA+PKA inhibitor. Basal and PKA+PKA inhibitor treated sample showed lower background, F-AC6 treated with PKA showed the strongest background and PKA treated (S674A)-F-AC6 PKA membranes showed moderate to high background. In order to decrease background, wt. and mutant (S674A) FLAG tagged AC6 enzyme were affinity purified using anti-FLAG-M2-gel. Briefly the expressed enzymes were phosphorylated by PKA in presence of [γ^{32}P] ATP, solubilized in modified RIPA buffer, extracted and purified by chromatography over the anti-FLAG-M2-gel. The gel elutates were resolved on SDS-PAGE and visualized by autoradiography (Fig.4-16). The result shows that only a single band in PKA treated F-AC6 is seen. These results indicate PKA is directly phosphorylated by PKA at Ser 674.

Effect of PKA treatment of F-AC6 and (S674A)-F-AC6

Membranes containing of F-AC6 and (S674A)-F-AC6 were treated with and without PKA and assayed for $G\alpha_s$ stimulation. (S674A)-F-AC6 showed a small increase in basal activity, but there is no difference in the level of (S674A)-F-AC6 stimulated AC6 activity between two enzymes. However, the

PKA inhibitory effect is totally abolished(Fig. 4-17). These data indicate that PKA negatively regulates $G\alpha_s$ stimulated AC6 activity by phosphorylation on residue S674. Mutation of this residue results in a total loss of PKA effects on AC6.

Figure legends

Fig. 4-1 AC activity in membranes from Hi-5 cells infected for varying times with AC6 and TPO bacoluvirus

A. Hi-5 cells were infected for increasing times from 48 h to 120 h. Infected cells were harvested and membranes were prepared. These cell membranes were assayed for basal, FSK and $G\alpha_s^*(\alpha_s^*)$ stimulated AC6 activities simultaneously.

B. For control, thyroid peroxidase(TPO) baculovirus was used to infect Hi-5 cells at the same time course as the AC6 virus. The TPO membranes were prepared in the same way as AC6 membranes, and assayed for basal, FSK and $G\alpha_s^*$ stimulated AC activity. In both assays FSK concentration was 50μM and $G\alpha_s^*$ concentration was 10nM.

Fig 4-2 Activity of AC6 as a function of incubation time

The linearity of AC6 activity during the in vitro incubation was tested. From 5-75min incubation both basal and $G\alpha_s^*$ stimulated activities were measured. $G\alpha_s^*$ concentration was 15nM.

Fig 4-3 Effect of PKA treatment on AC6 activity

AC6 membranes were treated with or without PKA, and then assayed for basal, $G\alpha_s^*$ and FSK stimulated AC6 activity. For control(without PKA), instead of PKA, Tris buffer was added. In these experiments $G\alpha_s^*$ concentration was 12nM and FSK concentration was 50μM.

Fig. 4-4 Effect of PKA on AC activity of uninfected and TPO virus infected Hi-5 cells

PKA treated uninfected Hi-5 and TPO virus infected Hi-5 cell membranes were assayed for AC activity. In these experiments the $G\alpha_s$* concentration was 15nM and FSK was 50μM and PKA concentration was 75nM.

Fig. 4-5 Effect of pH on PKA treatment of AC6 containing Hi-5 cell membranes

AC6 containing Hi-5 cells membranes were treated with PKA at three different pH conditions (pH 7.4, 7.5 and 8.0). In these experiments the $G\alpha_s$* concentration was 15nM. The PKA concentration was 75nM.

Fig. 4-6. Time course of PKA treatment

AC6 containing Hi-5 cell membranes were treated with PKA for 0 min., 10 min., 15 min. and 30 min. Basal and $G\alpha_s$* stimulated AC6 activities were then assayed. The concentration of PKA was 75 nM and the $G\alpha_s$* concentration was 15nM.

Fig. 4-7 effect of varying concentration of PKA on treatment of AC6 containing membranes

Four different concentrations of PKA (25, 50, 75 and 100nM) were used to treat AC6 membranes for 15 min at 35°C. Basal and $G\alpha_s$* stimulated AC6 activities were then assayed. The $G\alpha_s$* concentration was 15 nM.

Fig. 4-8 Effect of Mg^{2+} on PKA mediated regulation of AC6 activity

AC6 containing Hi-5 cell membranes were treated with or without PKA, then the $G\alpha_s$* stimulated AC6 activities were measured in presence of four different concentrations of Mg^{2+}. The $G\alpha_s$* concentration was 12 nM and PKA concentration was 75 nM.

Fig. 4-9 effect of WIPTIDE on PKA regulation of AC6

To verify that the PKA effect on $G\alpha_s$ stimulated AC6 activity was specific, a specific PKA inhibitor (WIPTIDE) was used. When required, membranes were preincubated with WIPTIDE for 10 min. Then AC6 containing membranes were treated with PKA for 15 min, basal, FSK and $G\alpha_s$* stimulated AC6 activity were measured. In these experiments, the $G\alpha_s$* concentration was 12nM, FSK was 50μM, PKA concentration was 75nM and WIPTIDE concentration was 50μM.

Fig. 4-10 Effect of PKA treatment on AC1 and AC2

AC1 and AC2 containing Hi-5 cell membranes were pretreated with PKA with the same procedure as AC6 membranes then tested for basal and $G\alpha_s$* stimulated AC activity. The $G\alpha_s$* concentration was 12nM and PKA concentration was 75nM.

Fig. 4-11 Effect of PKA on Mn^{2+} stimulated AC6 activity

AC6 containing membranes were treated with or without PKA. AC6 activities were measured in the presence of three different concentrations of Mn^{2+}. In these experiments the PKA concentration was 75nM.

Fig 4-12 Effect of PKA treatment on stimulation of AC6 by varying concentration of $G\alpha_s$*

AC6 containing Hi-5 cell membranes were treated in the presence and absence of PKA and then assayed in the presence of indicated concentrations of activated $G\alpha_s$*. Data were analyzed on a Sun work station by using the program PROPHET. The plots of the data points and the fitted curves were generated by PROPHET. The data best fit a two-site model, and the indicated constants were obtained from the two site fit. The printed plots were exported to CANVAS program in a Mac 8100. The plots were labeled within CANVAS and printed as CANVAS files. In these experiments PKA concentration was 75nM.

Fig. 4-13 Expression of 4-13 F-AC6 and (S674A)-F-AC6 in Hi-5 cells

Immunoblotting was used to detect F-AC6 and (S674A)-F-C6 expression. Both F-AC6 and (S674A)-F-C6 were expressed in Hi-5 cells. After baculovirus infection for 96 hours cell membranes were prepared. The solubilized membranes were resolved by SDS-PAGE, transfered to nitrocellulose membranes, blotted with anti-FLAG-M2-antibody, and then treated with horseradish peroxidase-conjugated goat anti-rabbit

secondary antibody. Bands were detected by the ECL system. In both lanes a protein of molecular weight about 135 kDa was detected.

Fig. 4-14 Comparison of the activities of AC6 and F-AC6

The F-AC6 and wt. AC6 activity were treated with or without PKA for 15 min, then basal, FSK and $G\alpha_s^*$ stimulated activities were assayed. FSK concentration was 50μM, $G\alpha_s^*$ was 15nM and PKA concentration was 75nM.

Fig. 4-15 Autoradiography of membranes containing F-AC6 and (S674A)-F-AC6 treated with or without PKA

F-AC6 and (S674A)-F-AC6 containing membranes were treated with PKA catalytic subunit for 30 min at 35°C. The PKA concentration was 75nM, PKA inhibitor concentration was 75μM. Fresh [γ-^{32}P]-ATP was used(For details see Methods). Lanes 1 to 3 were F-AC6 Lane 4-6 were (S674A)-F-AC6. The treatment condition for each lane was indicated. Lanes 1 and 4 were the negative control with no PKA treatment, Lanes 2 and 5 were treated with PKA. lanes 3 and 6 were treated with PKA in the presence of PKA inhibitor. Membranes were solubilized and proteins were resolved by electrophoresis, and phosphorylated bands were visualized by autoradiography.

Fig. 4-16 Autoradiography of affinity purified F-AC6 and (S674A)-F-AC6 after PKA treatment

F-AC6 and (S674A)-F-AC6 membranes were treated in the absence or presence of 75nM PKA or 75nM PKA and 75μM PKA inhibitor. After treatment F-AC6 or

(S674A)-F-AC6 membranes were solubilized separately, extracted, and purified by anti-FLAG-M2-affinity gel. F-AC6 and (S674A)-F-AC6 finally were eluted and the elutates were resolved by SDS gel and visualized by autoradiography. Lanes 1 to 3 were F-AC6, and lanes 4-6 were (S674A)-F-AC6. The different treatments of each lane were labeled as indicated.

Fig. 4-17 Activity of F-AC6 and (S674A)-F-AC6 with or without treatment of PKA

F-AC6 and (S674A)-F-AC6 activities were measured in samples treated with or without PKA. Basal and $G\alpha_s$* stimulated AC6 activities were measured. The $G\alpha_s$* was 15nM, and PKA concentration was 75nM and incubated at 35°C for 15 min.

Fig.4-1a

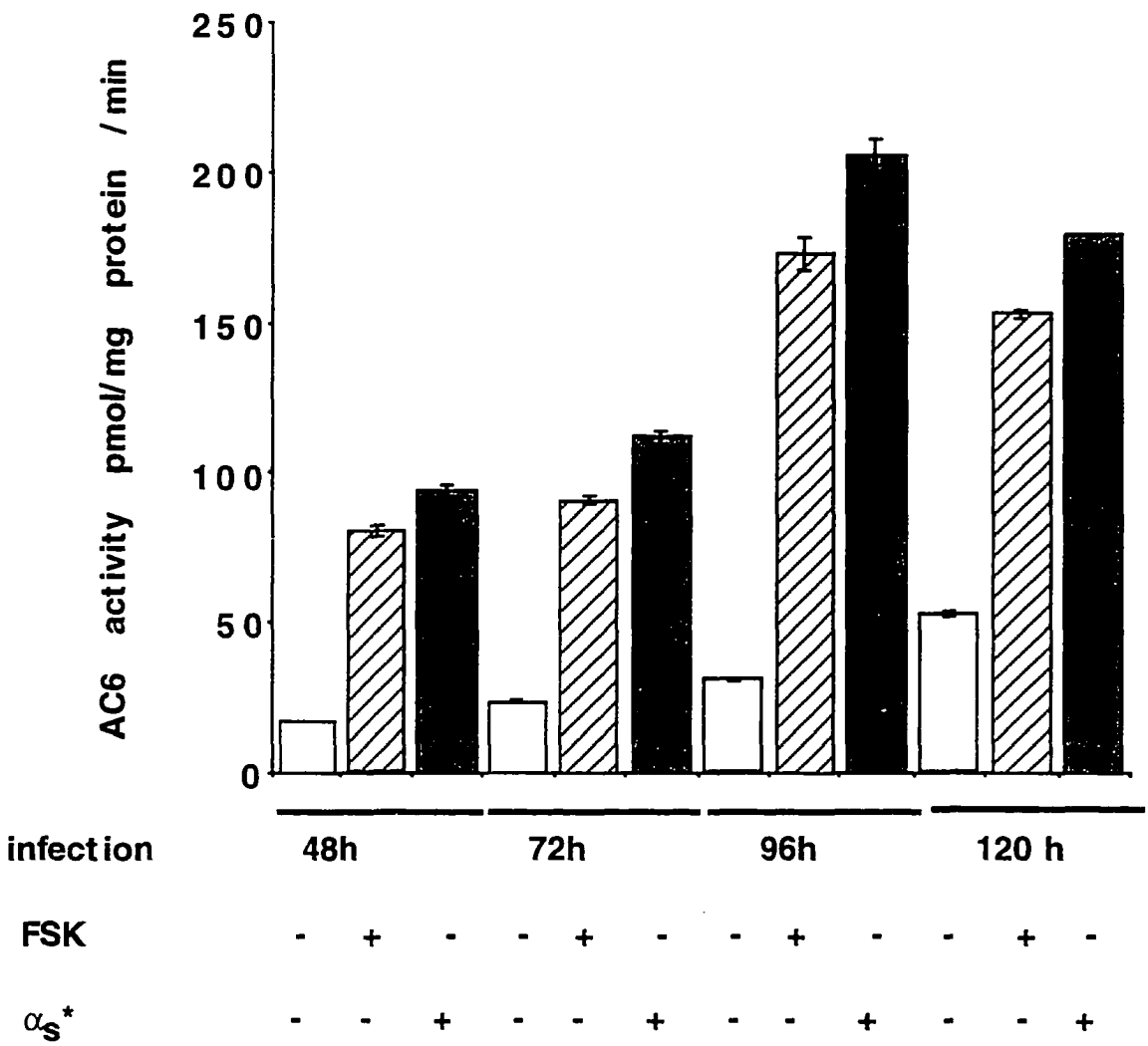


Fig. 4-1 b

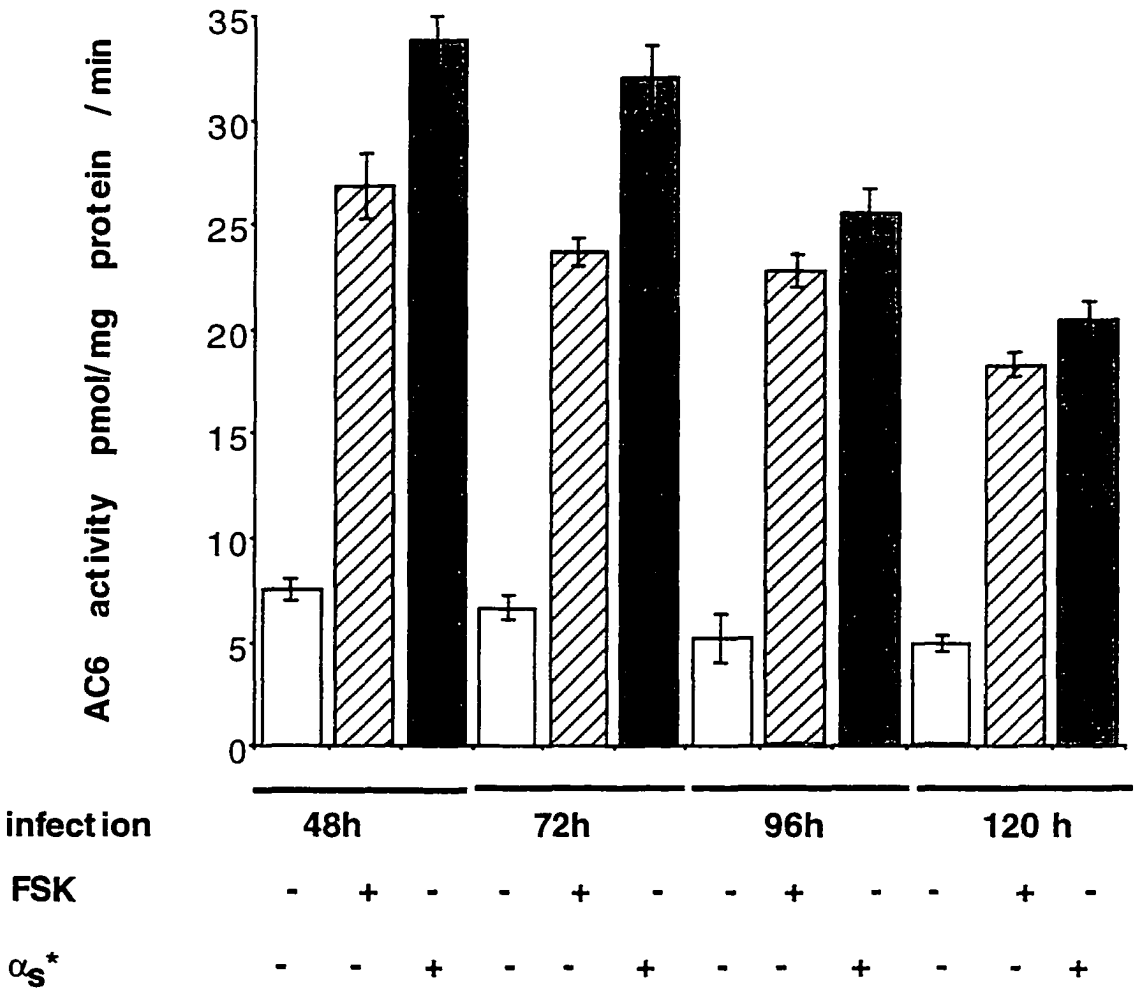


Fig.4-2

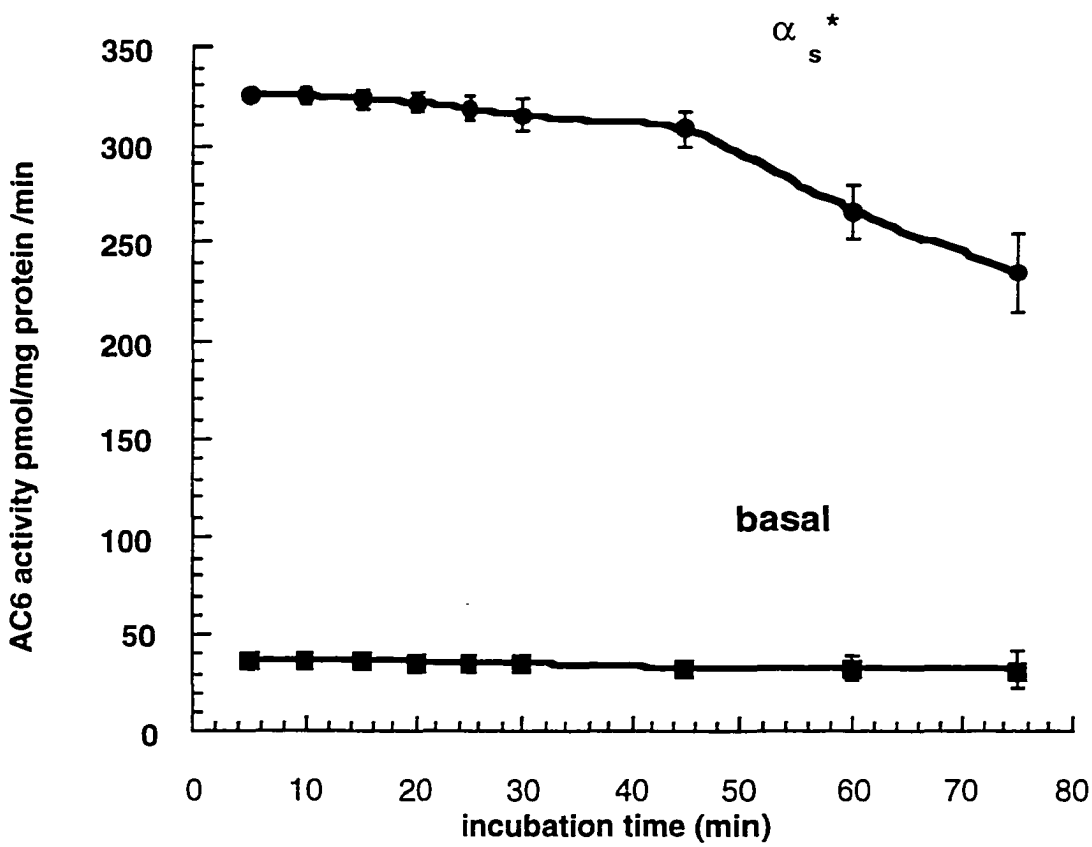


Fig.4 -3

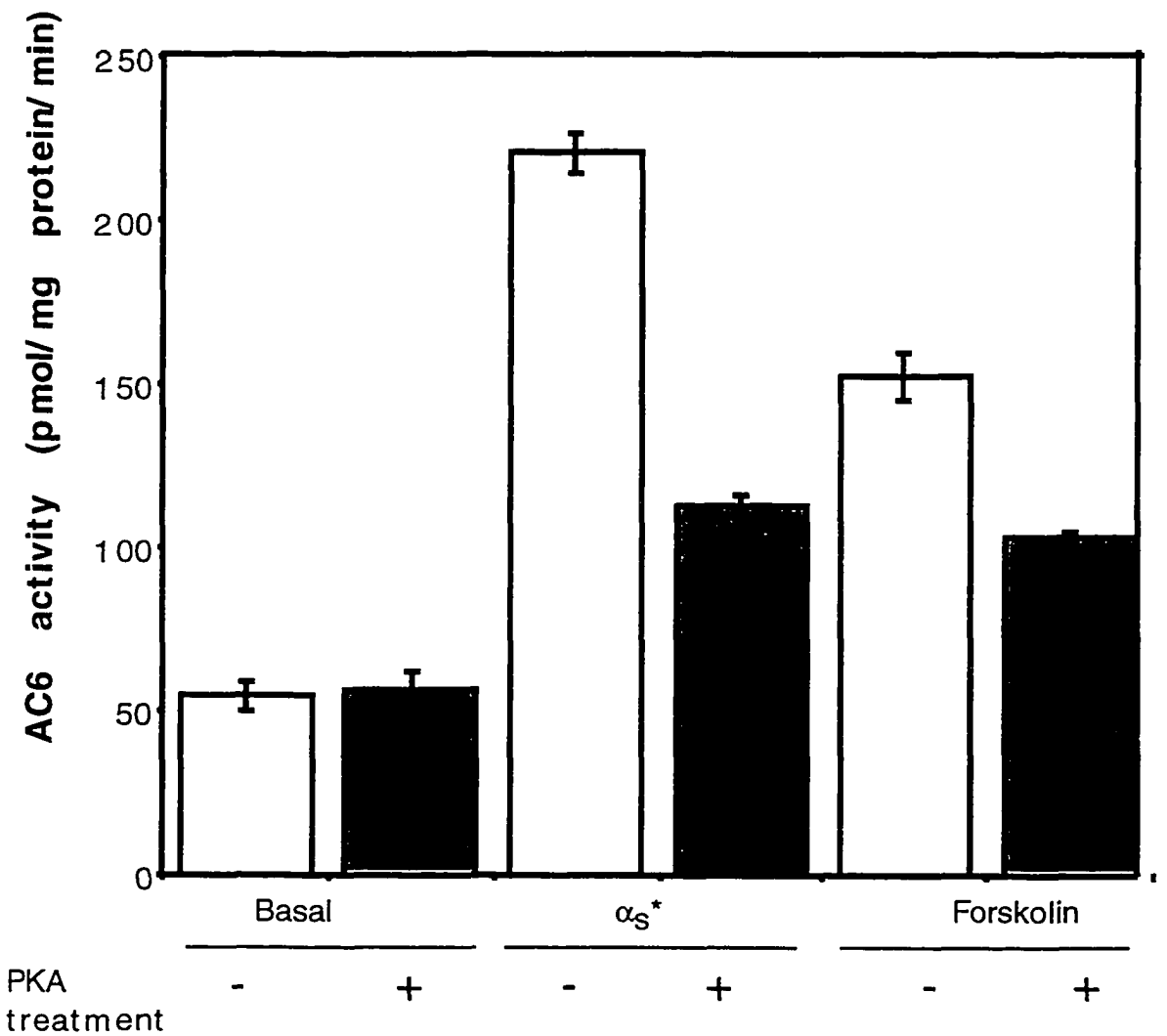


Fig. 4-4

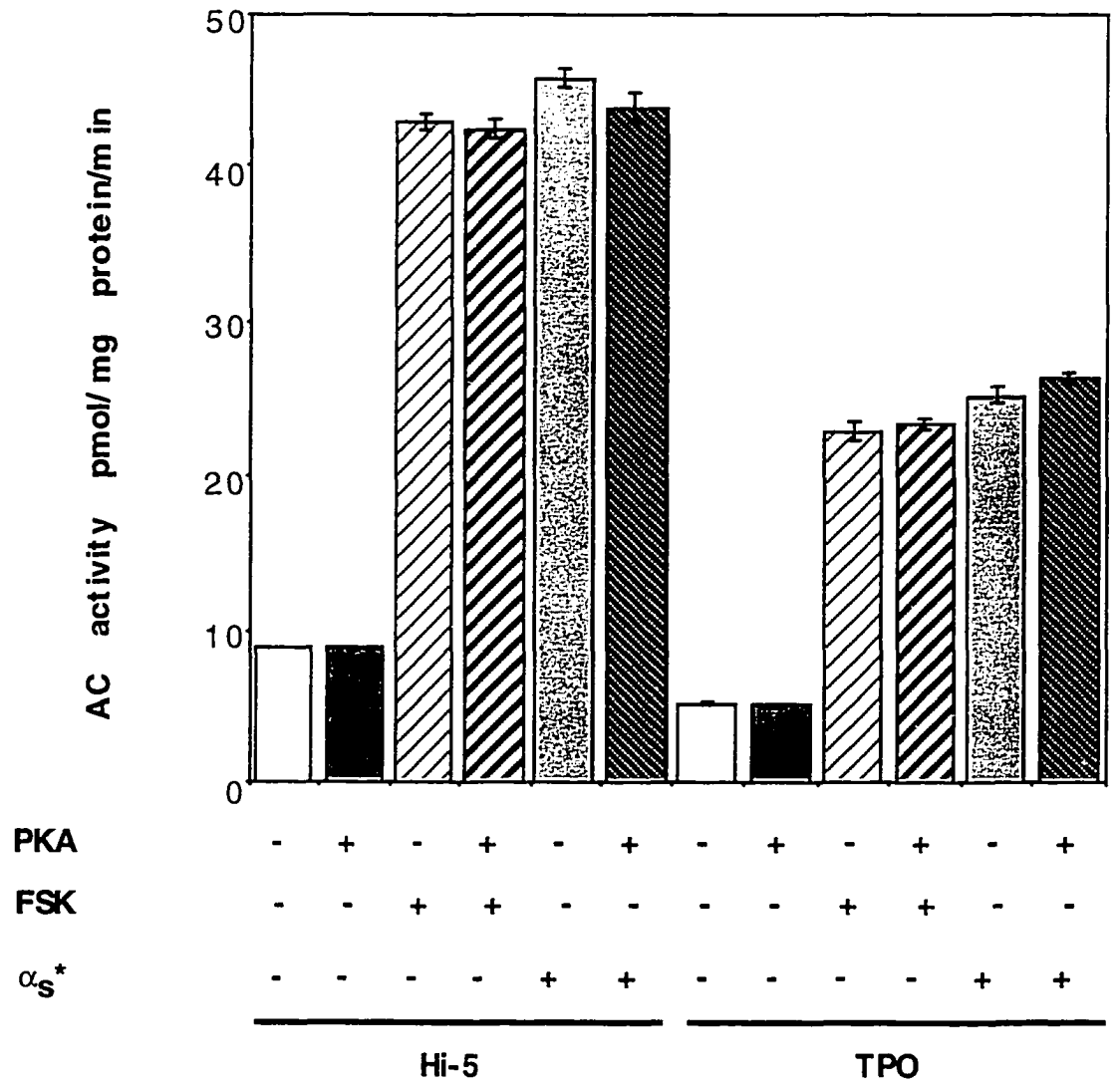
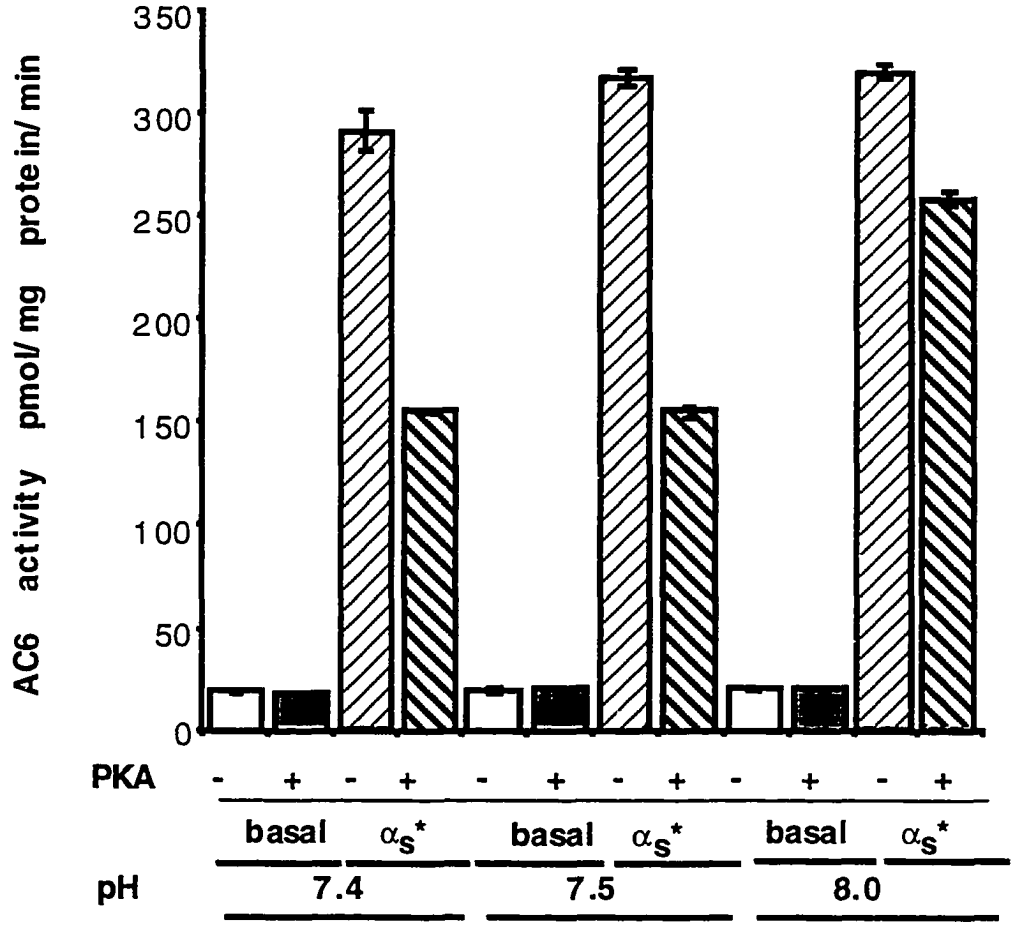


Fig.4-5



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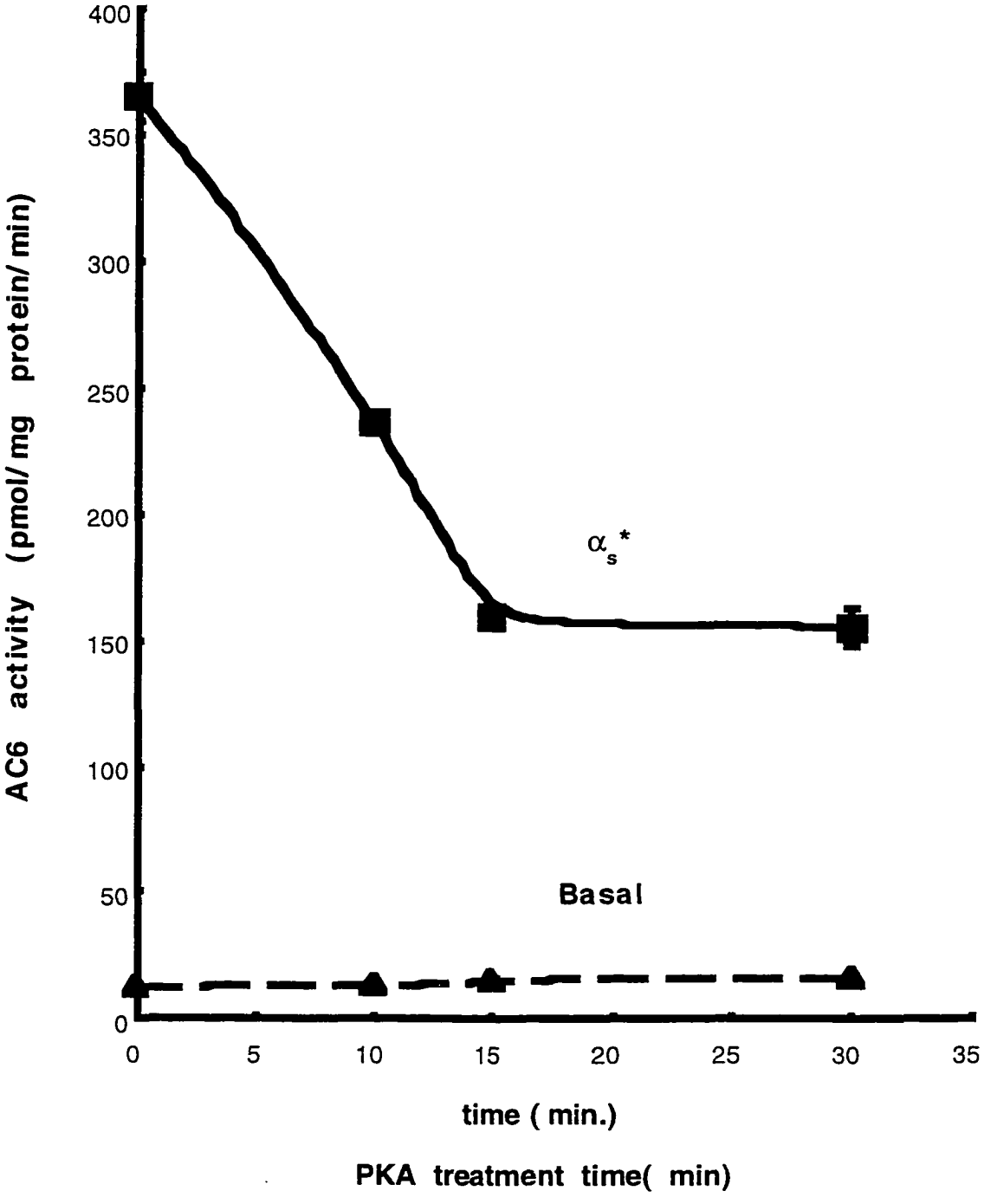


Fig.4-7

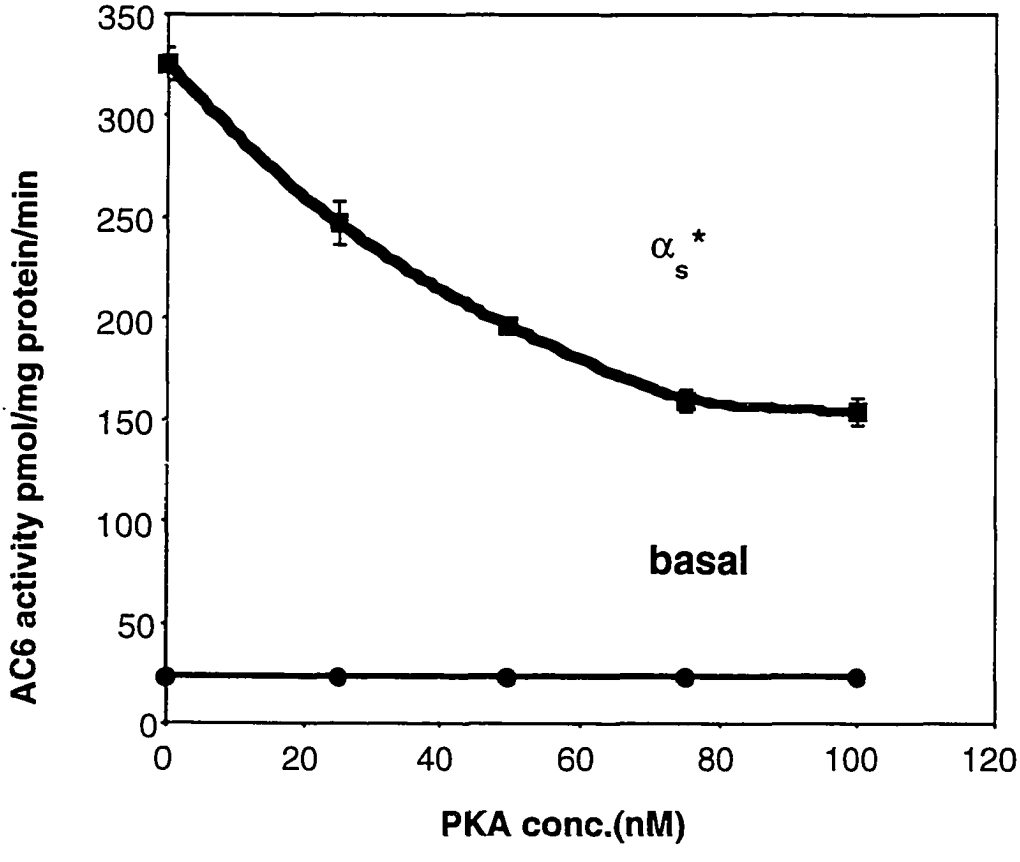


Fig.4-8

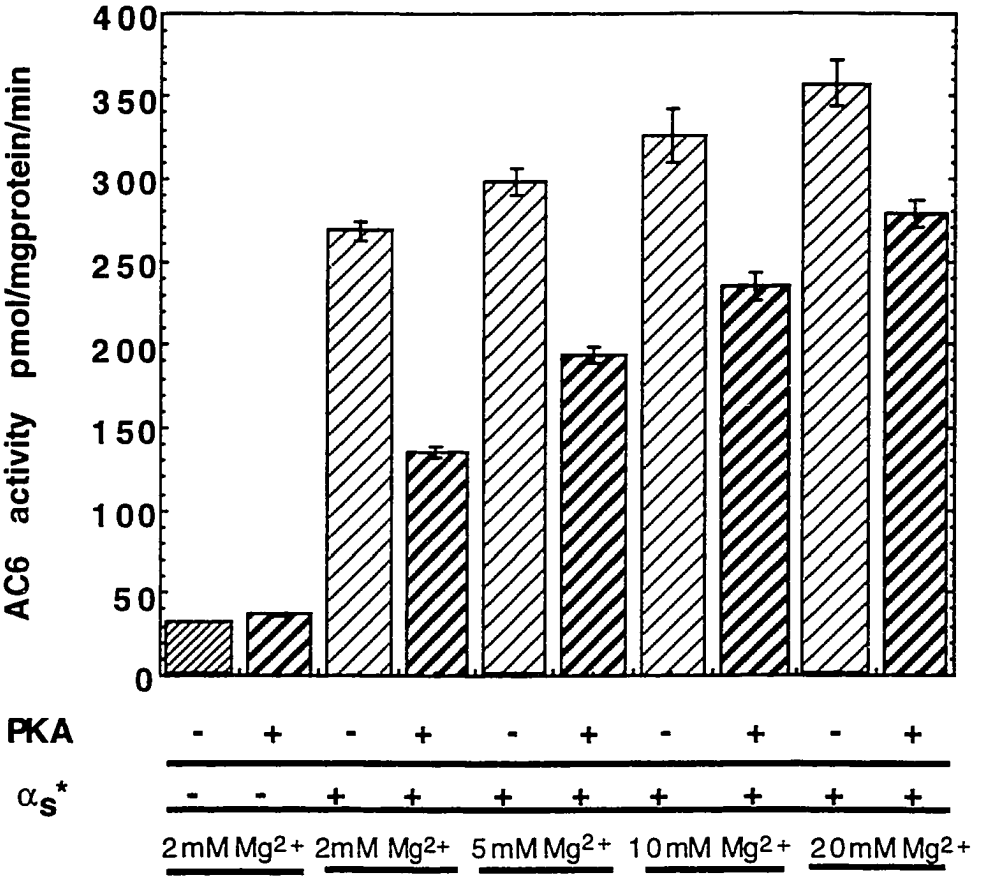


Fig. 4-9

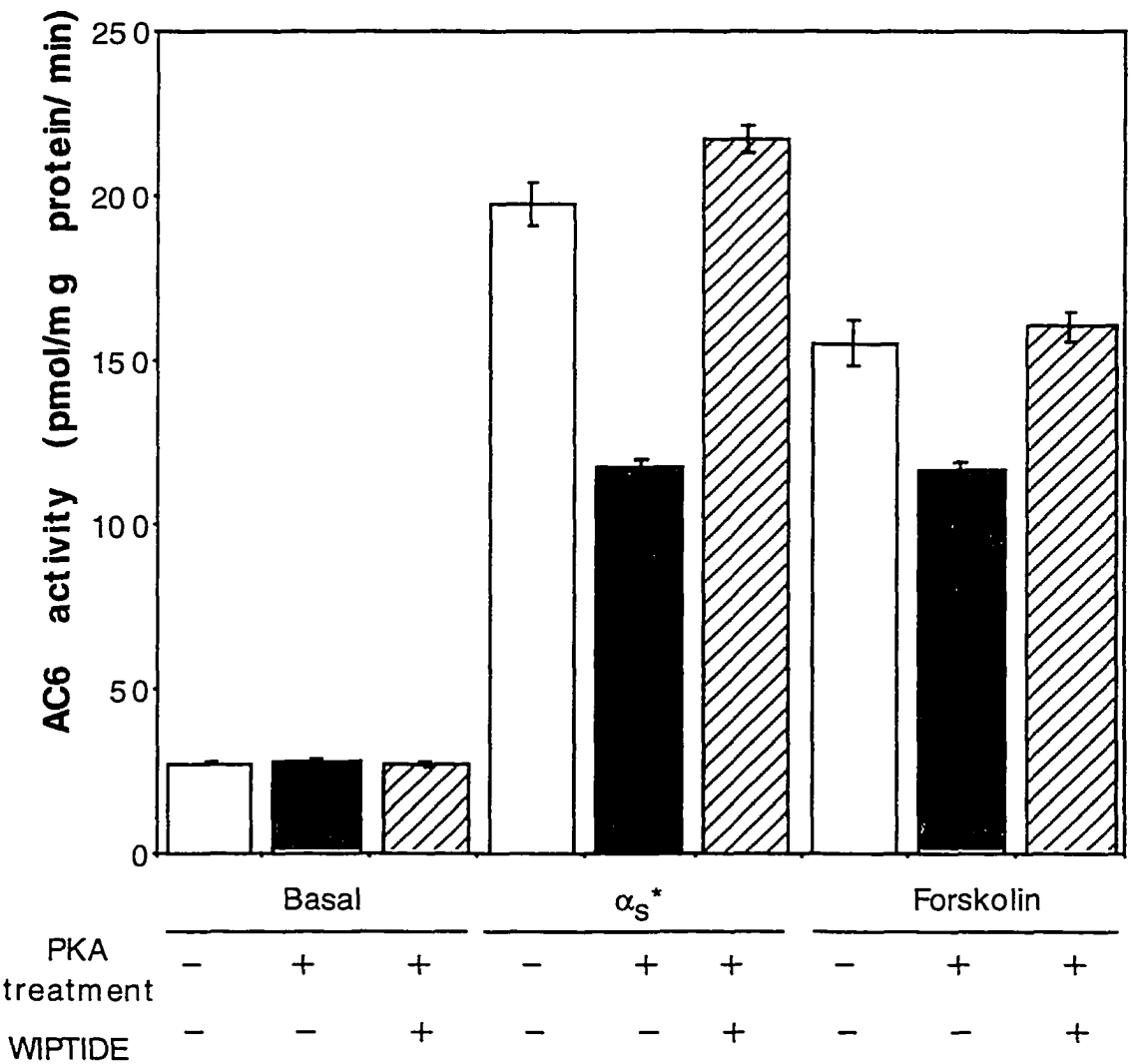


Fig.4-10

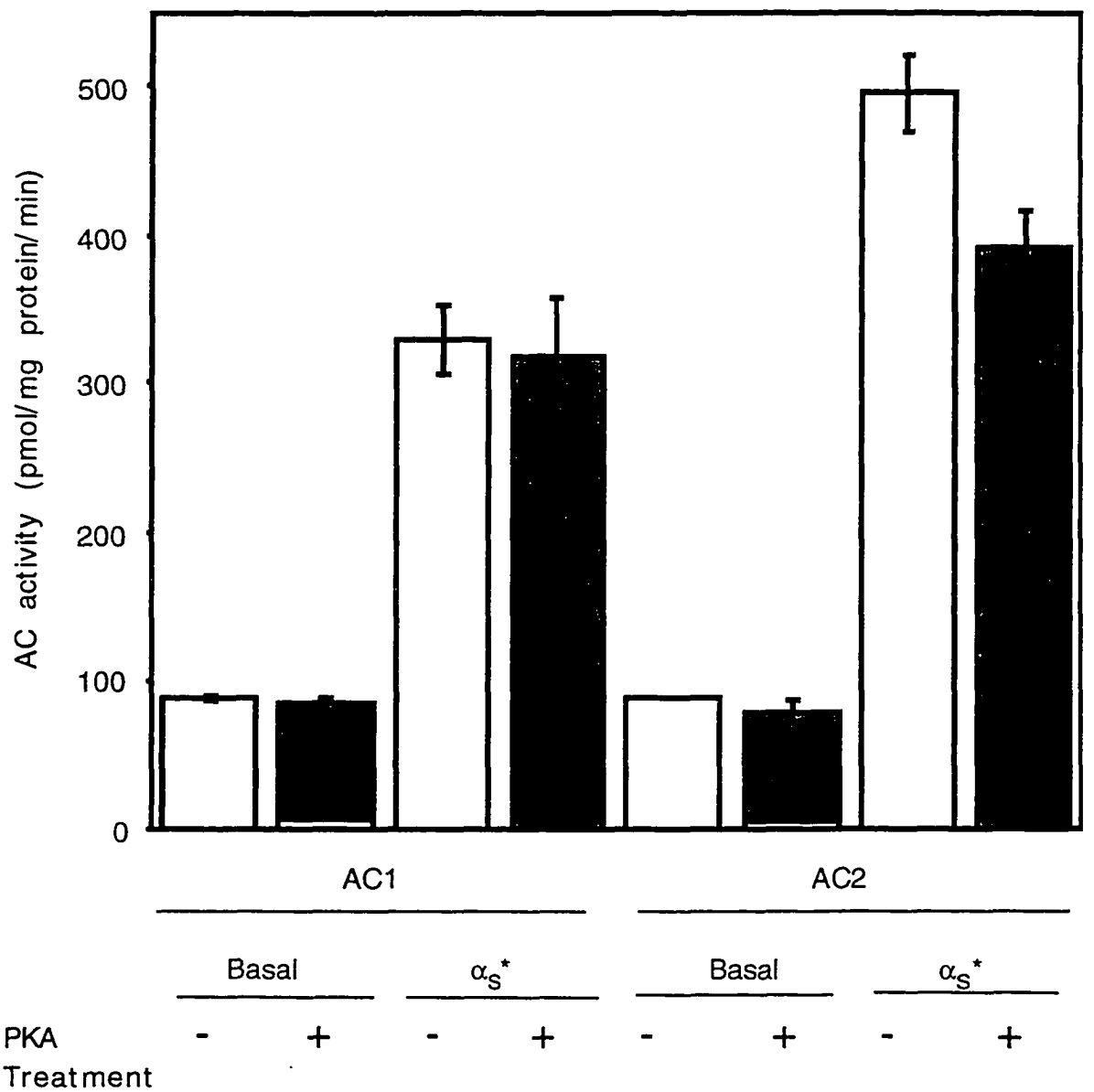


Fig.4-11

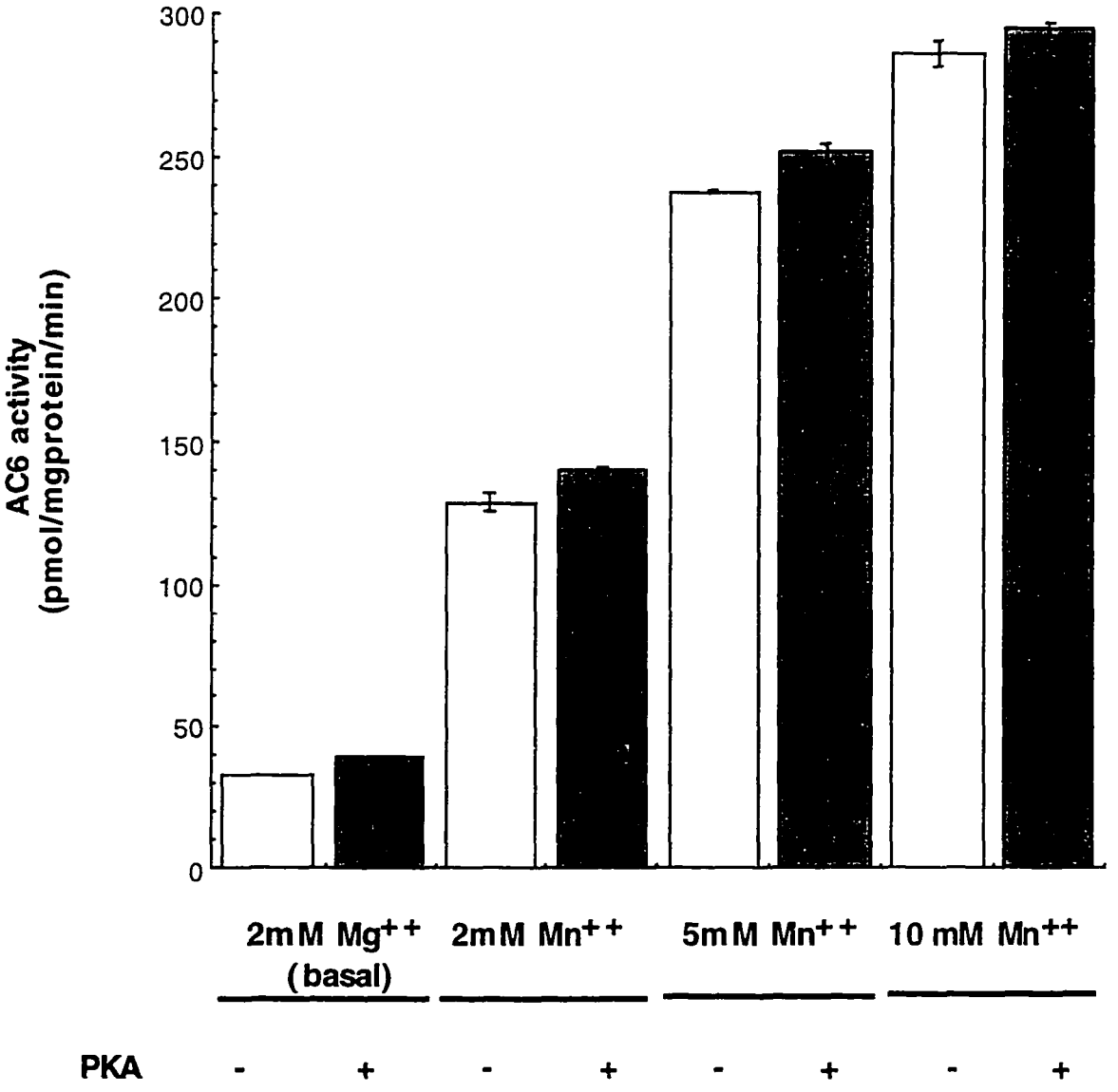
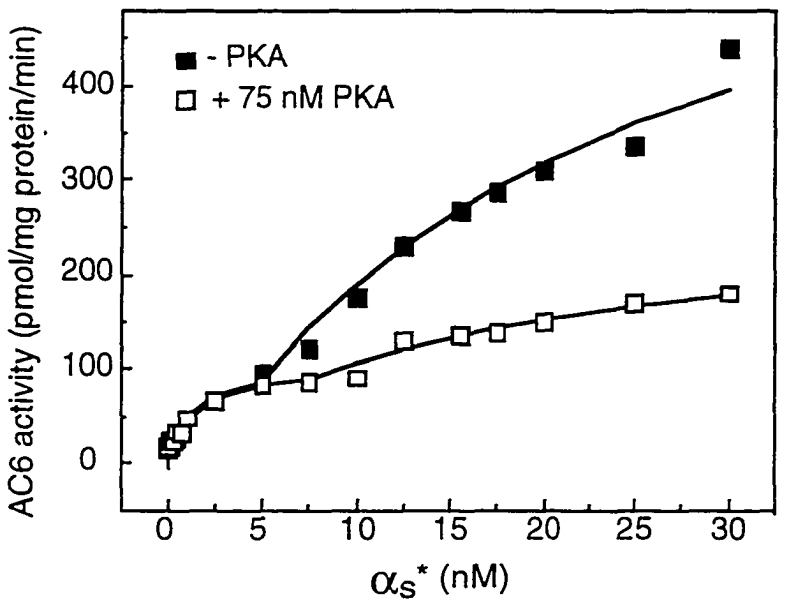


Fig.4-12



		V_{max}	
	app K_{act} (nM)	no PKA	with PKA
site1	1.2+0.9	114±24	108±7
site 2	28±10	620±23	179±9

Fig. 4-13

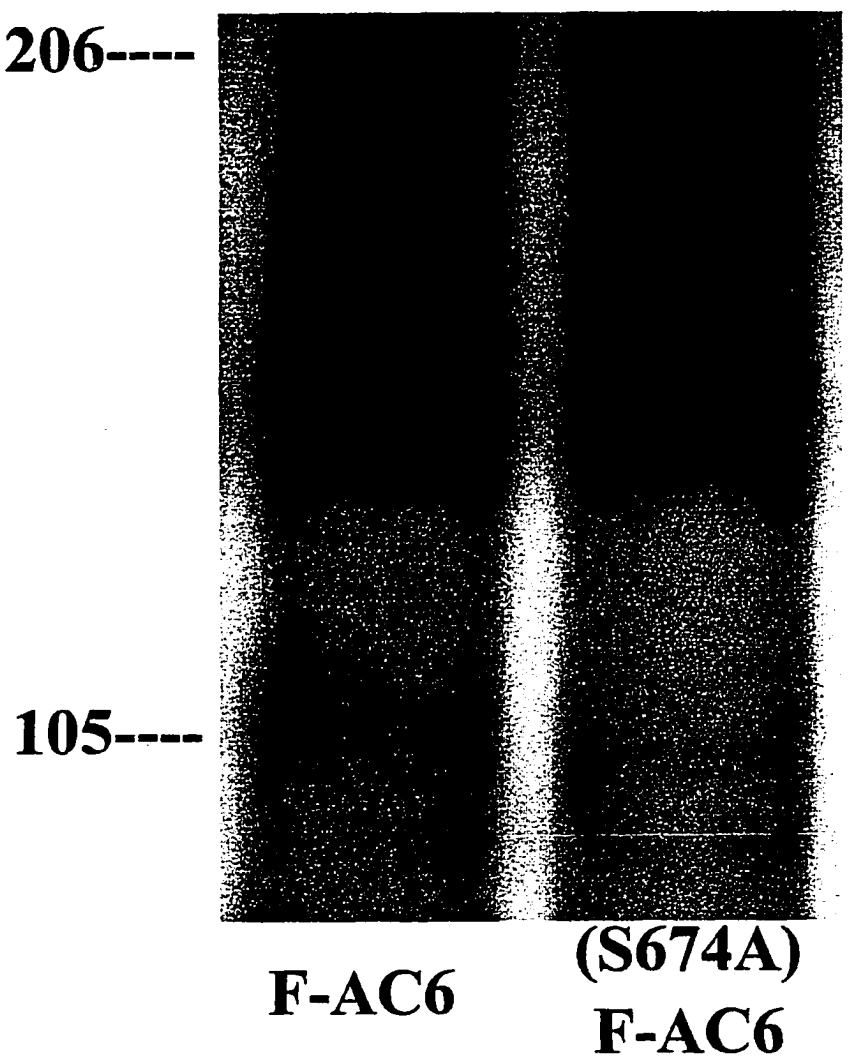


Fig.4-14

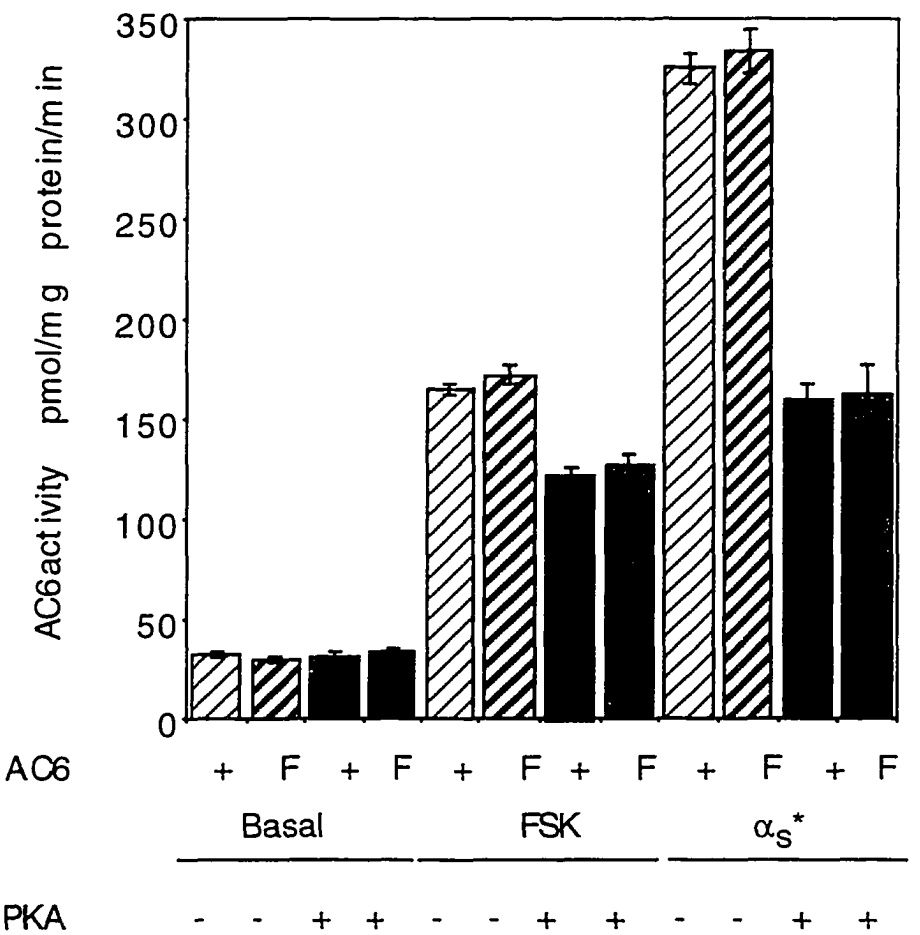
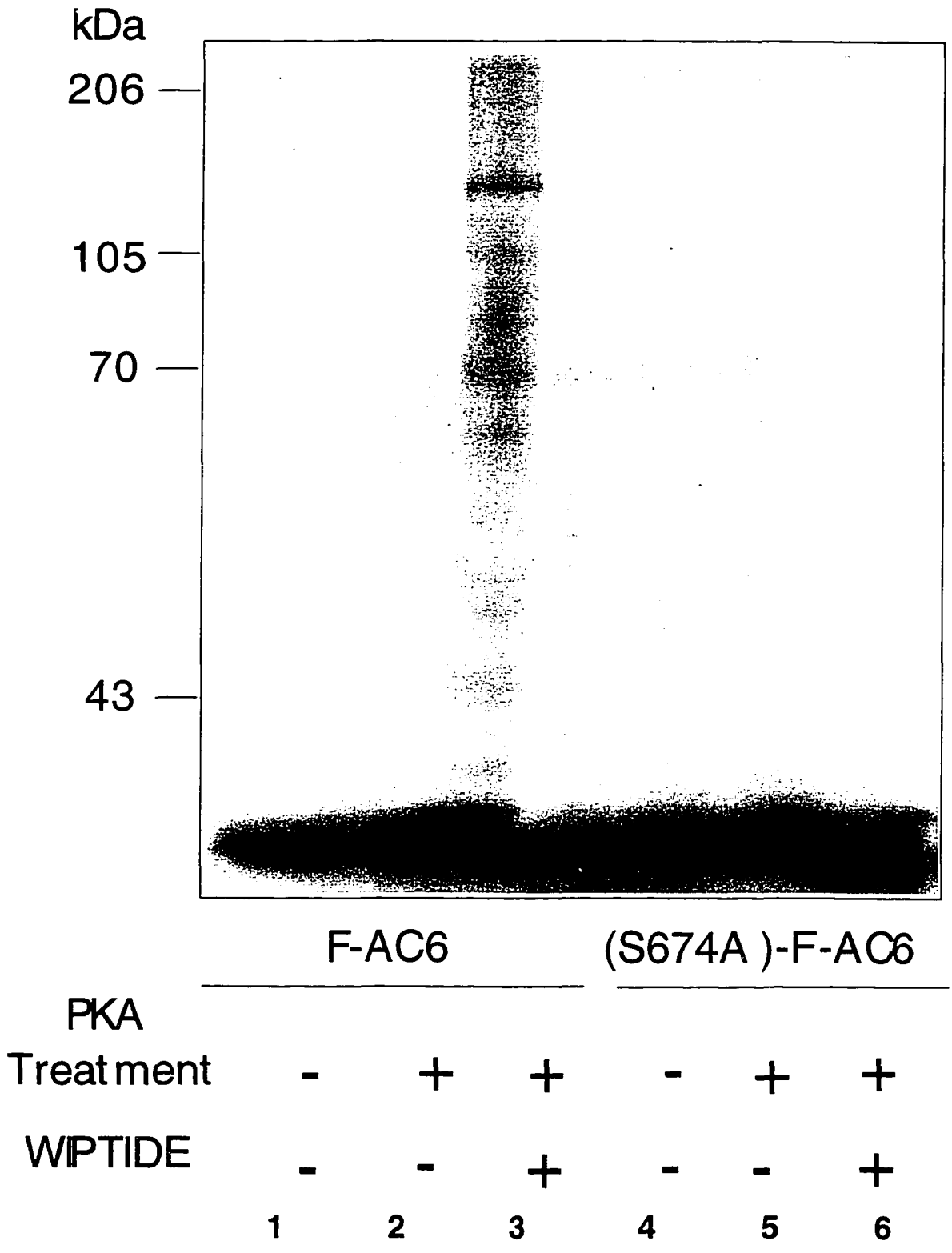


Fig.4-15



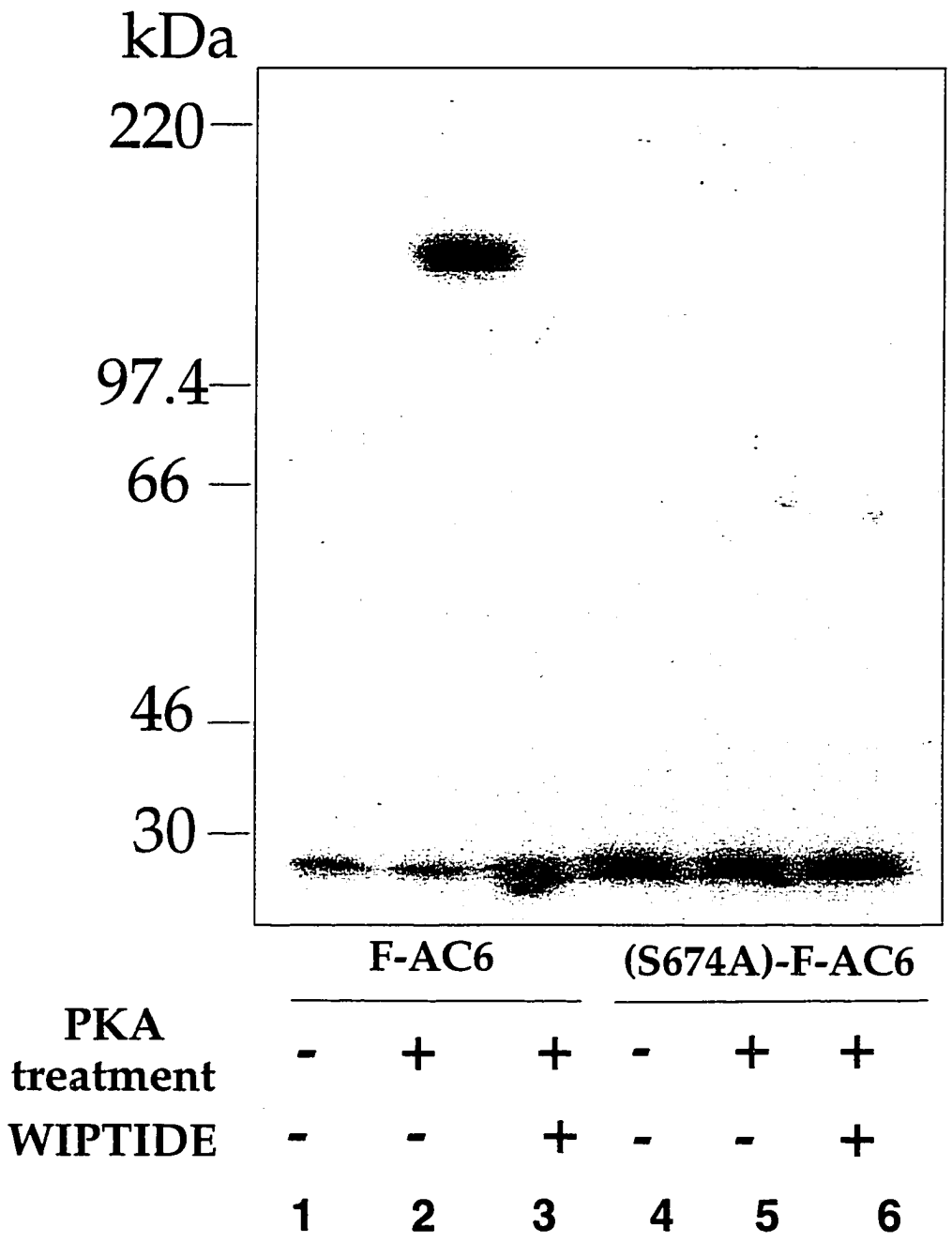
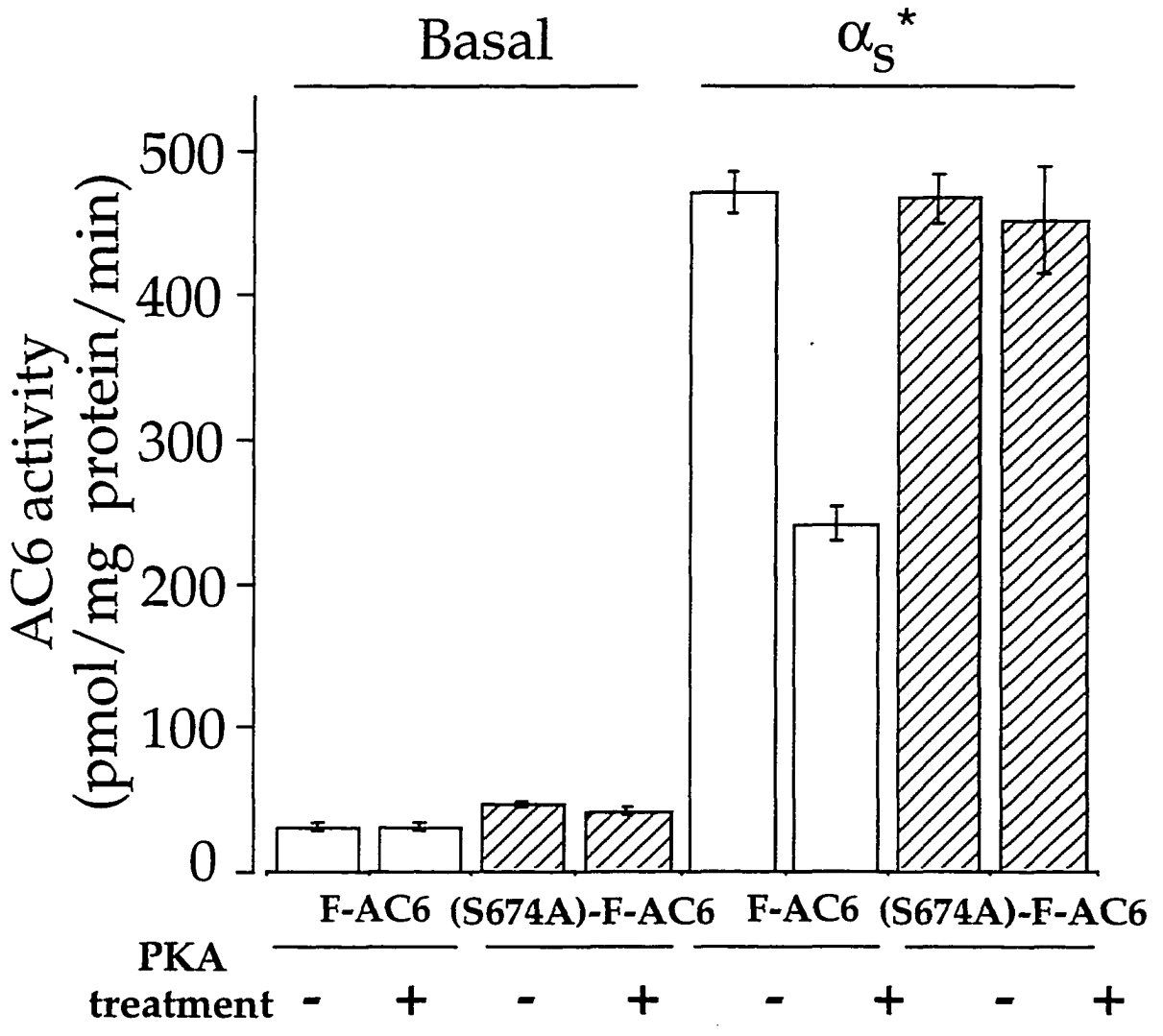


Fig.4-17



Chapter 5

Identification a region involved in regulation of AC6 by high concentration of $G\alpha_s$

Chapter 5

Identification a region involved in regulation of AC6 by high concentration of $G\alpha_s$

My second specific aim is to determine whether the AC6 phosphorylation site and low affinity G protein interaction site are in same region. After characterizing the PKA mediated negative regulation of $G\alpha_s$ stimulation of AC6, one question soon emerged: what is the molecular mechanism of AC6 phosphorylation that causes inhibition of $G\alpha_s$ and FSK stimulated AC6 activities? Several explanations are possible.

1) Phosphorylation of AC6 can change protein folding, change the active conformation, or cause a loss in AC6 activity. It is also possible that S674 is critical for maintaining the active conformation of AC6. Phosphorylation at this residue could change the AC6 active conformation and cause partial loss of AC6 activity, but my mutant AC6 regulatory activity data show that the (S674A)-F-AC6 mutation does not show a significant change in either $G\alpha_s$ or FSK stimulated activities when compared to wild type AC6. This indicates that the Ser residue at this site is not an indispensable residue since the Ser to Ala switch did not fundamentally change AC6 activity. The (S674A)-F-AC6 mutant also does not explain why phosphorylation does not change activity of AC6 at low concentrations of $G\alpha_s$.

2) Phosphorylation of AC6 may interrupt interactions between the two cytosolic domains. The KKYS motif is near the second transmembrane domain and is a good candidate as a linker to connect the interaction of the two cytoplasmic domains However Mn^{2+} stimulated activity is unaffected and this mechanism also does not explain why PKA phosphorylation does not affect AC6 activity in the presence of low concentrations of $G\alpha_s$.

3) Phosphorylation blocks $G\alpha_s$ or FSK interaction with AC6.

When I was thinking of a mechanism of how phosphorylation affects $G\alpha_s$ stimulated AC6 activity, Anya Harry in our laboratory found that there may be two $G\alpha_s$ interaction sites; a high affinity and a low affinity interaction site, (high affinity interaction site Kact=0.4-1.0nM , low affinity interaction site Kact= 8-22 nM).

My AC6 data are very similar to that obtained by A. Harry. The $G\alpha_s$ dose response curve of AC6 is typically biphasic. The $G\alpha_s$ dose response curve of AC6 following PKA treatment, on the other hand is almost monophasic. In this dose response curve it appears that PKA treatment completely blocks the low affinity AC6-$G\alpha_s$ interaction site, leaving only high affinity site intact. It is possible that the PKA phosphorylation site and $G\alpha_s$ "low" affinity $G\alpha_s$ interaction site might be in the same region. My hypothesis is that the phosphorylation of S674 by PKA creates both a charge and steric hindrance which blocks $G\alpha_s$ interaction with the low affinity interaction site of AC6 and

causes inhibition $G\alpha_s$ stimulated AC6 activity. If phosphorylation at this site causes a large decrease in $G\alpha_s$ affinity for AC6, then a ten fold increase in $G\alpha_s$ concentration would show only a little increase in $G\alpha_s$ stimulated AC6 activity, and if this region is involved in $G\alpha_s$ interaction then a peptide encoding this region might affect $G\alpha_s$ stimulation of adenylyl cyclase.

Identification of the low affinity $G\alpha_s$ interaction site in AC6

In order to investigate my hypothesis that the PKA phosphorylation site may be part of the $G\alpha_s$ low affinity interaction site, a number of experiments were designed. Chen JQ in our laboratory used a peptide to inhibit a $G\beta\gamma$ interaction with to AC2 and successfully found important determinants of the AC2-$G\beta\gamma$ binding site (Chen J. et al., 1995). I used a similar approach to map the low affinity $G\alpha_s$ interaction site, and two peptides were synthesized.

660 674 682
1)FLLTFQREDLE**KKY**S**R**KVDPRFG
2)FLLTFQREDLE**KKY**D**R**KVDPRFG

These two peptides contain AC6 a.a. residues from 660 to 682 (a 23mer). The peptide includes the full length PKA phosphorylation motif. The S674D-FLLT peptide has a single a.a. change S674D. Asp contains a negative charge similar to phosphorylated Ser. It is commonly used for replacing a phosphorylated

a.a. residue in protein structure-function research. Initially we attempted to synthesize the FLLT peptide with a phosphorylated Ser but technical difficulties resulted in a very low yield during peptide synthesis.

First, I wanted to verify that the FLLT peptide contained the $G\alpha_s$ low affinity interaction site. If this is the case, the peptide should be able to competitively interfere with the $G\alpha_s$-AC6 interaction. If the concentration of peptide is high enough, it should inhibit $G\alpha_s$ stimulated AC6 activity. I did not expect the peptide to affect AC6 activity at low $G\alpha_s$ concentrations, since phosphorylation of AC6 does not affect high affinity $G\alpha_s$ stimulation. 300μM FLLT was used in experiment (Fig. 5-1). This data shows FLLT strongly inhibits $G\alpha_s$* stimulated AC6 activity in presence of high concentrations of $G\alpha_s$*. The degree of inhibition is similar to that seen after PKA treatment. In the presence of 300μM of FLLT, the typical biphasic AC6 $G\alpha_s$* dose response curve is significantly changed. The low affinity phase of the curve is almost eradicated. However, at low concentrations of $G\alpha_s$* there is only a slight change in $G\alpha_s$* stimulated AC6 activity. In contrast, in control membranes increasing concentrations of $G\alpha_s$* causes increases in the activity of AC6. The maximum effect of AC6 in response to higher concentration of $G\alpha_s$* could not be reached due to limitation of the expression systems. In this experiment both apparent Kact (app Kact) and Vmax are estimated by these data. The app

Kact in site 1 is 1.2 +/- 0.5 nM and site 2 it is 26 +/- 8 nM, and Vmax at site 1 is 109 +/- 7 pmol/mg/min and at site 2 about 713 +/- 26 pmol/mg/min. In the presence of FLLT at site 1, Vmax is not significantly changed, but at site 2 Vmax is greatly reduced. The reduction of the Vmax at site 2 by FLLT qualitatively is very similar to the result of PKA treatment. This result supports the hypothesis that FLLT peptide and PKA phosphorylation may work through same mechanism.

In order to further demonstrate that the inhibition of $G\alpha_s$ stimulation of AC6 by PKA phosphorylation and addition of the FLLT peptide may work through the same mechanism, experiments combining peptide and PKA treatment were performed. If PKA and FLLT work through the same mechanism, then adenylyl cyclase activity in AC6 membranes that had been treated with PKA should not be further inhibited by FLLT peptide. However, if these two treatments work through different mechanisms then the AC6 membranes treated with these two conditions (PKA+FLLT) should show either synergistic or additive effects. AC6 membrane were treated with and without PKA and then assayed in the presence or absence of FLLT peptide. A typical experiment is shown in (Fig. 5-2). There is not much change in basal AC6 activities. For $G\alpha_s^*$ stimulated AC6 activity, either PKA treatment or FLLT addition during the assay showed strong inhibition, however compared to PKA treatment + FLLT peptide in the assay there was no significant difference in AC6 inhibition. These data indicate that the PKA and FLLT effects are nonadditive. That may imply that PKA and FLLT peptide may

work through the same mechanism to regulate AC6 activity. These data support the hypothesis that PKA regulation of $G\alpha_s$* stimulated AC6 activity is through phosphorylation of Ser 674, and that Ser 674 phosphorylation interferes with the $G\alpha_s$* interaction with AC6.

The synthetic peptide may interact with either $G\alpha_s$* or AC. This interaction may interfere or even block native protein interactions. However, when a peptide inhibits the activity of an enzyme, it does not necessarily mean that the peptide is capable of specifically interacting with the enzyme. If a peptide does not contain a specific region or motif which can interact with a specific target, it's regulation may not be specific. For a protein the conformation is decided by its primary structure. The primary structure determines secondary and tertiary structure, but after the discovery of prions and chaperonins this idea has been challenged. A great deal of evidence has demonstrated that a single a.a. mutation in the conserved region or critical region may cause a protein to totally or partially lose function. Such an effect from a single mutation can be explained by the mutation altering the correct protein folding or changing the active conformation of the protein. Some residues are important because they may directly contact other proteins. Changing those residues may cause the protein to lose the ability to interact with other proteins and that may cause a loss of function. These characteristics may also be true for "mini proteins", or small polypeptides. In order to test whether the inhibition of peptide FLLT displayed structural specificity for regulation of AC6, the D674S peptide was tested. If the function

of FLLT peptide is to interact with $G\alpha_s$*, and phosphorylation of Ser 674 disrupts this interaction then changing the Ser to Asp in peptide FLLT should lower the ability of the peptide to interact with $G\alpha_s$*. If the function of FLLT is to block the interaction of the C1 and C2 regions of AC6, such blocking effects should be structurally specific. Fig. 5-3 shows that when the Ser is changed to Asp in S674D- FLLT peptide, the peptide does not inhibit $G\alpha_s$* stimulated AC6 activity. This lack of activity is most likely not due to a change of the structural conformation such as folding, since in mutant S674 -AC6, Ser changed to Ala does not change $G\alpha_s$* stimulated AC6 activity. It is more likely that the change of the OH group to COO^- which produces a negative charge, causes a charge and/or steric hindrance, that interrupts the $G\alpha_s$* interaction with the S674D- FLLT peptide. This suggests that in this region of AC6 a neutral charge may be important for the $G\alpha_s$* interaction.

In order to further characterize FLLT inhibition of $G\alpha_s$* stimulated AC6 activity, the effect of varying concentrations of the FLLT peptide on $G\alpha_s$* stimulated AC6 activity was measured. Increasing concentrations of FLLT blocked $G\alpha_s$* stimulation with an IC_{50} of FLLT of about 50 +/- 10μM (Fig. 5-4). Increasing FLLT peptide concentration from 300 to 400μM results only in a small increase in inhibition meaning that maximal inhibition is near 400μM. In contrast, for the soluble hetero AC enzyme. FLLT can inhibit $G\alpha_s$*

stimulated AC activity more than 90%(Yan et al unpublished data). FLLT does not affect basal AC6 activity at concentrations lower than 300μM.

I next determined if the FLLT peptide inhibition was specific for $G\alpha_s$ stimulated activity. In the PKA treatment experiments, PKA phosphorylation does not affect Mn^{2+} stimulated AC6 activity. If FLLT works through the same mechanism as PKA does it should not affect Mn^{2+} stimulated AC6 either. This was tested in the experiment in Fig. 5-5 . Although 10mM Mn^{2+} extensively stimulates AC6, 300μM FLLT does not inhibit Mn^{2+} stimulated AC6 activity.

FSK stimulated AC6 activity can also be negatively regulated by PKA treatment. (Fig. 5-6) shows that FSK stimulated AC6 activity is also negatively regulated by FLLT peptide although it is not as effective in blocking forskolin stimulation.

The FLLT motif has high similarity between AC6 and AC5 but less similarity among other AC subtypes. However several a.a. residues in the peptide are conserved among all AC subtypes, such as L362, E371, K372 and D378. In order to test FLLT peptide inhibition of $G\alpha_s$ stimulated AC6 is selective or is observed with other ACs, the effect 300μM FLLT peptide on the stimulation of AC2 by varying concentrations of $G\alpha_s$ has been studied. As can be seen from the dose response curve in (Fig. 5-7) the FLLT peptide also inhibits $G\alpha_s$ stimulated AC2 activity by about 25-30% at high concentrations of $G\alpha_s$. Since AC2 does not have a BBXS motif in this region it may not be a target for PKA regulation, but this region is also important for AC2 and $G\alpha_s$

interactions. Thus all my data support the hypothesis that the FLLT peptide and PKA phosphorylation may work through the same mechanism.

Figure legends

Fig. 5-1 Effect of FLLT peptide on stimulated AC6 by varying concentration of $G\alpha_s$

AC6 containing Hi-5 cell membranes were assayed in presence of indicated concentrations of $G\alpha_s$* with or without the FLLT peptide. The data best fit a two-site model. Indicated constants obtained from the two-site fit are given in the inset.

Fig. 5-2 Effect of PKA treatment on inhibition of $G\alpha_s$ stimulated AC6 activities by FLLT peptide

AC6 containing H-5 cell membranes were treated in the absence or presence of PKA and then assayed for basal and 20 nM $G\alpha_s$* stimulated AC6 activities in the absence or presence of 300μM FLLT peptide. Values are mean +/- SD of triplicate determinations.

Fig. 5-3 Comparison of FLLT and S674D-FLLT peptides on AC6 activities

The effect of FLLT peptide and a modified peptide (S674D)-FLLT peptide where the Ser corresponding to Ser^{674} was substituted with an Asp(D) on activation of AC6 containing Hi-5 cell membranes. Basal and 20 nM $G\alpha_s$* stimulated AC6 activities were measured. Values are mean +/- SD of triplicate determinations.

Fig. 5-4 FLLT peptide dose response curve for basal and $G\alpha_s$* stimulated AC 6 activities

Effect of varying concentrations of FLLT peptide on stimulation of AC6 by 17.5 nM $G\alpha_s$ and on basal activity.

Fig. 5-5 FLLT peptide effect on Mn^{2+} stimulated AC6 activity

Mn^{2+} stimulated AC6 activities were measured at indicated concentrations of Mn^{2+}. Basal activity was the AC6 activity in presence of 2 mM $MgCl_2$. FLLT peptide concentration was 300μM.

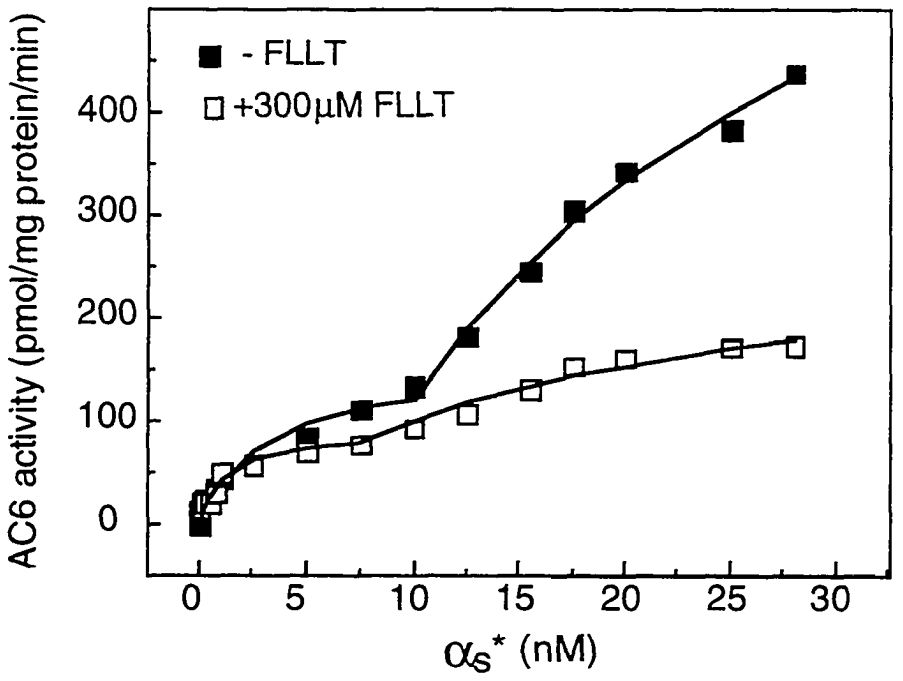
Fig. 5-6 Effect of FLLT peptide on FSK stimulated AC6 activity

FSK stimulated AC6 activity was measured in the absence or presence of 300μM of FLLT peptide. FSK concentration was 50μM. Values are mean +/- SD of triplicate determinations

Fig. 5-7 Effect of FLLT peptide on Gas stimulation of AC2

AC2 containing Hi-5 cell membranes were assayed in the presence of indicated concentrations of $G\alpha_s$ in the absence(o) or presence(**o**) of 300 μM FLLT peptide.

Fig.5 -1



	app K_{act} (nM)	V_{max} no FLLT	V_{max} with FLLT
site1	1.2+0.5	109±7	94±10
site 2	26±8	713±26	204±20

Fig.5-2

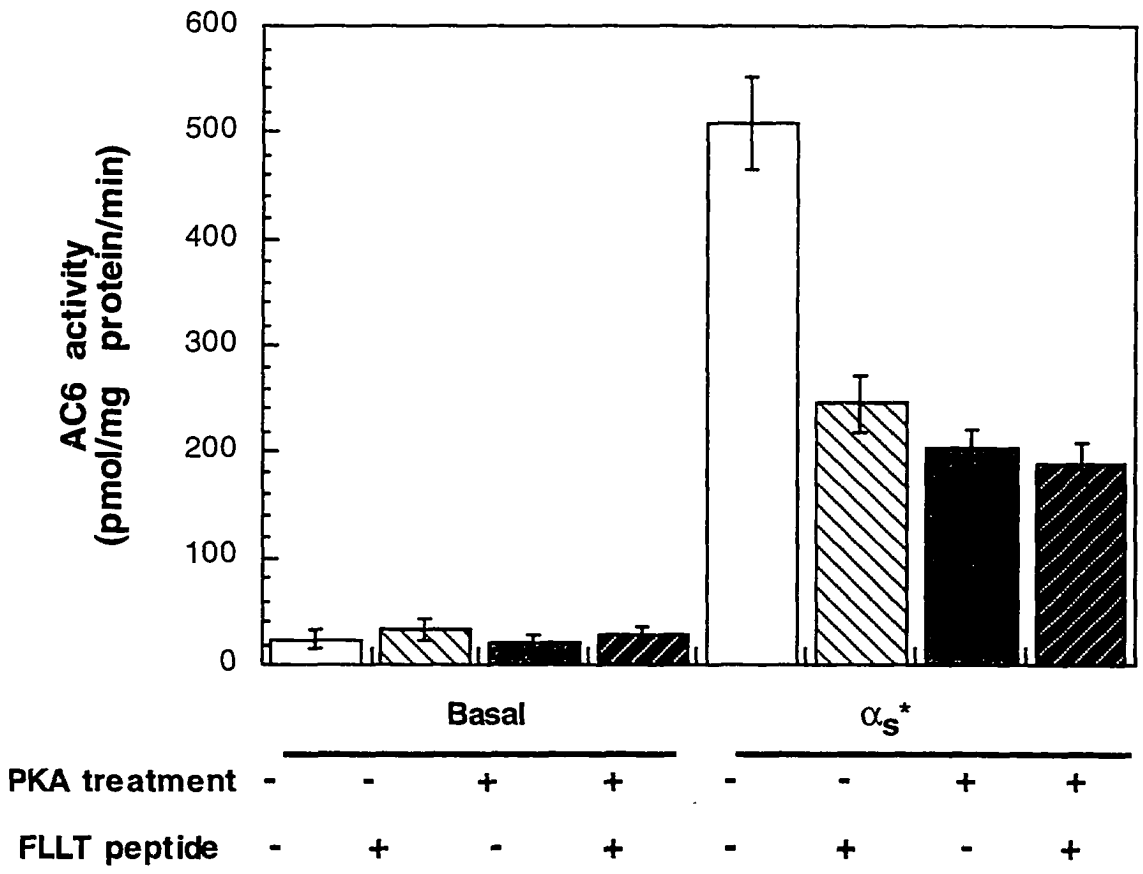


Fig. 5-3

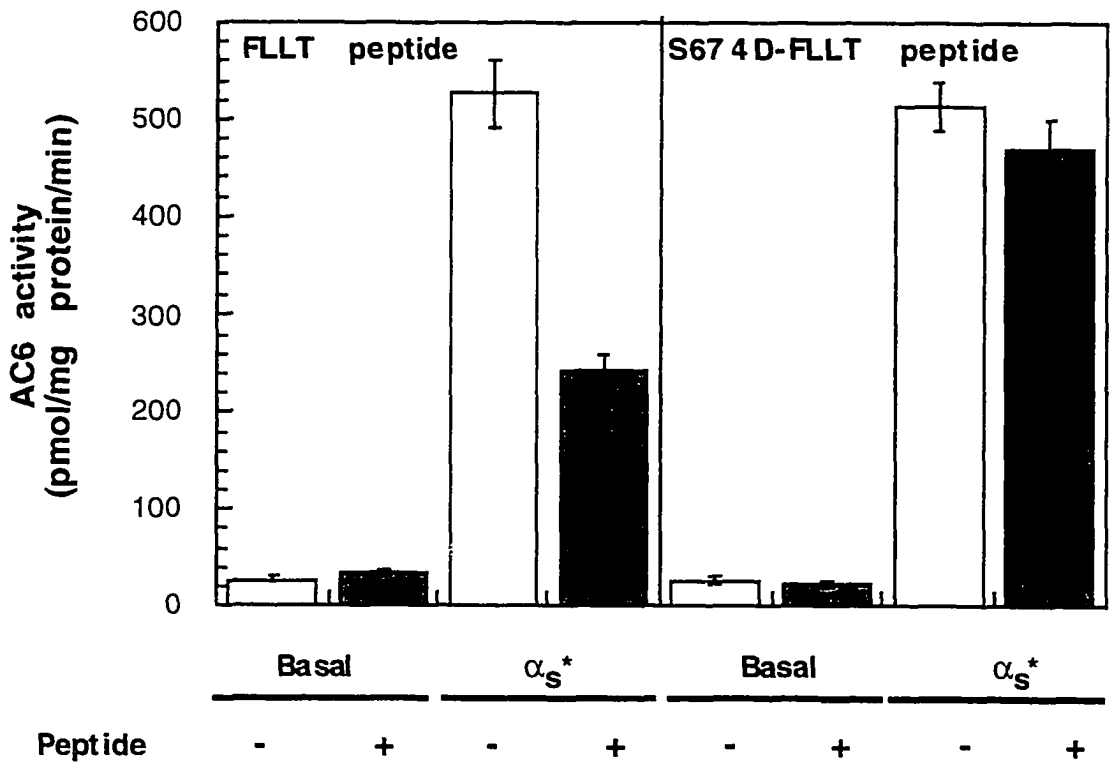


Fig. 5-4

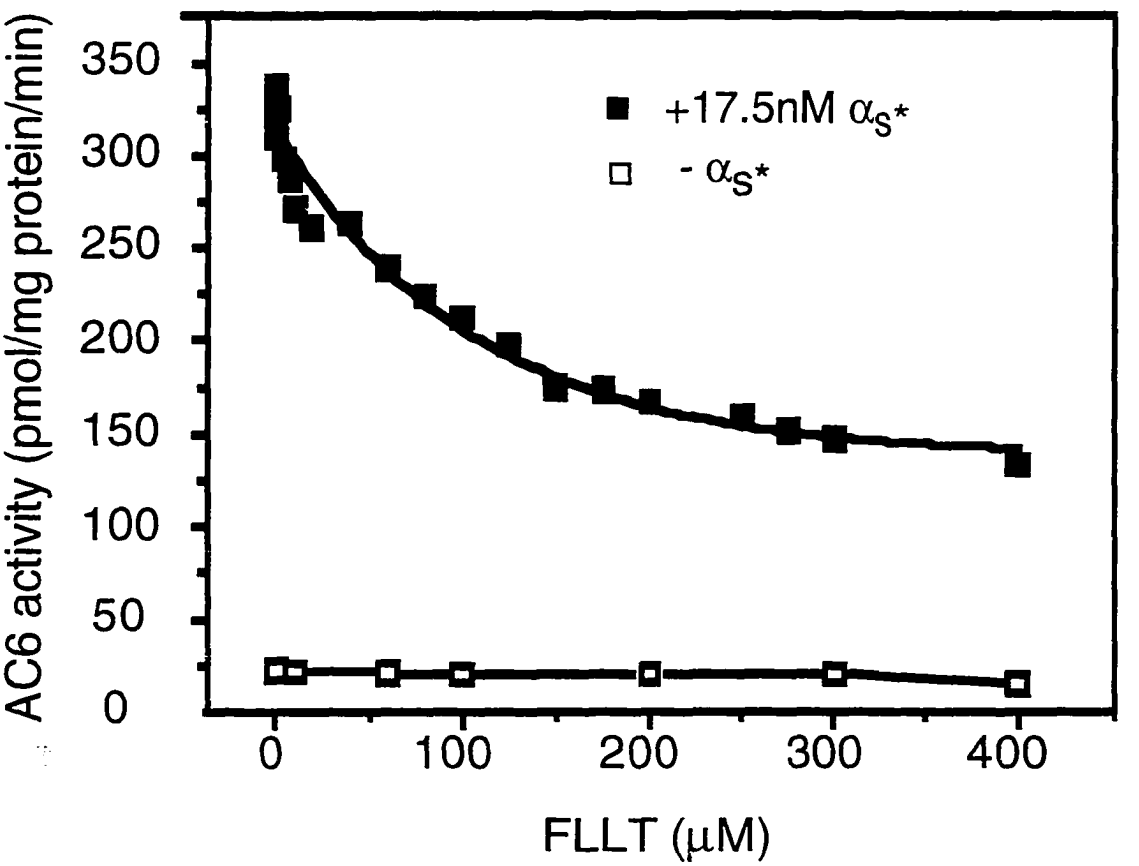


Fig.5-5

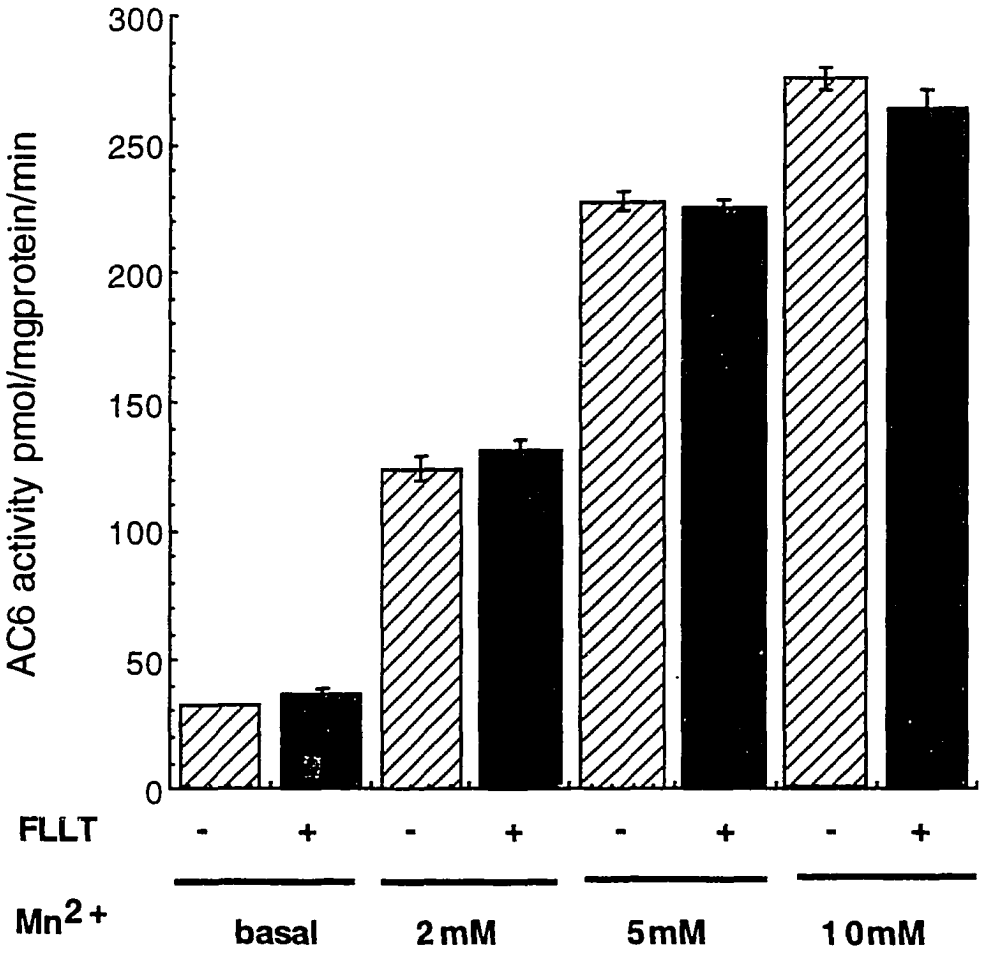


Fig.5-6

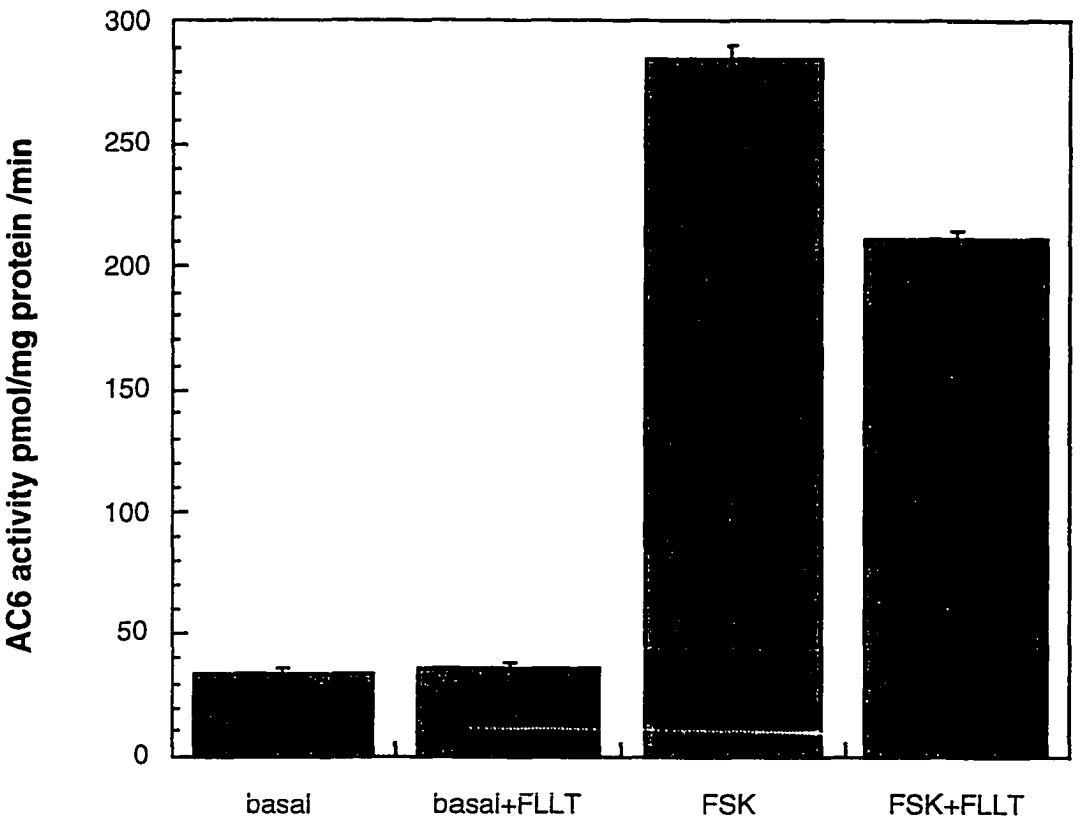
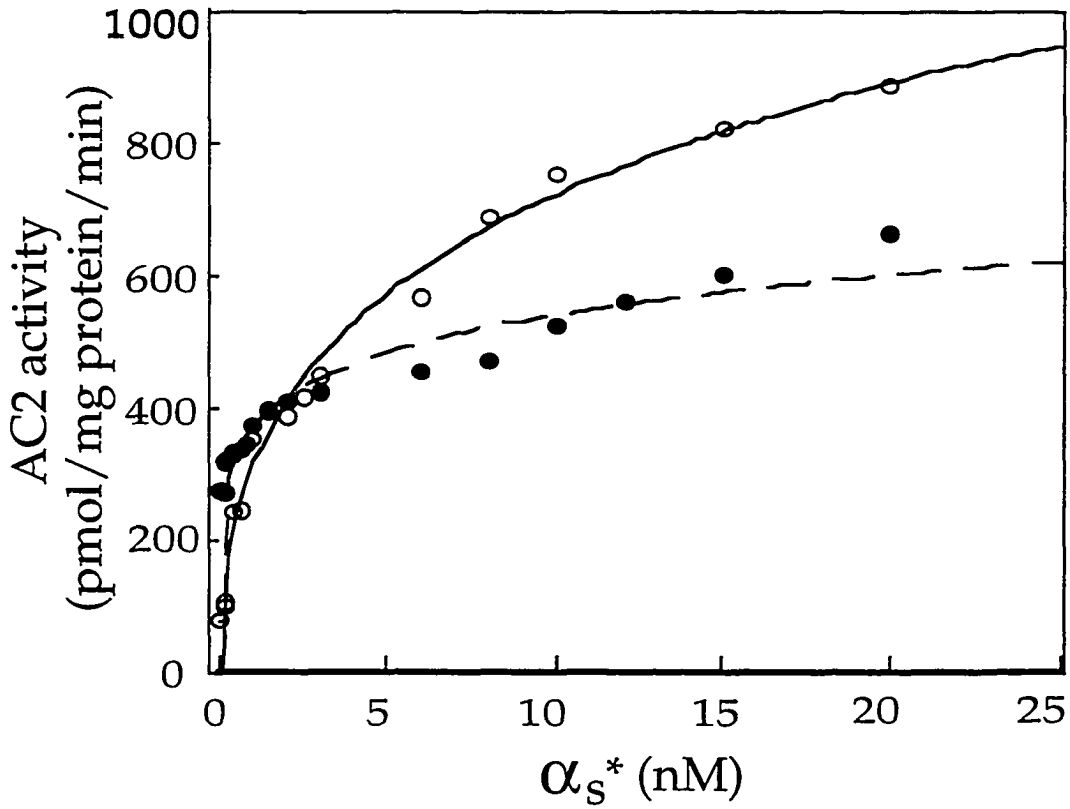


Fig.5-7



Chapter 6

Discussion

Chapter 6

Discussion

The results in this thesis show that AC6 is a target for PKA regulation. PKA phosphorylates AC6 at Ser^{674}, and this results in a reduced capability to be stimulated by $G\alpha_s$. Several aspects of this regulation are worthy of discussion.

1. Substrate specificity of PKA and AC6 phosphorylation

In my thesis research I have focused mainly on characterizing PKA regulation of $G\alpha_s$ stimulated AC6 activity and the AC6 low affinity $G\alpha_s$ interaction site. Anya Harry has characterized $G\alpha_s$ regulation of AC6 activity and compared it to other adenylyl cyclase isoforms. There appears to be two $G\alpha_s$ interaction sites in a fully functional AC6, high affinity and low affinity. My data showed that the low affinity $G\alpha_s$ interaction site and PKA phosphorylation site may share domains on AC6. Thus PKA may mediate feedback inhibition of A6 by phosphorylation which creates negative charge and steric hindrance to blocks $G\alpha_s$ interaction. The PKA phosphorylation would work as an on and off switch to control AC6 and downstream cellular elements cellular involved in eliciting downstream biological activity. This switch works only when AC6 activity is high and the intracellular cAMP level is greatly elevated.

What are the specificity and efficacy of PKA with AC6 as substrate?.

PKA has broad substrate specificity. Any substrate that has a BBXS motif can a be candidate substrate of PKA, where B stands for basic amino-acids, X for any amino acid and S for Ser. This does not necessarily mean the specificity of PKA is low. Within the general motif certain sequences are preferred more than others. For example, differing peptide sequences show differing rates of phosphorylation by PKA.

Peptide sequence	Vmax/Km,μmol/(min.μmX10^4)
LRRASLG	12500
LRKASLG	654
GGGGGGGGRRSGG	150
GGGGGGGRRGSGG	95000
GGGGGGRRGGSGG	200

LRRASLG is called Kemptide and is a very efficacious substrate of PKA. The sequence is derived from its natural parent protein pyruvate kinase. When one R is changed into a K, the peptide LRKASLG has a lower binding affinity and Vmax/Km is 20 fold reduced.(Kemp B. et al., 1977, Feramisco J., et al., 1980, Walsh D. and Patten S. 1994). If the basic a. a. residue R is replaced in either the right or left position both substitutions dramatically decrease susceptibility to be phosphorylated by PKA. This means that even though PKA has broad specificity for substrates it still can discriminate among substrates.

In AC6 the phosphorylation the sequence motif of PKA is

660 674 682

FLLTFQREDLEKKYSRKVDPRFG,

KKYS motif is not predicted to be a great substrate for PKA. In *in vitro* phosphorylation experiments, higher PKA concentrations and longer

incubation times may be required in order to see a sufficient phosphorylation effect of AC6 activity and visualize the ^{32}P phosphorylated AC6 band. On the other hand, the BBXS motif may not be the only motif for PKA phosphorylation. For instance, fructose bisphosphatase is a substrate of PKA, but it does not have a BBXS box. Instead it has a KAKS sequence. That might indicate that BBXS is not the only motif for PKA regulation. Although there are other Ser in AC6 , it appears that Ser-674 is the only one involved in PKA regulation since its conversion to Ala blocks the PKA effect. Thus, although AC6 may not be the best substrate for PKA, PKA phosphorylation leads to significant effects on the regulation of AC6.

2. Biological Relevance of AC6 regulation by PKA.

The characteristics of PKA phosphorylation profile of AC6 and the $G\alpha_s$ stimulation profile indicates that this mode of regulation may be physiologically relevant. The liver, where AC6 is expressed, is one organ where PKA regulation of AC6 may be important for glucagon regulation of blood glucose. Glucagon binds to liver glucagon receptors and stimulates the glycogenolytic pathway, resulting in a rapid build-up of blood glucose. Glucagon regulates blood glucose concentration by regulating key enzymes in glucose metabolism and gluconeogenic pathway. Glucagon is secreted by α cells of pancreatic islets, and this secretion is regulated by blood glucose levels. If there is too much glucagon present, blood sugar might increase to abnormal levels. For normal healthy persons the blood glucose levels are well controlled and fluctuate within a limited range. Such a range may be defined by PKA regulation of AC6. If too much glucagon is secreted more receptors are activated which in turn activate more $G\alpha_s$. The more activated $G\alpha_s$ can

stimulate AC6 to produce more cAMP. When cAMP goes up to certain levels (above the PKA threshold) PKA is activated. If the PKA is extensively activated it can phosphorylate AC6 and reduce cAMP production and thus block a continuous increase and prevent very high increase in blood glucose. Under normal physiological conditions, and moderate increases in glucagon may cause PKA to be activated for routine biological purposes since PKA has broad substrate specificity. In cells there are numerous PKA substrates. Some are "good" substrates in that they are easily phosphorylated since they have high affinity. There are some "bad" substrates since they have low affinity to PKA and need higher concentrations of activated PKA to be phosphorylated. From the sequence motif, AC6 may not be a good substrate for PKA. Only when there is too much glucagon and consequently increasing concentrations of glucose in blood, is PKA fully activated. Fully activated PKA will phosphorylate AC6 and negatively regulate the activity of AC6. The KKYS motif in AC6 makes it is possible for AC6 to be a low efficacy substrate fot phosphorylation. Only when cAMP is increased beyond a critical concentration will AC6 be phosphorylated and further increases in glucagon mediated cAMP formation be limited. This regulation arises from the nature of the $G\alpha_s$ stimulation of AC6. The $G\alpha_s$ dose response curve for AC6 is biphasic. Upon PKA phosphorylation, stimulation at low concentrations of $G\alpha_s$ is unaffected, however when adenylyl cyclase activity reaches the first steady state of the curve, further increases in $G\alpha_s$ do not show dramatic increases in AC6 activity. Thus, feedback regulation by PKA can curb the AC6 activity and cAMP production at certain levels, and thus, the possibility that increased hormone release will cause a blood glucose crisis is greatly reduced.

A similar physiological phenomenon may also occur in heart. The heart is another place where AC5 and AC6 are expressed extensively. The β-

adrenergic signaling system via β-adrenergic receptors regulates positive cardiac excitation to increases both force and rate of heart-beat. Activation of β adrenergic receptors, which via activated $G\alpha_s$, increases cAMP, which then activates PKA. Several cardiac proteins such as troponin I, phospholamban, sarcolemma p27 protein, phosphorylase kinase are phosphorylated by PKA(Walsh et al., 1979; Angelos KL. et al., 1987; England, PJ. et al., 1975; England et al., 1976; Huggins JP. et al., 1983). These proteins are involved in positive cardiac regulation. Maintaining high concentrations of cAMP can cause heart problems since sympathetic stimulation may cause extensive release of epinephrine. PKA mediated feedback inhibition at the level of AC6 could regulate cAMP production and may serve to keep heart rate and contraction in a normal range.

3. Mg^{2+} effect on catalytic activity of both PKA and AC6

Mg^{2+} is required for PKA mediated phosphorylation. It is also required for maintaining AC6 catalytic activity and Mg^{2+} may be required for all the enzyme catalytic reactions which use ATP as a substrate. Increasing the concentration of Mg^{2+} may facilitate PKA mediated substrate phosphorylation. Increasing concentrations of Mg^{2+} also increases basal, $G\alpha_s$, and FSK stimulated AC activities. PKA treatment does not affect basal activity of AC6 at different concentrations of Mg^{2+}. However with increasing concentrations of Mg^{2+} , PKA mediated inhibition of $G\alpha_s$ stimulated AC6 activity was decreased. One possible explanation is that after AC6 was treated with PKA, S674 was phosphorylated. The phosphate group on S^{674} has a strong negative charge(PO_3^{2-}), and that negative charge may prevent $G\alpha_s$ interaction with AC6, this results in inhibition of $G\alpha_s$ stimulated AC6 activity. In the AC assay

Mg^{2+} concentrations are higher than ATP. Beside Mg^{2+} binding to ATP to form the Mg^{2+}-ATP complex, increasing concentrations of Mg^{2+} may neutralize the negative charge of phosphorylated S674. This neutralization may allow for partial recovery of $G\alpha_s$ binding and thus partially reduce PKA mediated inhibition of $G\alpha_s$ stimulation.

4. PKA Regulation of AC6

The Ishikawa laboratory has shown that treating AC5 expressed in Sf9 cell membranes with PKA results in a phosphorylated AC5 band (Iwami, G., et al., 1995). AC5 and AC6 both have a conserved PKA site, but AC5 has a second consensus PKA phosphorylation site. Currently we do not know which sites are phosphorylated or if both sites are phosphorylated. However data from the Ishikawa laboratory have shown that PKA treatment of AC5 also results in inhibition of FSK and $GTP\gamma_s$ stimulated AC5 activity (Iwami, G. et al., 1995 b). Nevertheless we do not know whether such inhibitory effects are mediated by PKA phosphorylation of the cognate Ser in AC5. Since AC5 has more than one phosphorylation site, the question arises what if any is the function of each phosphorylation site? Do they work cooperatively or do they have no relation to each other at all? We still do not know the answer. The location of the second consensus phosphorylation site of AC5 is in the cytosolic loop between transmembrane domain 8 and 9. At this location AC6 does not have a consensus PKA phosphorylation site. The role of this region in adenylyl cyclase regulation remains to be explored.

5. Peptide versus mutation in AC structure studies

Using site-directed Ala screening mutagenesis Yan et al. (1997 b) have identifed regions on soluble AC1/AC2 heterodimer involved in interactions with $G\alpha_s$. Grishina and Berlot (1997) have defined the AC interaction site on $G\alpha_i$ and $G\alpha_s$. Using peptide competition approaches Chen JQ et al. (1995) in our laboratory found the $\beta\gamma$ binding site on AC2. These two techniques have been successfully used in AC and G protein research. I have used both techniques in my thesis research. The advantage of site-directed mutagenesis is that it can define the residues which are important for catalytic or regulatory function of AC or important for G protein binding and function. If that residue is important, mutation of the residue will cause significant alterations in AC or G protein biological activity. The disadvantage of using mutagenesis is that mutations may cause changes in protein folding, and the resulting loss of biological activity may be related to folding rather than to changes in protein-protein interactions. Alanine substitutions is a conservative approach, where the folding problem is greatly reduced. But other problems may arise, such as a lack of effect upon substitution. Nevertheless, a series of Ala scanning mutations generally are useful in defining interaction sites. For peptide competition methods the advantages are: a) The use of native proteins as a target, both stimulatory and inhibitory effects may be faithfully reproduced on the target protein. b) On the whole, the method is quite simple. The disadvantages include an unclear understanding of the limits of the region involved in the interactions. Often inhibitory effects can be easily observed but stimulation by peptides is not often seen. Further the mechanisms involved in peptide competition are still not very clear.

6. Current developments in structure-function studies on adenylyl cyclases and $G\alpha_s$.

During my preparation of this thesis several important papers on the structure of adenylyl cyclases and its regulation by G protein subunits have been published. Tang's laboratory showed that the C2 domain contained two key residues for catalytic activity: Asn^{1025} and Arg^{1029}(Yan SZ. et al., 1997 a). Mutation of each one caused a 30 to 100 fold reduction of K_{cat}; if both residues were mutated there was a 3000 fold reduction of K_{cat}, but the same mutations did not affect the K_m for ATP. Hence this region is thought to be part of the catalytic core of AC.

The second significant advance was the crystal structure of the AC2 tail. Since ACs are membrane proteins it is very difficult to obtain their crystal structures. Fragments of adenylyl cyclase which are soluble and still maintain function are good candidates for solving the structure of functional ACs. Recently, Hurley and his colleagues solved the AC2 tail IIC2 crystal structure(Zhang G. et al., 1997). Two IIC2 monomers are linked by two FSK molecules, and are arranged in a wreath-like dimer which has a central cleft. Two FSK molecules bind in hydrophobic pockets at ends of cleft. The central part of the cleft is lined by charged residues which may imply that this is the area for ATP binding but they had not shown which residues are possible to interact with ATP. It seems that the AC2 tail contains most of the elements for catalysis. They further predict that FSK appears to activate AC by promoting the assembly of the active dimer and by direct interaction with the catalytic cleft. The important contribution of this crystal structure is that it identified the FSK binding site for the first time. There are two sets of a.a. residues that form the FSK binding sites, Lys^{896}, Gly^{941}, and Ser^{942}; and Phe^{889},

Try^{1020} Val^{1024} and Met^{945}. These residues are tightly clustered within the 895-1020 region. A significant drawback in using the C2 homodimer crystal as a model for a functional adenylyl cyclase is that the C2 domain does not show significant catalytic function. So far no one has reported if the C2 tail by itself has stimulatable or regulatable catalytic activity by any common AC stimulator. However when the two cytosolic domains IC1 and IIC2 or VC1 and IIC2 are mixed they show strong regulatory AC activity.

A third significant paper was the finding of the $G\alpha_s$ binding site by Yan et al in Tang's lab. Using a large mutagenesis study they found two discrete regions in AC2 C2 that may form a $G\alpha_s$ binding site(Yan SZ. et al., 1997b). One region is encoded by a.a. residue from 910 to 925, another region 986 to 996 of AC2 tail. Since these residues are distal to the catalytic core(Yan SZ. et al., 1997a) they think that these residues may be part of the $G\alpha_s$ binding site. In preliminary experiments, I have confirmed one of these regions (Chen Yb et al., unpublished data). I have found that a peptide derived from this region can strongly inhibit $G\alpha_s$ stimulated AC2 and AC6 activity without affecting basal activity.

Another important finding was from the Gilman laboratory using the AC5-C1 and AC2-C2 chimeric soluble enzymes (Sunahara JJ. et al., 1997a). Full length AC2 tail was expressed but, only a partial AC5 C1 cytosolic domain, about 227 a. a. residue was expressed. However, it showed substantial regulatory and stimulated catalytic activity. In this paper an important finding was that the function of $G\alpha_s$ stimulation was to link C1 and C2 and promote their initial interaction. FSK could also facilitate such a link. Glimmer's data also showed that in $G\alpha_s$ stimulated chimeric soluble AC, $G\alpha_s$: 5C1: 2C2 of AC is 1:1:1 .

7. Crystal structure of ACV(aa residue 364-580) VC1 and ACII (aa residue 874-1081)IIC2 hetero soluble enzyme complexed with $G\alpha_s$· GTPγS

The most important progress in AC research was solution of the crystal structure of soluble AC composed of AC5-C1 and AC2-C2 (Tesmer JG. et al., 1997). This paper was published just a few weeks ago. The crystal structure of the VC1 and IIC2 together with $G\alpha_s$ ($G\alpha_s$ GTPγS-Mg^{2+}) and the FSK analog MPFSK, was described, at the same time as the crystal structure of $G\alpha_s$ itself was solved(Sunahara RK. et al., 1997 b). The crystal structure shows the $G\alpha_s$ binding site is basically at the same region as Yan et al., predicted by mutational analysis. Interestingly enough the FSK binding site is very similar to that seen in the AC2 homodimer. The only difference is that in the VC1 and IIC2 heterodimer, the FSK binding site is formed by Lys^{896}, Gly^{941}, and Ser^{942} in IIC2 domain that are the same as the homodimer crystal. The second binding site consisting of Phe^{889}, Try^{1020} Val^{1024} and Met^{945} in the homodimer is replaced in the heterodimer such that the second forskolin molecule does not bind to the heterodimer. The crystal also shows that the C1 and C2 contact interface is important for function. Preliminary data which I obtained (Chen Y-b unpublished data) shows that a peptide encoding a region of this interphase show strong inhibition of $G\alpha_s$ stimulated AC activity. The crystal shows the P-site inhibitor biding site, but whether this site is the same site for ATP is still not fully resolved, although the authors suggest that it is analogous. The reason for this question is simple since P-site inhibition is either non-or un-competitive inhibition. If both the P-site inhibitor and ATP bound to the same site, inhibition should be competitive. The crystal shows that C2 itself may not be enough to form the catalytic center. To contain the catalytic site it needs an ATP binding site. In the crystal this is formed by both

the C1 and C2 domains, and individual C1 or C2 cytoplasmic domains are unlikely to be catalytic (Tesmer JG, et al., 1997). Although the soluble AC crystal structure reveals a lot of information, it is doubtful that it is representative of the native enzyme. For instance, the crystal structure does not contain the region involved in PKA regulation. Further, since the P-site inhibitors are non or un- competitive inhibitors, whether the ATP binding site is the same site as 2'd3'AMP needs to be confirmed.

8. Speculations on the structural requirements for adenylyl cyclase function.

According to my data and all the information I have, I propose the following mechanism for adenylyl cyclase activity. In AC there are two cytosolic domains which are required for full catalytic and regulation of AC activity. AC contains a catalytic core which is formed by a heterodimer of C1 and C2 that has full catalytic capacity, but under basal condition AC catalytic core activity is inhibited by a negative regulatory elements on AC itself. The truncated C1 and C2 domains contain all major regulatory elements and at least one $G\alpha_s$ binding site, and one FSK binding site. The $G\alpha_s$ has high affinity to C2 and low affinity to C1, but when $G\alpha_s$ binds to C2, both C2 and $G\alpha_s$ initiate a conformational change that increases their affinity for C1. In the presence of $G\alpha_s$ they form a trimer complex. The interaction between C1 and C2 causes conformational changes and releases the negative regulatory element to form a high capacity catalytic functional center. Without stimulatory entities, the negative elements are not fully functional and do not block AC activity completely. There is some "leaky" basal AC activity. Since C1 and C2 contain all major regulatory elements, the function of $G\alpha_s$ is to bring the two domains together to making a two domain interaction.

Higher AC activity depends on the stability of the heterodimer formed. FSK synergises with $G\alpha_s$ to facilitate $G\alpha_s$ mediated C1 and C2 complex formation or to directly stabilize the catalytic center. AC may have more than one $G\alpha_s$ interaction site. For AC6; high affinity $G\alpha_s$ interaction works at low concentrations of $G\alpha_s$, and at increasing $G\alpha_s$ concentrations the low affinity regulation may also be involved, but this may involve dimerization of adenylyl cyclase. Each subtype of AC has its own $G\alpha_s$ binding site which should be highly consered since the primary sequence in these regions is very similar in all the ACs and they share many common characteristics.

$G\beta\gamma$ and Ca^{2+}/CaM may also work by the same mechanism to help form the C1C2 dimer. The difference may be only that their binding site is at a different locations in AC and occur only in selected subtypes. The structural basis for how these other regulators work needs to be explored.

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references

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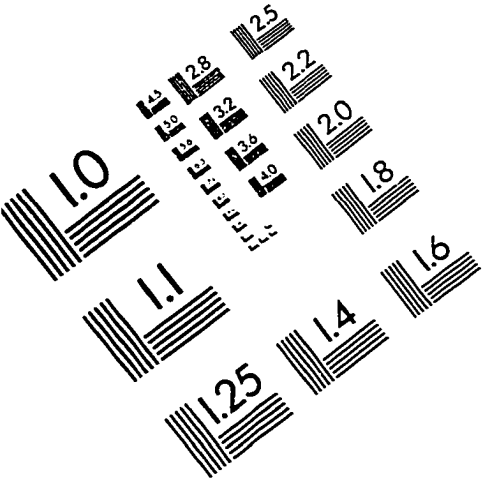
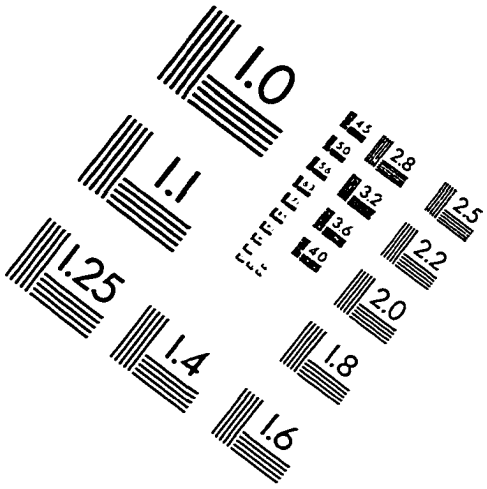
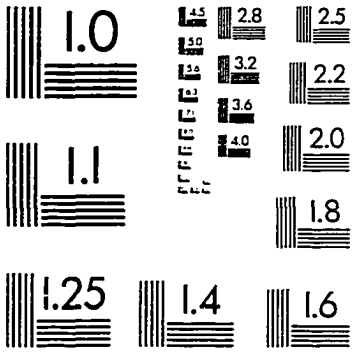
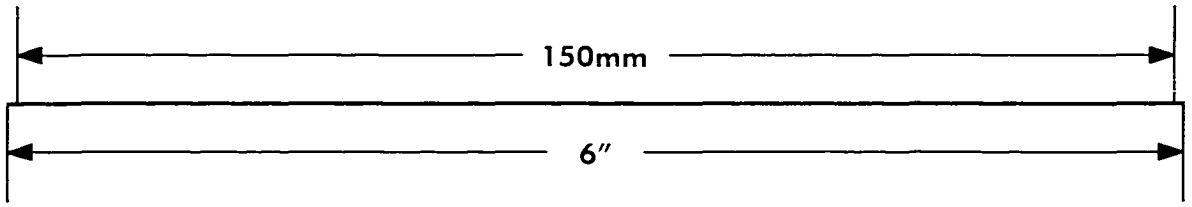
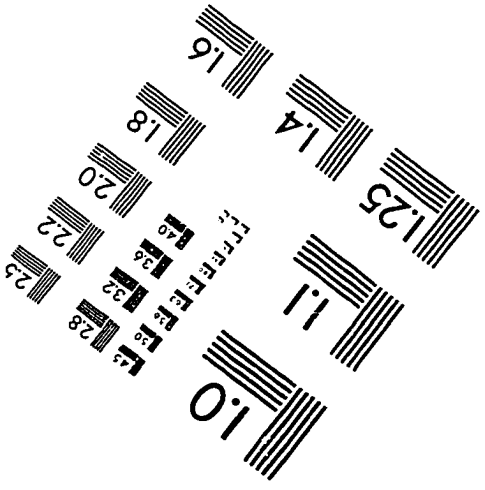
IMAGE EVALUATION TEST TARGET (QA-3)

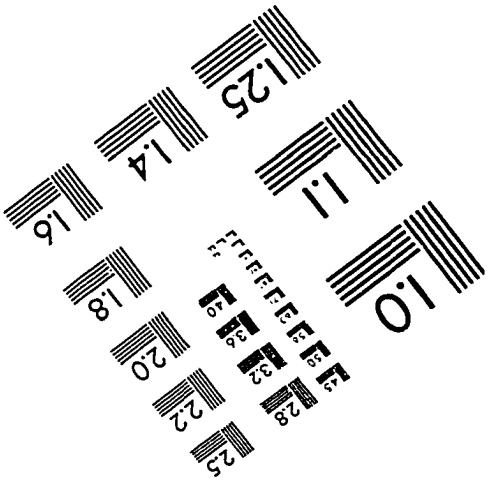












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