MOLECULAR BASIS FOR THE PHENOTYPE OF FORSKOLIN-RESISTANT Y1 ADRENAL CELLS

by

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A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy

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ABSTRACT

A family of forskolin-resistant mutants was isolated from the Y1 mouse adrenocortical tumor cell line on the basis of their resistance to the growth-inhibiting effects of forskolin. In these mutants, the adenylyl cyclase was partially resistant to forskolin and completely resistant to ACTH. The mutant phenotype was accompanied by decreased levels of Gsα and Gia subunits in plasma membrane and decreased Gβγ activity. These observations led to the hypothesis that a decreased Gβγ activity resulted in decreased levels of membrane-associated Gsα, which in turn, caused ACTH and forskolin-resistance. The present study was undertaken to further characterize the forskolin-resistant mutant phenotype. The ACTH resistance in the mutants is accompanied by markedly decreased levels of ACTH receptor transcript, suggesting that the loss of the ACTH-responsive adenylyl cyclase activity resulted from impaired expression of ACTH receptor gene in these cells. Transfection of mutant cells with genes encoding the B2-adrenergic receptor or ACTH receptor restored hormone-responsive adenylyl cyclase activity and the transformants showed normal receptor-G protein coupling. In addition, the levels of Gsα and Gia were increased in mutant transformants and approached the levels seen in parental Y1 cells. These results suggested that the decreased levels of Gα resulted from impaired expression of the ACTH receptor and that a functional G protein-coupled receptor seems to be required to maintain the normal levels of G protein α subunit in the plasma membrane. Transfection of mutant cells with genes encoding an epitope-tagged Gsα also restored the Gsα levels associated with the
plasma membrane. Both the receptor and Gsα transformants, however, retained forskolin-resistant adenylyl cyclase activity and were not rescued with respect to expression of endogenous ACTH-R transcripts, suggesting that the phenotypic change in forskolin-resistance mutants cells is not a direct result of low levels of membrane-associated Gsα.

To investigate the contribution of decreased Gβγ activity to the mutant phenotype, forskolin-resistant mutants were transfected with expression vectors encoding G protein β1 or β2, and γ2 subunits. The transformants expressed ACTH receptor transcripts and functional ACTH receptors as evidenced by the recovery of ACTH-stimulated cAMP accumulation and cell rounding. These results demonstrated a role for Gβγ in the regulation of ACTH receptor gene expression. The results also indicated that a defect in Gβ or Gγ may be responsible for the ACTH receptor deficiency in forskolin-resistant mutants, and that the impaired expression of ACTH receptor, in turn, results in decreased levels of Gsα and Gicα. The impaired Gβγ function may also accounts for forskolin-resistant adenylyl cyclase and cell growth in these mutants.
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<tr>
<td>5'-RACE</td>
<td>rapid amplification of cDNA ends</td>
</tr>
<tr>
<td>8BrcAMP</td>
<td>8-bromo-cAMP</td>
</tr>
<tr>
<td>ACTH</td>
<td>adrenocorticotropic hormone</td>
</tr>
<tr>
<td>Btk</td>
<td>Bruton tyrosine kinase</td>
</tr>
<tr>
<td>cAMP</td>
<td>cyclic adenosine 3',5'-monophosphate</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary deoxyribonucleic acid</td>
</tr>
<tr>
<td>CRE</td>
<td>cAMP-responsive element</td>
</tr>
<tr>
<td>ERK</td>
<td>extracellular signal regulated kinase</td>
</tr>
<tr>
<td>G protein</td>
<td>guanyl nucleotide-binding regulatory protein</td>
</tr>
<tr>
<td>Gα</td>
<td>G protein α subunit</td>
</tr>
<tr>
<td>GAP</td>
<td>GTPase-activating protein</td>
</tr>
<tr>
<td>GEF</td>
<td>guanine-nucleotide exchange factor</td>
</tr>
<tr>
<td>Gi</td>
<td>inhibitory G protein</td>
</tr>
<tr>
<td>GRF</td>
<td>guanine-nucleotide releasing factor</td>
</tr>
<tr>
<td>GRK</td>
<td>G protein-coupled receptor kinase</td>
</tr>
<tr>
<td>Gs</td>
<td>stimulatory G protein</td>
</tr>
<tr>
<td>Gβγ</td>
<td>G protein βγ subunits</td>
</tr>
<tr>
<td>HA-Gsα</td>
<td>epitope-tagged Gsα</td>
</tr>
<tr>
<td>HEPES</td>
<td>N-2-hydroxyethylpiperazine-N-ethanesulfonic acid</td>
</tr>
<tr>
<td>IP3</td>
<td>inositol-1,4,5,-trisphosphate</td>
</tr>
<tr>
<td>IPR</td>
<td>isoproterenol</td>
</tr>
<tr>
<td>IRS-1</td>
<td>insulin receptor substrates-1</td>
</tr>
<tr>
<td>JNK</td>
<td>c-Jun NH2-terminal kinase</td>
</tr>
<tr>
<td>LPS</td>
<td>lysophosphatidic acid</td>
</tr>
<tr>
<td>MAP-kinase</td>
<td>mitogen-activated protein kinase</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
</tr>
<tr>
<td>PAF</td>
<td>platelet-activating factor</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
<td>-------------</td>
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<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PGE</td>
<td>prostaglandin E</td>
</tr>
<tr>
<td>PH domain</td>
<td>pleckstrin homology domain</td>
</tr>
<tr>
<td>PI</td>
<td>phosphatidylinositol</td>
</tr>
<tr>
<td>PI3-kinase</td>
<td>phosphoinositide 3-kinase</td>
</tr>
<tr>
<td>PIP2</td>
<td>phosphatidylinositol-4,5-bisphosphate</td>
</tr>
<tr>
<td>PIPES</td>
<td>piperazine-N,N'-bis[2-ethanesulfonic acid]; 1,4-piperazinediethanesulfonic acid</td>
</tr>
<tr>
<td>PKA</td>
<td>cAMP-dependent protein kinase</td>
</tr>
<tr>
<td>PLA2</td>
<td>phospholipase A2</td>
</tr>
<tr>
<td>PLC</td>
<td>phospholipase C</td>
</tr>
<tr>
<td>PMSF</td>
<td>phenylmethysulfonyl fluoride</td>
</tr>
<tr>
<td>RACK</td>
<td>receptor for activated c-kinase</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>reverse transcriptase-PCR</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
</tr>
<tr>
<td>Sos</td>
<td>son of sevenless</td>
</tr>
<tr>
<td>β2AR</td>
<td>β2-adrenergic receptor</td>
</tr>
<tr>
<td>βARK</td>
<td>β-adrenergic receptor kinase</td>
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I. INTRODUCTION

A. ACTH RECEPTOR

1. Structure and function of the ACTH receptor

ACTH (adrenocorticotropic hormone) is a 39 amino acid peptide hormone that is produced by the pituitary gland from the large precursor of proopiomelanocortin (POMC). The target organ of ACTH is the adrenal cortex, which is responsible for the synthesis of steroid hormones, most importantly glucocorticoids and mineralocorticoid. ACTH acutely regulates steroidogenesis by binding to specific ACTH receptors in adrenocortical cell membranes and activating adenylyl cyclase. Over a longer period of time, ACTH increases glucocorticoid production by increasing the expression of several steroid metabolizing enzymes. In addition, ACTH increases adrenal cortex size and is crucial for the normal development of this tissue (Waterman and Keeney, 1996).

The ACTH receptor couples to adenylyl cyclase through the stimulatory G protein and therefore is a member of the G protein-coupled receptor family. The amino acid composition of the receptor is typical of the receptor family with seven putative transmembrane spanning regions, three extracellular and three intracellular loops, an extracellular amino terminus and an intracellular carboxyl terminus. Detailed studies on another member of this receptor family, the β2-adrenergic receptor (β2AR), revealed that the transmembrane domains all participate to the binding of specific ligands and the second and third intracellular loops (loop i2 and loop i3) are important for receptor-G protein coupling (Caron and Lefkowitz, 1993). On the other hand, the ACTH receptor also has some unusual structural topology. It has a short N-terminal extracellular domain and short C-terminal intracellular domain. The second extracellular loop of these receptors is so hydrophobic that it is possible that no significant extracellular domain exists (Cone and Mountjoy, 1993). In addition, the ACTH receptor lacks several amino acids that are conserved in most G protein-coupled receptors; two proline residues in the fourth and fifth transmembrane domains that are thought to introduce a bend in the α-helical structure of the transmembrane domains and to participate in the formation of the binding pocket, and two extracellular cysteines that are thought to form a disulfide bridge between the first and second extracellular loops (Mountjoy et al., 1992).
2. Regulation of ACTH receptor expression

ACTH stimulates expression of its own receptor. ACTH has been shown to induce a marked increase in ACTH receptor mRNA by 3 to 20-fold in mouse, human and bovine adrenal cells (Mountjoy et al., 1994, Lebrethon et al., 1994). This effect of ACTH is mimicked by other activators of the cAMP pathway such as cAMP analogs and forskolin (Schimmer et al., 1995), indicating that it is mediated by a cAMP-dependent mechanism. ACTH not only increases the transcription rate of the ACTH receptor but also the ACTH receptor mRNA half-life (Lebrethon et al., 1994). Although most cell surface receptors exhibit a reduction in binding after agonist stimulation as a result of sequestration, it is common to see some increase in receptor gene expression following activation by agonist. The characteristic feature of the ACTH receptor may be the extent of this compensatory expression of the gene which clearly results in a rapid increase in cell surface binding sites (Clark and Cammas, 1996). This phenomenon may also represent a teleological advantage for one of the principal stress hormone systems.

Angiotensin II (A-II), an important regulator of aldosterone secretion in adrenal cells, also stimulates the expression of the ACTH receptor. It induces ACTH receptor mRNA levels 3 to 17fold in mouse and human adrenocortical cells (Mountjoy et al., 1994, Lebrethon et al., 1994). The effect of A-II on ACTH receptor expression is blocked by a specific antagonist of the A-II type 1 receptor (AT1) (Lebrethon et al., 1994). AT1 couples to Gi/Gq protein and upon activation by A-II, stimulates phospholipase C, resulting in increased intracellular Ca^{2+} and activation of protein kinase C (PKC). The observations that ACTH receptor expression can be regulated by both cAMP and Ca^{2+}/PKC-dependent mechanisms indicate that the ACTH receptor gene is regulated by multiple signal transduction pathways.

The major site of expression of the ACTH receptor is the adrenal cortex (Mountjoy et al., 1992). The receptor also seems to be expressed in lipogenic cells (Boston and Cone, 1996). The genomic structures of the human and mouse ACTH receptor were characterized just recently. The human ACTH receptor consists of two exons of 49 bp and >1000 bp, -- the first exon (49 bp) and part of the second exon (128 bp) contain the 5'-untranslated region and the rest of the second exon contains the entire coding sequence (Naville et al., 1994). The mouse ACTH receptor gene consists of at least three exons of lengths 113 bp, 112 bp and >1000 bp (Cammas et al., 1997). An
additional exon of 57 bp resulting from alternate splicing is located between the 113 bp and 112 bp exons (Shimizu et al., 1997). The first three exons contain the 5'-untranslated regions while the fourth exon contains 96 bp of 5'-untranslated region and the entire coding sequence (Cammas et al., 1997). For both the human and mouse ACTH receptors, the promoter regions do not show any typical characteristics of TATA box, CAAT box, or GC-rich region. An initiator-like site that overlaps the transcription start site, however, has been identified (Fig. 1). The initiator element, first identified for viral transcriptional regulation, has been shown to be involved in transcription initiation of many TATA box-containing and TATA-less promoters (Weis and Reiberg, 1992).

The human ACTH receptor promoter also contains several putative cAMP-responsive elements (CREs) that respond to stimulation by forskolin with increased promoter activity and may be responsible for the stimulation by ACTH of its own receptor (Naville et al., 1996a). In contrast, the mouse ACTH receptor promoter does not contain a consensus CRE (Cammas et al., 1997).

Sequence analysis of the promoter region of the mouse ACTH receptor identified several putative sites for transcription factors including Oct B, Sp1, AP1, AP2 and SF-1 (Fig. 1). Of particular interest is the presence of a partial consensus sequence at position -25 bp for an SF-1 binding site. SF-1 has been shown to contribute to the tissue-specific expression of several steroidogenic enzymes (Parker and Schimmer, 1997). SF-1 also appears to be involved in the cAMP-regulated expression of the steroidogenic enzyme Cyp 17 (Bussis et al., 1995) and PKA-dependent expression of Cyp21 (Parissenti et al., 1993). Deletion and mutation studies on the promoter activity of the mouse ACTH receptor have shown that SF-1 or an SF-1-like factor is involved in the cell-specific expression of the ACTH receptor. SF-1 bound specifically to the putative SF-1 binding site in the ACTH receptor promoter and expression of SF-1 in a cell line that normally does not have any ACTH receptor promoter activity led to an induction of a reporter gene under control of the ACTH receptor promoter. In addition to an SF-1 binding site, an inhibitory element from position -1236 to -908 (Fig. 1) also seemed to be important for the tissue-specific expression of the ACTH receptor. ACTH receptor promoter activity was detected in mouse adrenocortical Y1 cells but not in mouse Leydig TM3 cells or fibroblast L cells. However, promoter activity became apparent in TM3 Leydig cells when the fragment at -1236 to -908 was deleted, but remained undetectable in fibroblast L cells. These observations suggest that the ACTH
Figure 1. Schematic Diagram of mouse ACTH receptor promoter. The binding sites for SF-1 and putative binding sites for other transcription factors (OctB, Sp1, AP1, and AP2) were indicated. The initiator element that overlaps transcription start site, the glucocorticoid response element (GRE), and the inhibitory region at position -1236 and -908 were also shown.
receptor promoter is potentially active in steroidogenic tissues other than the adrenal, but is totally inhibited by the binding of a factor to an element between position -1236 and -908 bp that would act as a repressor of expression in nonadrenal steroidogenic cells such as the Leydig cells (Cammas et al., 1997). Therefore, at least two factors appear to be important for the control of ACTH receptor expression: SF-1 or an SF-1-like factor may be responsible for steroidogenic cell-specific expression of the ACTH receptor, and an inhibitory factor may be responsible for silencing ACTH receptor expression in nonadrenal steroidogenic cells.

3. Mutations in the ACTH receptor

Mutations in ACTH receptor have been related to human diseases. Loss-of-function mutations in both alleles of this receptor gene cause familial glucocorticoid deficiency (FGD), an autosomal-recessive disorder characterized by ACTH resistance, low levels of serum cortisol, and high levels of plasma ACTH. Affected individuals may be compound heterozygotes or homozygotes for particular mutations. Among the mutations so far described are P27R, I44M, S74I, D107N, F119frameshift, S120R, R128C, R146H, L192frameshift, R201stop, G217frameshift, C251F, Y254C, and P273H (Clark and Cammas, 1996, Naville et al., 1996b, Elias et al., 1997, Stratakis et al., 1997). These mutations result in truncated receptor protein, or interfere with ligand binding and G protein coupling, as predicted by the structure-function relationship for G-protein-coupled receptors. On the other hand, not all FGD patients have mutations in the coding region of their ACTH receptor gene. In some patients with the disorder, no mutation was found in the receptor, but rather impaired binding of ACTH to the receptor and altered coupling to adenylyl cyclase were observed (Yamamoto et al., 1995). In addition, certain forms of FGD as well as another ACTH resistance disorder, the triple A syndrome, have no mutations in the coding region of ACTH receptor and seem to have defects at another locus mapped outside of the ACTH receptor gene (Weber and Clark, 1994, Tsigos et al., 1995). In these cases, ACTH unresponsiveness may have been caused by defect(s) affecting ACTH receptor expression. Decreased levels of ACTH receptor mRNA have been observed in some adrenal carcinomas and nonfunctioning adenomas, suggesting a deficiency in the expression of ACTH receptor (Allolio and Reincke, 1997).
B. GUANYL NUCLEOTIDE-BINDING REGULATORY PROTEINS (G PROTEINS)

1. G protein cycle

G proteins are heterotrimers comprised of three polypeptides: an α subunit that binds and hydrolyzes GTP, a β subunit, and a γ subunit. The β and γ subunits are tightly associated to form a functional βγ complex. To date, at least 16 α, 5 β and 7 γ have been identified in mammals (Neer, 1995). G proteins function as transducers of information across the cell membrane by coupling diverse receptors to effectors. They act as molecular switches with an "on" and "off" state governed by GTPase cycles. When GDP is bound, the α subunit associates with the βγ subunits to form an inactive heterotrimer that binds to receptor. When the receptor is stimulated by extracellular signals (e.g. hormones, neurotransmitters, odorants, and photons of light), binding of the ligand to the receptor results in a conformational change which promotes association of the receptor with the inactive GDP-bound heterotrimeric G protein. The interaction between activated receptor and G protein increases the exchange of GDP on the α subunit for GTP. The G protein then dissociates from the receptor and the GTP-bound α subunit dissociates from the βγ dimer. The separated α subunit and βγ dimer may then interact with effector proteins. The activated state lasts until the GTP is hydrolyzed to GDP by the intrinsic GTPase activity of the α subunit. Once GTP is cleaved to GDP, the α and βγ subunits reassociate, become inactive and return to the receptor. The reassociation inactivates both α and βγ subunits and primes the system to respond again. Figure 2 shows a schematic illustration depicting the GTPase cycle of a heterotrimeric G protein coupled to the adenylyl cyclase system.

Both Gα and Gβγ can independently activate or inhibit multiple effectors (Fig. 3), including adenylyl cyclase, phospholipase C (PLC), Ca^{2+} and K^{+} channels. Gα also activates cGMP phosphodiesterase; Gβγ, on the other hand, activates phospholipase A2, phosphoinositide 3-kinase (PI3-kinase), β-adrenergic receptor kinases (BARKs), and mitogen-activated protein kinase (MAP-kinase)(Neer, 1995). In the cases where both Gα and Gβγ regulate one type of effector, the regulation is usually specific for the effector subtype. Among the best-studied of the G protein effectors is the hormone-responsive adenylyl cyclases. Both the α subunit of stimulatory G protein (Gsα) and Gβγ regulate adenylyl cyclase. Type III, type V, and type VI adenylyl cyclase are activated by Gsα but not Gβγ, whereas type II and type IV are activated by...
Figure 2. The GTPase cycle of heterotrimeric G protein. Depicted is the GTPase cycle of a heterotrimeric G protein coupled to adenylyl cyclase. A = agonist, AC = adenylyl cyclase. Scheme adapted from Taylor, 1990 with modifications.
Figure 3. Signal transduction regulated by G protein $\beta\gamma$ subunits. $\beta\gamma$ directly activates several effectors including adenylyl cyclase (AC), PLC, and Ca$^{2+}$ and K$^+$ channels. $\beta\gamma$ also activates MAP-kinase through a Ras-dependent pathway, which, in part, reprises the receptor tyrosine kinase (FGF-R, EGF-R, PDGF-R) paradigm. $\beta\gamma$ stimulates c-Src kinase activity, resulting in the phosphorylation of a variety of cellular substrates, including the Shc adapter protein and receptor tyrosine kinases, thereby activating the signaling pathway leading to MAP-kinase activation. $\beta\gamma$ also activates MAP-kinase cascade via PI3-kinase (PI3K) that may act upstream of c-Src kinase. Scheme adapted from van Biesen et al., 1996 with modifications.
both Gsα and Gβγ, and type I is activated by Gsα and inhibited by Gβγ. Inhibition by Gβγ is independent of Gsα, whereas stimulation by Gβγ has an underlying prerequisite for the stimulation of adenylyl cyclase by Gsα (Tang and Gilman, 1991, Iyengar, 1994). Thus, the final signal that is transduced into a cell, not only will depend on the selective hormone, the specific hormone receptor, and the G protein that couples to the receptor, but also is regulated at the level of effector -- in this case, the relative levels of the specific isoforms of adenylyl cyclase present in the cells.

2. Structure and function of G protein subunits
   a. Gα subunit
      i. Gα subunit structure

The proteins representing the 16 different α subunits of G proteins that have been cloned can be divided into four major classes (Gsα, Gai, Gaq, and Ga12), according to the similarity of their amino acid sequences that range from 56% to 95% identity. Gα interacts with guanine nucleotides, the βγ complex, receptor and effectors. Certain regions of all α subunits that are very highly conserved serve common functions such as GTP binding and hydrolysis, whereas other regions are much more distinctive for a particular subtype, and presumably are involved in the protein-protein interactions with receptors and effectors. Site-directed mutagenesis, studies of naturally occurring mutants, construction of chimeric α subunits, biochemical studies, and finally the identification of the crystal structures of GTP and GDP-ligated transducin and Gαi1 (Noel et al., 1993, Lambright et al., 1994, and Coleman et al., 1994) helped to provide a detailed view of the relationship between Gα structure and function.

The Gα subunit consists of two domains: a GTPase domain that contains the guanine nucleotide-binding pocket as well as sites for binding receptors, effectors and βγ complex, and an α helical domain whose function is not clear. This α-helical domain may be involved in GTP hydrolysis and may contribute to the effector-binding site (Conklin and Bourne, 1993, Coleman et al., 1994). The GTPase domain consists of five helices (α1 - α5) and a surrounding six-stranded β sheet (β1 - β6). The guanine nucleotide-binding pocket is formed by the five helical regions in which the amino acid sequences are highly conserved not only across the Gα subunit subfamily, but also across the GTP-binding protein superfamily (Sprang, 1997). In addition to nucleotide
binding, these regions (particularly the α2 helix region) are also involved in the critical conformational switch that leads to the replacement of GDP with GTP and in the hydrolysis of bound GTP. Mutations of some of the critical amino acids in these regions resulted in either reduced GTPase activity and constitutive activation of effector pathways, or blockade of GTP-dependent activation (Masters et al., 1989, Sprang, 1997). The amino acid (Arg 201) that is responsible for the cholera toxin catalyzed ADP-ribosylation of Gsα is located in the α2 region of the guanyl nucleotide binding pocket.

The βγ subunits, effectors and receptors seem to bind to different surfaces of Gα subunits. The first 25 amino acids of the Gα subunit are essential for Gβγ binding. For certain Gα subunits, myristoylation of an amino-terminal glycine residue is required for high affinity interaction with the βγ complex (Linder et al., 1991). The Gβγ binding surface probably also includes the α2 helix because a cysteine on this helix can be chemically cross-linked to Gβγ (Thomas et al., 1993). The α2 helix region is in different conformational states in GDP and GTP-liganded Gα (switch II region, Lambright et al., 1994), consistent with the fact that Gβγ only binds to α-GDP with high affinity. The α2 helix is also important for effector-G protein binding (Conklin and Bourne, 1993). The effector binding site partially overlaps the putative βγ-binding surface. Therefore, it is unlikely that the Gα subunit can simultaneously bind effector and Gβγ. In the cases where both Gα and Gβγ activate the same effector, e.g. type II adenylyl cyclase, Gα and Gβγ bind to distinct sites on the enzyme (Chen et al., 1995).

At least three regions of Gα have been postulated to contact the receptor. The most important and well-identified region is the C-terminus. An activated receptor triggers intracellular responses by dramatically decreasing the affinity of the Gα subunit for GDP, perhaps by moving or twisting the C-terminal α helix to loosen the grip of the Gα subunit on GDP (Neer, 1995). This is supported by the observation that deletion of the C-terminal 14 residues of Goα produces a protein that appeared to release α-GDP more rapidly than did wild-type Goα (Denker et al., 1992). The extreme C-terminus of Gα may also have a major role in the specificity of G protein-receptor interaction. Replacement of three amino acids at the C-terminus of Gqα by the corresponding residues of Gic2 created αq-αi2 chimeras which mediated stimulation of PLC (the effector target
of Gαq) by two receptors (dopamine D2 and adenosine A1 receptors) that otherwise coupled exclusively to Gi (inhibition of adenylyl cyclase). The glycine at the -3 position (three residues from the C-terminus) was proposed to be involved in forming part of a β turn that constitutes signals for recognition by specific subsets of receptors (Conklin et al., 1993). In addition to the C-terminal region, the N-terminal and α5 helical region may also be important for receptor-G protein binding. An N-terminal peptide of Gt blocked the activation of Gt by photorhodopsin, whereas mutations at the α5 helical region resulted in decreased affinity of Gα (αs and αo) for GDP, which mimics the activation of G protein by receptors (Hamm et al., 1988, Iiri et al., 1994).

ii. Lipid modification of α subunits

All G protein α subunits are modified at or near their N-termini by covalent attachment of the fatty acids myristate and/or palmitate. While myristoylation is a stable modification, palmitoylation is a readily reversible process. Both palmitoylation and myristoylation contribute to membrane association of Gα; palmitoylation, due to its greater hydrophobicity, provides a stronger interaction with membrane lipids (Wedegaertner et al., 1995). Mutation of the cysteine (palmitoylation) or the glycine (myristoylation) near the N-termini of αs, αi, αq, and α11 resulted in significant increases in the amount of each protein in the cytosolic fraction. Beside functioning as membrane anchors, myristoylation is also required for high-affinity interaction of Gα (αo and αi) with the Gβγ complex (Linder et al., 1991). On the other hand, palmitoylation seems to be important for G protein-effector interaction. Mutation of the palmitoylation site of Gsα or Gqα resulted in reduction of cAMP accumulation and loss of PI synthesis in HEK293 cells when stimulated by respective agonists (Wedegaertner et al., 1993).

An important aspect of palmitoylation is its biological reversibility and consequent potential for regulation. Studies on Gs have shown that the turnover of palmitate attached to Gsα is dramatically affected by Gs activation. Activation of Gs by β2AR stimulation with the agonist isoproterenol in COS cells led to an increase in Gsα palmitate labeling. The effect of isoproterenol was blocked by a β2AR antagonist and was not mediated by cAMP, indicating a direct effect mediated by the activation of Gsα (Mumby et al., 1994, Degtyarev et al., 1993). Pulse-chase experiments showed that activation by β2AR induced rapid depalmitoylation of Gsα, which
correlates with, and is probably the mechanism for, activation-induced translocation of Gsα from membrane to cytosol (Levis and Bourne, 1992, Wedegaertner and Bourne, 1994). Since mutant non-palmitoylated Gsα could not mediate hormonal stimulation of its effector, adenylyl cyclase, depalmitoylation of Gsα may provide a physiologically relevant way to dampen or turn off G protein signaling, in addition to better established mechanisms (e.g. hydrolysis of GTP bound to Gα or desensitization of receptor)

b. βγ subunit

i. βγ complex

The βγ complex has the following important functions in receptor-G protein signaling: 1) It increases the affinity of the α subunits for GDP (Higashijima et al., 1987); 2) It facilitates deactivation of stimulated α subunits, promoting the inactive state of G protein heterotrimers (Sternweis, 1994); 3) It is required for high affinity coupling of G protein to receptor (Dolph et al., 1994); 4) It is important for the attachment of α subunits to plasma membrane (Sternweis, 1986); 5) It is a direct regulator of many effectors (Section I.B.1 and I.B.4). The five known mammalian Gβ subunits (B1-B5) are between 53% and 90% identical to each other. In contrast, the six Gγ (γ1-γ5, and γ7) are much more distinct in their individual sequences and are crucial for determining the functional specificity of the βγ subunits (Clapham and Neer, 1993). The β and γ subunits are tightly bound as complexes and act inside cells as heterodimers. The ability to form the βγ complex is different among different members of the β and γ families. For example, the B1 subunit is able to interact with γ1 and γ2, but the very similar B2 is able to form a dimer with γ2 but not γ1 (Garritsen and Simonds, 1994, Yan et al., 1996). So far there seems to be no difference in the ability of reconstituted βγ pairs to activate effectors or interact with α subunits, except for B1γ1, which sometimes is much less effective (Wickman et al., 1994).

ii. β subunit structure

The molecular structure of the β subunit contains two domains—an N-terminal region and seven repeating units of approximately 43 amino acids each (WD repeat, Simon et al., 1991). Based on the crystal structure of the βγ complex, the Gβ subunit forms a propeller composed of an N-terminal α helix and seven β sheets corresponding to the seven WD repeats. Each β sheet has four antiparallel strands that form the "blades" of a β-propeller structure (Lambright et al., 1996).
The Gβ subunit interacts with Gα and Gγ subunits, and with effectors. The docking of Gα to Gβγ involves extensive contacts: binding of the Gα N-terminal α helix to the side of the Gβ propeller parallel to its central tunnel and binding of the catalytic domain of Gα to the top surface of the β propeller. The β blades 1, 2, and 7 seem to be particularly important for this binding. On the other hand, Gγ stretches along the side and bottom of the Gβ subunit contacting blades 5, 6, 7, and 1. In addition, the α helix at the N-terminus of the Gβ is amphipathic and forms part of a coiled coil with a similar amphipathic helix from the N-terminus of the Gγ subunits. As for Gβ and effector binding, the four residues that are largely conserved within each of the seven WD repeats have been implicated in forming hydrogen bonds that are important for stabilizing the characteristic folded structure of the β strand. Mutations introduced in these residues affect Gα-mediated activation of effector either by disrupting the folding of the β strands or by disrupting the contact sites that participate in Gβ-effector interaction (Yan and Gautam, 1997). Since in some cases different Gβ subunits could determine the specificity and selectivity of G protein-receptor coupling (Kleuss et al., 1992), Gβ may also interact directly with receptors. The domain responsible for this interaction is not well identified but is likely located on blades 6 and 7 of the β structure (Wall et al., 1995).

Other than being involved in Gα binding and effector interaction, the WD repeats of Gβ may have other functions. The WD repeats consist of a conserved core of 23-41 residues usually bounded by Gly-His and Trp-Asp. The repeating units in Gβ are examples of a class of repeating sequences found in a family of proteins engaged not only in signal transduction, but also in control of cell division, transcription, processing of pre-mRNA, cytoskeletal assembly, and vesicle fusion. Examples include the HIR1 protein that acts as a repressor of histone gene transcription, and coronin that binds actin and co-precipitates with actin-myosin complex (Neer et al., 1994). The WD repeat proteins contain different numbers of repeats and some of these repeats seem to be important for their interactions with other proteins. In these interactions, no single repeating unit specifies a partner and each partner protein interacts with more than one repeating unit (Neer, 1995). Gβγ dimers interact with a variety of proteins (Section I.B.4). It is not clear how the WD repeats of Gβ might be involved in the interaction of Gγ with other proteins; however, based on their structural similarity, Gβ may share similar functions with other WD repeat proteins. Many
WD repeat proteins help to assemble macromolecular complexes (Neer et al., 1994). GB may also play such a role in that it could mediate the functions of Gβγ on the formation of a complex that includes a receptor and a specific receptor kinase that phosphorylates the liganded receptor (Pitcher et al., 1992). In addition, Gβγ has been shown to interact with many small GTP-binding proteins including Rho, Rac, and Cdc 42, which promote the polymerization of actin stress fibers and control the assembly of focal adhesion complexes (Harhammer et al., 1996). The effect of Gβγ in the regulation of actin filament organization may also be mediated by the WD repeat structure of GB. The WD repeats seem to enable GB to adopt multiple conformations, which could greatly expand the numbers of Gβγ signaling partners (Neer, 1995).

iii. γ subunit structure

Gγ forms complexes with GB and determines the selectivity of the dimer formation (Gautam et al., 1990, Kleuss et al., 1993, ). The selectivity of the γ subunits for different β subunits is determined by a stretch of 14 amino acids in the middle of the γ subunit (Spring and Neer, 1994). The crystal structures derived from transducin βγ and Giαβ1γ2 complexes (Wall et al., 1995, Sondek et al., 1996) revealed that Gγ contains two helices that have no inherent tertiary structure. The N-terminal helix of Gγ forms a coiled-coil with the N-terminal helix of GB whereas the remainder of Gγ interacts extensively with the β-propeller domain of GB. The coiled-coil contact is required to stabilize the dimer as shown by mutagenesis experiments (Garritsen et al., 1993). Gγ also interacts with Goα to regulate the assembly of functional distinct G protein heterotrimeric from particular combinations of α, β, and γ subunits. Both the N-terminal region and the prenylated C-terminal regions of Gγ are important for this interaction (Rahmatullah and Robishaw, 1994, Rahmatullah et al., 1995). The C-terminal region is also important for the interaction of Gγ and receptor and may contribute to the specificity of receptor-G protein interaction (Kisselev and Gautam, 1993, Kisselev et al., 1995). The interactions between Gγ and Goα, and Gγ and receptor, however, have not been confirmed by subsequent studies on the heterotrimeric crystal structures.

The Gγ subunits contain an invariant cysteine residue 4 amino acids from the C-terminal which provides a site for post-translational modification by isoprenoid lipids. The lipid
modification of $\gamma$ has been shown to be important not only for membrane association of the $\beta\gamma$ complex, but also for $\beta\gamma$ functions. $\beta\gamma$ dimers serve as membrane anchors which associate $G$ protein heterotrimeric complexes with the plasma membrane. This function of $\beta\gamma$ is mediated by prenylated $\gamma$ since mutation of the modified cysteine to serine in $\gamma_2$ leads to a redistribution of $\beta$ and $\alpha$ subunits from membrane to cytosol (Muntz et al., 1992). Although prenylation of $\gamma$ is not required for $\beta\gamma$ dimer formation, only $\beta\gamma$ complexes containing prenylated $\gamma$ were functional as measured by $\beta\gamma$ supported ADP-ribosylation, GDP-GTP exchange, and activation of adenylyl cyclase (Higgins and Casey, 1994, Iniguez-Lluhi et al., 1992). These observations indicate that the C-terminal modification of $\gamma$ is essential for high affinity interactions of $\beta\gamma$ with both $G\alpha$ and effectors.

3. Regulation of $G\alpha$ levels

Changes in the levels of $G$ proteins have been associated with cellular events such as development and differentiation, the aging process, and certain pathological states. $G$ protein levels also are modulated by the receptor that the $G$ protein couples to, generally in response to agonist stimulation. In response to agonist stimulation, $G$ protein levels could either decrease or increase depending on the agonist, length of stimulation, $G$ protein isoform, and cell type. Short term stimulation (less than 2 h) of the $\beta_2\alpha R$ in S49 lymphoma cells by isoproterenol caused the redistribution of $G\alpha_\alpha$ from the plasma membrane to the cytosol. The mechanism attributed to this modulation is the dissociation of $G\alpha_\alpha$ and $G\beta\gamma$ upon agonist activation since $G\beta\gamma$ is important for the attachment of $G\alpha$ to the plasma membrane. This hypothesis was supported by the observation that a $G\alpha_\alpha$ mutant that was not able to dissociate from $\beta\gamma$ did not respond to agonist-induced regulation (Levis and Bourne, 1992). In addition, $G$ protein palmitoylation is important for the membrane association of $G\alpha$. Activation of $\beta_2\alpha R$ in S49 cells by isoproterenol induced rapid depalmitoylation of $G\alpha$, which may have contributed to the translocation of $G\alpha$ from membrane to cytosol (Wedegaertner and Bourne, 1994). On the other hand, long-term exposure (more than 16 h) to agonists causes down-regulation of $G$ protein $\alpha$ subunits in both membrane and cytosol fractions. This phenomenon has been observed for almost all $G$ protein $\alpha$ subunits including $\alpha_s$, $\alpha_i$, $\alpha_q$, and $\alpha_{11}$ (McKenzie and Milligan, 1990, Palmer et al., 1995, Milligan et al., 1995a). The regulation of $G$ protein levels upon long-term agonist stimulation is specific for the isoform of $G$
protein with which a receptor preferentially couples, is independent of the related second messenger, and is not the result of reduced transcription since the mRNA levels for the respective G protein were not affected (Milligan et al., 1995b). In the case of Gs and Gq/G11, it is known that the mechanism for the down-regulation is enhanced proteolytic degradation (Mitchell et al., 1993).

In some cases, decreases in the levels of G protein also are accompanied by decreases in receptor levels. The time course and the dose-response relationship of receptor and G protein down-regulation were similar, suggesting that the losses of receptor and G protein were coupled (Adie et al., 1992, Mullaney et al., 1993). Down-regulation of receptors in response to prolonged exposure to agonist is a component of desensitization, wherein exposure of a cell to a hormone results in subsequent insensitivity to that hormone. The concomitant decrease of a G protein to which a receptor is normally coupled may therefore contribute to the overall process of desensitization. The agonist-mediated down-regulation of G protein also provides a potential mechanism for heterologous desensitization of hormone signaling events, in that the down-regulation of a particular G protein by agonist stimulation of one receptor would subsequently prevent activation of another receptor which utilizes the same G protein. Indeed, down-regulation of Gsα in NG108-15 cells by treatment with prostanoid agonists resulted in heterologous desensitization of all receptors that act to stimulate adenylyl cyclase (Kelly et al., 1990).

Receptor-mediated regulation may also result in induction of G protein levels. Chronic treatment of human neuroblastoma cells with morphine that specifically activates μ-receptors increased the levels of several G protein subunits including Gal, Gsα, Goα, Gzα, and Gβ. The up-regulation of G protein levels developed in a time and dose-dependent manner and was due to enhanced protein synthesis since concomitant treatment of the cells with cycloheximide prevented this effect. The effect was also specific for the agonist and its receptor since only the specific μ-receptor antagonist blocked it and the inactive enantiomer of morphine had no effect. The increase in Gsα levels resulted in elevated levels of basal, forskolin-stimulated, and prostaglandin-stimulated adenylyl cyclase activity (Ammer and Schulz, 1993). The authors suggest that the increased levels of multiple G protein subunits in response to long-term exposure to morphine may represent one potential mechanism underlying opioid tolerance/dependence.
In some cases, agonist treatment can lead to down-regulation of one G protein α subunit but up-regulation of another. In S49 cells, agonist activation of β2AR resulted in down-regulation of Gsα but up-regulation Giaα. The induction in Giaα2 was accompanied by a marked increase in Giaα2 mRNA and could be mimicked by the generation of cAMP in a receptor-independent manner (Hadcock et al., 1990). Since Gi has been shown to act as the inhibitory regulator of adenylyl cyclase in a number of systems, this effect of β2AR activation thus results in more effective inhibition of adenylyl cyclase and seems to provide one means for cellular adaptation to limit the effects of sustained β2AR occupancy.

Changes in G protein levels do not always result in coordinate changes in function of pathways known to be regulated by affected G protein, since in many tissues G proteins are usually present in substantial excess (Levis and Bourne, 1992, Post et al., 1995). Whether the G protein level is limiting may depend on the ratio of G protein to receptor, presence of interchangeable G protein subunits, and tissue type. An example of this apparent paradox can be found in the ob/ob mouse, where adipocyte Gia1 and Gia3 were reduced two-fold without any effect on the ability of adenosine to inhibit adenylyl cyclase, protein kinase activation, or lipolysis. The Gia2 level, however, remained unchanged and may have compensated for the loss of Gia1 and Gia3, resulting in normal receptor-G protein coupling (Gettys et al., 1994). In S49 lymphoma cells, where Gsα is 140 and 80 times in excess of β2AR and adenylyl cyclase, respectively, cholera toxin-treated cells maintained maximally elevated intracellular cAMP at a time when they lost close to 90% of their complement of Gsα (Chang and Bourne, 1989, Levis and Bourne, 1992). On the other hand, in other tissues including neuroblastoma x glioma hybrid cells, CHO cells, adipocytes, and HEK 293 cells, agonist-mediated down-regulation of Gsα, Gia, Gqα, or G11α (45 to 90%) resulted in loss of response which likely contributed to the desensitization process (Milligan et al., 1995a), whereas the up-regulation of Gsα (40%) in neuroblastoma cells in response to an opioid receptor agonist translated into an increase in adenylyl cyclase activity (Ammer and Schulz, 1993). Gsα levels also appear to be limiting in some physiological conditions. For examples, a 50% reduction in Gsα levels has been linked to Albright hereditary
osteodystrophy in humans (Weinstein et al., 1990) and to impaired lipolytic responses to β2AR in obese mice (Getty et al., 1991).

4. Proteins that interact with Gβγ

a. Adenylyl cyclase

At least ten isoforms of adenylyl cyclase have been identified by molecular cloning and six of them (type I to VI) have been well characterized. Gsα and forskolin activate all of the isoforms and type I and III are also sensitive to stimulation by Ca2+-calmodulin (Sunahara et al., 1996). Gβγ regulates adenylyl cyclase activity in an isoform-specific manner. It inhibits type I, stimulates types II and IV, and has no effects on types III, V and VI (Iyengar, 1993). The solubilized and purified type I and type II enzymes remain sensitive to Gβγ, indicating a direct interaction between adenylyl cyclase and Gβγ (Taussig et al., 1993a). While the inhibitory effects of Gβγ do not require the presence of Gsα, the stimulatory effects of Gβγ are dependent on coincidental activation by Gsα (Tang and Gilman, 1991). Gsα and Gβγ interact with the type II adenylyl cyclase at different sites (Chen et al., 1995). For the Gβγ-stimulated type II adenylyl cyclase, activation of protein kinase C (PKC) appears to relieve the requirement for the presence of activated Gsα, perhaps by phosphorylating the type II enzyme, and this modification somehow allows the βγ-interacting domain of the enzyme to become responsive (Tsu and Wong, 1996). The concentration of Gβγ required to modulate adenylyl cyclase activity is significantly greater than that of Gsα. Thus the Gs oligomer could not be the source of Gβγ involved in this regulation. Rather, it is assumed that the necessary concentrations of βγ are contributed by other G proteins, such as Go and Gi, which are far more abundant than Gs. This situation provides a clear mechanism for cross-talk between signaling pathways. At least three possible mechanisms for cross-talk between two signals can be postulated. The first is signal amplification. Signals that activate Go or Gi-coupled receptors alone cannot activate type II adenylyl cyclase, however, with release of βγ from Go or Gi, these same signals could greatly increase intracellular cAMP produced by the type II adenylyl cyclase that has been activated via Gs-coupled receptors. These circumstances were created by transfecting HEK 293 and COS-7 cells with expression plasmids encoding a constitutively active mutant Gsα, type II adenylyl cyclase, and the Gi-linked α2-adrenergic, dopamine, or adenosine receptors. Under these conditions, the inhibitory receptors are
indeed able to stimulate type II adenylyl cyclase, presumably by acting through Gβγ (Federman et al., 1992). The second is signal attenuation. The activity of type I adenylyl cyclase, which is stimulated by signals from Gs-coupled receptors or by elevation of intracellular Ca²⁺, can be attenuated by βγ release from Go (Tang and Gilman, 1991). Signal attenuation can also be accomplished by the release of activated Gicα, particularly with types V and VI adenylyl cyclases, and is presumed to be the major mechanism for the inhibition of adenylyl cyclase in non-neuronal tissues (Taussig et al., 1993b). The third is selected signal blockade. Signals that activate Go or Gi-coupled receptors could activate PLCβ via Gβγ, resulting in increased intracellular Ca²⁺ concentrations and consequent activation of calmodulin. The release of Gβγ from Go or Gi could prevent activation of type I adenylyl cyclase by Ca²⁺-calmodulin without interfering with other events resulting from the activation of PLCβ (Ueda and Tang, 1993).

b. Phospholipase C (PLC)

PLCs are divided into three subtypes: β, γ and δ. Only the PLCβ subclass (PLCβ1-4) is activated by Gq/G11α and Gβγ. Activation of PLC results in the production of diacylglycerol and inositol-1,4,5-trisphosphate (IP3), leading to the activation of PKC and the mobilization of intracellular Ca²⁺. The effect of Gqα/G11α and Gβγ on PLCβ are independent and not conditional on priming by either subunit. Experiments that examined regulation of PLC activity by combinations of Gqα and Gβγ and that analyzed truncated forms of the enzyme indicated that the site of βγ action is distinct from that for α subunits, with Gβγ binding to the N-terminal and Gα binding to the C-terminal region of PLC (Sternweis, 1994). As for the activation of adenylyl cyclases, higher concentrations of Gβγ are required for the stimulatory effect of Gβγ as compared to Gα (Muller and Lohse, 1995). PKA specifically inhibits Gβγ-activated PLCβ2 activity but not that of the Gα-activated PLC isoforms. The effect of PKA is mediated by phosphorylating serine residues of the PLCβ2 and is not mimicked by PKC isozymes (Liu and Simon, 1996). These observations provide a mechanism for the crosstalk between the two G protein-coupled signal transduction pathways. When receptors coupled to Gsα activate adenylyl cyclase, increase cAMP and activate PKA, the activated PKA can then phosphorylate PLCβ2 and prevent its activation. On the other hand, receptors coupled to Gicα subfamily inhibit adenylyl cyclase and PKA, thereby
synergizing with free Gβγ to activate PLCβ2. Such a mechanism would account for the specificity and communication between the cAMP and the phosphatidylinositol (PI) signaling pathways.

c. PH domain-containing proteins

The pleckstrin homology (PH) domain is a common structural motif found in more than 90 proteins including βARKs (βARK 1 and βARK 2), guanine-nucleotide exchange factors (GEFs), GTPase-activating proteins (GAPs), phospholipases, cytoskeletal proteins, and several protein tyrosine kinases. PH domains of several proteins, including βARKs, Ras-GAP, Ras-GRF, PLCγ, IRS-1, Db1, and Btk, reversibly bind with high affinity to Gβγ subunits in vitro. Transient expression of peptides corresponding to these PH domains inhibits Gβγ-mediated PI hydrolysis and the activation of P21 Ras and MAP-kinase, suggesting an interaction of Gβγ with these PH domain-containing proteins in vivo. However, binding of Gβγ is not a universal property of PH domains. The PH domain of spectrin, for example, does not detectably bind Gβγ in vitro. Furthermore, many Gβγ-binding proteins including Go, G protein-gated K+ channels, Raf-1 and phosducin lack PH domains (van Biesen et al., 1996). One general function of PH domains may be to anchor PH domain-containing proteins to the appropriate membrane compartment. The anchoring effect may be mediated by the signal-dependent interaction with WD-40 repeat regions of membrane-associated proteins, such as Gβγ, or more general Gβ-like proteins, such as RACK (receptor for activated C-kinase), as well as by the constitutive association of PH domains with PIP2 (Inglese et al., 1995).

Among the best-studied PH domain-containing proteins that interact with Gβγ are βARKs. βARKs are members of a family of G protein-coupled receptor kinases (GRKs) that phosphorylate agonist-activated G protein-coupled receptors. This initiates a process called homologous desensitization (Lefkowitz, 1993). βARKs exist as cytoplasmic proteins in unstimulated cells and are translocated to a variety of G protein-coupled receptors by forming a complex with Gβγ released upon activation of heterotrimeric G proteins. Therefore, the Gβγ appears to target βARKs to the plasma membrane and to position them in close proximity to the receptor, where they consequently phosphorylate the activated receptor (Pitcher et al., 1992). The structure of βARK that mediates the Gβγ interaction does not correspond to the entire PH domain, but rather overlaps
the carboxyl terminus of this domain (Touhara et al., 1994). Gβγ binds to BARK with much higher affinity than it binds to other PH domain-containing proteins such as GAP and PLCγ (Neer, 1995, Xu et al., 1996). Therefore, a peptide containing PH domain derived from BARK has been used as an inhibitor to study Gβγ-dependent pathways (Faure et al., 1994, Luttrell et al., 1995).

Gβγ also interacts with another group of PH domain-containing proteins, a family of protein tyrosine kinases including Btk, Tsk, Tec, and Bmx. Expression of these tyrosine kinases is generally limited to the hematopoietic system where they play crucial roles in B cell activation and development (Desiderio and Siliciano, 1994). Mutations in the PH domain region of the Btk gene have been implicated in X-linked agammaglobulinemia (Vihinen et al., 1995). The isolated PH domain of Btk can bind Gβγ subunits in vitro and antagonize Gβγ-dependent signaling when transiently overexpressed in intact cells. Both Btk and Tsk are activated and exhibit increased kinase activity upon addition of exogenous Gβγ (Tsukada et al., 1994, Langhans-Rajasekaran et al., 1995). These observations suggest that these tyrosine kinases may be regulated in a manner analogous to BARK and possibly provide a direct link between Gβγ subunits and tyrosine phosphorylation.

d. MAP-kinase

MAP-kinase is a family of closely related serine-threonine kinases that convert extracellular stimuli to intracellular signals which, in turn, control the expression of genes that are essential for many cellular processes, including cell growth and differentiation. MAP-kinases have been classified into three subfamilies: extracellular signal regulated kinases (ERKs), c-Jun NH2-terminal kinases (JNKs), and p38 kinases (Cano and Mahadevan, 1995). ERKs phosphorylate and regulate the activity of certain enzymes, including phospholipase A2 and p90rsk, and nuclear proteins such as the ternary complex factor p62TCF or ELK-1. The latter represents a critical event in controlling the expression of several genes, including c-fos. JNKs phosphorylate the amino-terminal transactivating domain of c-Jun and ATF2, thereby increasing their transcriptional activity. The functions of the p38 kinases are still unknown but may be related to inflammatory response (Coso et al., 1996).
The regulation of MAP-kinase activation by peptide growth factors (e.g. FGF, EGF, PDGF) has been studied extensively. The receptors for growth factors, upon ligand binding, autophosphorylate the tyrosine residues in their cytoplasmic domains. The resulting phosphotyrosine residues serve as docking sites to recruit components of the mitogenic signal complex to the receptor. This results in a cascade of events leading to ERK activation, which includes: formation of a Shc-Grb2-Sos complex, activation of p21 Ras triggered by GDP-GTP exchange catalyzed by Sos, activation of Raf-1 kinase, phosphorylation and activation of MAP kinase kinase (MEK), and finally, phosphorylation and activation of MAP kinase (ERKs, Fig. 3).

Gßγ activates MAP-kinase after stimulation of Gs, Gi and Gq-coupled receptors. In COS-7 cells, activation of M1 (Gq), M2 (Gi) muscarinic receptors, or lutropin receptor (Gs) resulted in the activation of ERK 1 and ERK2. The activation of ERKs could be blocked by co-transfecting cells with the α subunit of transducin, which acts to sequester Gßγ released upon stimulation from endogenous G proteins (Crespo et al., 1994, Faure et al., 1994). The effects of Gßγ were further confirmed by overexpressing Gßγ subunits in COS-7 cells, which led to a dramatic increase in basal ERK2 phosphorylating activity. These observations demonstrated that Gßγ can directly stimulate biochemical pathways leading to ERK activation. This effect of Gßγ was dependent on the ßγ dimer since coexpression of ß and γ, but not expression of individual subunits, was required, and coexpression of Gß and a ßγ mutant which was unable to form the Gßγ dimer with Gß did not result in ERK2 activation. The Gßγ-mediated activation of ERK was abolished by coexpression of a dominant negative mutant of Ras, indicating that Ras is required to transduce signals from G proteins to ERK2 (Crespo et al., 1994). Gßγ appears to direct the assembly of the Ras activation complex at the plasma membrane and this effect of Gßγ requires the activity of a protein tyrosine kinase that phosphorylates Shc (van Biesen et al., 1995). One of the candidates for this tyrosine kinase is c-Src. In COS-7 cells, stimulation of several Gi-coupled receptors led to a rapid increase in c-Src autophosphorylation, c-Src activity, and the formation of c-Src-Shc complex. The interaction of c-Src and Shc results in the tyrosine phosphorylation of Shc, which forms the complex with Grb2-Sos (Figure 3). These effects were inhibited by pertussis toxin and the BARK C-terminal peptide that contains the Gßγ-interacting PH domain and thus sequesters Gßγ, indicating that c-Src activity is required for Gßγ-mediated ERK activation (Luttrell et al.,
Another kinase that also is involved in Gβγ-mediated activation of MAP-kinase is PI3-kinase γ. Overexpression of PI3-kinase γ in COS-7 cells activated MAP-kinase in a Gβγ-dependent fashion, and expression of a catalytically inactive mutant of PI3-kinase γ abolished the stimulation of MAP-kinase by Gβγ or by the activation of the M2 muscarinic Gi protein-coupled receptor. Gβγ seems to localize PI3-kinase γ to the plasma membrane, thereby allowing its access to lipid substrates. Signaling from PI3-kinase γ to MAP-kinase appears to require a tyrosine kinase, Shc, Grb2, Sos, Ras, and Raf, suggesting that PI3-kinase γ may act upstream of the c-Src kinase (Lopez-llasaca et al., 1997, Fig. 3). Thus PI3-kinase γ also has a critical role linking G protein-coupled receptors and Gβγ to the MAP-kinase signaling pathway.

Gβγ may also activate MAP-kinase cascades through other mechanisms. These mechanisms include: 1) Direct Gβγ interaction with Raf-1 protein kinase. G protein-coupled receptor agonists, such as platelet-activating factor (PAF) and lysophosphatidic acid (LPS), stimulate Raf-1 and MAP-kinase kinase via a Ras-independent pathway. This stimulation appears to be mediated by Gβγ. In this case, Gβγ may act analogously to Ras, functioning to recruit Raf-1 to the plasma membrane (Pumiglia et al., 1995). 2) Gβγ activation of another Ras exchange factor, CDC25Mm or p140Ras-GRF. The increase in the specific activity of P140Ras-GRF is associated with an increase in its phosphorylation state. This pathway to Ras activation is thus distinct from the Shc-Grb2-Sos mechanism for tyrosine-dependent signaling, in which increased phosphorylation of Sos has been associated with down-regulation rather than activation (Mattingly and Macara, 1996). P140Ras-GRF binds calmodulin and is implicated in a calcium-dependent pathway for Ras activation in cultured cortical neurons (Farnsworth et al., 1995). 3) Gβγ-mediated phosphorylation of the protein-tyrosine phosphatase SH-PTP1 induced by LPS. In this case, SH-PTP1 seems to be involved in a positive signal between Gβγ and Ras, possibly involving activation of a Src-like kinase by dephosphorylation (Gaits et al., 1996). 4) Gβγ interaction with other PH-domain containing proteins including GAP, IRS-1, and PLCγ, thereby regulating the MAP-kinase pathway (van Biesen et al., 1996).

Gβγ has also been shown to interact with small G proteins of the Rho family (Rho, Rac, Cdc42) to activate JNK, a member of the MAP-kinase family. In COS-7 cells, stimulation of either
muscarinic receptors (M1 or M2) led to JNK activation; this effect was not mimicked by expression of activated forms of Gsα, Giα and G13α. In contrast, overexpression of Gβγ potently induced JNK activity. The effect of Gβγ was blocked by the BARK peptide and by co-expression of dominant-negative mutants of Ras, Rac and Cdc 42 (Coso et al., 1996). The Gβ subunit seems to play a more important role than the Gγ subunit in Gβγ-mediated JNK activation. While ERK activation requires βγ dimers, JNK can be activated by Gβ2 alone, since a mutant Gβ2 that lost its ability to form dimers with Gγ could stimulate JNK to the same extent as co-expression of Gβ and Gγ (Yamauchi et al., 1997). Mutations at the C-terminal regions of Gβ1 or Gβ2 subunits resulted in the loss of the Gβγ effect on the activation of ERK; however, the activation of JNK by Gβγ was not affected (Zhang et al., 1996a, Yamauchi et al., 1997). In addition, Gβ5, when complexed with γ2, γ4, γ5, and γ7 in βγ dimers, can activate PLCβ2, but not ERK and JNK (Zhang et al., 1996b). These observations suggest that it is the Gβ subunit which determines the pattern of downstream signaling and the regulation of the MAP-kinase pathway may require some specific structural features of the Gβ subunit.

e. Phosducin

Phosducin is a cytosolic protein that is most abundantly expressed in the retina and pineal gland but whose mRNA has been found in many other organs. It has high affinity for Gβγ subunits. The binding of phosducin to Gβγ is regulated by the phosphorylation state of phosducin. Upon phosphorylation by PKA, the affinity of phosducin for Gβγ decreases (Hawes et al., 1994). Phosducin appears to regulate G protein signaling by two mechanisms. First, it inhibits the intrinsic GTPase activity of several different G proteins including transducin (T), Gs, Go and Gi (Bauer et al., 1992). Second and possibly more important, it binds and sequesters Gβγ, thereby inhibiting Gβγ-mediated effects. In bovine rod outer segments, phosducin decreases the GTPase activity of transducin and the subsequent activation of cGMP phosphodiesterase. This inhibition is abolished by addition of excess βγ subunits of transducin (Tβγ), suggesting that phosducin regulates the amount of Tβγ available to bind to Tα, thereby inhibiting recycling of the Tαβγ complex (Lee et al., 1992, Muller et al., 1996). Phosducin also inhibits BARK phosphorylation of the β2AR and GRK3 phosphorylation of odorant receptors by competing for binding sites on the
Gβγ complex (Hekman et al., 1994, Boekhoff et al., 1997). In addition, expression of phosducin in COS-7 cells inhibits Gβγ-stimulated PI hydrolysis and cAMP production, possibly through similar mechanisms (Xu et al., 1995). These effects of phosducin can be inhibited by PKA-mediated phosphorylation of phosducin. Thus, phosducin may have dual functions in G protein signaling: in its non-phosphorylated state it may dampen G-protein mediated signaling but may at the same time reduce βARK-mediated receptor desensitization.

C. FORSKOLIN

Forskolin is a natural diterpene extracted from the plant Coleus forskohlii Briq. The ability of forskolin to stimulate adenylyl cyclase in membranes and in intact cells was discovered in 1981 (Seamon et al., 1981). Forskolin activates adenylyl cyclase rapidly and reversibly to cause an increase in intracellular cAMP. This effect of forskolin is observed even in the absence of a functional Gs protein and therefore distinguishes the action of forskolin from those of hormones and other activators of adenylyl cyclase such as sodium fluoride (NaF), guanine nucleotides, and cholera toxin. It also suggests that forskolin acts directly on adenylyl cyclase. On the other hand, the ability of forskolin to stimulate adenylyl cyclase is markedly affected by the presence and functional state of G proteins that mediate the stimulation and inhibition of adenylyl cyclase by hormones and other effectors. Specifically, hormonal agonists that act through Gs markedly potentiate the effect of forskolin, and vice versa. Conversely, stimulation of adenylyl cyclase by forskolin is markedly reduced by hormonal agonists that act through Gi (Seamon and Daly, 1986).

Maximal stimulation of adenylyl cyclase by forskolin requires the presence of Gs. In a mutant cell line of S49 mouse lymphoma cells (S49 Cyc-) that do not express Gsα and therefore lack functional Gs (Miller et al., 1988), forskolin stimulated adenylyl cyclase approximately 20-fold. However, when erythrocyte Gsα was added to the Cyc- membrane, forskolin stimulated adenylyl cyclase activity approximately 45-fold (Downs and Aurbach, 1982). In the presence of hormones that stimulate adenylyl cyclase through receptor-Gs interaction, forskolin caused synergistic stimulation of adenylyl cyclase and production of cAMP (Insel et al., 1982), also indicating a role for activated Gsα in the action of forskolin. High affinity binding sites for forskolin have been detected in membranes from a variety of tissues and cells using [3H]
forskolin. The number of high affinity binding sites for forskolin was increased under conditions that promoted activation of adenylyl cyclase by Gs, — for example, in the presence of hormones that couple to Gs (Post et al., 1995) or in the presence of NaF or Gpp(NH)p that directly activate Gs (Nelson and Seamon, 1985). Under the same condition, however, high affinity binding sites for forskolin were not observed in membranes from Cyc- cells that do not contain Gsα (Seamon and Clark, 1983). Therefore, it was proposed that the high affinity sites correspond to ternary complexes of forskolin, adenylyl cyclase and Gs. This model suggests that in the absence of Gs, forskolin binds to adenylyl cyclase with lower affinity. The binding affinity of forskolin to adenylyl cyclase is increased by activation of Gs and binding of Gsα to the catalytic subunit of adenylyl cyclase (Mittag and Tormay, 1993). Forskolin seems to bind to adenylyl cyclase at a site different from that to which the Gsα is bound, since adenylyl cyclase that is associated with activated Gs still binds to forskolin affinity columns (Seamon and Daly, 1986). The ternary complex model also predicts that the Gs will remain in an active state longer with forskolin bound to the adenylyl cyclase, since the binding of forskolin to the complex appeared to inhibit the reassociation of Gsα with Gβγ (Barber and Goka, 1985).

Although forskolin elicits large increases in intracellular cAMP in almost all mammalian tissues and cells, the potency and efficacy of forskolin with respect to increasing cAMP levels, the additive and synergistic activation of adenylyl cyclase by hormones and forskolin, and the affinity of forskolin for its binding sites, all vary among tissues. The differences likely result from differences in the amounts and functional states of Gs and Gi and the presence of different isoforms of adenylyl cyclase. All isoforms studied so far can be activated to different extents by forskolin as well as by activated Gsα; however, the interaction among forskolin, Gsα and various isoforms of adenylyl cyclase show different characteristics. For example, there was little effect of Gsα on the calmodulin-sensitive (type I) adenylyl cyclase in the presence of maximally effective concentrations of forskolin (Tang et al., 1991), since the activation of type I adenylyl cyclase by forskolin and Gsα was not greater than additive. In contrast, Gsα and forskolin synergistically activated the type II, V and VI enzymes. Gsα potentiated the effect of forskolin on type II adenylyl cyclase to the greatest extent (Sutkowski et al., 1994). Consistent with these observations, only low affinity forskolin binding sites were detected in membranes expressing type II adenylyl cyclase.
in the absence of Gsα, and exogenous Gsα greatly increased the high affinity binding sites in the membrane preparation. Similarly, but to a lesser extent, increases in the forskolin high affinity binding sites also were observed in type V and VI adenylyl cyclases in the presence of Gsα. On the other hand, high affinity forskolin binding sites were detected in membranes expressing type I adenylyl cyclase in the absence of Gsα, and the specific binding was not enhanced by the addition of exogenous Gsα. Ca\(^{2+}\)/calmodulin or Gβγ had no effect on the forskolin binding sites detected in type I and type II adenylyl cyclase in the absence or presence of Gsα (Sutkowski et al., 1994). Thus, in membranes expressing type II as well as type V and VI adenylyl cyclases, the high-affinity forskolin binding sites appear to be dependent upon the formation of ternary complexes of forskolin, adenylyl cyclase and Gsα, as previously proposed (Seamon and Daly, 1986).

The mechanism of action of forskolin on type I adenylyl cyclase seems to be different from most adenylyl cyclase isoforms. Although forskolin bound to type I adenylyl cyclase with high affinity in the absence of Gsα, this high affinity binding did not seem to be required for forskolin activation of type I adenylyl cyclase (Sutkowski et al., 1996). It has been suggested that forskolin normally interacts with adenylyl cyclase at two sites: the low affinity site that mediates the stimulation of adenylyl cyclase in the absence of Gsα, and the high affinity site that requires the presence of functional Gs protein. In the case of type I adenylyl cyclase, forskolin may bind to the two sites with the same affinity. The significance of the different forskolin binding properties of type I adenylyl cyclase is not clear. Since the type I adenylyl cyclase is expressed at high levels in brain and similar high-affinity binding sites were detected only in brain and platelets, the high affinity binding sites may be related to the specific function of type I adenylyl cyclase in these tissues.

The crystal structure of a rat type II adenylyl cyclase in complex with forskolin has been reported recently (Zhang et al., 1997). Mammalian adenylyl cyclase consists of twelve hydrophobic membrane-spanning regions, the short loops that link them and two cytoplasmic regions which contain both variable and conserved sequences. The catalytic activity of adenylyl cyclase resulting from forskolin and G protein stimulation is conferred by two well-conserved domains designated C1 and C2, that also are homologous to each other. The structure of the C2 catalytic region of type II adenylyl cyclase has an α/β class fold in a wreath-like dimer, which has a
central cleft. Two forskolin molecules bind in hydrophobic pockets at the ends of the cleft. The central part of the cleft is lined by charged residues implicated in ATP binding. Forskolin appears to activate adenylyl cyclase by promoting the assembly of the active dimer and by direct interaction within the catalytic cleft (Zhang et al., 1997). Although the crystallization study revealed the structure of adenylyl cyclase involved in forskolin binding, it did not provide information on the interaction of adenylyl cyclase with Gsα, or on the interaction of forskolin with Gsα.

Other than its direct stimulatory effects on adenylyl cyclase, forskolin also interacts with other membrane-associated proteins through a mechanism that does not involve the production of cAMP. Forskolin competitively inhibits glucose transporter, increases the rate of acetylcholine-induced desensitization of the nicotinic acetylcholine receptor, modulates voltage-dependent K+ channels and GABA_A-gated chloride channels, and reverses adriamycin resistance in S180 murine sarcoma cells that are multidrug-resistant (Laurenza et al., 1989, Morris et al., 1991a). Forskolin exerts its cAMP-independent effects by directly binding to several membrane transport proteins including the glucose transporter, ligand and voltage-gated ion channels, and P-glycoprotein. The interaction of forskolin with adenylyl cyclase and other membrane proteins could be distinguished by utilizing the naturally occurring analog of forskolin, 1,9-dideoxy-forskolin, which does not activate adenylyl cyclase, but is able to reproduce the cAMP-independent effects of forskolin on other proteins. Furthermore, some synthesized forskolin derivatives are able to specifically bind to adenylyl cyclase but not to other proteins, and vice versa (Appel et al., 1992, Sutkowski et al., 1993). For example, N-(3-(4-azido-3-phenyl)-propionamide)-6-aminoethylcarbamylforskolin (6-AIPP-forskolin) only binds to adenylyl cyclase, whereas 7-AIPP-forskolin only binds to glucose transporter (Morris et al., 1991a). The proteins that forskolin binds to, including adenylyl cyclase and other membrane transporter proteins, all seem to have similar structures that include one or two six transmembrane helices and an ATP binding site. The forskolin binding site in these proteins seems to be composed of transmembrane helices and the specific characteristics of the binding site, e.g. the selective binding to different forskolin derivatives are determined by the amino acid residues in the transmembrane helices (Morris et al., 1991b). Determination of forskolin-binding characteristics in structurally related proteins should provide more insight into the mechanism of action of forskolin on adenylyl cyclase.
D. FORSKOLIN-RESISTANT MUTANTS

The Y1 mouse adrenocortical tumor cell line originated from a mouse adrenal tumor (Yasumura et al., 1966) and retains functional properties similar to normal adrenocortical cells (Schimmer, 1989). It responds to ACTH with increased adenyl cyclase activity and steroidogenesis, changes in cell shape and growth arrest at the G1 phase of the cell cycle (Schimmer and Zimmerman, 1976, Armelin et al., 1977, ). The concentration of ACTH required to stimulate the adenyl cyclase and steroidogenic activities were virtually the same in the Y1 cell line as in normal isolated adrenal cells (Schimmer and Zimmerman, 1976). The similarity of Y1 cells to normal isolated adrenal cells has enabled researchers to use it to examine numerous aspects regarding the function and regulation of the adrenal cortex. Among the advantages offered by the Y1 cell line is the availability of mutants with specific lesions in hormone-stimulated pathways. Elucidating the biochemical bases for these mutations has facilitated the identification of obligatory components in the actions of ACTH. For example, mutants with specific defects in cAMP-dependent protein kinase have helped to establish obligatory roles for the enzyme in the steroidogenic response to ACTH (Wong et al., 1992, Olson et al., 1993).

In order to understand forskolin's mechanisms of action, a family of forskolin-resistant mutants was isolated from the Y1 adrenocortical tumor cell line based on the ability to resist the growth inhibitory and morphological effects of forskolin. Y1 cells respond to forskolin with increased adenyl cyclase activity, steroid hormone biosynthesis, growth inhibition, and changes in cell morphology. These effects of forskolin are mediated by forskolin's action via the cAMP signaling cascade, as these effects are attenuated in Kin mutants of the Y1 cell line, which have impaired PKA activity (Schimmer and Schulz, 1985). In the presence of forskolin, parental Y1 cells assumed a rounded morphology and detached from the culture plate. Forskolin-resistant mutants are characterized by their resistance to the growth inhibitory and morphological effects of forskolin. The forskolin-resistant cells, however, remained sensitive to the growth inhibitory effects of cAMP analog, 8BrcAMP, indicating that the resistance to forskolin results from a lesion affecting cAMP accumulation rather than cAMP action (Schimmer and Tsao, 1984). The resistance to forskolin in these mutants occurred as a spontaneous mutational event rather than as an adaptive response to the selection procedure (Schimmer and Tsao, 1985). The calculated mutation rate (2 x
10^-6 per cell per generation) is close to the frequency expected for a mutational event at a single genetic locus in bacteria and far greater than the value of 10^-12 predicted for two distinct mutational events (Lewin, 1977), suggesting that the phenotypic changes observed in forskolin-resistant cells, as will be described in detail next, result from a single mutational event.

In the forskolin-resistant mutants, adenylyl cyclase is partially resistant to activation by forskolin. Adenylyl cyclase activity in response to forskolin in mutant cells was approximately 2.5-fold less than the activity measured in parental Y1 cells. The impaired response to forskolin could not be attributed to the degradation of cAMP since cAMP-phosphodiesterase activity in mutant cells was lower than in parent Y1 cells (Schimmer and Tsao, 1984). The adenylyl cyclase activity in response to the guanyl nucleotide analog, Gpp(NH)p, was also reduced to 50% of levels obtained in Y1 cells. Strikingly, adenylyl cyclase activity in forskolin-resistant cells was completely insensitive to ACTH; however, the enzyme remained fully responsive to NaF. The forskolin-resistant phenotype was not readily explained in terms of a primary defect in adenylyl cyclase. Since ACTH, Gpp(NH)p, and NaF all regulate adenyly cyclase activity through Gs, a postulated defect in the catalytic subunit of adenylyl cyclase would not account for the complete loss of ACTH-regulated activity, the partial loss of Gpp(NH)p-stimulated activity, and the retention of fluoride-sensitive activity. On the other hand, as a common factor involved in the actions of all the activators of adenylyl cyclase including ACTH, Gpp(NH)p, NaF, and forskolin, Gs seemed to be a possible site of defect in the forskolin-resistant mutants.

As assessed by immunoblotting, the forskolin-resistant cells exhibited 70% reductions in the levels of Gsα and Giα subunits in purified plasma membrane fractions. The levels of Gsα and Giα RNA, however, were essentially unaltered. The levels of GB1 were not different and the levels of GB2 varied independently of the mutation (Schimmer and Tsao, 1990). Furthermore, the activity of βγ complexes purified from mutant cells was decreased 50% as measured by the ability of βγ to support either rhodopsin-catalyzed guanyl nucleotide exchange on Gtα or pertussis toxin-catalyzed ADP-ribosylation of Gtα (Mitchell et al., 1992).

The forskolin-resistant phenotype thus appeared to be extremely complex. Inasmuch as the decreases in Gsα and Giα were not accompanied by corresponding decreases in the levels of RNA encoding these subunits, the mutation to forskolin resistance may have affected either the post-
translational processing of these subunits or their incorporation into the plasma membrane. On the other hand, βγ complexes have been shown to play important roles in the association of Gα subunits with the plasma membrane (Sternweis, 1986). Therefore, to accommodate these observations in a model that could be explained by a single gene defect and that was consistent with the role for Gα in forskolin action, it was suggested that forskolin resistance may have resulted from mutations in either the β or γ subunits of G proteins, which in turn, affected the incorporation of Gα subunit into the plasma membranes (Schimmer et al., 1987, Schimmer and Tsao, 1990, Mitchell et al., 1992). The decreased levels of membrane Gα might account for forskolin resistance in the mutant cells. The decreased levels of Gα also were postulated to explain the loss of responsiveness to ACTH and guanyl nucleotides seen in these mutants.

E. RESEARCH OBJECTIVE

In studies from other laboratories, the action of forskolin on adenylyl cyclase activity was examined in a mutant cell line S49 Cyc-. The Cyc- cells do not express Gα and as a result, their adenylyl cyclase is rendered completely insensitive to stimulation by catecholamines, guanyl nucleotides, and NaF (Johnson et al., 1979, Miller et al., 1988). In this mutant cell line, forskolin stimulated adenylyl cyclase activity appreciably, but not to the same level achieved in parent S49 cells and not with the same kinetics (Seamon and Daly, 1986). The forskolin-resistant mutants, however, are apparently different from the Cyc- mutant. In the forskolin-resistant mutants, the adenylyl cyclase was only partially resistant to activation by guanyl nucleotide analogs, and fully responsive to NaF. The levels of Gα and Giα subunits associated with the plasma membrane were decreased and the activity of βγ complexes was impaired. These observations have led to the hypothesis that decreased levels of Gα and Giα in these mutants were secondary to a mutation in either the β or γ subunits of G proteins. Based on studies from other laboratories indicating a regulatory role for Gα in forskolin’s action on adenylyl cyclase, the secondary decrease in levels of membrane Gα may have accounted for the forskolin resistance of the mutant cells.

Although this hypothesis seemed to accommodate most of the phenotypic changes observed in the forskolin-resistant mutants, several observations remained unexplained. First was the impact of low levels of Gα and Giα on the mutant phenotype. In the forskolin-responsive Y1
cells, forskolin appeared to act through two sites with different apparent affinities; the high affinity site accounted for approximately 25% of forskolin's total activity. The high affinity site has been suggested to result from the cooperative binding of Gsα to the catalytic subunit of adenylyl cyclase in the presence of forskolin (Seamon and Daly, 1986). In parent Y1 cells, no apparent synergistic effect was observed between forskolin and ACTH, Gpp(NH)p, or NaF in the activation of adenylyl cyclase, and the affinity of forskolin for the enzyme was not notably increased upon the inclusion of ACTH, Gpp(NH)p, and NaF in the assay (Schimmer et al., 1987). Therefore, activation of Gs by these agonists seemed not to markedly increase the fraction of forskolin-sensitive adenylyl cyclase in the high-affinity state. In forskolin-resistant mutant cells, the low levels of Gsα did not seem to cause selective changes in the apparent affinity of the enzyme for forskolin at either site. Rather, the mutation was associated with reduced responsiveness to forskolin at both sites (Schimmer et al., 1987). These characteristics of forskolin-resistant Y1 mutants are different from the Gsα-defective S49 Cyc- cells, in that the mutation in Gsα resulted in selective loss of the high affinity site on the enzyme for forskolin as well as the complete loss of sensitivity to Gpp(NH)p and NaF (Johnson et al., 1979). Thus, the decreased levels of Gsα could not satisfactorily explain some of the phenotypic changes in forskolin-resistant mutant cells.

In a number of systems, including Y1 cells, the divalent cation Mn2+ can substitute for Mg2+ at the catalytic site and at the allosteric regulatory site of adenylyl cyclase (Birnbaumer et al., 1983). Mn2+ also uncouples Gs from adenylyl cyclase at concentrations greater than 5 mM and uncouples Gi from adenylyl cyclase at concentrations in the range of 0.5 to 2 mM (Limbird et al., 1979, Abramowitz and Campbell, 1984). The effects of Mn2+ could thus be used to further evaluate the possible contributions of Gs and Gi to the mutant phenotype. The effects of Mn2+ on adenylyl cyclase from the forskolin-resistant mutants were similar to their effects on the parent enzyme over a broad concentration range, and the adenylyl cyclase activity in the presence of MnCl2 instead of MgCl2 did not reverse the enzyme's resistance to forskolin or to ACTH (Schimmer et al., 1987). These observations indicated that the decreased levels of Gsα and Giα in mutant cells are not likely to explain the mutation to forskolin resistance.

The decreased levels of Giα also do not explain the complete loss of response to ACTH response and an unaffected response to NaF in the mutant cells. Although ACTH and NaF activate
adenylyl cyclase through different mechanisms, both require a functional $G_{s\alpha}$ for their actions. ACTH exerts its effect through binding to the ACTH receptor which is coupled to adenylyl cyclase through $G_{s}$. NaF exerts its effect by forming AlF$4^-$, which binds to GDP-$G_{s\alpha}$ and the $G_{s\alpha}$-GDP-AlF$4^-$-H$_2$O complex mimicks the $\gamma$-phosphate of GTP in its pentavalent transition state during GTP hydrolysis (Sondek et al., 1994). The stabilizing interaction keeps $G_{s\alpha}$ at its active state, similar to GTP-$G_{s\alpha}$, which goes on to activate adenylyl cyclase. In the forskolin-resistant cells, although the $G_{s\alpha}$ levels decreased to only the 30% levels of the wild-type Y1 cells (Schimmer and Tsao, 1989), adenylyl cyclase was only partially resistant to forskolin and the guanyl nucleotide analog, indicating that at least part of $G_{s}$ function is preserved. The fact that NaF stimulates adenylyl cyclase to the full extent further indicates the presence of functional $G_{s\alpha}$ protein. Thus, a complete loss of ACTH response could not be explained by the low levels of $G_{s\alpha}$ in the mutant cells; rather, a deficiency in ACTH receptor is implicated.

The decreased activity of $\beta\gamma$ complex may play a more important role in the mutant phenotype. Although $\beta\gamma$ is important for the membrane attachment of $G_{\alpha}$, it is not the only factor that contributes to this process. As described in Section I.B.2.a. ii., the palmitoylation ($G_{s}$ and $G_{i}$) and myristoylation ($G_{i}$) of $G_{\alpha}$ subunits, seem to play essential roles in the association of $G_{\alpha}$ with membrane fractions. On the other hand, the $\beta\gamma$ dimer is capable of directly regulating adenylyl cyclase activity and can either inhibit or activate the enzyme depending on the specific isoforms present (Taussig and Gilman, 1995). In addition to regulating adenylyl cyclase activity, $G_{\beta\gamma}$ also modulates the activities of other signaling pathways such as PLC and PLA2, $K^+$ and $Ca^{2+}$ channels, and MAP-kinase (Neer, 1995, Fig.3). Therefore, mutations affecting $G_{\beta\gamma}$ activity might be expected to impact on multiple signaling pathways.

The research objective of this study was to understand the molecular basis for forskolin-resistance in the Y1 adrenal cell mutants. Specifically, this study attempted to: 1) determine the basis for the ACTH-resistance that accompanies the forskolin resistance mutation. 2) determine the contribution of $G_{s\alpha}$ to the forskolin-resistant mutant phenotype. 3) determine the effect of $G_{\beta\gamma}$ on the forskolin-resistant phenotype. 4) localize the mutation leading to forskolin resistance by cloning and sequencing G protein subunits from mutant cells.
II. MATERIALS AND METHODS

A. CELL CULTURE, PLASMIDS, AND GENE TRANSFER

The Y1 mouse adrenocortical tumor cell line, the forskolin-resistant Y1 mutants, 10r6 and 10r9, and the spontaneous ACTH receptor-resistant Y1 mutants Y6 and OS3, were cultured as monolayers in Nutrient Mixture F10 (Ham, 1963) supplemented with 15% heat-inactivated horse serum, 2.5% heat-inactivated fetal bovine serum, 200 U/ml penicillin G, and 270 µg/ml streptomycin sulfate as described in detail previously (Schimrner, 1985). For transfection, supercoiled plasmid DNA was isolated by alkaline lysis method as described by Maniatis et al. (1982) and purified through CsCl gradient. Cells were transfected with plasmid DNA using LIPOFECTIN Reagent (Canadian Life Technology, Burlington, ON). Cells were grown to approximately 50% confluence, washed twice with Alpha-Minimum essential medium (Alpha-MEM; Canadian Life Technology) and incubated with 3 ml Alpha-MEM plus 30 µl Lipofectin for 1 hour. Then supercoiled plasmid DNA (25 µg) and pSV2-Neo (7.5 µg), which encodes a neomycin-resistant gene (Southern and Berg, 1978, Figure 4), were added and the incubation was continued for another 6 hours. Plates were washed with Alpha-MEM and grown overnight in Nutrient Mixture F10 with sera and antibiotics. Cells were replated at 1/4 dilution and grown for two to three weeks in F10 growth medium containing the neomycin analog, G418 (100 µg/ml (active concentration; Canadian Life Technologies). Neomycin-resistant colonies were isolated with cloning cylinders to establish clonal cell lines. This transfection procedure was used for the introduction of the following plasmids into various adrenal cell lines: a mouse β2 adrenergic receptor genomic clone (pβ2AR, from Richard Palmiter, University of Washington, Seattle, WA, Figure 5), a human ACTH receptor cDNA clone (from Roger Cone, University of Oregon, OR, Figure 6), and a rat HA-tagged Gsα cDNA clone (from Henry Bourne, University of California, San Francisco, CA, Figure 7).

Alternatively, for the transfection of G protein β and γ subunits (from William Simonds, NIDDK, National Institute of Health, Bethesda, MD, Figure 8), the calcium phosphate precipitation technique developed by Graham and Van der Eb (1973) was used. Cells were grown to approximately 30 to 40 % confluence, rinsed and maintained in Alpha-MEM supplemented with
Figure 4. Map of plasmid encoding neomycin resistance gene. The plasmid pSV2-neo was used to confer resistance to neomycin analogs to transformed cells. The neomycin phosphotransferase gene was cloned into the bacterial vector pBR 322 and was flanked by the SV 40 promoter, the SV40 small t antigen intron and the SV40 polyadenylation signal.
Figure 5. Plasmid encoding β2 adrenergic receptor (pβAR). The plasmid pβAR contains a 12.9 kb genomic clone of the mouse β2AR gene subcloned into the bacterial vector pUC 19 at the Sal I sites. The β2AR coding region is flanked by 3.5 kb 5' and 7.4 kb 3' sequences.
Figure 6. Plasmid encoding ACTH receptor. The plasmid pACTH-R contains cDNA encoding human ACTH receptor was cloned into the expression vector pcDNA I Neo. The ACTH-R cDNA is flanked by the CMV promoter and SV40 polyadenylation segment. The expression vector also contain the neomycin phosphotransferase gene that is flanked by the RSV promoter and SV40 polyadenylation signal.
Figure 7. Plasmid encoding epitope-tagged Gsα subunit. The plasmid pHA-Gsα contains a 1.2 kb Sal I and Hind III fragment of the long form of rat Gsα cDNA. The cDNA was modified to contain an epitope tag (HA-Gsα) and was cloned into the expression vector pcDNA 1d. The Gsα cDNA is flanked by the SV40 promoter and SV40 polyadenylation segment.
Figure 8. Plasmid encoding G protein β and γ subunits. A. The plasmid pGβ contains cDNAs encoding either bovine Gβ1 or human Gβ2 that were cloned into the expression vector pCDM8.1. The Gβ cDNA is flanked by the CMV promoter and SV40 polyadenylation signal. B. The plasmid pGγ contains a human Gγ cDNA and is flanked by the CMV promoter and SV40 polyadenylation signal.
15% horse serum and 2.5% fetal bovine serum, 200 U/ml penicillin G, and 270 μg/ml streptomycin sulfate. Plasmid DNA in 250 mM CaCl₂ was added dropwise to an equal volume of double strength solution of Hepes-buffered saline (HBS) containing 50 mM Hepes-NaOH (pH 7.1), 250 mM NaCl, and 1.5 mM Na₂HPO₄.NaH₂PO₄ (pH 7.0). The DNA-CaCl₂ precipitates were formed at room temperature over a 30 min period and were then added dropwise over cell monolayers. Cells were incubated with DNA for 24 h at 37 C and the medium containing DNA was then removed. Cells were rinsed and incubated for an additional 24 h in Alpha-MEM plus sera, and were then grown in F10 growth medium containing the neomycin analog G418. G418-resistant colonies was tested for change of cell shape in response to ACTH (24 nM) and clones that rounded up in the presence of ACTH were isolated using cloning cylinders.

For the transient transfection of G protein β and γ subunits, cells were incubated with DNA for 24 h at 37 C using the calcium phosphate precipitation technique and then incubated in the DNA-free medium for an additional 48 to 72 h. After this period, total cellular RNA was prepared from the transfected cells using the RNAeasy Mini kit (Qiagen Inc. Santa Clarita, CA), as described later.

B. ADENYLYL CYCLASE ACTIVITY

Adenylyl cyclase activity was measured in cell homogenates by measuring the conversion of [2, 8-³H]ATP to [2, 8-³H]cAMP, as previously described (Schimmer, 1972). Cell monolayers were rinsed with a Tris buffer (1mM MgCl₂, 20 mM Tris-HCl, pH 7.7) plus 250 mM sucrose, scraped into the Tris buffer containing 1 mM DTT, and homogenized at 0 C in a Dounce homogenizer (Wheaton, Millville, NJ) with a tight pestle. Cell homogenates containing approximately 120 μg total protein were added to a reaction mixture containing 1 mM [2,8-³H]ATP (approximately 1.6 x 10⁶ cpm; Dupont, Canada, Markham, ON), 2 mM MgCl₂, 6 mM theophylline, 12.5 mM Tris-HCl, pH 7.7, in a final volume of 85 μl. The reaction was carried out for 5 min at 37 C and terminated by adding 0.1 ml of recovery mix (40 mM disodium ATP and 12.5 mM cAMP) and boiling the mixture for 3 min. Labeled cAMP was separated from other labeled compounds by chromatography on AG 50W-X4 ion exchange resin (100-200 mesh, hydrogen form; BioRad Laboratories Ltd., Mississauga, ON) and treatment with BaSO₄. Aliquots
of cell homogenates were taken for protein determination by the method of Lowry (Lowry et al., 1951)

C. ASSAY OF CAMP ACCUMULATION

Cells were plated in 60 mm dishes and grown to confluence. Endogenous ATP was labeled by prior incubation of cells with [2-3H] adenine 8 μCi for 2 h in Alpha-MEM plus sera. After the labeling period, cell monolayers were rinsed 5 times with 1 ml of Alpha-MEM and maintained in 1 ml of Alpha-MEM plus 0.1% trypsin inhibitor. ACTH was added to the medium and cells were incubated for 30 min at 37 °C. At the end of the incubation, medium was collected from the dishes and 0.2 ml of a recovery mix containing 40 mM disodium ATP and 12.5 mM cAMP were added to the collected sample. Labeled cAMP was separated by chromatography on AG 50W-X4 ion exchange resin and treatment with BaSO4. For protein determination, 1 ml of 1N NaOH was added to the cell monolayer and incubated for 1 h at 37 °C. Aliquots of lysate were taken for protein determination by the method of Lowry (Lowry et al., 1951)

D. LIGAND BINDING ASSAYS

Ligand binding assays were carried out on crude membrane fractions as described (Olson and Schimmer, 1994). Cells were grown on 150 mm tissue culture dishes to confluence, then incubated in Alpha-MEM plus 1% fetal calf serum for 18 -24 h. Cell monolayers were rinsed with a Tris buffer (1mM MgCl2, 20 mM Tris-HCl, pH 7.7) plus 250 mM sucrose, scraped in ice-cold binding buffer (5 mM MgCl2, 5 mM EDTA, 5 mM KCl, 1.5 mM CaCl2, and 50 mM Tris-HCl, pH 7.4), and homogenized at 0 °C in a Dounce homogenizer with a tight pestle. Cell homogenates were centrifuged for 5 min at 800 g and 4 °C, the resultant supernatant was centrifuged for 15 min at 40,000 g and 4 °C. The pellet (crude membrane) was resuspended in binding buffer and frozen at -70 °C. For binding assays, crude membranes (100 μg) were incubated with 40 pM [125I]iodocyanopindolol (100,000 cpm, 2,000 Ci/mmol; Amersham Canada, Ltd., Oakville, ON) and various concentrations of isoproterenol in binding buffer for 30 min at 37 °C. Reactions were terminated by rapid filtration through glass fiber filter mats (Skatron, Inc, Sterling, VA). The
amount of radioactive ligand bound was measured by gamma scintillation spectrometry. Binding parameters were determined using the program LIGAND (Munson and Rodbard, 1980).

E. PREPARATION OF TOTAL CELLULAR RNA

Total cellular RNA was used in RT-PCR, Northern blot analysis, and ribonuclease protection assays. RNA was extracted from cells using guanidinium thiocyanate (Chirgwin et al., 1979). Cells were rinsed twice with 5 ml phosphate-buffered saline and scraped in 2 ml guanidinium thiocyanate solution (4 M guanidinium thiocyanate, 0.5% sodium-N-lauroylsarcosine, 25 mM sodium citrate, 0.1% antifoam A, 0.1 M 2-mercaptoethanol). Extracts from two 150 mm dishes of cells were combined, layered over 2.0 ml of CsCl (5.7 M), and centrifuged at 80,000 g for 22 h at 15 C. The RNA pellet was dissolved in 360 µl DEPC-treated water, ethanol precipitated, and re-dissolved in 50 µl of sterile H2O. The amount of recovered RNA was quantitated by UV spectrophotometry.

Alternatively, total cellular RNA was prepared using an RNAeasy Mini kit (Qiagen Inc. Santa Clarita, CA) according to the manufacture's specifications. Cells were lysed in guanidinium thiocyanate solution. Cell lysates were mixed with an equal volume of 70% ethanol and loaded onto RNeasy spin columns. Columns were washed with wash buffer and RNA was eluted in 30-60 µl of H2O. The amount of recovered RNA was quantitated by UV spectrophotometry.

F. REVERSE TRANSCRIPTASE POLYMERASE CHAIN REACTION (RT-PCR)

To synthesize cDNA from RNA, RNA (1-10 µg) was first mixed with 10-50 pmols of gene-specific primer or oligo-dT primer and heated for 10 min at 70 C. A reaction solution containing 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 3 mM MgCl₂, 10 mM DTT, 400 µM each dATP, dCTP, dGTP, dTTP, and 200 U Superscript II Reverse Transcriptase (Canadian Life Technology, Burlington, ON) were added to RNA samples and the mixture was incubated for 1 h at 42 C. Reverse transcriptase was inactivated by heating samples for 15 min at 70 C. RNase H (Canadian Life Technology) was added to eliminate RNA template and samples were incubated for 10 min at 55 C.
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The positions of all primers listed are based on the relative position to the initiator codon ATG where A is at position +1. All sequences are in reference to GeneBank sequences with access numbers <sup>a</sup>S50461, <sup>b</sup>D31952, <sup>c</sup>S65874, <sup>d</sup>M36430, <sup>e</sup>U29055, respectively.
The synthesized cDNA was added to a PCR reaction mixture containing 1X reaction buffer (10 mM Tris-HCl, 50 mM KCl, 1.5 mM MgCl₂, and 0.01% gelatin), 200 μM of each dNTP (dATP, dGTP, dTTP, and dCTP), and 50 - 100 pmol oligonucleotide primers (Table 1) in a total volume of 50 or 100 μl. Samples were overlaid with 50-100 μl of mineral oil and heated at 94 C for 5 min. Taq DNA polymerase (2.5 unit, Boehringer Mannhein Canada, Laval, PQ) was then added and the reaction mixture was subjected to 25 -35 cycles of PCR using a DNA Thermal Cycler (Perkin Elmer Cetus, Norwalk, CT). For amplification of Gsα and Gβ, the timing was 1 min at 94 C, 1.5 min at 60 C, and 2 min at 72 C per cycle. For amplification of ACTH receptor and SF1, the timing was 1 min at 94 C, 1 min at 60 C, and 1 min at 72 C per cycle. An additional 10 min at 72 C was carried out at the end the PCR reaction. SF-1 cDNA was examined in all experiments in which ACTH receptor cDNA was amplified and served as an internal control to ensure that equivalent amounts of cDNA were used in each sample. The amount of SF-1 cDNA and the cycle number for SF-1 amplification were tested to ensure a linear relationship between the input of cDNA and PCR products.

G. NORTHERN BLOT ANALYSIS

Total cellular RNA was fractionated by electrophoresis on a 1.2% agarose/2.2 M formaldehyde gel. RNA (25 μg) was combined with gel running buffer (250 mM Heps, pH 7.8, 50 mM sodium acetate, 50 mM EDTA), formaldehyde (15%), and deionized formamide (30%) in a total volume of 27 μl. The mixture was heated for 5 min at 60 C to denature RNA and was applied to the gel. The sample was electrophoresed for 4 h at 65 V, rinsed with H₂O and incubated overnight in H₂O with gentle rocking to remove formaldehyde. RNA was transferred to a Nytran nylon filter (Xymotech Biosystems, Toronto, ON) by capillary action (Southern, 1975) for 16 h at room temperature using 10X SSPE (20X SSPE containing 3.6 M NaCl, 20 mM EDTA, 0.2 M NaH₂PO₄) as transfer buffer. The filter was baked for 1 h in a vacuum oven at 80 C.

For preparation of ³²P-labeled cDNA probe, a nick translation reaction kit (Canadian Life Technology) was used to introduce [α-³²P]-dCTP into cDNA fragments. A solution (5 μl) containing dTTP, dATP, dGTP (0.2 mM each of the dNTPs in 500 mM Tris-HCl, pH 7.8, 50 mM MgCl₂, 100 mM 2-mercaptoethanol, 100 μg/ml nuclease-free BSA), 100 ng DNA fragment,
and 50 μCi [α-32P]-dCTP were combined in a 1.5 ml eppendorf tube. The reaction mixture was brought to 40 μl with sterile H2O and 5 μl of enzyme solution containing DNA polymerase/DNase I (100 units) was added. The tube was incubated for 1 h at 15 C and the reaction was terminated by addition of 5 μl of 300 mM Na2EDTA, pH 8.0. The labeled DNA was separated from unincorporated nucleotides by chromatography on a 0.9 x 9 cm column of Sephadex™ G-50 fine, equilibrated with TNE buffer (10 mM Tris-HCl, pH 8.0, 50 mM NaCl, and 0.1 mM EDTA, pH 8.0).

The RNA blot was prehybridized for 2-4 h at 42 C in a solution containing 50% formamide, 5X Denhardt’s solution (prepared from 50X Denhardt’s stock solution containing 1% bovine serum albumin, 1% polyvinyl pyrrolidine, 1% ficoll 400), 5X SSPE, 0.5% SDS and 100 ng/ml sheared heat-denatured salmon sperm DNA. Incubation was carried out in a heat-sealable polyethylene bag. The prehybridization buffer was then removed and replaced with hybridization buffer, a solution containing the same ingredients plus 10 % dextran sulfate with approximately 1-5 x 10^6 cpm/ml of nick-translated DNA probe. The hybridization reaction was allowed to proceed for 18-20 h at 42 C. The blot was washed twice in 2X SSPE, 0.1 % SDS for 20 min at room temperature and twice in 0.1X SSPE, 0.1% SDS for 20 min at 55 C. Blots were exposed to X-ray film and analyzed using the Molecular Phosphoimager from BioRad Laboratories Ltd.

H. RIBONUCLEASE PROTECTION ASSAYS

Ribonuclease protection assays were carried out using a MAXIscript labeling kit and an RNase protection assay kit (RPAII) from Ambion (Austin, TX). For preparation of radio-labeled RNA probe, plasmid DNA (pBluescript, Stratgene, La Jolla, CA) containing T7 promoter and a 273 bp ACTH receptor cDNA insert was linearized by digestion with restriction enzyme Sal I. The cDNA fragment was transcribed in vitro in a solution containing 1 μg of plasmid DNA, 1X transcription buffer, 500 μM each of ATP, CTP and GTP, 50 μCi [α-32P]-UTP, 10 mM DTT, 25 U RNase inhibitor, and 10 U T7 RNA polymerase. The mixture was incubated for 1h at room temperature. DNA template was then destroyed by adding 2 U RNase-free DNase I and incubating for 15 min at 37 C. At the end of the DNase I incubation, an equal volume of gel loading buffer
(80% formamide, 0.1% xylene cyanol, 0.1% bromophenol blue, 2 mM EDTA) was added to the reaction, and the tube was heated for 5 min at 90 C. The labeled antisense RNA was gel purified and eluted in a buffer containing 0.5 M ammonium acetate, 1 mM EDTA, and 0.2% SDS. An aliquot of sample was counted to determine specific activity.

Ribonuclease protection assays were carried out using RNase protection assay kit (RPAl) from Ambion. RNA (1 µg) and 32P-labeled probe (1 x 105 cpm) were combined, co-precipitated from 0.5 M ammonium acetate with ethanol, and resuspended in 20 µl of hybridization buffer (80% deionized formamide, 100 mM sodium citrate, pH 6.4, 300 mM sodium acetate, pH 6.4, and 1 mM EDTA). The sample was heated at 90 C for 3 min and then incubated overnight at 42 C. RNase (1 to 100 and 1 to 200 dilution of RNase A/RNase T1 mixture) was added and samples were incubated for 30 min at 37 C. The reaction was stopped by adding RNase inactivation/precipitation mixture (from RPAl kit). Samples were precipitated, resuspended in gel loading buffer, and heated for 3 min at 90 C. The heated samples were loaded on a 5% polyacrylamide/8M urea gel, and electrophoresed at 250 volt for about 1 h. The gel was covered with plastic wrap and exposed to X-ray film.

I. GENOMIC DNA PREPARATION AND SOUTHERN BLOT ANALYSIS

High molecular weight genomic DNA was prepared as described by Wigler (Wigler et al., 1979a). Briefly, cell monolayers were rinsed with PBS and scraped in 2-5 ml ice-cold PBS. Cells then were centrifuged at 800 g for 5 min and the supernatant was removed. Cell pellets were well resuspended in 0.6 ml digestion buffer (100 mM NaCl, 10 mM Tris-HCl, pH 8.0, 25 mM EDTA, pH 8.0, 0.5% SDS, and 0.3 mg/ml proteinase K) and incubated overnight at 50 C with constant shaking. Samples were then extracted with phenol and precipitated with ethanol. DNA was resuspended in 0.6 ml TE buffer (10 mm Tris-HCl, pH 8.0, 1 mM EDTA) and incubated at 60 C with constant shaking until completely dissolved.

DNA samples (10 µg each) were digested with restriction endonucleases and electrophoresed through agarose at low voltage (20 volt) for 16 h. DNA was then fragmented in 0.25 N HCl for 10 min and blotted onto Hybond+ nylon membranes (Amersham Canada Ltd.)
0.4 M NaOH blotting buffer. Blots were hybridized with $^{32}$P-labeled cDNA probes as described in detail for Northern blot analysis.

J. NUCLEAR RUN-OFF ASSAYS

Nuclear run-off transcription assays were performed as described (Ausubel et al., 1994). Cells were grown on 150 mm tissue culture dishes to confluence. Cell monolayers were rinsed, scraped in ice-cold PBS, and centrifuged for 5 min at 800 g and 4 C. The cell pellets were then resuspended in buffer containing 10mM Tris-HCl (pH 7.4), 3 mM CaCl$_2$, 2mM MgCl$_2$ and re-centrifuged for 5 min at 800 g and 4 C. Cells were resuspended at 5 x 10$^7$ cells/ml in the same buffer. An equal volume of NP-40 lysis buffer (10mM Tris-HCl, pH 7.4, 10 mM CaCl$_2$, 10 mM NaCl, 3mM MgCl$_2$, and 0.5 % NP-40) was added and the mixture was homogenized at 0 C in a Dounce homogenizer with a loose pestle. The homogenates were centrifuged for 5 min at 800 g and 4 C. Pelleted nuclei were resuspended at 5 x10$^7$nuclei/200 µl in glycerol storage buffer (50mM Tris-HCl, pH8.3, 40% glycerol, 5 mM MgCl$_2$, and 0.1 mM EDTA) and stored at -70 C.

Nuclear run-off reactions were performed for 30 min at 30 C in a transcription buffer containing 5 x 10$^7$ nuclei, 10 mM Tris-HCl, pH 8.0, 5 mM MgCl$_2$, 0.3 M KCl, 5 mM DTT, 1 mM each ATP, CTP, GTP, and 100 µCi [$\alpha$-$^{32}$P]UTP (760 Ci/mmol, Dupont, Canada). Samples were treated with RNase-free DNase I and proteinase K to remove DNA and protein. Labeled RNA was extracted with phenol/chloroform and precipitated with 50% TCA/30 mM sodium pyrophosphate. The TCA precipitates were filtered through 0.45 µm Millipore HA filters. RNA was again treated with RNase-free DNase I and eluted from the filters by adding SDS and heating at 65 C. $^{32}$P-labeled RNA was treated with proteinase K, phenol/chloroform extracted and ethanol precipitated, and resuspended in TES solution (10 mM N-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid, pH 7.4, 10 mM EDTA, 0.2 % SDS).

Labeled RNA was hybridized for 36 hr at 65 C to a nitrocellulose membrane strip containing an immobilized 1.1 Kb mouse ACTH receptor cDNA (starting 38 bp upstream of the initiator ATG) in pBluescript. A 1.9 Kb cDNA fragment of mouse transketolase in pBluescript (Schimmer, 1995) and the pBluescript vector (Stratagene, La Jolla, CA) were also immobilized on the membrane strip as positive and negative controls respectively. Membrane strips were then
washed with 2XSSC (prepared from 20XSSC containing 3M NaCl and 3.3M NaCitrate, pH7.4),
treated with RNase A, and exposed to x-ray film.

K. WESTERN BLOT ANALYSIS

Cells were grown on 150 mm tissue culture dishes to confluence. Cell monolayer were
rinsed with PBS, scraped in ice-cold scraping buffer ( 50 mM Tris-HCl, pH 7.4, 1 mM MgCl2, 1
mM EDTA, and 1mM DTT), and homogenized at 0 C in a Dounce homogenizer with a tight pestle.
For crude membrane preparations, cell homogenates were centrifuged for 5 min at 800 g and 4 C,
the resultant supernatant was centrifuged for 15 min at 40,000 g and 4 C. The pellet (crude
membrane) was resuspended in scraping buffer and stored at - 70 C. For separation of membrane
and cytosol fractions, cell homogenates were centrifuged for 5 min at 800 g and the resultant
supernatants were centrifuged for 45 min at 150,000 g and 4 C. The membrane fractions (pellets)
resuspended in scraping buffer and the cytosol fractions (supernatants) were stored at -70 C. For
Western blot experiments, cell homogenates or membranes were solubilized in 50 mM Tris-HCl
(pH 6.8), 100 mM DTT, 2% SDS, and boiled for 5 min. Equivalent amounts of membrane protein
(typically 50 to 100 µg/sample) were electrophoresed on 10% polyacrylamide gels in Tris-glycine
buffer containing 0.1% SDS and blotted onto nitrocellulose using a BioRad transblot apparatus.
Western blot analysis was carried out using a chemiluminescent detection system (Amersham
Canada Ltd, Oakvilla, ON ). Briefly, the blots were blocked with buffer (20 mM Tris-HCl pH 7.6,
137 mM NaCl, 0.05% Tween 20) containing 5% skim milk powder and incubated for 1-2 h at
room temperature with peptide-specific antiserum diluted in blocking buffer. Antibody interactions
with target proteins were visualized by chemiluminescence after incubating the blots with
horseradish peroxidase-labeled secondary antibodies (Amersham Canada Ltd). The specific
antisera and the dilutions used in this study were: A-572 (1:500)—recognizes Gsα; A-569
(1:1500)—interacts with Giα; U-49 (1:2000)—recognizes Gβ1 (from Susan Mumby, University of
Texas Southwestern Medical Branch, TX).
L. IMMUNOPRECIPITATION

Cell monolayers were rinsed with PBS, scraped in ice-cold lysis buffer containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1 mM DTT, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, and protease inhibitors (2 mM PMSF, 10 μg/ml aprotinin, and 10 μg/ml leupeptin), and incubated in the lysis buffer for 1 h at 4°C. The lysates were clarified by centrifugation for 10 min at 12,000 g and 4°C. For immunoprecipitation, cell lysates (200 μl) were pre-cleared by adding 30 μl protein A sepharose (50% slurry in lysis buffer) and incubating for 1 h at 4°C. Supernatants were collected, antibody was added, and samples were then incubated overnight at 4°C with gentle rocking. Protein A sepharose was then added and incubation was continued for another 2 h. The protein A sepharose beads containing antibody-antigen complex were washed three times with lysis buffer and two times with lysis buffer without detergents. Loading buffer (50-80 μl, 120 mM Tris-HCl, pH 6.8, 15% sucrose, 2% SDS, 50 mM DTT, and 0.01% bromophenol blue) was added to the protein A sepharose beads and the samples were boiled for 10 min. Supernatants were then collected and applied to polyacrylamide gels for Western blot analysis.

M. LIBRARY SCREENING

A cDNA library prepared from total mouse adrenal RNA was used to clone wild-type G protein β subunit. This cDNA library was custom-made in a unidirectional orientation in the Uni-Zap™ XR cloning vector (Stratagene, La Jolla, CA). This library contained 10 x 10¹⁰ pfu/ml and was a gift from Dr. Keith L. Parker (Duke University, Durham, NC). In order to screen the cDNA library, phage were plated at approximately 10,000 plaque/100 mm dish. Lower plaque densities were used for subsequent rounds of screening. A single colony of E. coli XL1-blue cells (bacterial host) was picked and grown overnight at 37°C in 5 ml LB medium supplemented with 0.2% maltose and 10 mM MgSO₄. Cells were pelleted, resuspended in 10 mM MgSO₄ and adjusted to OD₆₀₀ = 0.5/ml. XL1-blue cells (300 μl), phage buffer (300 μl; 50 mM Tris-HCl, pH 7.5, 10 mM MgSO₄, 100 mM NaCl and 0.1% gelatin), and the λ Zap adrenal cDNA library (50,000 pfu) were combined and incubated for 15 min at 37°C. Top agar (85 mM NaCl, 8 mM MgSO₄-7H₂O,
0.5% yeast extract, 1% casein enzymatic hydrolysate (N-Z-Amine A), and 0.7% agarose; melted and kept at 48°C) was then added and the mixture was spread evenly onto a 15 x 150 mm NZY plate (same ingredients as in top agar except that 0.7% agarose was replaced by 1.5% agar). The plate was incubated at 37°C overnight and placed in 4°C for at least 1 h before lifting plaques.

Plaques were lifted using nitrocellulose membrane filters (Schleicher & Schuell). Filters were placed on the surfaces of the plates so that the entire membrane was in contact with the agarose. Phage DNA was allowed to transfer to the membrane filters for 5 min at room temperature. The membrane and agar were pricked with a needle for orientation. The blots were then denatured with 0.5 N NaOH, 1.5 M NaCl for 3 to 5 min, neutralized with 0.5 M Tris-HCl (pH 8.0), 1.5 M NaCl for 3 to 5 min and rinsed with 2X SSC. The blots were dried in air and baked at 80°C for 1.5 h.

The filters were prehybridized at 42°C for 2 h in a solution (2.5 ml per filter) containing 50% formamide, 2X PIPES (prepared from a 10X PIPES stock containing 4 M NaCl and 0.1 M PIPES, pH 6.5), 0.5% SDS, and 100 µg/ml denatured salmon sperm DNA. Incubation was carried out in a covered glass jar. The prehybridization solution was replaced by hybridization solution (2.5 ml per filter) with the same composition plus nick-translated cDNA probe (approximately 1 x 10^6 cpm/ml). The hybridization reaction was allowed to proceed for 18 to 20 h at 42°C. Membranes were washed twice at room temperature in 2X SSC, 0.1% SDS for 20 min, twice at 55°C in 0.2X SSC, 0.1% SDS for 20 min, and exposed to Kodak X-AR film with one intensifying screen.

N. DNA SEQUENCING

Double-stranded DNA template for sequencing was prepared using the Wizard Plus Minipreps DNA Purification System (Promega, Madison, WI) according to manufacturer's specifications. Briefly, 3 ml bacterial cultures were centrifuged for 2 min at 10,000g and the pellets were resuspended in cell resuspension solution containing 50 mM Tris-HCl, pH 7.5, 10 mM EDTA, and 100 µg/ml RNase A. Cells were lysed in a solution containing 0.2 N NaOH and 1% SDS and the cell lysates were neutralized with 3 M potassium acetate and 11.5% glacial acetic acid. The mixtures were vortexed, incubated on ice for 5 min, and centrifuged for 5 min to
precipitate chromosomal DNA and membrane proteins. The supernatants containing the plasmid DNA were applied to a column composed of Wizard minopreps DNA purification resin and DNA was eluted with TE buffer (10 mM Tris-HCl, pH 7.5 and 1 mM EDTA). Plasmid DNA (1-2 μg) in 20 μl was denatured by adding 2 μl of a freshly prepared solution containing 2 N NaOH and 2 mM EDTA at room temperature for 5 min, and neutralized by adding 8 μl of 1 M Tris-HCl (pH 4.5). The sample was adjusted to contain 0.3 M sodium acetate and DNA was precipitated by adding 75 μl of absolute ethanol. DNA pellets were washed with 70% ethanol and resuspended in 10 μl H2O.

Single-stranded DNA template was prepared according to the protocol provided by Stratagene (pBluescript Exo/Mung DNA Sequencing System). Miniprep DNA was transfected into E. coli JM 101 cells. A single colony of transformed JM 101 cells was picked and grown at 37 C overnight in 4 ml of M9 medium (43 mM NaH2PO4, 22 mM KH2PO4, 0.85 mM NaCl, 18 mM NH4Cl, 2 mM MgSO4, 0.1 mM CaCl2, 0.2% glucose) supplemented with 1 mM thiamine and 50 μg/ml ampicillin. A 200 μl aliquot of the culture was transferred into a new tube containing 2 ml of LB medium supplemented with 50 μg/ml ampicillin and was grown at 37 C with vigorous shaking until the culture reached an OD600 0.3 to 0.8. VCR-M13 helper phage (10 μl) (Stratagene) was then added to the tube and the culture was grown at 37 C for an additional 1 h. A 200 μl aliquot of the culture was transferred into a fresh tube containing 3 ml of LB medium supplemented with 100 μg/ml of kanamycin and grown at 37 C overnight. The next day, cells were pelleted by centrifugation and 1.2 ml of the supernatant containing single-stranded DNA was collected in an Eppendorf tube. A solution containing 3.5 M ammonium acetate and 2% polyethylene glycol (300 μl) was added to the tube. DNA was precipitated on ice for 30 min and pelleted by centrifugation. The DNA pellet was resuspended in 200 μl TE buffer, extracted twice with an equal volume of phenol/chloroform. The solution was adjusted to contain 0.3 M sodium acetate and DNA was precipitated with absolute ethanol. The precipitated DNA was resuspended in 12 μl H2O. A 2 μl aliquot was electrophoresed in a 1% agarose gel to visualize the yield of single strand DNA.

Single-stranded and double-stranded DNA templates were sequenced with T3, T7 (Pharmacia) and gene-specific primers by the chain termination method of Sanger et al. (1977) using Sequenase 2.0 (United States Biochemical, Cleveland, OH). Briefly, 2 μl of sequencing
primer (2 pmol) and 2 µl of annealing buffer were added to a tube containing 10 µl of DNA template. The mixture was incubated at 37 C for 20 min (for double-stranded DNA) or at 60 C for 10 min (for single-stranded DNA). Labeling mix (2 µl), 0.1 M DTT (1 µl), α-35S-dATP (10 µCi in 1 µl; Amersham Canada Ltd.), and diluted Sequenase or T7 DNA polymerase (3 units) were added to the annealed mixture and incubated at room temperature for 2 to 5 min. Aliquots of the labeling reaction mixture (4.5 µl) were transferred to each of four termination mix tubes (containing 14 µM of each ddGTP, ddATP, ddTTP, ddCTP and prewarmed at 37 C) and incubated at 37 C for 5 min. Stop solution (5 µl; 97.5% deionized formamide, 10 mM EDTA, pH 7.5, 0.3% xylene cyanol, 0.3% bromophenol blue) was added to each tube. The mixture containing the completed reaction was heated at 80 C for 2 min and was electrophoresed on 6% polyacrylamide/urea sequencing gels.
III. RESULTS

A. THE BASIS FOR THE ACTH-RESISTANCE IN FORSKOLIN-RESISTANT MUTANTS

1. Forskolin-resistant mutants do not express ACTH receptor transcripts

The observation that the forskolin-resistant mutants were completely resistant to ACTH did not seem to be consistent with the fact that the mutant cells still retained some levels of Gsα in their plasma membrane and their adenylyl cyclase was only partially resistant to activation by forskolin and guanyl nucleotide analogs, but fully responsive to NaF. To further understand the basis for ACTH resistance, several approaches were used to assess the expression of ACTH receptors in the mutant cells. Northern blot analyses were performed to examine ACTH receptor transcripts using a 273 bp cDNA probe corresponding to the region between transmembrane 3 and transmembrane 6 of the ACTH receptor. As shown in Figure 9, the mutation to forskolin resistance is accompanied by a loss of ACTH receptor transcripts. Whereas a 2 kb ACTH receptor transcript was detected in parental Y1 cells on Northern blots, ACTH receptor transcripts were not detected in two independently isolated forskolin-resistant mutants, 10r6 and 10r9 (Fig. 9A). These results were verified in more sensitive RNase protection assays, where an antisense RNA probe for the ACTH receptor generated a 230 bp protected fragment using RNA from parental Y1 cell but not from mutant 10r6 and 10r9 cells (Fig. 9B). The protected fragment was somewhat smaller than the 273 bp fragment corresponding to the antisense RNA probe because the 273 bp cDNA was generated by PCR using degenerate oligonucleotide primers (Mountjoy et al., 1994) and resulted from mismatches between the mouse ACTH receptor transcripts and primer regions of the probe. Results from the Northern blot analysis and RNase protection assays showed that the forskolin-resistant mutants lost the ability to express ACTH receptor transcripts, implying that these mutants are ACTH receptor-deficient.

RT-PCRs were performed to further estimate the deficiency of ACTH receptor transcripts in the mutant cells. cDNAs were synthesized from RNA prepared from parent Y1 and mutant 10r6 and 10r9 cells, and a region that spanned a 1.6 kb intron in the ACTH receptor gene was amplified (Fig.10A). The choice of this region distinguished ACTH receptor signals specifically derived
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For 18 hours, preparations from Qin et al., 1996, Mol Endocrinol 10:1708-1718, with
was prepared from cells treated with 3 mM 8-BrcAMP or 2 mM isoproterenol (IPR)
derived from the 273 bp ACTH receptor cDNA fragment. Where indicated, mRNA
analyzed for ACTH transcripts by RNase protection using an antisense probe
the mouse ACTH receptor (Munjoy et al., 1992. B. Total RNA (20 ng) was
bp cDNA probe corresponding to the regions spanning transcriptional 3 and 6 of
mammalian cells transfected with the β2AR gene (101-962-2) was hybridized with a 273
preparations from parental Y1 cells, the forskolin-resistant mutants 10R and 10F, and

**Figure 3.** ACTH Receptor Transcripts. A Northern blot of total RNA (25 µg)

![Image of a gel with bands labeled B and C, with arrows indicating 30 bp and 2.0 Kb.](image-url)
Figure 10. Amplification of ACTH receptor transcripts by RT-PCR. A. Schematic diagram showing the primers used for cDNA synthesis (oligo-dT primer) and PCR amplification (P30 and P31). B. cDNA was synthesized from parent Y1 cells, forskolin-resistant 10r6 and 10r9 cells, and mutant cells transfected with β2AR using an oligo-dT primer. ACTH receptor transcripts were amplified by PCR at 94 C for 1 min, 60 C for 1 min, and 72 C for 1 min for 30 cycles with a hot start as described in Materials and Methods. A transcription factor, SF-1, was also amplified from the same cDNA samples and served as an internal control. C. ACTH receptor transcripts were amplified by PCR for 40 cycles. PCR products were digested with Pst I to confirm the specific amplification of ACTH receptor transcripts.
from mRNA (240 bp) from signals derived from genomic DNA (approximately 1.8 kb). As shown in Figure 10B, a 240 bp PCR product was seen in Y1 cells but not in 10r6 and 10r9 cells. With extended cycles of amplification (over 40 cycles), however, signals were also seen in 10r6 and 10r9 cells (Fig. 10C). The signals reflected ACTH receptor transcripts since they could be digested with the restriction enzyme Pst I, which cleaves a unique restriction site in the amplified region of ACTH receptor cDNA, resulting in fragments of expected sizes (134 bp and 106 bp, Fig. 10C). The signals from the mutant cells, however, were considerably less intense than that generated from parent Y1 cells, confirming that mutant cells were deficient in ACTH receptor transcripts. SF-1, the transcription factor that is involved in the steroidogenic cell-specific expression of the ACTH receptor (Parker and Schimmer, 1997), was also amplified from the cDNAs that were used to amplify ACTH receptor transcript. The SF-1 transcripts were seen in both parent Y1 cells and mutant cells and their levels were comparable (Fig 10B), indicating that equal concentrations of transcript were analysed in the RT-PCR experiments and that the failure to express ACTH receptor did not result from the absence of SF-1 in the mutant cells.

Southern blot analysis was carried out to test the presence of the ACTH receptor gene in these cells. Genomic DNA from the parent and mutant cells was digested with three different restriction endonucleases (Hind III, BamH I, and Kpn I) and the resultant restriction patterns were examined by Southern blot hybridization using an ACTH receptor cDNA probe. Single hybridizing bands were generated from Hind III, BamH I (Fig. 11) and Kpn I (data not shown) digestion; in each case, the ACTH receptor genes from parent and mutant cells gave the same restriction pattern, suggesting that the loss of ACTH receptor transcripts in 10r6 and 10r9 mutants did not result from gene deletion or gross gene rearrangement.

In order to determine if the absence of ACTH receptor transcripts in the forskolin-resistant mutants was associated with a defect in ACTH receptor gene transcription, nuclear run-off assays were carried out using nuclei from parent Y1 cells and the forskolin-resistant mutant 10r9. As seen in Figure 12, hybridization of the labeled run-off transcripts from Y1 nuclei to ACTH receptor cDNA showed a clear signal. In contrast, when labeled run-off products from mutant 10r9 cells were hybridized to ACTH receptor cDNA, a signal was virtually undetectable. These results indicate that ACTH-resistance in 10r9 cells is associated with a defect in ACTH receptor gene
Figure 11. Analysis of the ACTH receptor gene. Genomic DNA from Y1 and forskolin-resistant mutant cells was digested with *Hind III* and *BamHI* restriction endonucleases and probed with the mouse ACTH receptor cDNA. Fragment sizes were estimated using *Hind III*-digested, end-labeled bacteriophage λ DNA as a standard. Reprinted from Qiu et al., 1996, Mol Endocrinol 10:1708-1718 with permission from The Endocrine Society.
Figure 12. ACTH receptor gene transcription measured by nuclear run-off assay. Run-off assays were performed using nuclei prepared from parent Y1 cells or mutant 10r9 cells. Labeled run-off products were hybridized to nitrocellulose filters containing immobilized cDNA clones of the mouse ACTH-receptor in pBluescript, the mouse transketolase in pBluescript or the empty pBluescript vector as indicated. After hybridization, the nitrocellulose filters were exposed to X-ray film with two intensifying screens for two weeks. Results are representative of three independent experiments. Reprinted from Qiu et al., 1996, Mol Endocrinol 10:1708-1718 with permission from The Endocrine Society.
transcription. In the same experiments, equivalent signals were obtained when run-off transcripts from parent and mutant cells were hybridized to a control mouse transketolase cDNA and no background signal was obtained when transcripts were hybridized to the vector pBluescript.

2. Effects of the forskolin-resistance mutation on receptor-G protein coupling

To determine the extent to which the absence of receptor transcripts contributed to the loss of hormone-responsive adenylyl cyclase activity in forskolin-resistant mutants, clones 10r6 and 10r9 were transfected with an expression vector encoding the mouse β2-adrenergic receptor (β2AR). Stable transformants were isolated and screened for isoproterenol-stimulated cell rounding, indicative of isoproterenol-stimulated increases in cAMP levels (Olson et al., 1991). Approximately 45-55% of transformants exhibited morphological responses to isoproterenol as estimated by phase contrast microscopy. Ten morphologically responsive clones were isolated from each transformed cell line and assayed for isoproterenol-stimulated adenylyl cyclase activity. As shown in Table 2, the 10r6 and 10r9 transformants exhibited isoproterenol-responsive adenylyl cyclase activity that averaged 310 ± 55 and 370 ± 65 pmol cAMP accumulated/5 min/mg protein respectively. These activities reflected 4.6 ± 0.7 fold over basal activity and were similar to the values obtained for parental Y1 cells transfected with the mouse β2AR (Table 2, Olson et al., 1991). The extent of coupling of the expressed β2AR to the adenylyl cyclase system was further evaluated by examining the dose-response relationships for isoproterenol-stimulated adenylyl cyclase activity in these transformants and the ED50s were determined by a curve fitting program (De Lean et al., 1978). As shown in Fig. 13, the β2AR-transformed 10r6 and 10r9 mutants responded to isoproterenol with average ED50 values of 0.24 ± 0.03 μM. These ED50 values were similar to the values obtained using parental Y1 cells transformed with the β2AR gene (0.27 ± 0.09 μM), suggesting that the β2AR was efficiently coupled to adenylyl cyclase in the forskolin-resistant mutants.

Ligand binding analyses also were performed on crude membranes prepared from the forskolin-resistant mutants transformed with the β2AR gene to determine if receptor-G protein coupling was impaired in the mutant clones. The β2AR has been shown to exhibit both high and low affinity agonist-binding sites; the high affinity binding sites are proposed to represent hormone
Table 2. Adenylyl cyclase activity in forskolin-resistant cells transfected with β2AR.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Basal</th>
<th>Isoproterenol</th>
<th>ACTH</th>
<th>NaF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Y1DS4-2</td>
<td>55 ± 30</td>
<td>310 ± 60*</td>
<td>766 ± 30</td>
<td>933 ± 100</td>
</tr>
<tr>
<td>10r6</td>
<td>70 ± 25</td>
<td>55 ± 10</td>
<td>81 ± 15</td>
<td>870 ± 25</td>
</tr>
<tr>
<td>10r6 + β2AR</td>
<td>90 ± 15</td>
<td>310 ± 55*</td>
<td>80 ± 30</td>
<td>520 ± 65</td>
</tr>
<tr>
<td>10r9</td>
<td>65 ± 5</td>
<td>70 ± 10</td>
<td>80 ± 5</td>
<td>1310 ± 190</td>
</tr>
<tr>
<td>10r9 + β2AR</td>
<td>70 ± 5</td>
<td>370 ± 65*</td>
<td>85 ± 10</td>
<td>1050 ± 105</td>
</tr>
</tbody>
</table>

Adenylyl cyclase activity was measured in cell homogenates prepared from Y1 cells transfected with β2AR (Y1DS4-2), mutant 10r6 and 10r9 cells and β2AR transformants of 10r6 and 10r9. Isoproterenol (100 µM), ACTH (20 µM), and NaF (15 mM) were added as indicated. Results were compiled from ten β2AR transformants of 10r6 and nine β2AR transformants of 10r9. Adenylyl cyclase activity in Y1DS4-2 cells was averaged from four independent experiments. Values are expressed as pmols cAMP accumulated/5min/mg protein ± S.E.M. The * denotes isoproterenol-stimulated adenylyl cyclase activities that are significantly elevated over unstimulated controls (p ≤ 0.001) but not significantly different from each other (p ≥ 0.5) as determined using a multiple comparison statistical test (Harper, 1984).
Figure 10. Concentration-dependent activation of adenylyl cyclase in the β2AR transformants. Cell homogenates were prepared from the β2AR transformants of Y1 (Y1DS4-2; closed circles), 10r6 (10r682-1; open squares) and 10r9 (10r982-2; open triangles) and assayed for adenylyl cyclase activity in the presence of different concentrations of isoproterenol (IPR) as indicated. Results are averaged from two experiments each performed in duplicate and expressed as a percentage of the maximum response obtained with each cell line. Reprinted from Qiu et al., 1996, Mol Endocrinol 10:1708-1718 with permission from The Endocrine Society.
receptors coupled to G proteins, whereas the low affinity binding sites are thought to reflect receptors uncoupled from G proteins (Kent et al., 1980). Guanyl nucleotides shift the entire population of receptors to a low affinity state, presumably due to the uncoupling of receptors from their G proteins (Lefkowitz et al., 1976). As shown in Figure 14, there were no apparent differences in the agonist displacement curves generated using membranes prepared from β2AR-transformants of parental Y1 cells or transformants of forskolin-resistant mutants 10r6 and 10r9. The guanyl nucleotide (Gpp(NH)p acted similarly in each case, shifting the population of receptors to a low affinity state (Fig. 15). The proportions of high and low affinity sites as well as the estimated Kd values were similar in parent and mutant cells (Table 3). Thus, the receptor binding data support the conclusion that forskolin-resistant mutants transformed with a functional β2AR do not exhibit defects in receptor-G protein coupling.

3. Effects of β2AR expression on Gα levels
a. Gα levels in β2AR transformed forskolin-resistant mutant cells

The observations suggesting that receptor-G protein coupling in the β2AR transformed mutants were normal came as somewhat of a surprise, since previous studies indicated that the plasma membrane fractions from four independent forskolin-resistant mutants had markedly reduced levels of Gsα and Giα compared to parental Y1 cells (Schimmer and Tsao, 1990). Therefore, Western blot analyses were performed to determine the levels Gsα and Giα in the forskolin-resistant mutants following transfection with the β2AR gene.

Crude membrane fractions were prepared from untransfected parent Y1 cells, forskolin-resistant mutant 10r6 and 10r9 cells, and Y1 and mutant cells transfected with the β2AR gene. Two lymphoma cell lines—S49 which expresses Gsα, and the S49 Cyc- mutant which is deficient in Gsα (Bourne et al. 1975, Harris et al., 1985)—also were included in the experiment as controls to test the specificity of the Gsα antibody. As shown in Figure 16, specific signals at 52 KDa and 45 KDa were obtained for Gsα using Gs purified from rat liver membranes and membranes from S49 cells. These signals represent the long and short splice variant forms of Gsα described previously (Robishaw et al., 1986, Bray et al., 1986) Signals were not detected using membranes from Cyc- cells, establishing the specificity of the antiserum for Gsα in crude membrane fractions.
Figure 14. Agonist displacement curve using membranes from β2AR transformants of Y1 and forskolin-resistant cells. Membrane preparations from β2AR-transfected Y1(Y1DS4-2, closed circles), 10r6 (10r6β2-2, open squares), and 10r9 (10r9β2-2, open triangles) were analyzed in a competition binding assay using the β2AR agonist IPR as unlabeled competitor and the antagonist ICYP as the labeled ligand. Data are presented as percentage of specifically bound 125I-labeled ligand ICYP versus the concentrations of competing unlabeled IPR.
Figure 15. Effects of Gpp(NH)p on the agonist displacement curves using membranes from Y1 and 10r9 transformants. Binding assays were performed on membranes from β2AR transformed Y1 (triangles) and 10r9 mutant cells (squares) in the presence (open symbols) and absence (closed symbols) of 200 μM Gpp(NH)p. Data are presented as a percentage of specifically bound $^{125}$I-labeled ICYP versus the concentrations of competing unlabeled IPR.
Table 3. Binding parameters of $\beta_2$AR in transformants of Y1 and forskolin-resistant mutant cells.

<table>
<thead>
<tr>
<th>Cell Lines</th>
<th>Kd(nM)</th>
<th>Receptor density (fmol/mg.protein)</th>
<th>High affinity sites (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>High</td>
<td>Low</td>
<td></td>
</tr>
<tr>
<td>Y1DS4-2</td>
<td>5.8 ± 3.1</td>
<td>66 ± 20</td>
<td>23 ± 3</td>
</tr>
<tr>
<td>10r6+$\beta_2$AR</td>
<td>3.7 ± 2.6</td>
<td>59 ± 9</td>
<td>30 ± 9</td>
</tr>
<tr>
<td>10r9+$\beta_2$AR</td>
<td>3.2 ± 0.7</td>
<td>50 ± 3</td>
<td>14 ± 2</td>
</tr>
</tbody>
</table>

Binding assays were performed on membranes from $\beta_2$AR transformants of parent Y1, mutant 10r6 and mutant 10r9 cells. Binding parameters were determined using the program LIGAND and values are expressed as means ± S.E.M. For Y1DS4-2 cells, results were averaged from three separate experiments. For the 10r6 and 10r9 transformants, binding parameters represent the average values obtained from four and three independent isolates respectively. Using a multiple comparison statistical test (45), the percentage of receptors in the high affinity state was not significantly different among transformants ($p \geq 0.18$). Similarly, the Kd values for the high and low affinity binding sites each did not differ significantly among transformants ($p \geq 0.6$ and $p \geq 0.5$ respectively).
Figure 16. Gsα levels in β2AR transformants. Crude membrane fractions were prepared from parent Y1 cells, Y1 cells expressing β2AR (clones Y1DS4-2 and Y1βar2), mutant 10r9 cells and their β2AR transformants (10rβ2-1 and 10rβ2-2). Membrane proteins were Western blotted onto nitrocellulose filters and probed using an anti-Gsα antibody (A572). S49 lymphoma cell membranes and purified Gsα from rat liver were used as positive controls, S49 Cyc- membranes were used as negative controls. Bands at approximately 75 KDa were seen in all membrane samples and their levels were not consistent with the 52KDa Gsα signal. This band therefore may be a result of nonspecific interaction of a membrane protein with the anti-Gsα antibody. A. 10r9 transformants; B: additional 10r9 transformants and 10r6 transformants; C. Y1 transformants.
Membranes from parent Y1 cells and the mutant 10r9 and 10r6 cells contained only the large splice variant form of Gsα. Forskolin-resistant mutants contained approximately 50% less Gsα than did parental Y1 cells. After transfection with the β2AR gene, Gsα levels in the membrane fractions from the mutant cells rose and approached the levels seen in parental Y1 cells (Figs. 16A and 16B). These results suggest that normal receptor-G protein coupling observed following transfection of forskolin-resistant mutants with the β2AR gene may be associated with the recovery of Gsα levels in mutant cell membranes. To validate the apparent increases in Gsα levels in β2AR transformants, Western blot analysis was performed to examine the relationship between the amount of membrane protein and the observed signals. Different amounts of membrane protein ranging from 20 μg to 100 μg prepared from Y1, 10r9 mutant cells, and their β2AR transformants were analyzed. As seen in Figure 17A, the Gsα levels in these samples showed an increase in signal corresponding to increasing amounts of membrane used. The Gsα signal in these cells were further quantitated by using scanning densitometry and the NIH Image program. All displayed a linear relationship between protein concentrations (from 40 to 100 μg) and the relative density of the bands (Fig. 17B). All Western blot analysis described in this thesis used protein concentrations in this range. Therefore the changes in Gsα signals observed in Western blot assays represented increases in Gsα levels following β2AR expression.

b. Gαi and GB1 levels in β2AR transformants

Previous studies have shown that the levels of Gαi in the forskolin-resistant cells also were decreased, but the levels of GB1 were not affected (Schimmer and Tsao, 1990). The effect of β2AR expression on the levels of Gαi and GB1 were investigated by Western blot analysis using specific antibodies. As shown in Figure 18, the 41 KDa Gαi was detected in the crude membrane fractions of Y1, 10r9, and their transformants. The Gαi levels in the mutant cells were reduced approximately 60 to 70% as compared to wild-type Y1 cells, but were increased to different extents following transfection with β2AR as assessed in four independent transformants. The levels of GB1, however, did not differ in amount among parent Y1 cells, the forskolin-resistant mutants, and the β2AR transformants (Fig. 18B). These results suggested that the increase in Gα levels following β2AR transfection did not result from a nonspecific induction of all G protein subunits, but rather represented a specific modulation of the level of G protein α subunits.
Figure 17A. Linear relationship between amount of membrane and observed Gsα signal. A. Membrane fractions from Y1 and 10r9 cells, and their β2AR transformants in amount ranging from 20 to 100 μg were analyzed using anti-Gsα antibody. Purified Gsα from rat liver was used as positive control.
Figure 17B. Linear relationship between amount of membrane and observed Gsα signal. B. Gsα signals from Western blot analysis were quantitated using scanning densitometry and the NIH image program. Curves were fitted by linear regression with $r^2$ values ranging from 0.95 to 0.997.
Figure 18. Giα and Gβ1 levels in β2AR transformants. Giα and Gβ1 levels were measured on crude membrane fractions prepared from Y1, 10r6 and 10r9 cells, and their β2AR transformants. Giα levels also were determined in membranes from the spontaneous ACTH-resistant mutants, Y6 and OS3. A. Giα level was probed using an antibody that recognizes the α subunit of several G proteins (A569). Purified Giα from rat brain was used as positive control. B. A Western blot was probed using a mixture of the Gα antiserum A569 and a specific Gβ1 antibody (U49).
c. Possible mechanism of the increase in Gsα following β2AR expression

The increases in Gsα appeared to be a general response to transfection with the β2AR and were observed, not only in transformed mutant cells, but also in transformed parental Y1 cells. As shown in Fig. 16C, two β2AR transformants isolated from parental Y1 cells also exhibited increased levels of Gsα (30%).

The changes in Gsα levels seen here following transfection with the β2AR were not accompanied by corresponding changes in Gsα transcripts (Fig. 19). Northern blot analysis was performed using a 1.6 Kb Gsα cDNA probe and the Gsα signal was normalized by subsequently probing the same blot with a tubulin cDNA fragment. The relative densities of the Gsα transcripts were similar among Y1 and 10r9 cells, and their transformants, indicating that the increase in Gsα protein levels did not result from an increase in Gsα RNA levels.

Although cAMP increases Gsα levels in some cell lines (Dib et al., 1994, Tasken et al., 1995), the changes in levels of Gsα observed here were not secondary to increases in cAMP levels resulting from the functional expression of β2AR. In fact, treatment of untransfected parent and mutant cells with 8Br-cAMP resulted in a decrease in Gsα levels rather than an increase; treatment of β2AR-transformed parent or mutant cells with isoproterenol or 8Br-cAMP also decreased the levels of Gsα (Fig. 20). Together, these observations suggest that the increases in Gα seen following the expression of β2AR may result from a receptor-mediated stabilization of Gα levels in the membrane fraction rather than from receptor-mediated second messenger events. It is possible that a G protein-coupled receptor is required to maintain the normal levels of Gsα in the membrane. Although the basis for changes in Gicα were not explored, similar mechanisms might regulate Gicα levels.

4. Effect of β2AR expression on other manifestations of the forskolin-resistant phenotype

Since ACTH receptor deficiency seemed to be the major cause of both hormone-insensitive adenylyl cyclase activity and the reduced levels of membrane-associated Gsα in the forskolin-resistant mutants, it was of interest to determine the extent to which receptor deficiency contributed to other aspects of the forskolin-resistant phenotype.
Figure 19. Gsα mRNA levels in the β2AR transformants. Total RNA (25 µg) was analyzed by Northern blot hybridization using a Gsα cDNA fragment as probe. The same blot was stripped and reprobed with a tubulin cDNA fragment. The densities of the hybridization signals for Gsα were quantitated using a phosphoimager and normalized to the signal obtained with the tubulin probe. Data are from three independent experiments and expressed as means ± S.E.M. Reprinted from Qiu et al., 1996, Mol Endocrinol 10:1708-1718 with permission from The Endocrine Society.
Figure 20. Effects of 8BrcAMP and isoproterenol on Gsα levels. Membrane fractions from parent Y1 cells, a β2AR transformant of Y1 (Y1DS4-2), mutant 10r9 cells and a β2AR transformant of 10r9 (10r9β2-1) were Western blotted and probed with an anti-Gsα antibody. Cells were untreated or treated with 3 mM 8BrcAMP (cAMP) or 2 μM isoproterenol (IPR) for 18 h as indicated before membranes were isolated. As determined from multivariate analysis (Harper, 1984), Gsα levels in all the cells treated with 8BrcAMP or all the transformants treated with IPR were significantly lower (p<0.05) than in untreated cells. Reprinted from Qiu et al., 1996, Mol Endocrinol 10:1708-1718 with permission from The Endocrine Society.
a. Growth regulation

The growth of parental Y1 cells was markedly inhibited by 2 μM and 10 μM forskolin as well as by 0.4 mM 8BrcAMP (Fig. 21). In contrast, the forskolin-resistant mutant 10r9 was resistant to these concentrations of forskolin, but remained sensitive to the growth-inhibitory effects of 8BrcAMP, indicating that forskolin-resistance resulted from a defect in forskolin-stimulated cAMP accumulation. β2AR transformants of mutant 10r9 cells retained their resistance to the growth-inhibitory effects of forskolin.

b. Adenylyl cyclase activity

As reported previously, the growth inhibitory effect of forskolin in Y1 cells is associated with an increase in adenylyl cyclase activity, is dependent upon a functional cAMP-dependent protein kinase, and therefore reflects a cAMP-mediated inhibition of cell proliferation (Schimmer and Tsao, 1984). In mutant 10r9 cells, forskolin stimulated adenylyl cyclase activity much less effectively than in parental Y1 cells. Expression of β2ARs in the forskolin-resistant mutants did not change the efficacy with which forskolin activated adenylyl cyclase (Fig. 22).

c. ACTH receptor transcripts

As shown in Figures 9 and 10, expression of the β2AR gene in the forskolin-resistant mutants did not restore their ability to express ACTH receptor transcripts, despite the recovery of Gsα and Gia levels in the membrane fraction. These results indicate that the failure to express the ACTH receptor likely is a down-stream consequence of the mutation to forskolin-resistance. Furthermore, treatment of the forskolin-resistant mutants with 8BrcAMP or the β2AR transformants with isoproterenol to raise intracellular levels of cAMP did not restore ACTH receptor expression (Fig. 9), suggesting that the mutation affects a regulatory pathway for ACTH receptor expression that is cAMP-independent.

5. The spontaneous ACTH receptor-deficient mutants, Y6 and OS3, also are forskolin-resistant

Two spontaneous mutants isolated previously from the Y1 adrenal cell line, clones Y6 and OS3, also have been characterized as ACTH-resistant clones that fail to accumulate ACTH receptor transcripts (Schimmer et al., 1995) and consequently are unresponsive to ACTH (Schimmer,
Figure 21. Effect of $\beta_2$AR gene expression on the growth inhibitory effect of forskolin. Parent Y1 cells, mutant 10r9 cells and the $\beta_2$AR transformants 10r9B2-2 and 10r9B2-3 were plated at $2 \times 10^4$ cells/well and grown for 2 weeks in the presence of varying concentrations of forskolin or 8Br-cAMP as indicated. Survivors were fixed and stained with 1% methylene blue in 70% 2-propanol. Reprinted from Qiu et al., 1996, Mol Endocrinol 10:1708-1718 with permission from The Endocrine Society.
Figure 22. Regulation of adenylyl cyclase activity in the β2AR transformants.

Homogenates of Y1 cells (closed circles), the Y1 transformant 8ar2 (open circles), mutant 10r-9 cells (closed triangles), and the 10r9 transformants 10r9β2-1 (open triangles) and 10r9β2-2 (open squares) were assayed for adenylyl cyclase activity in the presence of different concentrations of forskolin. Results shown are representative of two independent experiments, each carried out in duplicate. Data are expressed as a percentage of the response obtained with 15 mM NaF to normalize the data for variations in recovery of adenylyl cyclase activity in the various samples (Watt and Schimmer, 1981). Reprinted from Qiu et al., 1996, Mol Endocrinol 10:1708-1718 with permission from The Endocrine Society.
1969, 1972). As evaluated by RT-PCR (Fig. 23), ACTH receptor transcripts were detected in Y6 cells at levels that were considerably lower than in parent Y1 cells. No ACTH receptor transcripts were found in OS3 cells. Inasmuch as these characteristics are similar to those associated with mutations to forskolin-resistance, experiments were performed to determine if the spontaneous mutants, Y6 and OS3, also were forskolin-resistant.

a. Y6 and OS3 are forskolin-resistant

As shown in Figure 24, the Y6 and OS3 mutants also exhibited a forskolin-resistant phenotype. In contrast to parental Y1 cells that were markedly growth-inhibited by various concentrations of forskolin, Y6 and OS3 cells grew well in the presence of forskolin up to 2 μM but did not survive in the presence of 10 μM forskolin. In this respect, the mutants Y6 and OS3 were not as resistant to forskolin as were the mutants 10r6 and 10r9 which survived in the presence of 10 μM forskolin (Fig. 21).

Like the forskolin-resistant mutants, the Y6 and OS3 cells exhibited significantly lower (p < 0.05) forskolin-responsive adenylyl cyclase activity than did parent Y1 cells (Fig. 25), and contained 50% less Gsα (Fig. 26A). The OS3 mutant also exhibited a low level of Giα (85% reduction, Fig. 26B). Unlike the forskolin-resistant mutants (Fig. 18), however, Y6 seemed to contain higher levels of Giα as compared to parent Y1 cells (40% more than Y1, Fig. 26B). Y1, Y6, and OS3 mutants all contained the same levels of Gβ1 (Fig. 23B). These results demonstrate that the Y6 and OS3 mutants resemble the forskolin-resistant mutants in several aspects, and suggest that the forskolin-resistance phenotype and ACTH receptor deficiency seem to be closely related in the mouse adrenal Y1 cell line.

b. Expression of the ACTH receptor in mutant Y6 and OS3 cells

Transfection of Y6 and OS3 cells with an expression vector encoding the human ACTH receptor (Mountjoy et al., 1992) led to the recovery of transformants that responded to ACTH with increased adenylyl cyclase activity (Table 4). The Y6 and OS3 transformants exhibited ACTH-responsive adenylyl cyclase activity with increases of 10.3 ± 2.6 and 10.5 ± 1.2 fold over the basal levels, respectively. The fold increase in adenylyl cyclase activity in the ACTH receptor transformants was similar to the values obtained for parental Y1 cells with endogenous ACTH
Figure 23. Amplification of ACTH receptor transcripts from Y1, Y6, and OS3 cells. cDNAs were synthesized using RNA prepared from parent Y1 cells and from mutant Y6, and OS3 cells. ACTH receptor transcripts were amplified by PCR at 94 C for 1 min, 60 C for 1 min, and 72 C for 1 min for 30 cycles with a hot start as described in Materials and Methods. SF-1 serves as an internal control. The results shown are representative of 3 experiments carried out using two independent RNA samples from each cell line.
Figure 24. Effects of forskolin on the growth of Y6 and OS3 cells and their ACTH receptor (ACTH-R) transformants. Cells (2 x 10^4/well) were grown for two weeks in the presence of various concentrations of forskolin as indicated. Survivors were fixed and stained with 1% methylene blue in 70% 2-propanol. Reprinted from Qiu et al., 1996, Mol Endocrinol 10:1708-1718 with permission from The Endocrine Society.
Figure 25. Effects of ACTH receptor expression on forskolin stimulated adenyl cyclase activity in Y6 and OS3 cells. Homogenates from Y1, Y6 and OS3 cells, and Y6 and OS3 transformants expressing the human ACTH receptor (ACTH-R) were assayed for adenyl cyclase activity in the presence of 100 μM forskolin. Data were expressed as a percentage of the response obtained with 15 mM NaF and represent the averaged values from at least five separate determinations. The asterisks denote activities significantly different (p < 0.05) from the activity of parental Y1 cells as determined by multivariate analysis (Harper, 1984). Reprinted from Qiu et al., 1996, Mol Endocrinol 10:1708-1718 with permission from The Endocrine Society.
Figure 26. Effects of ACTH receptor expression on Gsα and Giα levels in Y6 and OS3 cells. Membranes from Y6 cells, OS3 cells and four independent isolates of Y6 and three independent isolates of OS3 transformed with the human ACTH receptor gene (ACTH-R) were Western blotted. A. Gsα levels were probed using an anti-Gsα antibody (A572). Purified Gs from rat liver was included as a control. B. Giα (41 KDa) and GB1 (36 KDa) levels were probed using a mixture of anti-Gα common (A569) and anti-GB1 antibodies (U49).
Table 4. Adenylyl cyclase activity in Y6 and OS3 cells transfected with the human ACTH receptor gene.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Basal</th>
<th>ACTH</th>
<th>NaF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Y1</td>
<td>115 ± 60</td>
<td>1030 ± 170</td>
<td>1490 ± 215</td>
</tr>
<tr>
<td>Y6 + ACTH-R</td>
<td>190 ± 60</td>
<td>870 ± 140</td>
<td>1820 ± 225</td>
</tr>
<tr>
<td>OS3 + ACTH-R</td>
<td>135 ± 20</td>
<td>1510 ± 220</td>
<td>1730 ± 195</td>
</tr>
</tbody>
</table>

Adenylyl cyclase activity was measured in cell homogenates prepared from Y1 cells, and from Y6 and OS3 transformants expressing the human ACTH receptor gene (ACTH-R). ACTH (20 μM), and NaF (15 mM) were added as indicated. Results were compiled from fourteen Y6 transformants and nine OS3 transformants. Adenylyl cyclase activity in Y1 cells was averaged from 5 independent experiments. Values are expressed as pmol cAMP accumulated/5min/mg protein ± S.E.M. In all cell lines, adenylyl cyclase activity measured in the presence of ACTH or NaF was increased significantly over basal levels (p ≤ 0.001) using a multiple comparison statistical test (Harper, 1984). Reprinted from Qiu et al., 1996, Mol Endocrinol 10): 1708-1718 with permission from The Endocrine Society.
receptor (10.8 ± 2.3 fold), suggesting that the transfected ACTH receptor was efficiently coupled to adenylyl cyclase in the Y6 and OS3 mutants.

RNA from these transformants did not contain transcripts corresponding to the mouse ACTH receptor as evidenced by the absence of a 230 bp protected fragment in RNase protection assays using a mouse ACTH receptor-specific RNA probe (Fig. 27). Human ACTH receptor transcripts, however, were present in these transformants as evidenced by protected fragments of smaller size. These results thus demonstrate that the ACTH-responsive adenylyl cyclase activity in these transformants resulted from expression of the transfected human ACTH receptor gene rather than from expression of the endogenous mouse gene.

c. Gα levels and forskolin-resistance in ACTH receptor-transformed Y6 and OS3 cells.

The normal receptor-G protein coupling in Y6 and OS3 mutants suggested that Gsα levels may have increased following ACTH receptor transfection. As shown in Figure 26A, the ACTH receptor-transformed Y6 and OS3 cells exhibited increased levels of Gsα in association with ACTH receptor expression, similar to the forskolin-resistant cells transfected with β2AR. In the two Y6 transformants (clone #1 and #2, Fig. 26A), the Gsα reached the levels that were 30% more than the Gsα levels in parent Y1 cells, while the Gsα levels in OS3 transformants were similar to those of Y1 cells. The Gîα levels also increased but not as markedly as the Gsα levels. A 20% increase in Gîα in Y6 and a 10% increase in Gîα in OS3 were observed following ACTH receptor expression. Gβ1 levels, meanwhile, remained unchanged (Fig. 26B). These results combined with the results obtained from β2AR transformants showed that both G protein-coupled receptors (ACTH receptor and β2AR) increased levels of Gsα and Gîα in mutant cells. Thus, the low levels of Gsα and Gîα may have resulted from lack of expression of G protein-coupled receptors in these mutants. These receptors may regulate Gα levels through a common mechanism, perhaps by stabilizing Gα subunits in the membrane fraction.

The ACTH receptor transformants, nonetheless, remained resistant to the growth-inhibiting effects of forskolin (Fig. 24) and to the effects of forskolin on adenylyl cyclase activity (Fig. 25). These results suggest that the loss of ACTH receptor and the consequent decrease in Gα levels in mutants cells is not the cause of forskolin-resistance. Instead, the mutation that led to forskolin-resistance may have resulted in ACTH receptor deficiency.
Figure 27. ACTH receptor transcripts in Y6 and OS3 cells transfected with the human ACTH receptor gene. Total RNA (20 μg) from Y1 and two independent ACTH receptor (ACTH-R) transformed Y6 and OS3 cells were analyzed for ACTH receptor transcripts by RNase protection using a mouse ACTH-R specific RNA probe as in Figure 6. The positions of the ACTH-R probe and the 230 bp protected fragment associated with mouse ACTH-R transcripts are indicated. The smaller sized protected fragments associated with human ACTH-R transcripts are indicated and arise from partial mismatches with the probe.

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B. THE EFFECT OF Gsα ON THE FORSKOLIN-RESISTANT MUTANT PHENOTYPE

1. Expression of HA-Gsα in Y1 and forskolin-resistant mutant cells

Parent Y1 cells and the forskolin-resistant mutants 10r6 and 10r9 were transfected with an expression vector encoding an epitope-tagged Gsα (HA-Gsα, Levis and Bourne, 1992) to further evaluate the contribution of decreased Gsα levels to the forskolin-resistant mutant phenotype.

Alternative splicing of Gsα transcripts produced two 52 kD products which contain either a 15 or 16 amino acid insert (Bray et al., 1986). Part of this insert (residues 71-81) was changed to a well-defined peptide epitope of the influenza virus that is recognized by the monoclonal antibody, 12CA5 (Green et al., 1982). The modified Gsα appears to be functionally equivalent to unaltered Gsα in its membrane distribution and its ability to regulate adenylyl cyclase activity (Levis and Bourne, 1992). Expression vectors encoding this modified Gsα cDNA and a neomycin-resistance gene were transfected into parent Y1 cells and forskolin-resistant mutants 10r6 and 10r9. Stable transformants were recovered by selective growth in the medium containing the neomycin analog, G418. Expression of the HA-Gsα gene in the transformants was examined by Northern blot analysis and RT-PCR using total RNA from both transfected and untransfected Y1 and mutant cells. Northern blot hybridization was performed using a 1.6 kb fragment containing the coding region of HA-Gsα as probe. As shown in Figure 28A, a transcript at 1.9 kb was detected in all transfected and untransfected 10r6 cells. An extra band at 2.8 kb was obtained in some 10r6 transformants but not in the untransfected 10r6 mutant. This extra band apparently is specific for the HA-Gsα transcript since cells transfected with the neomycin resistance gene alone did not show this transcript. Additional Y1 and 10r9 transformants that express HA-Gsα are shown in Figure 28B.

Further confirmation of the expression of the HA-Gsα gene in the transformants was obtained by RT-PCR. cDNA was synthesized from total RNA isolated from both transfected and untransfected parent Y1 cells and mutants by reverse transcriptase, and amplified by PCR using Gsα primers that flanked the HA-tag. Two PCR products at sizes 330 and 290 bp, representing the long and short forms of Gsα, were obtained from all cells (Fig. 29). To ensure that the amplification was from mRNA and not from any contaminating DNA, RNA samples were either
Figure 28. Northern blot analysis of Gsα transcripts in Y1 and forskolin-resistant cells. A. Total RNA from mutant 10r6 cells and the HA-Gsα transformants was probed using a Gsα cDNA fragment. Cells transfected only with neomycin-resistance gene were used as a negative control. B. RNA from additional Y1 and 10r9 HA-Gsα transformants probed with Gsα cDNA fragment. The numbers (#1, #2, #3 ......) indicate independent clones isolated from HA-Gsα transfected cells. For example, 10r6 + HA-Gsα #1 equals 10r6Gs1, as shown in the text and through figures 26 to 32.
Figure 29. Amplification of Gsα transcripts by RT-PCR. cDNA was synthesized from RNA prepared from mutant 10r6 cells and a 10r6 HA-Gsα transformant (10r6Gs6) using gene-specific primer P8, and amplified using the primer set P1 and P2 (Table 1) at 94 C for 1 min, 60 C for 1.5 min, and 72 C for 2 min for 25 cycles. Full length HA-Gsα plasmid cDNA was also amplified as a positive control. As indicated, some RNA samples were treated with either RNase (55 C, 10 min) or DNase (37 C, 10 min) before the reverse-transcriptase reaction.
DNase or RNase treated before cDNA synthesis. As shown in Figure 29, DNase treatment had no effect whereas RNase treatment abolished the formation of the PCR products. The altered region of HA-Gsα contains an Mlu I site which is not present in the long form of wild-type Gsα. On the other hand, the wild-type Gsα contains an Ava II site in the same region which has been removed in HA-Gsα. These unique restriction sites were used to distinguish the HA-Gsα cDNA from endogenous Gsα (Fig. 30, 10r6Gs6). Ava II digestion generated fragments of 200 and 135 bp in both transfected and untransfected mutant cells (Fig. 30A, lanes 5 and 8), characteristic of the endogenous Gsα. In contrast, Mlu I digestion generated fragments of 185 and 150 bp only in transfected 10r6 cells that appeared positive for HA-Gsα on Northern blots (Figs. 28 and 30, clone #1 and #6; Fig. 30A, lane 4, Fig 30B) but not in untransfected cells or in clones negative for HA-Gsα on Northern blot (Figs 28 and 30A, clone #4; Fig. 30A, lanes 2 and 7); indicating that these transfected 10r6 transformants contain transcripts from the HA-Gsα gene. Similarly, Y1 transformants (e.g. Y1Gs9) that appeared positive for HA-Gsα on Northern blot (Fig. 28B) also generated fragments of 185 and 150 bp upon Mlu I digestion (Fig. 30B), indicating the expression of HA-Gsα in the Y1 transformant.

The HA-Gsα protein was detected from whole cell lysates of the transformants by immunoprecipitation with anti-Gsα antibody and then Western blotting with 12CA5 (Fig. 31A). Similarly, HA-Gsα also was detected by immunoprecipitation with 12CA5 and then Western blotting with anti-Gsα antiserum (Fig. 31B). The HA-Gsα migrated slower than untagged-Gsα, probably owing to the addition of a negative charge (Levis and Bourne, 1992). The protein was expressed at high levels in the 10r6 transformant (10r9Gs1 and 10r9Gs6). On the other hand, HA-Gsα expression in Y1 transformants seemed to be relatively low (Fig. 31A), despite the high expression of HA-Gsα in their mRNA transcript (Fig. 28B).

2. Effect of HA-Gsα expression on the forskolin-resistant mutant phenotype

The transformants from mutant cells, although expressing the HA-Gsα, did not recover ACTH receptor gene expression or ACTH-stimulated adenylyl cyclase activity. As shown by RT-PCR in Figure 32, similar to the untransfected 10r9 cells, mutant 10r6 and 10r9 cells that expressed HA-Gsα did not accumulate ACTH receptor transcripts. Treatment of the HA-Gsα
Figure 30. Restriction enzyme digestion of RT-PCR products amplified from HA-Gsα transformants. A. RT-PCR products amplified from mutant 10r6 cells and 10r6 cells transfected with HA-Gsα (10r6Gs6 and 10r6Gs4) were digested with either restriction endonuclease Ava II or Mlu I as indicated. B. RT-PCR products amplified from HA-Gsα transfected parent Y1 (Y1Gs9 and Y1Gs11) and mutant 10r6 cells (10r6Gs1) were digested with restriction endonuclease Mlu I. HA-Gsα plasmid DNA was also included as control.
Figure 31. Gsα levels in an HA-Gsα transformed forskolin-resistant mutant.

A. HA-Gsα in whole cell lysates as determined by immunoprecipitation with anti-Gsα antiserum (A572) and Western blotting with anti-HA antibody (12CA5).

B. HA-Gsα in whole cell lysates as determined by immunoprecipitation with 12CA5 and Western blotting with A572. The higher molecular bands that appear in all samples are the heavy chain of immunoglobulin.
Figure 32. Amplification of ACTH receptor transcripts from forskolin-resistant mutant cells transfected with HA-Gsa. cDNA was synthesized from RNA using oligo-dT primer and ACTH receptor transcripts were amplified by PCR at 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min for 30 cycles. The transcription factor, SF-1, also was amplified from the same cDNA sample and served as an internal control.
transformants with ACTH did not cause any cell rounding which is mediated by cAMP and thus is indicative of functional coupling of ACTH to adenyl cyclase (data not shown).

The HA-Gsα transformants remained forskolin-resistant. As shown in Figure 33, the growth of parental Y1 cells was markedly inhibited by forskolin and by 8Br-cAMP. In contrast, the growth of forskolin-resistant cells 10r6 cells and mutant cells expressing HA-Gsα (10r6Gs1 and 10r6Gs6) was not affected by forskolin, although it could be inhibited by 8Br-cAMP. As compared to parent Y1 cells, adenyl cyclase in the mutant 10r6 was approximately 2.5-fold less responsive to forskolin (Fig. 34). Transfection of these cells with HA-Gsα did not restore the responsiveness of the adenyl cyclase system to forskolin, the transformants remain resistant to the effect of forskolin on adenyl cyclase activity, consistent with their resistance to forskolin-mediated growth inhibition. These results suggested that decreased levels of Gsα were not responsible for the resistance of these mutants to ACTH and forskolin, and supported the conclusion reached in studies with B2AR and ACTH receptor transformants.

3. Gsα distribution in forskolin-resistant mutants

The distribution of Gsα was compared among whole cell, membrane and cytosol fractions of Y1 cells and HA-Gsα transformed 10r6 mutant cells using an anti-Gsα antibody. As shown in Figure 35, 10r6 contains only 36% of the Gsα found in parent Y1 cells. Expression of HA-Gsα increased the Gsα level in 10r6 transformants to 64% of the level found in Y1 cells. When the membrane and cytosolic fractions were separated, all of the Gsα was found in association with the membrane fraction. Gsα was not detected in the cytosolic fractions of Y1 or mutant cells (Fig. 35), indicating that the decreased level of Gsα in mutant cell membranes did not result from a redistribution of Gsα to the cytosol. The levels of Gsα in the membrane fractions of parent Y1 cells and mutant 10r6 cell transformed with HA-Gsα were comparable, suggesting that the HA-Gsα was incorporated into the membrane. These results demonstrated that transfection of forskolin-resistant mutants with the HA-Gsα restored the levels of membrane associated Gsα, but did not correct the forskolin-resistant phenotype, indicating that the decrease in Gsα levels in mutant cells did not cause of the forskolin resistance phenotype.
Figure 33. Growth inhibition of HA-Gsα transformed 10r6 cells by forskolin.

Y1, mutant 10r6, and HA-Gsα transformed 10r6 cells (10r6Gs1 and 10r6Gs6) were plated and then grown for 2 weeks in the presence of forskolin or 8 bromo-cAMP. Survivors were fixed and stained with 1% methylene blue in 70% 2-propanol.
Figure 34. Adenylyl cyclase activity in response to forskolin in the HA-Gsa-transformed forskolin-resistant cells. Homogenates from Y1 cells (closed circles); mutant 10r6 cells (open circles); and HA-Gsa transformed 10r6 cells (10r6Gs1, open triangles) were assayed for adenylyl cyclase activity in the presence of different concentrations of forskolin. Shown is a representative of two independent experiments, each carried out in duplicate. Results are expressed as pmoles of cAMP accumulated per 5 min per mg protein.
Figure 35. Distribution of Gsα in membrane and cytosolic fractions. The membrane and cytosolic fractions were separated by centrifugation of cell homogenate at 150,000 g. The Gsα levels were determined by Western blot using anti-Gsα antibody.
C. THE EFFECTS OF Gβγ ON THE FORSKOLIN-RESISTANT PHENOTYPE

The results described in Sections III.A and III.B showed that the loss of ACTH receptor expression accompanied the mutation to forskolin resistance, and that decreased levels of Gsα were not responsible for this effect. We therefore explored the possibility that another phenotypic change observed in the forskolin-resistant cells, impairment of βγ function, was responsible for the loss of ACTH receptor gene expression.

1. Stable transfection with the genes encoding Gβ and Gγ subunits

Forskolin-resistant mutant cells were transfected with expression vectors encoding human β1, bovine β2, and bovine γ2 cDNA. Specifically, cells were transfected with the combinations of either β1γ2 or β2γ2 cDNAs, plus a neomycin-resistance gene. G418 resistant clones were tested for change of cell shape in response to ACTH (24 nM) and clones that rounded up in the presence of ACTH were isolated. Expression of the Gβ and Gγ cDNAs in the transformants was examined by Northern blot analysis. As shown in Figure 36, when using a bovine γ2 cDNA fragment as probe, a 1.5 kb transcript (Gautam et al., 1989) was detected in six independent transformants (Gβ1γ1 clones #1 and #1a; Gβ2γ2 clones #2-4 and #6). This transcript was not seen in untransfected Y1 or 10r9 cells, indicating that it is the result of expression of transfected bovine γ2 cDNA. The transcript also was not detected in one of the Gβ2γ2 clones, clone #5 (Fig. 36), suggesting that this clone did not express the Gγ2 cDNA. On the other hand, using human β1 or bovine β2 cDNA fragments as probes, transcripts at 3.3 and 1.8 kb (Fong et al., 1987; Sugimoto et al., 1985) were detected in both untransfected and transfected cells, indicating that the endogenous mouse β subunits cross-hybridized with the human and bovine β cDNA probes. Note that in one of the Gβ2γ2 clones, clone #6, the levels of β1 and/or β2 transcripts were much higher as compared to the levels seen in Y1 or 10r9 cells and likely resulted from expression of transfected β2 cDNA (Fig. 36).

The Gβγ transformants were isolated on the basis of rounding in response to ACTH, indicating that these cells may have recovered the ability to express ACTH receptors that functionally coupled to adenylyl cyclase. Therefore, RT-PCR was carried out to test for the expression of ACTH receptor transcripts in these transformants. A gene specific primer (P29) for
Figure 36. Northern blot analysis of transcripts of G protein β and γ subunits. RNA from untransfected Y1 and 10r9 cells, and seven independent 10r9 Gβγ transformants were analyzed using the β1, β2 and γ2 cDNA fragments as $^{32}$P-labeled probes. The same blot was stripped and reprobed with a tubulin cDNA fragment. Two independent Gβ1γ2 transformants (Clone #1 and #a) and five Gβ2γ2 transformants (Clone #2-6) are shown.
the ACTH receptor was used for first-strand cDNA synthesis. Two primers (P30 and P31) that flanked a 1.6 kb intron in the ACTH receptor gene, were then used to amplify a 240 bp cDNA fragment (Fig. 37A). As shown in Fig. 37B, the 240 bp fragment representing the ACTH receptor cDNA was detected in parent Y1 cells, and was undetectable in untransfected 10r9 cells. In contrast, ACTH receptor transcripts were seen in the five independent 10r9 mutants transformed with G protein βγ subunits (clone #1, 2, 3, 5, 6), although none approached the levels seen in Y1 cells. One of the clones, clone #5, which did not express Gγ2 transcripts by Northern blot analysis (Fig. 36), was positive for ACTH receptor transcripts by RT-PCR (Fig. 37B), suggesting that co-expression of Gβ and Gγ2 cDNA may not be required for the recovery of ACTH receptor transcripts. Since these transformants were transfected with the combination of Gβ and Gγ, it seemed possible that the expression of Gβ alone might be sufficient for the ACTH receptor expression. This was confirmed by introducing individual Gβ2 or Gγ vectors into mutant cells, as will be described in a later section.

ACTH receptor transcripts were detected in parent Y1 cells and in the βγ transformants using a gene-specific primer for cDNA synthesis (P29) as well as using an oligo-dT primer (Figs. 37A and 38A). To ensure that equivalent amounts of cDNA from Y1, 10r9, and transformants were used for the PCR reaction, the transcript for SF-1 was also amplified from the oligo-dT primed cDNA samples (Fig. 38B). The PCR products generated in each case reflected ACTH receptor transcripts since they contained the Pst I site which is present in the region between the two amplification primers (P30 and P31, Figs. 37A and 38C). The recovery of ACTH receptor transcripts in these transformants was specifically dependent on the Gβγ expression vectors since transfection of mutant cells with vectors encoding HA-Gsα or β2AR did not lead to expression of ACTH receptor transcripts (Figs. 10 and 32). The expression vector encoding HA-Gsα (pcDNA1) also served as a negative control to exclude a nonspecific effect of the Gβ or Gγ vector (pCDM8.1) since pcDNA1 and pCDM8.1 are identical except for additional multiple cloning sites in pcDNA1. Results from RT-PCR experiments indicated that transfection of cells with a combination of G protein β and γ subunit genes led to the recovery of the expression of ACTH receptor transcripts in forskolin-resistant cells. Combinations of β1 and γ2 or β2 and γ2 behaved the same since transformants from both combinations expressed ACTH receptor transcripts (Fig. 38A).
Figure 37. Amplification of ACTH receptor transcripts from 10r9 cells transfected with G protein βγ subunits. A. Schematic diagram showing the primers used for cDNA synthesis (oligo-dT and P29) and PCR amplification (P30 and P31). The sequences of primers were given in Table 1. The region between P30 and P31 spans a 1.6 kb intron and a restriction site Pst I. B. cDNA was synthesized using P29 and amplified using P30 and P31 at 94 C for 1 min, 60 C for 1 min, and 72 C for 1 min for 30 cycles. Six independent Gβγ transformants are shown.
Figure 38. Amplification of ACTH receptor transcripts by RT-PCR. A. cDNAs were synthesized using either the gene-specific primer P29 or an oligo-dT primer as indicated and amplified using the primers P30 and P31 (Table 1, Fig. 37). B. cDNAs synthesized using oligo-dT primer (from panel A) were amplified using the SF-1 specific primers P41 and P59 (Table 1). C. PCR products (from panel A) were digested with Pst I. Three independent G8γ transformants (#1: G81γ2; #3 and 6: G82γ2) are shown.
The changes in cell shape seen in the Gβγ transformants in response to ACTH treatment also were indicative of elevated intracellular levels of cAMP. The ability of ACTH to stimulate cAMP accumulation was tested to determine if the accumulation of ACTH receptor transcripts detected by RT-PCR was accompanied by the synthesis of the receptor protein and functional coupling of ACTH receptors to adenyl cyclase. As shown in Table 5, Y1 cells exhibited a 14 ± 5 fold increase in cAMP accumulation in response to 24 nM ACTH. In four independent Gβγ transformants, ACTH increased cAMP accumulation by an average of 6.0 ± 1.5 fold. The amount of cAMP accumulated in response to ACTH in each individual clone seemed to correlate with the levels of recovery of ACTH receptor transcripts (Fig. 37B and 38A). These results suggested that over-expression of G protein βγ subunits in forskolin-resistant cells led to the recovery of ACTH receptor gene expression. As a consequence, the transfected mutant cells exhibited ACTH-stimulated cAMP accumulation and cell rounding, indicating the synthesis of functional ACTH receptor protein.

The recovery of ACTH responsiveness, however, was unstable and declined rapidly as the transformants were kept in culture for a period of time. Four weeks after the initial thawing of frozen cells, the transformants lost 50% of their ACTH responsive adenylyl cyclase activity (Fig. 39). Consistent with the decreased adenylyl cyclase activity, less than 40% of cells exhibited rounding in response to ACTH (Fig. 40). It is possible that over the interval in culture, cells eventually lost the β and/or γ cDNA or the cDNAs may have been otherwise silenced (Wigler et al., 1979b). The loss or silencing of the transfected gene may also account for the discrepancies observed between the expression levels of transfected G protein subunits and the extent of recovery of ACTH receptor in some of the transformants. For example, Northern blot analysis showed that clone #4 expressed transfected Gγ2 cDNA (Fig.36), however, clone #4 appeared to be negative in ACTH receptor transcripts in RT-PCR (Fig. 37B). On the other hand, Clone #6 expressed high levels of Gβ2 and Gγ2 subunits (Fig. 36), but only exhibited moderate recovery of ACTH receptor transcripts (Fig 37B). These clones were initially isolated on the basis of their rounding response to ACTH treatment. RNA isolated from these clones was first used for Northern blot analysis. At a later date, more RNA was isolated for RT-PCR. It is possible that
Table 5. cAMP accumulation in forskolin-resistant cells transfected with β and γ subunits of G protein.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>cAMP Accumulation (cpm/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Basal</td>
</tr>
<tr>
<td>Y1</td>
<td>484 ± 86</td>
</tr>
<tr>
<td>10r9</td>
<td>688 ± 21</td>
</tr>
<tr>
<td>10r9 + Gβ1γ2 #1</td>
<td>363 ± 31</td>
</tr>
<tr>
<td>10r9 + Gβ2γ2 #2</td>
<td>282 ± 24</td>
</tr>
<tr>
<td>10r9 + Gβ2γ2 #3</td>
<td>557 ± 50</td>
</tr>
<tr>
<td>10r9 + Gβ2γ2 #6</td>
<td>313 ± 29</td>
</tr>
</tbody>
</table>

cAMP accumulation was measured in cultures of Y1, forskolin-resistant 10r9 cells and 10r9 cells transfected with the β (81 or 82) and γ2 subunits of G protein. ACTH (24 nM) was added to the tissue culture media and cells were incubated at 37 C for 30 min.
Figure 39. ACTH receptor stimulated-cAMP accumulation in Gβγ-transfected mutant 10r9 cells. Cells were thawed from frozen stocks, grown to confluence and then plated out for the assay. ACTH (24 nM) was used to stimulate the accumulation of cAMP. Data are compiled from six independently isolated 81γ2 and 82γ2 transformants and presented as the mean ± SEM fold increase over control (basal level of unstimulated samples). The fold increase at day 41 was not significantly different from unstimulated control.
Figure 40. The effect of ACTH on the morphology of Y1, 10r9, and 10r9 cells transfected with Gβ2 and Gγ2. Cells were treated with ACTH (24 nM) for 4 h and photographed under phase contrast at the magnification indicated in the upper left panel. A. Y1 cells; B. 10r9 cells; C. 10r9 cells transfected with Gβ2 and Gγ2, clone #3. For the transformant, a picture was taken 27 days after initial thawing.
during the period that these clones were kept in culture, the $\beta$ and/or $\gamma$ cDNAs were eventually lost or silenced, resulting in a loose correlation between expression levels of G protein subunit(s) and the expression of the ACTH receptor gene.

2. Transient transfection of the combination of G$\beta$γ subunits

The effects of G$\beta$γ on ACTH receptor expression in the forskolin-resistant mutants also were evaluated in transient transfection assays. Transient transfection assays are not biased by a selection process that examines only cells that rounded in response to ACTH. These experiments measure changes in ACTH receptor expression that are observed only in mutant cells exposed to the expression vectors encoding G$\beta$ and Gγ. These changes, if any, would most likely be the direct result of the G$\beta$ and/or Gγ expression. Since the transformants would only be kept in culture for a short time, the transient transfection experiments also overcome the difficulties of instability associated with the stable transfection (Fig. 39 and 40). One disadvantage associated with transient transfection experiments is that only a small number of cells in the population are able to take up and express cDNAs in these assays. The RT-PCR method, however, proved to be sensitive enough to measure increases in the levels of ACTH receptor transcripts in a small fraction of the cell population.

10r9 cells were transfected with expression vectors encoding G protein $\beta$2 and $\gamma$2 cDNAs. At different time points post-transfection (48-120 h), cells were harvested and RNA was isolated using a Qiagen mini-RNA kit. cDNA was synthesized using the ACTH receptor specific primer (P29) and amplified with primers that flank the 1.6 kb intron in the ACTH receptor gene (Fig. 37A). As shown in Figure 41, untransfected 10r9 cells seemed to contain trace amounts of the 240 bp product consistent with the size of ACTH receptor transcripts. 10r9 cells transfected with G$\beta$2γ2 showed increased levels of this product, which was clearly visible 48h post-transfection and was constant for at least another 48 h (Fig. 41). The levels of ACTH receptor transcript were further induced in the G$\beta$2γ2 transfected cells following pretreatment with ACTH (24 nM for 18h, Fig. 41B), consistent with the observation that ACTH stimulates the expression of its own receptor (Mountjoy et al., 1994). These results further confirmed the findings from the stable transfection experiments in that upon transfection with G$\beta$γ subunits, the forskolin-resistant mutant cells
Figure 41. Amplification of ACTH receptor transcripts from mutant cells transiently transfected with vector encoding Gβ and Gγ subunits. Time course of expression of ACTH receptor transcripts in mutant cells transiently transfected with a combination of Gβ2 and Gγ2. RNA was prepared using a Qiagen RNA purification kit and cDNA was synthesized using the gene-specific primer P29 (Figure 37). ACTH receptor transcripts were amplified by PCR as described in Figure 37. A. Transient transfection using lipofectin reagent. B. Transient transfection using calcium phosphate method. Cells were treated with ACTH (24 nM) 18h before harvesting.
recovered the ability to express ACTH receptor transcripts. Thus, G protein β and/or γ subunits appear to play an important role in ACTH receptor expression in the forskolin-resistant mutants.

3. Transfection with individual Gβ or γ subunits

To determine if β or γ both were required for the expression of ACTH receptor transcripts, Gβ2 and Gγ2 subunits were transfected individually into the mutant cells and stable transformants were isolated on the basis of their response to ACTH treatment. Specifically, 10r9 mutant cells were transfected with an expression vector encoding either Gβ2 or Gγ2, plus a neomycin-resistance gene. G418 resistant clones were tested for change of cell shape in response to ACTH (24 nM) and clones that rounded up in the presence of ACTH were isolated. Expression of ACTH receptor transcripts in these clones were then measured by RT-PCR. As shown in Figure 42, the 240 bp fragment representing ACTH receptor cDNA was seen in parent Y1 cells, and was undetectable in untransfected 10r9 cells. In contrast, the ACTH receptor transcripts were seen in the four Gγ2 transformants and two Gβ2 transformants. In one of the Gγ2 transformants, clone #1, the ACTH receptor transcripts approached the levels seen in parent Y1 cells. On the other hand, a Gβ2 clone, clone #1, did not seem to contain any ACTH receptor transcripts, despite the fact that it was initially isolated based on its rounding in response to ACTH treatment and presumably contained functional ACTH receptors. Nevertheless, these results showed that transfection of either one of the subunits, Gβ or Gγ, could readily restore the expression of ACTH receptors in mutant cells, suggesting that the effects of these subunits may be mediated by a common mechanism, perhaps by forming functional βγ heterodimer with endogenous β or γ subunits.

As was observed in previous transfection experiments with combinations of Gβγ cDNAs, the effects of Gβ or Gγ on the recovery of ACTH receptor expression seemed to be unstable. All Gβ or Gγ transformants were isolated on the basis of changes in cell shape in response to ACTH treatment, an indication of ACTH-induced adenylyl cyclase activity. These transformants, however, became less and less responsive to ACTH treatment as they were kept in culture for a period of time. Figure 43 showed pictures taken one day before the cloning of the single G418-resistant colonies that were treated with ACTH (24 nM) for 4 hours. For these colonies, around 60 to 70% of the cell population responded to ACTH with rounding (Fig. 43). After these colonies
Figure 42. ACTH receptor transcripts from mutant cells transfected with expression vectors encoding either Gβ2 or Gγ2. A. RNA was extracted from mutant cells transfected with either β2 or γ2 subunit of G protein. cDNA was synthesized using oligo-dT primer and ACTH receptor transcripts were amplified using P31 and P30 primer (Figure 37). B. SF-1 transcripts were also amplified using P41 and P59 (Figure 37) to serve as controls using the same cDNA samples. Three β2 transformants and four γ2 transformants showed different levels of recovery of ACTH receptor transcripts.
Figure 43. The effect of ACTH on the morphology of 10r9 cells transfected with either Gβ2 or Gγ2 subunits. Cells were treated with ACTH (24 nM) for 4 h and photographed under phase contrast at the magnification indicated in Figure 39. A. 10r9 cell transfected with Gβ2, clone #2. B. 10r9 cells transfected with Gγ2, clone #1.
were picked up and grown in culture for several days, the proportion of cells that responded to ACTH declined even further (data not shown). In some of the clones (e.g., GB\textsubscript{8} transformant, clone #1, Fig. 42), the absence of ACTH receptor transcript may also reflect the instability in the expression of ACTH receptor gene. These transformants did not respond to ACTH in cell rounding and did not show ACTH receptor transcripts at the time of the RT-PCR experiment despite the earlier morphological response to ACTH.

D. CLONING AND SEQUENCING OF WILD-TYPE AND MUTANT GB\textsubscript{81} cDNA IN AN ATTEMPT TO LOCALIZE THE MUTATION LEADING TO FORSKOLIN RESISTANCE

The results presented above suggest that GB\textsubscript{8}γ may be a candidate target for the forskolin-resistant mutation. The mutation may reside in either β or γ subunits. Among the five GB subunits that have been identified, β3 has been inferred to be the β subunit associated with a cone photoreceptor-specific γ subunit type and β5 is expressed only in brain. Therefore, only β1, β2, and β4 are expressed ubiquitously in peripheral tissues (Yan et al., 1996). Previous studies have shown that γ1 and forskolin-resistant cells contained both GB1 and GB2 subunits (Schimmer and Tsao, 1990). The levels of GB1 were not different and the levels of GB2 varied independently of the mutation as evaluated by Western analysis. In an attempt to further define the forskolin-resistant mutation, the wild-type GB\textsubscript{1} was cloned and the sequence was compared with the GB\textsubscript{1} isolated from mutant cells.

The wild-type mouse GB\textsubscript{1} cDNA was cloned by a combination of library screening and 5'-RACE. A Balb/c mouse adrenal cDNA library was screened with a human β1 cDNA probe spanning 85% of its coding region (Codina et al., 1986). Five independent, overlapping cDNA clones were isolated that covered 1340 bp of the mouse GB\textsubscript{1} cDNA including the 3'-untranslated region (Fig. 44A). The missing sequences were isolated by 5'-RACE (rapid amplification of 5' cDNA ends; Frohman et al., 1988) using nested primers (Fig. 44B, Table 1). The mouse β1 cDNA contained an open reading frame of 1020 bp that was 90% identical to human and bovine β1 cDNA (Fig. 45), and less similar to mouse β2 (76%), human β3 (75%), mouse β4 (74%) or mouse β5 (51%) cDNA (Watson et al., 1994). The predicted protein sequence of 340 amino acids was completely identical to human and bovine β1 (Sugimoto et al., 1985; Codina et al., 1986).
Figure 44. Schematic Diagram of strategies for cloning mouse G protein β1 subunit. A. Mouse β1 clones isolated from mouse adrenal cDNA library using a Bgl II fragment of human β1 cDNA as probe. B. Amplification of the 5'-end of mouse β1 cDNA. cDNA was synthesized from total Y1 RNA using the gene-specific primer P5. The cDNA was then (dC)n-tailed and the 5'-end of the β1 cDNA was amplified by PCR using P4 and a (dG/dI)n-anchor primer (from 5' RACE System, Canadian Life Technologies). C. Primers for amplification and sequencing of β1 cDNA from forskolin-resistant cells. cDNA was synthesized using P13, amplified using P9 and P13, and sequenced using P9, P10, P12, P13 (Table 1).
Figure 45. Alignment of nucleotide sequences of the mouse (m), human (h), and bovine (b) G protein β1 subunits. Points indicate identical nucleotides. The 3’-untranslated region is presented in lower case letters and the polyadenylation signal sequence (AATAAA) is highlighted in bold type. The mouse β1 nucleotide sequence has been deposited in the GeneBank data base with the accession number U29055.

Two sequence variants of mouse B1 cDNA were isolated that differed in the 5' untranslated region (Fig. 46). A 49 bp insert at -40 relative to the start of translation was identified in three of the eight clones isolated. Consensus splice sites were found flanking the beginning (5' AG) and end (3' A) of the insert (Stryer, 1989), suggesting that the insert resulted from an alternative RNA splicing event. The 3'-untranslated region of mouse B1 contained 351 bp including one polyadenylation site (AATAAAA) plus the poly(A) tail. Inasmuch as human and bovine B1 cDNAs have longer 3'-untranslated regions (1788 bp and 1664 bp respectively) and have two polyadenylation sites, it is possible that the mouse adrenal gland produces another B1 transcript with a longer 3'-end and alternate polyadenylation signal. Consistent with this suggestion, we find that Y1 mouse adrenocortical tumor cells produce two B1 transcripts (1800 bp and 3300 bp) in Northern blot hybridization analysis (Fig. 36), consistent with the presence of two polyadenylated RNA species.

The common regions of the two alternatively spliced clones were used to design primers for selective amplification of Gβ1 cDNA from mutant Gβ1 by RT-PCR. Primers were generated from sequences including the 5' and 3'-untranslated regions of normal mouse B1 cDNA (P9 and P13, Fig. 44C, Table 1) in order to ensure specific amplification of B1 cDNA, since these regions are most different among β subunits of G protein. A 1.1 kb amplification product which covers the whole coding region of Gβ1, was obtained from RNA of forskolin-resistant mutant 10r9 cells. Sixteen independent clones were sequenced and analyzed. The B1 sequences obtained from the mutant cells were compared with the wild-type B1 cDNA to determine if there was any consistent difference in bases that may indicate a mutation. None of the clones, however, displayed any consistent base change among the sixteen clones examined. Some single base changes were detected, but each appeared only once in the sixteen clones and did not lead to an amino acid change, suggesting that the changes may result from PCR artifact. The results suggest that the forskolin-resistant mutation is unlikely to reside in the mouse B1 subunit gene.
Figure 46. The 5'-noncoding region of mouse G protein B1 subunit. Lower case
letters indicate the insert associated with the variant B1 transcript. Nucleotides are
numbered with reference to the A of the translation start codon ATG which is set
at +1. Reprinted from Qiu and Schimmer, 1996, GENE 175:275-277 with permission
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IV. DISCUSSION

The results presented in this study indicate that the ACTH unresponsiveness observed in forskolin-resistant cells results from reduced expression of the ACTH receptor gene, rather than from an impairment in receptor-G protein coupling secondary to decreased levels of Gsα and Giα, as hypothesized previously (Schimmer and Tsao, 1990). As demonstrated by Northern blot analysis and RNase protection assays (Fig. 9), forskolin-resistant 10r6 and 10r9 cells do not accumulate ACTH receptor transcripts. The loss of ACTH receptor transcripts seems to result from impaired ACTH receptor gene transcription as shown by nuclear runoff assay (Fig. 12). Using RT-PCR with extended cycles of amplification, weak signals of ACTH receptor transcripts could be detected in the mutant cells (Fig. 10). The signals, however, are considerably less than that generated from parent Y1 cells, indicating that the expression of ACTH receptors was markedly reduced, although not completely blocked. The extremely low levels of ACTH receptor transcript in mutant cells are unlikely to produce sufficient amount of functional ACTH receptor protein and therefore seem to account for the ACTH resistance of these mutants.

The spontaneously isolated ACTH receptor-deficient mutants, Y6 and OS3, are also forskolin-resistant, though not to the same extent as the mutant 10r6 and 10r9 cells. These cells do not express ACTH receptor transcripts as demonstrated by Northern blot analysis and RNase protection assays (Schimmer et al., 1995). Results from this study showed that the Y6 and OS3 cells also exhibit a forskolin-resistant phenotype, including resistance to the growth-inhibiting effects of forskolin and to the effects of forskolin on adenylyl cyclase (Figs. 24 and 25), and decreased levels of Gsα (Fig. 26). The observations that forskolin-resistant cells contain decreased levels of ACTH receptor and the spontaneous ACTH receptor-deficient mutants are forskolin-resistant suggest that the forskolin resistance and the impaired expression of the ACTH receptor may be genetically linked. As described in Section I.D, the forskolin-resistant phenotype likely resulted from a single mutational event. Therefore, different forskolin-resistant mutants may have mutations at a single locus or at different loci that regulate a common pathway responsible for both the ACTH deficiency and forskolin-resistance.
Although the Y6 and OS3 cells share many similar characteristics with the 10r6 and 10r9 mutants, they are also different in several ways: 1) Y6 cells express higher levels of ACTH receptor transcript than do the other forskolin-resistant mutants as determined by RT-PCR, but the levels are still considerably lower than in parent Y1 cells (Fig. 23). 2) Y6 and OS3 cells are not as resistant to the growth inhibitory effects of forskolin as are the 10r6 and 10r9 clones (Figs. 21 and 24). The Y6 and OS3 cells were resistant to the growth inhibitory effects of 2 μM concentration of forskolin but did not survive in 10 μM forskolin. In contrast, the mutant 10r6 and 10r9 cells survived in the presence of 10 μM forskolin. 3) The Gia levels in Y6 cells are not lower but higher than in parent Y1 cells. In contrast, the Gia levels are decreased as much as 85% in OS3 cells (Fig. 26B) and approximately 60% to 70% in forskolin-resistant 10r6 and 10r9 cells (Fig. 18). 4) Y6 and OS3 cells do not respond to ACTH with increased steroidogenesis but do respond to cAMP (Schimmer, 1976). On the other hand, the forskolin-resistant cells do not produce steroids after stimulation with ACTH or with cAMP analogs (Schimmer and Tsao, unpublished data).

These observations suggest that the mutations that lead to forskolin resistance and ACTH receptor deficiency in Y6, OS3, 10r6 and 10r9 cells may be different. It is possible that the phenotypic differences among the mutants reflect mutations of different severity at the same locus or mutations at different loci.

The Y1 adrenal cell mutants with impairment in the expression of ACTH receptor resemble a class of ACTH-resistant mutants that are also seen in some clinical cases. ACTH resistance has been identified in human subjects with familial glucocorticoid deficiency (FGD) and with adrenal tumors. In 40% of patients with FGD, point mutations in the coding region of the ACTH receptor gene accounts for hormone resistance (Clark and Cammas, 1996, Naville et al., 1996b). In 60% of FGD disorders as well as in another ACTH resistance disorder, the triple A syndrome, no mutations were found in the coding region of the ACTH receptor gene (Clark and Weber, 1994). The ACTH-resistance in these patients may result from defect(s) affecting ACTH receptor gene expression that are similar to the forskolin-resistance mutation. In some adrenal carcinomas and non-functioning adenomas, ACTH receptor mRNA is low or missing (Allolio and Raincke, 1997), suggestive of defects in ACTH receptor gene expression that, again, resemble the mutations in the forskolin-resistant cells. Factors that control ACTH receptor gene expression in the adrenal cortex
are not well understood at the present time. Analysis of ACTH receptor promoter regions identified an SF-1-binding element that may contribute to adrenal-specific expression of the ACTH receptor. Several consensus CREs (human ACTH receptor) and an AP2 binding site (mouse ACTH receptor) that may be important for ACTH and cAMP-mediated regulation also were identified (Naville et al., 1996a, Cammas et al., 1997). It is possible that the decreased expression of ACTH receptor in forskolin-resistant cells is caused by deficiency in SF-1 or cAMP-mediated regulation of ACTH receptor gene expression. The mutant cells contain the same levels of SF-1 as do parent Y1 cells (Figs. 10 and 23), indicating that the impaired expression of ACTH receptor did not result from the absence of SF-1, although the function of SF-1 in these cells was not evaluated. On the other hand, while it is possible that low basal levels of cAMP may have caused the deficiency in expression of ACTH receptor, my results indicate that this is not the case. As shown in Tables 2 and 4, basal adenylyl cyclase activity was similar in parent Y1 cells and mutant cells. Expression of the mouse β2AR (10r6 and 10r9, Figs. 9-10) or human ACTH receptor (Y6 and OS3, Fig. 27) in mutant cells restored hormone-sensitive adenylyl cyclase activity but did not turn on the expression of endogenous ACTH receptor. Treatment of mutant cells with cAMP did not induce ACTH receptor expression in the mutants (Fig. 9, Schimmer et al., 1995). The regulatory pathway for ACTH receptor downstream of cAMP, however, seems to be intact in these cells. In the Gβγ transformants that express ACTH receptor, the transcript levels of ACTH receptor increased in response to the treatment of ACTH (Fig. 41B), indicating a cAMP-dependent induction of ACTH receptor expression.

The ACTH receptor gene appears to be intact in Y6 and OS3 cells, and 10r6 and 10r9 cells, as demonstrated by Southern blot analysis (Fig. 11, Schimmer et al., 1995). Furthermore, the observations that transfection of the 10r9 mutant with G protein β and γ subunits stimulated ACTH receptor gene expression and led to the recovery of functional ACTH receptor (Figs. 37-38, 41-42), also point to the integrity of the ACTH receptor gene. In Y6 and OS3 cells, ACTH receptor expression was restored by growth of these cells as tumors in isogenic LAF1 mice (Schimmer, 1976, Schimmer et al., 1995). This effect of animal passage on Y6 and OS3 cells was stable and persisted over large numbers of cell doublings that extensively diluted the original tumor population (Schimmer, 1976). These observations further suggest that the ACTH receptor gene in
these mutants is intact but is silenced by a modification that is reversible upon passage of the cells as tumors. The molecular mechanisms involved in silencing the ACTH receptor gene are undefined. DNA methylation has been implicated in the silencing of 21-hydroxylase (Cyp-21) gene in Y1 cells (Szyf et al., 1989, Schimmer and Parker, 1992, Ramchandani et al., 1997). The extent of methylation of ACTH receptor gene, however, was not different among the Y1 parent and mutant Y6 and OS3 cells. In addition, demethylation of the ACTH receptor gene did not enhance its expression (Schimmer et al., 1995), suggesting that DNA methylation is not likely a major factor in silencing the ACTH receptor gene in these mutants.

The impaired expression of the ACTH receptor seems to be responsible for the reduced levels of membrane-associated Gα subunits in the forskolin-resistant mutants. Transfection of mutant cells with the gene encoding the G protein-coupled receptors (the mouse β2AR for 10r6 and 10r9 cells, and ACTH receptor for Y6 and OS3 cells) led to the recovery of functionally active receptors that formed high-affinity complexes with G proteins (Table 3, Figs. 14-15) and that activated adenylyl cyclase (Table 2, 4, Fig. 13) in a manner that was indistinguishable from forskolin-sensitive parental Y1 cells. The results suggest that these G protein-coupled receptors are efficiently coupled to adenylyl cyclase and that adenylyl cyclase is not impaired in the mutant clones, confirming previous conclusions (Schimmer and Tsao, 1984). In all the receptor transformants, the levels of Gsα and Giα also increased and approached the levels seen in parental Y1 cells (Fig 16, 18, and 26). Factors that contribute to the membrane attachment of Gα include the Gβγ subunit, lipid modification of Gα subunits (palmitoylation and myristoylation), and for Gocα, the N-terminal amino acids 11-14 that mediate membrane binding independent of Gβγ and lipid modification (Busconi et al., 1997). Deficiency in any of these factors may cause a redistribution of Gα from membrane to cytosol (Levis and Bourne, 1992, Iiri et al., 1996). A recent study has suggested that for Gsα, palmitate plays a dual role, increasing both the hydrophobicity of Gsα and its affinity for Gβγ. GTP-induced dissociation of Gβγ increases susceptibility of Gsα to depalmitoylation; the resulting loss of palmitate decreases the intrinsic avidity of Gsα for the lipid bilayer, promoting its transfer from membrane to cytosol (Iiri et al., 1996). According to this model, Gβγ binding and palmitoylation of Gsα reciprocally potentiate the attachment of Gsα to the membrane. In the forskolin-resistant cells, both endogenous Gsα and
transfected HA-Gsα were not detected in the cytosol fraction (Fig. 35), suggesting that redistribution of Gsα away from the membrane is not likely responsible for the decreased levels of Gsα and Giα in the mutants. Rather, transfection of G protein-coupled receptors (β2AR or ACTH receptor) resulted in an increase in Gsα and Giα levels (Figs. 16, 18, and 26) without changes in their transcript levels (Fig. 19). The up-regulation of Gsα was dependent upon receptor expression but was independent of activation with agonist and was not secondary to increased cAMP accumulation (Fig. 20). Based on these observations, I suggest that G protein-coupled receptors may increase Gα levels in Y1 cells by stabilizing the subunits, perhaps as membrane-associated heterotrimeric with the G protein βγ complex. These findings thus implicate an important role for receptors in the membrane attachment of Gα.

Regulation of Gα levels by receptor-mediated mechanisms has been seen in other systems, but generally occurs in response to agonist stimulation. Short-term stimulation of β2AR by isoproterenol causes the redistribution of Gsα from the plasma membrane to the cytosol, which may result from the dissociation of Gsα from Gβγ, and Gsα depalmitoylation (Levis and Bourne, 1992, Wedegaertner and Bourne, 1994). On the other hand, long-term (i.e., 16 to 24 h) exposure to agonist has been reported to cause both increases and decreases in the levels of various G subunits depending upon the agonist and cell type (Section I.B.3). Receptor-mediated regulation of Gα levels is generally independent of the generation of related second messengers and is not accompanied by an increase in the levels of mRNA (Milligan et al., 1995b). For the regulation of Gsα levels, for example, treatment of neuroblastoma x glioma cells with prostaglandin E1 (PGE1) reduced Gsα levels by 40-70%. This effect of PGE1 was not mimicked by forskolin and the Gsα mRNA levels remained unchanged (Mckenzie and Milligan, 1990, Adie et al., 1992). In addition, decreases in the levels of Gsα protein also are accompanied by the decreases of the prostanoid receptor levels. The time course and the dose-response relationship of receptor and G protein down-regulation were similar, suggesting that the losses of receptor and G protein were coupled (Adie et al., 1992). It is possible that the decrease in the receptor levels consequently results in decreased levels of Gsα, supporting my suggestion that receptor may stabilize the G protein it couples to at the plasma membrane in its unstimulated state.
Expression of G protein-coupled receptors increased the levels of Gsα in the Y1 adrenal cells mutants. On the other hand, agonist or cAMP treatment of these cells decreased Gsα levels. Treatment of β2AR transformants with isoproterenol (Fig. 20) and treatment of Y1 cells with ACTH (data not shown) resulted in decreased levels of Gsα. The effects of these agonists were mimicked by treatment with 8BrcAMP, indicating a cAMP-dependent regulation (Fig. 20). The agonist-dependent regulation of Gsα subunit in Y1 adrenal cells is different from that in primary cultures of bovine adrenocortical cells (Begeot et al., 1991). In bovine adrenal cells, treatment with ACTH for 72 h increased the levels of Gsα. The different effects of ACTH on Gsα levels in Y1 mouse adrenal cells and bovine adrenal cells may reflect difference between mouse and cow, between tumor cells and primary cultures of normal adrenal cells, or between different periods of incubation with ACTH (18 h vs. 72 h). In the bovine adrenal system, the effects of ACTH were mimicked by angiotensin II and correlated with changes in inositol phosphate accumulation and calcium uptake, suggesting a cAMP-independent mechanism of action.

The decreased levels of Gsα and Giα in the forskolin-resistant cells do not account for the forskolin-resistance in the mutant cells. Results from this study show that the mutant cells remain resistant to the growth inhibiting effects of forskolin and retain forskolin-resistant adenylyl cyclase activity after transfection with either the β2AR or the ACTH receptor (Figs. 21-22 and 24-25), despite the fact that the levels of Gsα in the membrane fractions of these transformants were partially or completely restored. In the mutant cells that were transfected with HA-Gsα, Gsα levels also were increased (Figs. 31 and 35), but the transformants remained forskolin-resistant (Figs. 33-34). These observations indicate that forskolin resistance in the mutant cells is not caused by the decreased levels of Gsα. Inasmuch as expression of the ACTH receptor and the restoration of Gsα levels in mutant transformants did not reverse the forskolin resistance, the results further suggests that the loss of ACTH receptor expression and decreased levels of Gsα are the consequence and not the cause of the mutation to forskolin resistance. As shown previously, the mutation that leads to forskolin resistance seemed to result from a defect in the ability of forskolin to activate adenylyl cyclase and increase cAMP formation in Y1 cells (Schimmer and Tsao, 1984). The absence of ACTH receptors and consequent reduction in Go levels associated with the forskolin-resistant
phenotype, however, are not the result of defects in cAMP accumulation per se, since treatment of the mutants with agents that raise intracellular cAMP levels did not restore ACTH receptor expression or elevate Gα subunits (Figs. 9-10 and 20).

A candidate target for the forskolin-resistance mutation is the G protein βγ complex which, as shown previously, has reduced activity in the forskolin-resistant mutants (Mitchell et al., 1992). Transfection of expression vectors encoding Gβ and Gγ subunits into the forskolin-resistant cells resulted in the recovery of ACTH receptor transcripts (Figs. 37-38, and 41), indicating that Gβγ plays a regulatory role in ACTH receptor gene expression. The effect of Gβγ on ACTH receptor expression is specific, since transfection of mutant cells with vectors encoding β2AR and HA-Gsα did not lead to expression of ACTH receptor transcripts (Figs. 10 and 32). In addition, expression of the human ACTH receptor in Y6 and OS3 cells also did not result in the expression of the endogenous mouse ACTH receptor gene (Fig. 27). The expression of ACTH receptor transcripts in the Gβγ transformants was accompanied by the recovery of ACTH-stimulated adenylyl cyclase activity and cAMP accumulation (Table 5), indicating the synthesis of functional ACTH receptor protein. The expression of ACTH receptor transcripts was further induced by treatment of the Gβγ transformants with ACTH (Fig. 41B), confirming an ACTH-induced, cAMP-dependent regulation of ACTH receptor gene expression. These results are consistent with the hypothesis that the loss of ACTH receptor expression in the forskolin-resistant cells results from a deficiency in Gβγ function. One of the possible causes of altered Gβγ function is a mutation in a gene encoding either Gβ or Gγ. It needs to be cautioned that although the results demonstrated a role for Gβγ in the expression of the ACTH receptor gene, they do not prove that the decreased levels of ACTH receptor transcripts in the mutant cells result from Gβγ deficiency or is caused by a Gβ or Gγ mutation. It remains possible that the mutation to forskolin-resistance may reside in a factor other than Gβ/Gγ subunit and that the involvement of Gβγ in ACTH receptor gene expression is a coincidental and fortuitous finding.

Interestingly, transfection of mutant cells with vectors encoding Gβ or Gγ alone could also restore ACTH receptor expression (Fig. 42). As described in Section I.B.2.b.i., the β and γ subunits are tightly bound as a complex and act inside cells as a heterodimer. In COS-7 cells, co-
transfection of Gβ and Gγ but not transfection of individual subunits, was required for the membrane targeting of βγ complex and βγ-mediated activation of MAP-kinase (Simonds et al., 1991, Faure et al., 1994). On the other hand, in human embryonal kidney 293 cells, transfection of either Gβ or Gγ as well as Gβ and Gγ resulted in complex formation of βγ and Raf-1 protein kinase (Pumiglia et al., 1995), indicating that the transfected Gβ or Gγ subunits function by forming βγ dimers with endogenous γ or β subunits, respectively. In the forskolin-resistant mutant cells, the transfected Gβ/Gγ may also function as in 293 cells.

As described in Section I.D, the forskolin-resistant phenotype likely resulted from a single mutational event. If the impaired ACTH receptor expression in mutant cells is caused by a Gβγ defect, the mutation might be located in either Gβ or Gγ. It seems surprising that the introduction of either one of the G protein subunits into the mutant cells could result in partial correction of the mutant phenotype. In this regard, I offer a model to explain these results, as described in Figure 47. The model is based on the presumption that the mutation that leads to the silencing of the ACTH receptor gene is located in one of the Gγ subunits, although it is also possible that the mutation is located in one of the Gβ subunits. In the latter case, a similar model could still apply. The model is also drawn on the presumption that the mutation in Gβ or Gγ is dominant, and is supported by the fact that the presumed single mutational event that caused the severe forskolin-resistant phenotype arose in a nearly-diploid cell line (Schimmer et al., 1985). As shown in Figure 43, I suggest that mutant cells contain two Gγ alleles — a mutant allele (Gγ*) and a wild-type allele (Gγ). Both the mutant Gγ* and the wild-type Gγ are expressed; however, Gγ* preferentially binds with Gβ to form mutant βγ* complexes. Expression of additional wild-type Gγ displaces the mutant Gγ* from mutant βγ* complexes, resulting in recovery of βγ function. On the other hand, expression of additional wild-type Gβ provides extra copies of Gβ in the cells. The excess transfected Gβ is able to rescue wild-type Gγ to form wild-type βγ complexes. The accumulation of the wild-type βγ complex thereby accounts for the recovery of βγ function. A similar model has been offered to explain the results with mutants that harbor a mutation in the regulatory subunit of PKA. In the PKA mutants, transfection with expression vectors encoding either the regulatory
**Figure 47. Hypothesis depicting wild-type and mutant G\(\beta\) and G\(\gamma\) subunit interactions.** The model is based on the presumption that the deficiency of \(\beta\gamma\) activity in mutant cells results from a mutation in the \(\gamma\) subunit of G protein. A similar model would also apply if the mutation is located in G\(\beta\). a) The mutant cells synthesize equal amounts of mutant \(\gamma^*\) and wild-type \(\gamma\) subunits. However, the mutant \(\gamma^*\) combines preferentially with the \(\beta\) subunits and excludes wild-type \(\gamma\) from the complex. The resultant \(\beta\gamma^*\) complex therefore exhibits decreased activity. b) Transfection of mutant cells with wild-type \(\gamma\) subunit results in a effective competition with the mutant \(\gamma^*\) for the \(\beta\) subunit, forming a normal \(\beta\gamma\) complex. c) Transfection with wild-type \(\beta\) subunit rescues wild-type \(\gamma\) and forms a normal \(\beta\gamma\) complex. These transformants most likely contain the mutant forms of \(\beta\gamma^*\) complex as well.
subunit or the catalytic subunit of the enzyme restored cAMP-stimulated PKA activity (Wong and Schimmer, 1989, Steinberg et al., 1991, Wong et al., 1992).

The mechanism of regulation of ACTH receptor gene expression by Gβγ is presently unknown. Gβγ directly interacts with many signaling proteins including adenylyl cyclase, PLC, Ca2+ and K+ channels, calmodulin, and MAP-kinase (Section I.B.4). Therefore, mutations affecting Gβγ activity could disrupt multiple signaling pathways. The end points of these signal transduction pathways include activation of transcription factors and regulation of expression of genes which may include the ACTH receptor gene. Gβγ may regulate gene expression through several pathways: 1) Gβγ dimer is capable of directly regulating adenylyl cyclase activity and can either inhibit or activate the enzyme depending on the specific isoforms present (Taussig and Gilman, 1995). The activation of adenylyl cyclase causes increases in intracellular cAMP and activation of PKA. Expression of the ACTH receptor is regulated by cAMP (Schimmer et al., 1995, Clark and Cammas, 1996); however, the impaired expression of ACTH receptors in the forskolin-resistant mutants appears to result from a defect that is not corrected by elevating intracellular levels of cAMP (Figs. 9 and 10), and thus likely reflects an impairment in βγ function that is independent of cAMP formation. 2) Gβγ may regulate gene expression through a Ca2+-dependent mechanism. Ca2+ is known to interact with many proteins including transcription factors (e.g. c-fos and CREB) and kinases, and regulate their function (Ginty, 1997). Angiotensin II is known to induce the expression of ACTH receptor, probably through a Ca2+/PKC-dependent mechanism (Mountjoy et al., 1994, Lebrethon et al., 1994). Gβγ may regulate Ca2+-dependent signaling pathways through: i) Activation of PLCβ, which causes the production of diacylglycerol and IP3, leading to the activation of PKC and increase in intracellular Ca2+ (Muller and Lohse, 1995). PKC could also regulate transcription by phosphorylating and activating many transcription factors (Liu, 1996). ii) Direct interaction with Ca2+ channels. Gβγ inhibits several types of voltage-dependent Ca2+ channels. The effect is antagonized by the PKC-dependent phosphorylation of residues within the Gβγ binding site of the Ca2+ channel (Waard et al., 1997, Zamponi et al., 1997). iii) Interaction with calmodulin. Gβγ directly binds to calmodulin, a Ca2+-activating protein (Katada et al., 1987). Calmodulin activates calmodulin-dependent protein kinase which phosphorylates several transcription factors including CREB (Clapham, 1995). 3) Gβγ
stimulates MAP-kinase (ERKs and JNKs) by acting on different steps of the MAP-kinase activation cascade (Section I.B.4.c.). The activation of MAP-kinase results in phosphorylation and activation of many cellular proteins and transcription factors (Cano and Mahadevan, 1995). One of the major functions of MAP-kinase is to regulate cell growth and differentiation. In Y6 and OS3 cells, ACTH receptor expression was restored by growth of these cells as tumors in isogenic LAFl mice (Schimmer, 1976, Schimmer et al., 1995), further suggesting that changes in cell growth may be related to the regulation of ACTH receptor gene expression. 4) Gβγ binds to the PH domain of many proteins and regulates their functions (Section I.B.4.b.). PH domains have been identified in at least 90 proteins, and some of the proteins may be transcription factors themselves and some are protein kinases that can activate transcription factors (Gibson et al., 1994, Ingley and Hemmings, 1994). 5) Gβγ may regulate ACTH receptor gene expression through affecting the function of SF-1, the transcription factor that is important for the adrenal-selective expression of ACTH receptor (Cammas and Clark, 1997). SF-1 also is known to regulate the cell-specific expression of several steroidogenic enzymes including Cyp11b (11β-hydroxylase) (Parker and Schimmer, 1993). Forskolin-resistant 10r6 and 10r9 cells do not contain the transcripts of Cyp11b as shown by Northern blot analysis (data not shown), indicating a possible deficiency of SF-1 function in these mutants. 6) Gβγ may directly regulate ACTH receptor gene expression. Gβ belongs to a family of WD-40 containing proteins that include several transcription factors (Neer et al., 1994). Although at the present time, no direct evidence has been found for Gβγ acting as a transcription factor, Gβγ has been shown to regulate the promoter activity of the prolactin gene in a transient transfection system. In GH4 cells, transfection of β1γ2 or β2γ2 caused significant inhibition of the prolactin promoter (30 to 35%). This inhibition was blocked by co-expression of retinal transducin which can sequester free Gβγ complexes. On the other hand, in COS-7 cells, β1γ2 or β2γ2 activated the prolactin promoter in a transducin-sensitive, promoter-selective manner (2.6 and 4.3 fold, respectively), reminiscent of dopamine D2 receptor regulation of the prolactin promoter in these two cell types (Lew and Elsholtz, 1995). These observations demonstrate the specificity and potential importance of Gβγ in the regulation of mammalian gene expression. Taken together, Gβγ could regulate gene expression through several independent mechanisms. At the
present time, we do not know which mechanism is more important in the regulation of ACTH receptor expression, since very little is known about the factors involved in the process.

Results from this study demonstrated that transfection of Gβ and/or Gγ into forskolin-resistant mutant cells restored ACTH receptor expression and ACTH-stimulated adenylyl cyclase activity. The recovery of ACTH responsiveness, however, was unstable and declined rapidly as the transformants were kept in culture for a period of time (Figs. 39-40 and 43). The reason for the instability is not known but there are several possibilities. It is possible that over the interval in culture, cells eventually lost the Gβ and/or Gγ cDNA. Cells are cultured in G418 containing media and the selection is only for the neomycin resistance plasmid. In many co-transfection experiments, only one selective marker (at lower concentration) seems to be required for the selection of transformants that stably express both genes. For example, mouse L cells were co-transfected with the viral thymidine kinase gene (tk, the selective marker) and bacteriophage DNA, φx174 at a concentration ratio of 1:1000 (tk: φx174). Of the 16 clones selected for the tk+ phenotype, 14 contained φx174. The φx174 sequence was integrated into the genome and φx genotype was stable through many generations in culture (Wigler et al., 1979a). In my study, the concentration ratio of the neomycin-resistant gene and Gβ or Gγ was 1:3, and only 15% of G418-resistant clones responded to ACTH with rounding. The difference on transfection efficiency, the plasmids that encode different cDNA (target cDNA), and the concentration ratio of selective marker to target cDNA may account for the discrepancies among these results. In fact, the transfection experiments introducing either the β2AR or HA-Gsα into forskolin-resistant cells used the same ratio (1:3) of the neomycin-resistance gene to the β2AR gene or HA-Gsα gene. In these experiment, 15% (HA-Gsα) or 30% (β2AR) of the G418-resistant clones expressed the target cDNA and the transformants were stable for many generations. These observations may reflect the difference of transfection efficiency as affected by different target cDNAs and the plasmid encoding the cDNAs.

On the other hand, in mouse L cells transfected with thymidine kinase gene and genomic DNA from cultured cells (CHO, LH2b, and Hela) and then selected for the recovery of adenine phosphoribosytransferase (aprt), two populations of transformants were isolated: stable transformants that retained the ability to grow in selection media and unstable transformants that did not. The rate of loss of aprt activity in the unstable transformants was as high as 27% per
generation (Wigler et al., 1979b). The mechanism for this phenomenon is not known. The Gβγ transformants, nevertheless, may resemble this unstable population of transformants. The Gβ and/or Gγ cDNA may not be integrated into the cellular DNA of the mutant cells and without the selective pressure, the transformants may have lost the cDNA. The eventual loss of recovery of ACTH receptor expression, on the other hand, suggests that the changes observed in the mutant transformants do result from the expression of Gβγ and not from spontaneous reversion of the cells to an ACTH receptor positive state. The transformation efficiencies were estimated as 1 x 10⁻³ in Y1 adrenal cells stably transfected with only the neomycin-resistance gene (pSV2-neo, Fig. 4, Wong et al., 1986). In my transfection experiments, given the observation that approximately 15% of G418-resistant clones responded to ACTH with rounding, the fraction of cells that responded to ACTH was calculated as 1.5 x 10⁻⁴. Spontaneous reversion, on the other hand, is usually stable and happens at a much lower rate (3 x 10⁻⁸ in mouse L cells, Wigler et al., 1979b).

Because of the instability associated with the Gβγ transformants, we could not determine if the Gβγ also reversed forskolin resistance in the Gβγ transformants. These transformants quickly lost the ACTH response in culture, resulting in a heterogeneous population (Fig. 40 and 43). The heterogeneity prevented an accurate measurement of the recovery of forskolin-mediated growth inhibition and forskolin-responsive adenylly cyclase activity in the mutant transformants. It thus remains possible that a mutation affecting Gβγ activity may also be responsible for the resistance of adenylly cyclase to forskolin. The Gβγ dimers can either inhibit or activate the enzyme depending on the specific isoforms present. It, however, does not directly influence the interaction of forskolin with different adenylly cyclase isoforms (Sutkowski et al., 1994). While forskolin exerts optimal stimulation on adenylly cyclase only in the presence of Gsα, no synergistic effect of Gβγ and forskolin was observed in the activation of type II adenylly cyclase (Tang and Gilman, 1991). Therefore, it is unlikely that altered βγ activity is directly responsible for the resistance to forskolin. Instead, another factor that is regulated by Gβγ may play a role in forskolin action. PKC phosphorylates and activates type II adenylly cyclase, and the effect of phosphorylation is synergistic with that of forskolin and Gsα (Yoshimura and Cooper, 1993, Jacobowitz and Iyengar, 1994). Therefore, depending on the distribution of specific adenylly cyclase isoform,
PKC, in addition to Gsα, may play an important role in the forskolin action on the regulation of type II adenylyl cyclase in some tissues. Gβγ is known to activate PLCβ and consequently regulate PKC activity (Muller and Lohse, 1995). At the present time, I do not know which adenylyl cyclase isoform is expressed in Y1 adrenal cells. It is possible that the type II adenylyl cyclase is the major isoform expressed in these cells and Gβγ could regulate forskolin-dependent activation of the type II isoform through PKC. Nevertheless, the current model for forskolin action is that forskolin acts via a ternary complex involving adenylyl cyclase, activated Gsα and forskolin (Section I.C). The data presented in this study suggest that this model is not sufficient to account for the mutation that leads to forskolin-resistant adenylyl cyclase. An additional component, possibly linked to altered Gβγ activity, may be required for maximal activation of adenylyl cyclase by forskolin. At the present time, I do not have evidence that the Gβγ deficiency accounts for forskolin-resistance. It remains possible that the mutation to forskolin-resistance may reside in a factor other than Gβ/Gγ subunit. This presumed factor may be important not only for forskolin action, but also for ACTH receptor gene expression. The factor may act upstream of Gβγ in a Gβγ -dependent signaling cascade or may act through a Gβγ-independent pathway.

Since adenylyl cyclase is the target enzyme of forskolin, the possibility of a mutation located in adenylyl cyclase could not been completely excluded. Previous and present results, however, suggest that this possibility is unlikely: 1) The adenylyl cyclase is fully responsive to NaF. In addition, agonist stimulation of ACTH receptor and β2AR activates the enzyme in parent Y1 cells to the same extent as in forskolin-resistant mutant cells. 2) The impaired expression of the ACTH receptor was not corrected by treatment with cAMP, indicating that the mutant phenotype did not result from a deficiency in cAMP production secondary to a mutation in adenylyl cyclase. It remains possible, however, that the forskolin-resistant mutation may reside in a specific isoform or a specific domain of adenylyl cyclase, with which forskolin and the Gβγ interact. This mutation may cause forskolin-resistance. The same mutation, on the other hand, may in some way affect the accessibility of Gβγ in the mutant cells, resulting in a deficiency in βγ signaling and consequently impaired expression of ACTH receptor.
I have attempted to localize the mutations that lead to forskolin-resistance by cloning and sequencing the GB cDNA from parent and forskolin-resistant cells. No mutation, however, was found in the GB1 subunit that could account for forskolin-resistance. The three GB subunits (β1, β2, β4) that are expressed ubiquitously in peripheral tissues share close to 90% identity among each other (Von Weizsacker et al., 1992) and also seem to function in the same manner. This is also supported by our observation that transfection of either GB1γ2 or GB2γ2 into forskolin-resistant cells resulted in the similar recovery of ACTH receptor expression (Fig. 37-38). Among the six Gγ subunits, only γ2, γ5 and γ7 are expressed ubiquitously in peripheral tissues and are very different from each other (Yan et al., 1996). Depending on the nature of the mutation, the forskolin-resistant mutation may reside in either a β or γ subunit of G protein.

Results from the present study are consistent with the hypothesis that decreased activity of Gβγ in forskolin-resistant cells may contribute to the mutant phenotype. My results, however, do not identify the locus of the forskolin-resistant mutation. Since expression of wild-type GB or Gγ has been shown to restore ACTH receptor gene expression in the mutant cells, it is possible that wild-type GB and Gγ may also restore forskolin-responsive adenylyl cyclase activity and forskolin-mediated growth inhibition. Homogeneous populations of stable transformants expressing GB or Gγ are required to study this possibility and would establish a role for Gβγ in the regulation of forskolin action. Restoration of forskolin-responsiveness upon transfection with GB or Gγ would suggest that the forskolin-resistant mutation resides in one of the GB or Gγ subunits or in a factor that regulates Gβγ function. On the other hand, it remains possible that the forskolin-resistant mutation may reside in another component that functions via a Gβγ-independent mechanism. To specifically identify the underlying mutation in these cells, a method based on expression cloning that does not discriminate among any of the possibilities may be used to identify the gene responsible for the mutant phenotype. A similar approach has been used to define a mutation in SREBP cleavage-activating protein (SCAP) in sterol-resistant CHO cells (Hua et al, 1996)

The present study demonstrated a role for Gβγ in ACTH receptor gene expression; however, the mechanism through which Gβγ exerts this effect remains undefined, although there are several possibilities (described above). It may be possible to dissect the signaling pathway
responsible for Gβγ-regulated expression of the ACTH receptor by identifying ACTH receptor promoter elements responsive to Gβγ, the transcription factors that interact with these elements and the modification of the transcription factors that are associated with Gβγ regulation of their activities.
V. CONCLUSION AND PROPOSED MODEL

The forskolin-resistant mutant phenotype is very complex. In these mutants, the adenylyl cyclase was partially resistant to forskolin, completely resistant to ACTH and fully responsive to fluoride. Mutant cells contain decreased levels of Gsα and Gia subunits in plasma membrane and exhibit decreased βγ activity. Results from this study demonstrated that these mutants do not express ACTH receptor transcripts, accounting for the loss of ACTH-responsive adenylyl cyclase activity in these mutants. Since forskolin-resistant cells do not express ACTH receptor and the spontaneous ACTH receptor-deficient mutants are forskolin-resistant, the results suggest that forskolin resistance and failure to express the ACTH receptor are closely linked in Y1 mouse adrenal cells.

Transfection of mutant cells with genes encoding the mouse β2AR or the human ACTH receptor restored hormone responsive adenylyl cyclase activity. In addition, the levels of Gsα and Gia were increased and approached the levels seen in parental Y1 cells. The increases in Gα were not accompanied by changes in Gα mRNA and were not secondary to changes in endogenous levels of cAMP. These results demonstrate that the decreased levels of membrane-associated Gsα and Gia are caused by the loss of ACTH receptors, and that G protein-coupled receptors are required to maintain normal levels of Gsα and Gia in the plasma membrane.

The receptor transformants and Gsα transformants remained forskolin-resistant and did not recover the ability to express endogenous ACTH receptor transcripts. Thus forskolin resistance is not caused by reduced levels of Gsα; instead, the decreased levels of Gsα seem to be secondary to the mutation affecting forskolin-resistance and the expression of ACTH receptors, rather than a primary determinant of the phenotype. The forskolin-resistant mutation appears to be the cause and not the consequence of ACTH receptor deficiency and consequent reduction in Gsα levels.

Transfection of expression vectors encoding Gβ and/or Gγ subunits into the forskolin-resistant cells resulted in the recovery of ACTH receptor transcripts, indicating that Gβγ plays a regulatory role in ACTH receptor gene expression. Inasmuch as Gβγ activity is
impaired in the mutant cells, Gβγ may be a candidate for the forskolin-resistant mutation that silences the expression of the ACTH receptor gene. Gβγ can regulate gene expression through many different mechanisms. It may also directly or indirectly affect forskolin action.

A proposed model for the interaction of ACTH receptor, Gα, Gβγ, forskolin, and adenylyl cyclase is illustrated in Figure 48. Gβγ regulates ACTH receptor gene expression. One candidate mechanism for this effect is the regulation of a signal transduction pathway modifying a protein kinase, which phosphorylates transcription factors (Fig.48, A). The expression of ACTH receptor is required to maintain the Gα levels associated with the plasma membrane (Fig.48, B). The current model for forskolin action is that forskolin acts via a ternary complex involving adenylyl cyclase, activated Gsα and forskolin. Results from this study suggest that an additional component (X), possibly linked to altered Gβγ activity, may be required for maximal activation of adenylyl cyclase by forskolin (Fig.48, C).
Figure 48. Proposed model for the interaction of ACTH receptor, Gα, Gβγ, forskolin and adenylyl cyclase. AC = adenylyl cyclase, X = a component that may link to Gβγ activity and may be important for forskolin action.
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