0026-895X/91/0702-09$03.00/0

MOLECULAR PHARMACOLOGY, 39:702-710

Expression and Characterization of the Long and Short Splice Variants of $G_{s\alpha}$ in S49 *cyc*$^-$ Cells

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Received November 15, 1990; Accepted March 13, 1991

SUMMARY

The α subunit of the guanine nucleotide-binding regulatory protein G_s mediates stimulation of adenylyl cyclase activity. This subunit, $G_{s\alpha}$, exists as two molecular weight forms, termed long and short, that differ by 14 or 15 amino acids. A physiological distinction between these two forms has yet to be defined. To compare the activities of these $G_{s\alpha}$ isoforms, long and short forms of rat $G_{s\alpha}$ were expressed in the *cyc*$^-$ variant of S49 murine lymphoma cells, which is deficient in endogenous $G_{s\alpha}$ expression. By immunoblot analysis, the level of recombinant proteins in the clones expressing the long form of $G_{s\alpha}$ was about twice that present in the clones expressing the short form of $G_{s\alpha}$ or in the S49 wild-type cells. Both recombinant $G_{s\alpha}$ proteins were sensitive to cholera toxin-catalyzed ADP-ribosylation, although the short form was labeled preferentially in both recombinant and S49 wild-type cell lines. In whole-cell assays, the clones expressing the long and short forms of $G_{s\alpha}$ and the S49 wild-type cells gave comparable responses for stimulation of cAMP accumulation after challenge with (−)-isoproterenol, cholera toxin, or forskolin. In adenylyl cyclase assays with partially purified membranes, clones expressing the long form of $G_{s\alpha}$ gave approximately twice the levels of cAMP in response to isoproterenol, guanosine-5′-*O*-(3-thio)triphosphate, NaF, or forskolin, compared with membranes from the clones expressing the short form of $G_{s\alpha}$ or the S49 wild-type cells. However, when maximal adenylyl cyclase activity was normalized to the level of $G_{s\alpha}$ protein in S49 wild-type cells, the cAMP productions were similar between all of the cell lines. In other membrane-based assays, the long and short forms of $G_{s\alpha}$ were also equivalent in their dose response to isoproterenol and GTP, their kinetics of guanine nucleotide exchange and GTPase activity, and the induced high and low affinity states of the β-adenergic receptor in response to isoproterenol. In the latter radioligand binding analysis, membranes from the two clones expressing the long form of $G_{s\alpha}$ consistently gave a greater proportion of the agonist high affinity state; however, this variation likely reflects the greater expression levels of $G_{s\alpha}$ in these membranes. Thus, we conclude that the long and short forms of $G_{s\alpha}$ expressed in S49 *cyc*$^-$ cells are very similar in their ability to stimulate adenylyl cyclase activity and to couple to β-adrenergic receptors.

G proteins are a family of homologous proteins that transduce extracellular signals from hormone or neurotransmitter receptors into cellular responses (for recent reviews, see Refs. 1–3). G proteins are heterotrimers composed of α, β, and γ subunits arranged in order of decreasing mass. The α subunits contain a GTP-binding site, have intrinsic GTPase activity, and are the substrates for covalent ADP-ribosylation catalyzed by microbial toxins. The α subunits of different G proteins share significant amino acid sequence homology, particularly in the GTP-binding and hydrolysis domains, but display diversity that is likely responsible for receptor and effector specificity. Multiple forms of the $\beta\gamma$ subunits copurify as a tightly associated heterocomplex. These $\beta\gamma$ subunits appear to be functionally interchangeable, help to anchor the G protein at the cytoplasmic face of the cell membrane, and, as recently suggested, may directly regulate effectors (4, 5).

One member of the G protein family mediates β-adrenergic receptor-dependent stimulation of adenylyl cyclase and has been termed G_s (1). Agonist interaction with the β-adrenergic receptor promotes the exchange of GTP for GDP on the α subunit of G_s and consequent dissociation of the α subunit from the $\beta\gamma$ complex. The GTP-bound α subunit associates with the catalytic unit of adenylyl cyclase to stimulate conversion of ATP into the second messenger cAMP. Cessation of adenylyl cyclase stimulation occurs when the α subunit hydrolyzes GTP to GDP and the heterotrimer is reformed. Utilizing

This work was supported in part by National Institutes of Health Grant GM 34781.

ABBREVIATIONS: G protein, guanine nucleotide-binding regulatory protein; bp, base pairs; DPBS, Dulbecco's phosphate-buffered saline; Gpp(NH)p, guanosine 5′-(β,γ-imido)triphosphate; G_s, stimulatory guanine nucleotide-binding regulatory protein; GTPγS, guanosine-5′-*O*-(3-thio)triphosphate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; ^{125}ICYP, [^{125}I]iodocyanopindolol; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate.

G_s in this manner, signals originating at the cell surface are rapidly amplified at the intracellular level.

Radioligand binding assays for β-adrenergic receptors demonstrate the existence of a heterogeneous population of two binding states for agonist in broken cells (6). The high affinity state is associated with a receptor putatively complexed with G_s at the cytoplasmic face of the cell membrane, whereas the low affinity state represents the free receptor. In the presence of agonist, all of the receptors can be converted into a homogeneous population of low affinity state by the addition of high concentrations of guanine nucleotide, which promotes the dissociation of the receptor-G_s complex. The fraction of β-adrenergic receptors that can be converted into the high affinity state as a result of agonist binding correlates with the extent of cAMP production by adenylyl cyclase (6).

There are four forms of the $G_{s\alpha}$ subunits, which result from differential mRNA splicing of a single gene, and these multiple forms are conserved between rat and human (7, 8). These four forms of $G_{s\alpha}$ differ by 14 or 15 amino acid residues, which are coded for by exon 3 of the $G_{s\alpha}$ gene (8). Both the M_r 46,000 (exon 3 present; designated long or L) and M_r 44,500 (exon 3 deleted; designated short or S) derivatives of $G_{s\alpha}$ exist as two subtypes, which differ from each other by a single serine residue, a result of an alternate splice acceptor site (8). In $G_{s\alpha_{S1}}$ and $G_{s\alpha_{L1}}$ a serine is absent and in $G_{s\alpha_{S2}}$ and $G_{s\alpha_{L2}}$ a serine is present. The long and short forms of $G_{s\alpha}$ are expressed in most cells; however, their ratios vary (9). For example, in cardiac cells the short form is more abundant, whereas in skeletal muscle the long form is the major species. The relative proportions of the S1 and S2 or the L1 and L2 isoforms in these tissues are not known.

The evolutionary conservation of multiple forms of $G_{s\alpha}$ suggests differential functional roles of these proteins and has raised the question of why both forms of $G_{s\alpha}$ continue to be expressed in many different tissues. Studies with partially purified long and short forms of $G_{s\alpha}$ from rabbit liver indicated that the long form of $G_{s\alpha}$ reconstituted hormone-stimulated adenylyl cyclase more efficiently and was activated by GTP analogs more rapidly (10, 11). Similar results were reported for recombinant long and short forms of $G_{s\alpha}$ expressed in *Escherichia coli*. (12, 13). However, the adenylyl cyclase activity in reconstituted membranes was 1 order of magnitude lower for recombinant as compared with native $G_{s\alpha}$. This decreased adenylyl cyclase activity was attributed to a posttranslational modification of $G_{s\alpha}$ that was not supplied in bacteria and that is essential for high affinity interaction of the G protein with adenylyl cyclase.

The present study was initiated to investigate differences between the long and short forms of $G_{s\alpha}$ in stimulating adenylyl cyclase, using $G_{s\alpha}$ expression in a mammalian system. A long and short form of $G_{s\alpha}$ were expressed in S49 *cyc*$^-$ cells, a line lacking endogenous $G_{s\alpha}$ (14), and a detailed biochemical and kinetic comparison of their activities was performed utilizing both whole-cell and membrane-based assays. In comparison with bacterially produced $G_{s\alpha}$, this system should provide any posttranslational modifications required for proper $G_{s\alpha}$ function. A similar approach was recently described by Jones *et al.* (15).

Materials and Methods

Reagents

[α-^{32}P]ATP, [^{32}P]NAD, and ^{125}ICYP were purchased from New England Nuclear. Forskolin and pyruvate kinase were purchased from Calbiochem. GTPγS was from Boehringer Mannheim Biochemicals, and cholera toxin was obtained from List Biological Laboratories, Inc. All other biochemicals were purchased from Sigma.

Cell Lines

The murine S49 wild-type and *cyc*$^-$ cells were propagated in Dulbecco's modified Eagle's medium (Difco), supplemented with 10% heat-inactivated horse serum (Difco), in an atmosphere of 5% CO_2 at 37° (11, 12). DPBS used to wash the cells did not contain calcium or magnesium.

Vector Construction

The methods utilized in the construction of the $G_{s\alpha}$ expression vectors were described by Maniatis *et al.* (16). In all cases, extensive restriction analysis was used to confirm each of the constructs. The plasmids pGEM2Gsar and pGEM2Gsar/sh (17), containing the $G_{s\alpha_{L1}}$ and $G_{s\alpha_{S2}}$ cDNAs, respectively, were kindly provided by R. Reed (John Hopkins). The parental expression vector, DZBApal, is a derivative of the pDSP1 expression vector described by Pfarr *et al.* (18), with the bacterial *galK* gene inserted between the SV40 early gene promoter and bovine growth hormone polyadenylation signal sequences. For the construction of Dzb$G_{s\alpha}^+$, pGEM2Gsar was digested with *Afl*II and end-filled with the Klenow fragment of DNA polymerase. The 1506-bp fragment containing the $G_{s\alpha}$-coding sequence was excised by digestion with *Hind*III and inserted into DZBApal between a filled-in *Eco*RI site and a *Hind*III site, thereby replacing the *galK* gene. The resulting plasmid, Dzb$G_{s\alpha}^+$ (6223 bp), was used in the transfections that produced the $G_{s\alpha}$L1.1 cell line. A derivative of this vector, Dzb$G_{s\alpha}$, was used in the transfections that produced the $G_{s\alpha}$L1.2 cell line. Dzb$G_{s\alpha}$ differs from Dzb$G_{s\alpha}^+$ by a deletion of 279 bp in the 5′-untranslated region of the $G_{s\alpha}$ cDNA, which was created to remove some of the noncoding sequence between the promoter and the ATG translation initiation codon. For construction of Dzb$G_{s\alpha}$sh, pGEM2Gsar/sh was digested with *Nco*I and *Bgl*II to generate an 841-bp fragment that contains the first two thirds of the $G_{s\alpha}$-coding sequence, encompassing the region where the splice variants occur. This fragment was used to replace the corresponding region in Dzb$G_{s\alpha}$. Dzb$G_{s\alpha}$sh was used to produce both the $G_{s\alpha}$S2.1 and $G_{s\alpha}$S2.2 cell lines.

DNA Transfection

The $G_{s\alpha}$ expression vectors were introduced in S49 *cyc*$^-$ cells by a modification of the electroporation procedure described by Nukada *et al.* (19). Cells were harvested in late logarithmic stage, at an approximate concentration of 1×10^6 cells/ml, and washed twice in DPBS before electroporation. In a typical transfection, $1–5 \times 10^7$ S49 *cyc*$^-$ cells were suspended in 0.5 ml of DPBS and incubated on ice for 30 min. The cells were then mixed with 25 μg of *Apa*I-digested vector DNA and immediately subjected to a short electrical pulse of 750 V, using a Bio-Rad GenePulser electroporator. Capacitance was set to the maximum of 25 μF. Cells were placed on ice for 10 min to recover from the electrical shock and then diluted to 5 ml with medium. After 24 hr in culture, cell viability was 5–10%, as assessed by exclusion of 0.4% trypan blue.

Clone Selection

For clonal selection after electroporation, cells were seeded in 96-well plates at a concentration of approximately 2000 viable cells/200 μl of Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated horse serum, 20% conditioned medium, and 750 μg/ml G418 (45–55% active compound; Difco). Every 2–3 days, 50% of the culture medium was replaced. In 2–3 weeks, several G418-resistant clones were observed, and these were carefully expanded without conditioned medium. After the clones were in culture for at least 1 month and $G_{s\alpha}$ expression was verified (see below), the concentration of G418 was decreased to a maintenance dose of 250 μg/ml.

DNA Blots

High molecular weight DNA was isolated from each of the cell clones, using the procedure described by van der Straten *et al.* (20). The entire 1300-bp $G_{s\alpha}$ cassette (Fig. 1) was nick-translated with α-^{32}P-nucleotides (ICN) and used to probe the blots according to the procedure of Southern (21). Quantitation of the copy number was assessed by comparison with known amounts of $G_{s\alpha}$ insert.

Immunoblots

Immunoblots were performed essentially as described (22). Typically, 10–20 μg of membrane protein were electrophoresed on SDS-PAGE gels (23), transferred to nitrocellulose, and incubated with a 1:500 or 1:1000 dilution of the anti-$G_{s\alpha}$ antibodies. Bands were visualized by autoradiography, after incubation of the blots with ^{125}I-Protein A (ICN). Gels exposed to autoradiography were scanned with an LKB Ultroscan XL laser densitometer, and the $G_{s\alpha}$ expression levels of the clones were normalized to the expression of the total long plus short forms of $G_{s\alpha}$ found in wild-type cells. Rabbit polyclonal antiserum was prepared against the long form of $G_{s\alpha}$, essentially as described for other proteins, using $G_{s\alpha}$ fused to the C-terminus of the first 81 amino acids of the influenza NS1 protein (24, 25). This antiserum did not cross-react with any other membrane protein from S49 *cyc*$^-$ cells, including $G_{i\alpha}$, but recognized both the long and short forms of $G_{s\alpha}$.

Cholera Toxin-Catalyzed ADP-Ribosylation

Cholera toxin-catalyzed ADP-ribosylation of $G_{s\alpha}$ proteins in membranes has been described previously (26). In our reactions, 15–25 μg of membrane protein were labeled with approximately 1 μCi of [^{32}P] NAD, in the presence of dithiothreitol-activated cholera toxin. The final concentration of the cholera toxin was 50 μg/ml. Reactions were then run on SDS-PAGE, dried, and exposed for autoradiography.

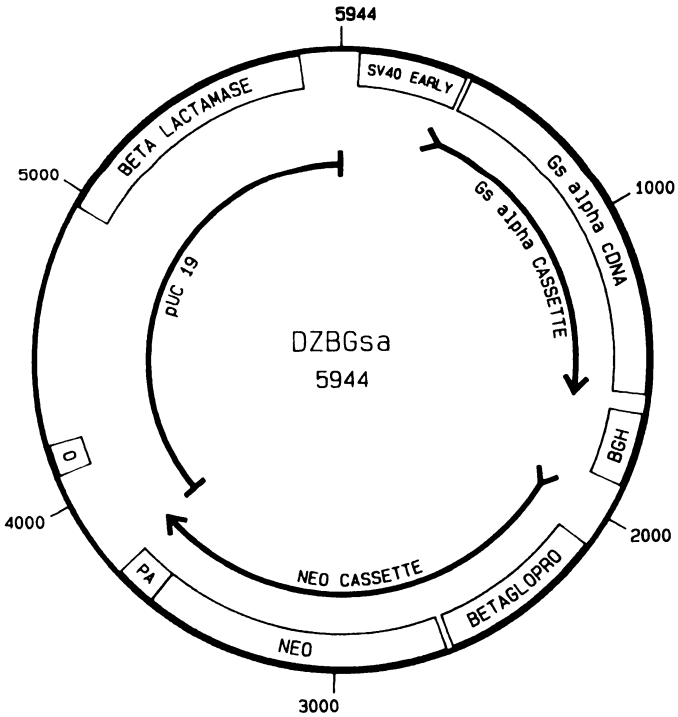

Fig. 1. Mammalian expression vector. $G_{s\alpha}$ cDNAs were inserted into the vector between the SV40 early gene promoter and the bovine growth hormone polyadenylation signal sequences. *BETAGLOPRO*, human β-globin promoter; *NEO*, neomycin phosphotransferase gene; *PA*, SV40 polyadenylation signal sequences; *O*, bacterial origin of replication. $DzbG_{s\alpha}$ is 5944 bp long and codes for $G_{s\alpha_L}$. Note that the vector used to generate the L1.1 cell line had an additional 279 bp of noncoding sequence between the promoter and the translation initiation ATG (see text).

Whole-Cell Assays

The accumulation of cAMP in whole cells was quantitated by using a Gammaflo automated radioimmunoassay instrument, as described in the manufacturer's instructions (27). Each reaction included 1×10^6 cells and was incubated for 10 min at 37°, except for that with cholera toxin, which was incubated for 90 min. All of the reactions contained 1 mM levels of the phosphodiesterase inhibitor isobutylmethylxanthine. For the steady state assays, the final concentrations of the stimulators were as follows: basal, no activator; (−)-isoproterenol, 10 μM; cholera toxin, 500 ng/ml; and forskolin, 100 μM. The concentrations of isoproterenol (1 nM to 10 μM) or forskolin (100 nM to 100 μM) were varied accordingly for the dose-response analysis.

Membrane Preparations

Membranes were isolated from each of the clones, using a modification of the procedure of Ross *et al.* (28). The 20–30% and 30–40% sucrose gradients were combined into one 10–40% step. Final concentrations of the membrane proteins ranged from 1.5 to 7.5 mg/ml. For screening of potential $G_{s\alpha}$-expressing clones, the procedure was adapted as follows. Approximately 30 ml of cells, at a density of $1–2 \times 10^6$ cells/ml, were pelleted at 500–600 × *g* for 5 min at 4°. The cell pellet was washed twice with DPBS and resuspended in 2 ml of lysis buffer (5 mM Na-HEPES, 1 mM $MgSO_4$, 1 mM EDTA, pH 7.5). Cells were placed on ice for 10 min and then lysed with 10–20 strokes in a 2-ml Dounce homogenizer. The nuclei and unbroken cells were removed by centrifugation at 900 × *g* for 5 min at 4°. The membranes were pelleted at 39,000 × *g* for 20 min at 4° and resuspended in 100–200 μl of membrane buffer (20 mM Tris·HCl, 1 mM EDTA, 1 mM dithiothreitol, 250 mM sucrose, pH 7.5). Samples were then analyzed by immunoblotting for $G_{s\alpha}$ expression, using 20–25 μg of protein/lane. Protein concentration in the membranes was quantitated by the method of Bradford (29), using bovine serum albumin as the standard. Relative concentrations were confirmed visually after Coomassie staining of SDS-PAGE gels.

Membrane-Based Assays

Adenylyl cyclase assays using 10 μg of membrane protein were carried out at 30°, as described by Salomon *et al.* (30) and modified by Hoffman *et al.* (31).

Steady state assays. For the steady state assays, the reaction time was 15 min and concentrations of the stimulators were as follows: basal, no activator; isoproterenol, 100 μM; GTPγS, 100 μM; NaF, 10 mM; and forskolin, 10 μM. The concentrations of isoproterenol (0.5 nM to 100 μM), GTP (1 nM to 100 mM), or Mg^{2+} (0–100 mM) were varied accordingly for the dose-response analysis.

Kinetics of k_{on}. Membranes were preincubated for 5 min at 30°. At $t = 0$ 0.1 mM GTPγS and [α-^{32}P]ATP were added to the reaction, and aliquots were removed at 1- (up to 10 min) or 2-min (after 10 min of elapsed time) intervals (32). Data were fit to the equation: pmol of cAMP = $(V_{max}t) - (V_{max}/k_{on})(1 - e^{-k_{on}t})$, where t is the time in min, V_{max} is the slope of the line at infinite time, and k_{on} is the x-intercept, which represents 1/lag time, an indirect measure of GDP/GTP exchange (33).

Kinetics of k_{off}. Membranes were preincubated for 5 min at 30° with nonradioactive ATP, in the presence or absence of isoproterenol (100 μM) to activate adenylyl cyclase production of cAMP (34, 35). At $t = 0$, the β-adrenergic receptor antagonist (−)-alprenolol (10 μM) and [α-^{32}P]ATP, or [α-^{32}P]ATP alone, were added. Aliquots were removed at 10-sec intervals. Data were fit to the equation: pmol of cAMP = $(V_o/k_{off})(1 - e^{-k_{off}t})$, where t is the time in sec, V_o is the initial rate of adenylyl cyclase stimulation (calculated from the data with isoproterenol alone), and k_{off} is a measure of the GTPase reaction rate (34).

Receptor Binding Assays

Radioligand binding assays for β-adrenergic receptors in membrane preparations, using the specific radiolabled antagonist ^{125}ICYP, were performed as previously described (36). ^{125}ICYP (40 pM) was incubated with 3 μg of membrane protein and varying concentrations of isopro-

terenol (0.5 nM to 100 μM), in the absence and presence of 300 μM Gpp(NH)p or GTPγS, for 60 min at 37°. Data were analyzed by computer-assisted nonlinear least squares fitting, using the LUNDON 2 software program (Lundon Software, Inc., Cleveland, OH).

Results

Expression of $G_{s\alpha}$ Subtypes in S49 cyc⁻ Cells

For comparative analysis of the long and short subtypes of $G_{s\alpha}$, vectors containing cDNAs encoding these proteins were constructed for expression in mammalian cells (Fig. 1). DZBG$_{s\alpha}$ encodes for the long form of $G_{s\alpha}$ ($G_{s\alpha_{L1}}$), which lacks a serine residue at the splice junction of exons 3 and 4 of the $G_{s\alpha}$ gene. DZBG$_{s\alpha}$sh encodes for the short form of $G_{s\alpha}$ ($G_{s\alpha_{S3}}$), which includes the serine residue at the splice junction of exons 2 and 4 where exon 3, coding for only 14 amino acid residues, is deleted. Vectors also carry the neomycin phosphotransferase gene, which confers resistance to the antibiotic G418 for selection of stable transformants in eukaryotic cells.

The cDNAs encoding the $G_{s\alpha}$ proteins were transfected by electroporation into the *cyc*⁻ variant of the S49 murine lymphoma cell line. G418-resistant clones were expanded and assayed for expression of the transfected rat $G_{s\alpha}$ proteins. The $G_{s\alpha}$ proteins were detected by whole-cell cAMP accumulation assays (see below), cholera toxin-catalyzed [^{32}P]ADP-ribosylation of crude membrane preparations, and immunoblot analysis using a rabbit polyclonal antiserum raised against a purified $G_{s\alpha}$ (long) fusion protein expressed in *E. coli* (see Materials and Methods). Approximately half of the G418-resistant cell lines expressed $G_{s\alpha}$. The copy number of the transfected cDNA sequences was determined by Southern blot analysis of high molecular weight DNA isolated from each of the clones. A unique fragment, corresponding to the entire $G_{s\alpha}$ cassette, was observed in each of the long and short $G_{s\alpha}$ cell lines (results not shown). This fragment was not present in DNA isolated from either the S49 wild-type or *cyc*⁻ cell lines. The transfected cell lines contained one to three copies of $G_{s\alpha}$ cDNA/cell.

Whole-Cell Studies

Cell clones expressing $G_{s\alpha_L}$ (L1.1 and L1.2) and $G_{s\alpha_S}$ (S2.1 and S2.2) were expanded for a more detailed analysis of adenylyl cyclase stimulation. Adenylyl cyclase activity was measured using a whole-cell cAMP accumulation assay, in the presence of the phosphodiesterase inhibitor isobutylmethylxanthine. Cells were treated with the adenylyl cyclase activators (−)-isoproterenol, cholera toxin, or forskolin, and the accumulation of cAMP was measured. Isoproterenol stimulates adenylyl cyclase through the β-adrenergic receptor and stimulation is dependent on $G_{s\alpha}$ mediation. Cholera toxin specifically ADP-ribosylates $G_{s\alpha}$, resulting in inhibition of the intrinsic GTPase activity and consequent continuous activation of adenylyl cyclase. Forskolin is a direct activator of adenylyl cyclase, an effect that is enhanced in the presence of $G_{s\alpha}$. Production of cAMP in clones expressing the long and short forms of $G_{s\alpha}$ were similar to each other and to the S49 wild-type cells for each of the three stimulators (Fig. 2). Overall, the relative responses under these conditions were forskolin > cholera toxin > isoproterenol. A dose-response analysis was carried out with isoproterenol and forskolin. Each of the cell lines showed a similar concentration-dependent stimulation of adenylyl cyclase for each activator (data not shown). We conclude from these whole-cell assays that the transfected $G_{s\alpha}$ cDNAs express

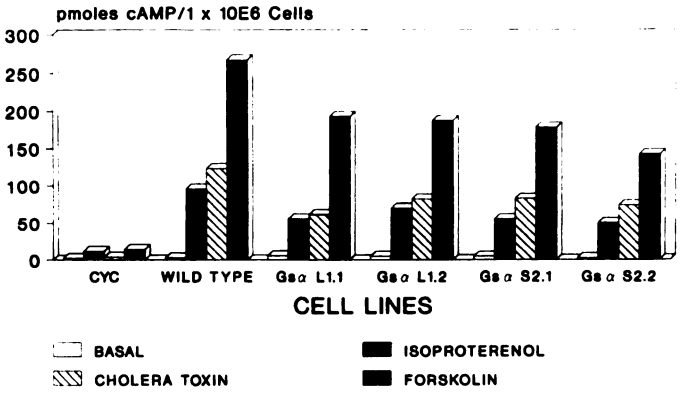

Fig. 2. Whole-cell cAMP accumulations. Cells (1×10^6) were incubated for 10 min (90 min for cholera toxin) at 37° with no drug (basal), 10 μM isoproterenol, 500 ng/ml of cholera toxin, or 100 μM forskolin. cAMP accumulations were determined using an automated radioimmunoassay procedure. *Data points* represent the average of three experiments done in duplicate, and the interexperimental variability was ≤10%.

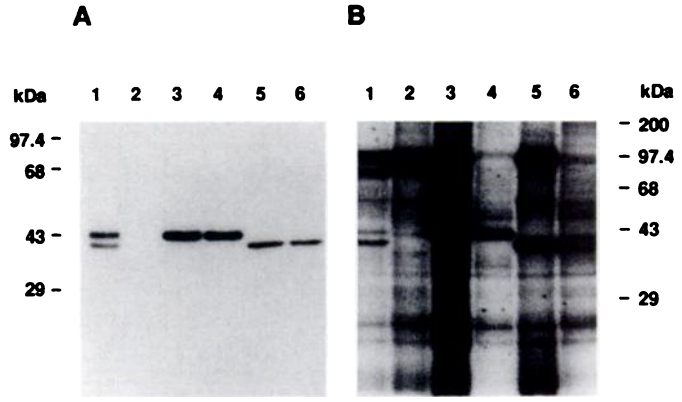

Fig. 3. *A*, Immunoblot of $G_{s\alpha}$ expression. Ten micrograms of partially purified membrane protein were run on an SDS-PAGE gel, transferred to nitrocellulose, and reacted with a 1:1000 dilution of anti-$G_{s\alpha}$ antibodies. $G_{s\alpha}$ antibodies were detected with ^{125}I-Protein A. The apparent molecular weights of the long and short forms of $G_{s\alpha}$ (44,000 and 40,000, respectively) comigrate with the internal controls of the long and short forms of $G_{s\alpha}$ found in the S49 wild-type cells. *Lane* 1, wild-type; *lane* 2, *cyc*⁻; *lane* 3, L1.1; *lane* 4, L1.2; *lane* 5, S2.1; *lane* 6, S2.2. *B*, ADP-ribosylation of $G_{s\alpha}$. Fifteen micrograms of membrane protein were subjected to cholera toxin-catalyzed [^{32}P]ADP-ribosylation, run on an SDS-PAGE gel, dried, and exposed for autoradiography. Lanes were the same as in A.

a functional protein that restores β-adrenergic receptor coupling and direct G protein-dependent stimulation of adenylyl cyclase in S49 *cyc*⁻ cells and that the long and short forms of $G_{s\alpha}$ are similarly competent in this process.

Membrane Studies

Steady state assays. Membrane were prepared from the four cell lines expressing $G_{s\alpha}$, to more closely examine the biochemical properties of the long and short forms of $G_{s\alpha}$. The relative amounts of $G_{s\alpha}$ in the membrane preparations were quantitated by immunoblot analysis and by cholera toxin-catalyzed ADP-ribosylation. Immunoblots with antiserum prepared against a recombinant $G_{s\alpha}$ protein demonstrated that the long and short forms of $G_{s\alpha}$ had apparent molecular weights of 44,000 and 40,000 and co-migrated with the authentic long and short forms of $G_{s\alpha}$ from S49 wild-type membranes (Fig. 3A). $G_{s\alpha}$ levels in the two representative L1 cell lines were 2–3-fold greater than the total $G_{s\alpha}$ protein (long plus short forms) in

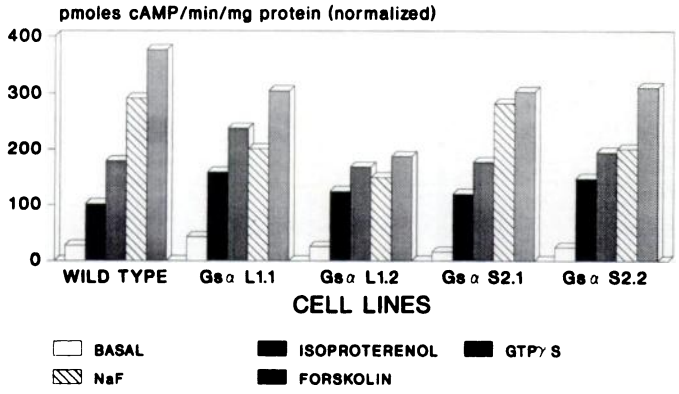

Fig. 4. Adenylyl cyclase assays. Adenylyl cyclase activators were added to 10 μg of membrane protein for 15 min at 30° with no activator (basal), 100 μM isoproterenol, 100 μM GTPγS, 10 mM NaF, or 10 μM forskolin. cAMP was determined by standard chromatographic methods (see Materials and Methods). *Data points* represent the mean of at least two experiments done in duplicate. The variability among experiments was ≤20%. Data were normalized to $G_{s\alpha}$ expression, as determined by immunoblot analysis (see text). For this set of experiments the normalization factors were: wild-type = 1.0, L1.1 = 2.35, L1.2 = 2.65, S2.1 = 1.03, and S2.2 = 0.78.

S49 wild-type cells, whereas in the two representative S2 clones the levels were about equivalent to the total $G_{s\alpha}$ protein in S49 wild-type cells. Comparable results were obtained with antipeptide antibodies specific for $G_{s\alpha}$ (data not shown). In contrast, cholera toxin-mediated ADP-ribosylation gave a greater relative abundance of $G_{s\alpha_S}$ in both the S49 wild-type and the transfected cell lines, indicating that this form is ADP-ribosylated more efficiently than $G_{s\alpha_L}$ (Fig. 3B).

Membranes were first assessed for their response to the β-adrenergic receptor agonist isoproterenol. Membranes from L1 clones stimulated the production of approximately twice the levels of cAMP, compared with membranes from either the S2 or wild-type cells. However, when the data were normalized to the relative level of $G_{s\alpha}$ expression in the S49 wild-type cells, the maximal responses were similar in membranes prepared from L1, S2, and wild-type clones (Fig. 4). Forskolin, GTPγS (a nonhydrolyzable GTP analog), and NaF (a direct stimulator of $G_{s\alpha}$ in membrane-based assays) also stimulated adenylyl cyclase production of cAMP to equivalent levels in each of the membranes preparations, following normalization of the data to the level of $G_{s\alpha}$ (Fig. 4). Dose-response analyses for isoproterenol, GTP, and Mg^{2+} were carried out on membranes from L1 and S2 clones and the S49 wild-type cells. With isoproterenol, the EC_{50} values were similar for the L1 and S2 clones, 54 and 88 nM, respectively (Fig. 5). The S49 wild-type membranes used in this study had an unusually high EC_{50} value of 250 nM; others reported EC_{50} values closer to the values found for the long and short $G_{s\alpha}$ membranes (15, 37). The EC_{50} estimates for GTP dependence were essentially identical for the L1, S2, and S49 wild-type cells (Table 1). The Mg^{2+} dependence of adenylyl cyclase stimulation by isoproterenol was also comparable for all three cell lines (data not shown). Thus, these steady state experiments did not reveal any difference between the long and short $G_{s\alpha}$ proteins.

Kinetic assays. In a recent study with recombinant $G_{s\alpha}$ proteins produced in bacteria, Graziano *et al.* (13) reported a 2-fold greater rate of GDP/GTP exchange for the long form of $G_{s\alpha}$. We thus asked whether the $G_{s\alpha}$ proteins expressed in the S49 *cyc*$^-$ cells exhibited this same difference. In the absence of β-adrenergic receptor agonist, the nonhydrolyzable GTP analogs GTPγS and Gpp(NH)p stimulate adenylyl cyclase with a lag time representative of the dissociation of GDP and the binding of the GTP analog (32, 33). Agonist binding to the β-adrenergic receptor abolishes this lag time, as a consequence of

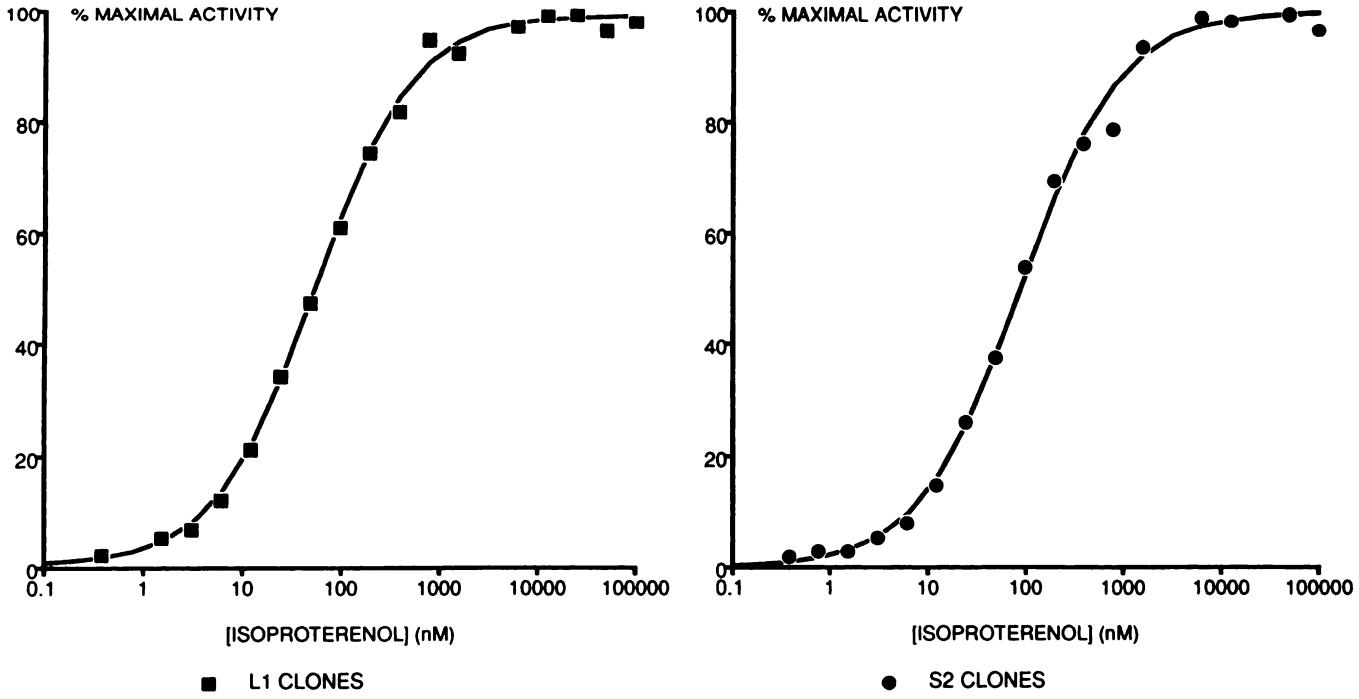

Fig. 5. Dose response with isoproterenol. Varying concentrations of isoproterenol were added to 10 μg of membrane protein for 15 min at 30°. cAMP was determined by standard chromatographic methods (see Materials and Methods). *Data points* represent the average of experiments using individual $G_{s\alpha_L}$ and $G_{s\alpha_S}$ clones, done in triplicate.

TABLE 1

Kinetic and receptor binding parameters

EC_{50} values for isoproterenol (Fig. 5) and GTP (dose response curve not shown) were calculated from dose response analysis of partially purified membranes. Each *data point* represents the average of triplicate (isoproterenol) or duplicate (GTP) determinations of a representative experiment. The k_{on} and k_{off} kinetic constants from 10 μg of membrane protein were determined as described in Materials and Methods and the legends to Figs. 6 and 7. Receptor binding constants were determined as described in Materials and Methods. Values represent the results of nonlinear least squares fitting, utilizing the LUNDON 2 software program, of a representative experiment done in duplicate. All values are representative of two or three experiments.

	Wild-type	L1.1	L1.2	S2.1	S2.2
Adenylyl cyclase kinetic parameters					
EC_{50}, isoproterenol (nM)	250	72	45	89	87
EC_{50}, GTP (μM)	0.23	0.13	0.18	0.13	0.12
k_{on} (min^{-1})	0.14	0.16	0.16	0.12	0.12
k_{off} (min^{-1})	ND[a]	2.2	2.2	2.9	2.3
β-Adrenergic receptor binding parameters					
−Gpp(NH)p (control)					
K_H (nM)	14	18	11	14	5
K_L (nM)	794	1070	583	773	301
R_H (%)	35	58	53	31	22
+Gpp(NH)p (300 μM)					
K_L (nM)	520	419	300	748	250

[a] ND, not done.

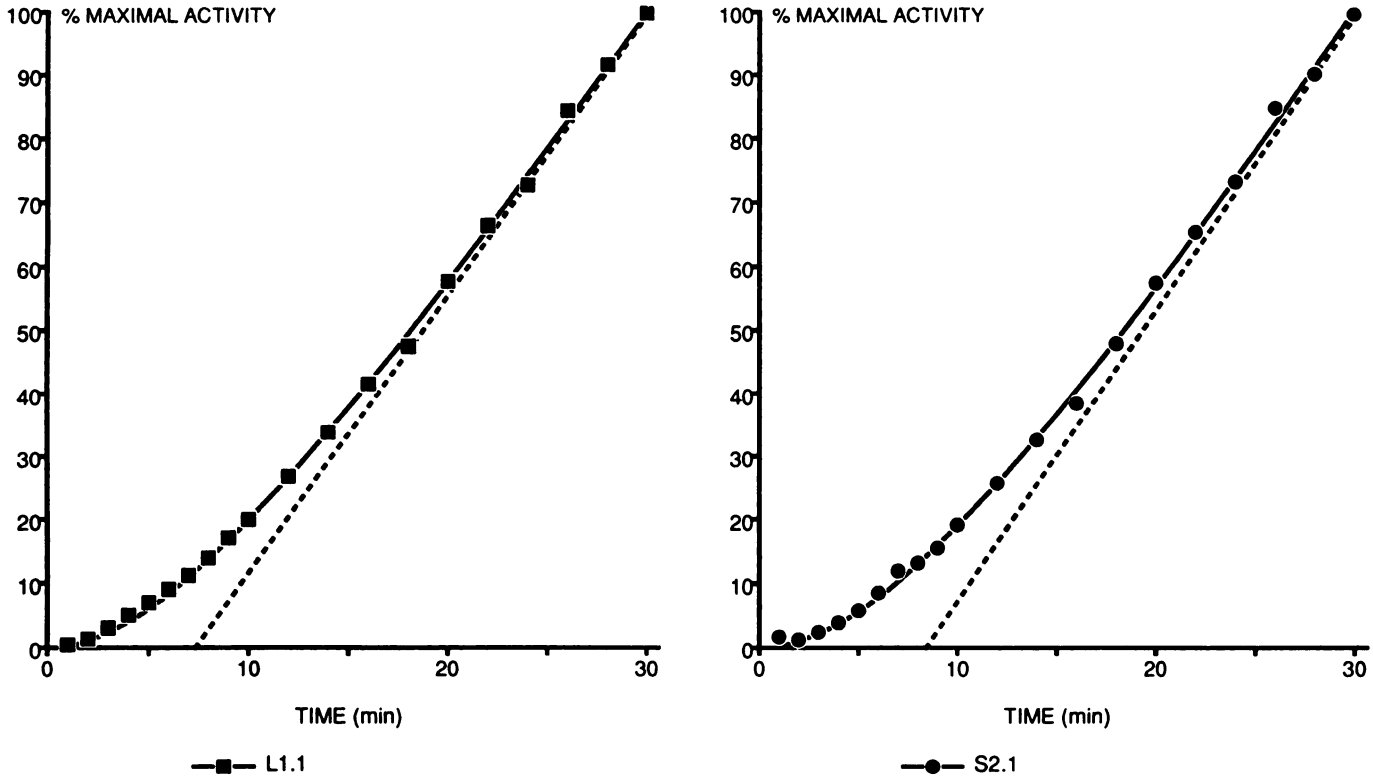

Fig. 6. Kinetics of k_{on}. Membranes (10 μg) were preincubated for 5 min at 30°. At $t = 0$ GTPγS (0.1 mM) was added, and aliquots were taken at the indicated times. cAMP was determined by standard chromatographic methods (see Materials and Methods). *Data points* are the mean of duplicate determinations from a representative experiment.

receptor-induced nucleotide exchange. In our studies, the hysteretic stimulation of adenylyl cyclase mediated by the $G_{s\alpha}$ subtypes was assessed using 50 μM GTPγS in the absence of a receptor agonist. The membranes from the L1, S2, and wild-type cell lines each showed similar kinetics for GTPγS-stimulated adenylyl cyclase activation (Fig. 6). This similarity is reflected in the close agreement of the k_{on} values for each of the cell lines (Table 1). Thus, the GDP exchange rates we observed in the absence of agonist are similar for membranes prepared from the L1, S2, and wild-type cell lines.

The intrinsic GTPase activity of the α subunits leads to the termination of adenylyl cyclase stimulation. The k_{off} for the GTPase activity was measured indirectly from the decay of isoproterenol- plus GTP-activated adenylyl cyclase activity upon antagonist binding to the β-adrenergic receptor (34, 35). Membranes from L1, S2, and S49 wild-type cells were incubated in the presence of isoproterenol and GTP to establish a steady state activation of adenylyl cyclase. At $t = 0$, the β-adrenergic antagonist alprenolol and ^{32}P-labeled ATP were added to the isoproterenol-preactivated system, to prevent further stimulation of adenylyl cyclase. Each of the membranes responded similarly, as shown in Fig. 7. The k_{off} for the GTPase reaction was calculated from the decay of cAMP formation for each of the membranes (see Materials and Methods). Calculated values

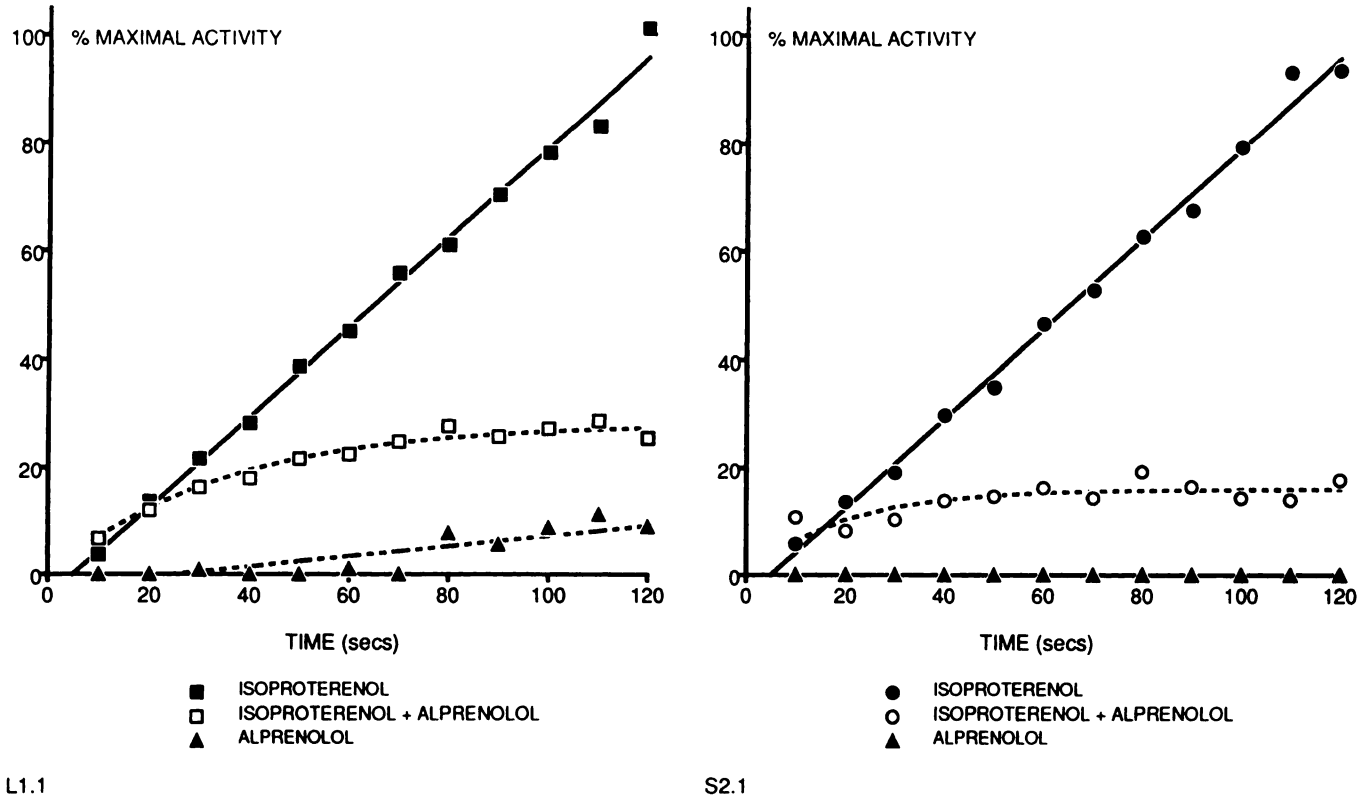

Fig. 7. Kinetics of k_{off}. Membranes (10 μg) were preincubated at 30° for 5 min with nonradioactive ATP, in the presence or absence of isoproterenol (100 μM). At t = 0 alprenolol (10 μM) and [α-^{32}P]ATP, or [α-^{32}P]ATP alone, were added, and aliquots were removed at the indicated times. cAMP was determined by standard chromatographic methods (see Materials and Methods). Data points are the mean of duplicate determinations from a representative experiment.

for the k_{off} reaction for each of the membrane preparations were equivalent, thus indicating that the long and short forms of $G_{s\alpha}$ do not differ in their intrinsic GTPase activity (Table 1). We conclude from these studies that under our conditions the long and short forms of $G_{s\alpha}$ are very similar, if not identical, in their kinetics of GDP/GTP exchange and intrinsic GTPase activity.

Receptor Binding Studies

To investigate agonist binding to the β-adrenergic receptor, varying concentrations of (−)-isoproterenol were used to compete with the high affinity antagonist ^{125}ICYP for the β-adrenergic receptor. Both the long and short forms of $G_{s\alpha}$ couple with the β-adrenergic receptor in the presence of an agonist and promote the formation of a high affinity receptor-G_s protein complex. When excess amounts of Gpp(NH)p (300 μM) were added to the binding assays, virtually all of the high affinity binding sites were converted into low affinity binding sites, as expected (Table 1). Similar results were obtained when GTPγS (300 μM) was used as the guanine nucleotide (data not shown). Receptors from the membranes of the $G_{s\alpha}$ long and short clones have comparable binding constants for the high (K_H) and low (K_L) affinity states, which are similar to the values for membranes from the S49 wild-type cells (Table 1). In the absence of added GTP analogs, the greater fraction of high affinity state (R_H) for the L1 clones likely reflects the higher level of $G_{s\alpha}$ protein in these membranes (Table 1). From these receptor binding studies, we conclude that the long and short forms of $G_{s\alpha}$ couple to the β-adrenergic receptor in a similar manner.

Discussion

We conducted a biochemical analysis of long and short forms of $G_{s\alpha}$, in an attempt to identify a differential role of these proteins in mediating stimulation of adenylyl cyclase. For this purpose, recombinant forms of rat $G_{s\alpha}$ were expressed in the murine S49 *cyc*$^-$ cell line, which lacks endogenous $G_{s\alpha}$. Of the four putative splice variants of $G_{s\alpha}$, the L1 and S2 forms were selected because they appear with greater frequency in both rat (17) and human (38) cDNA libraries. Both L1 and S2 proteins were localized to the cell membrane and were sensitive to activation by cholera toxin-catalyzed ADP-ribosylation. Clones expressing L1 or S2 responded similarly to isoproterenol, forskolin, or cholera toxin in whole-cell assays of cAMP accumulation. Biochemical parameters affecting $G_{s\alpha}$ stimulation of adenylyl cyclase and β-adrenergic receptor ligand binding assays were addressed using partially purified membrane preparations of the L1 and S2 cell lines. In steady state adenylyl cyclase assays, the L1 membranes consistently generated a greater response to a β-adrenergic agonist and to activators of $G_{s\alpha}$. However, when $G_{s\alpha}$ levels were normalized to the levels of $G_{s\alpha}$ in S49 wild-type cells, the long and short form activities were comparable. Indirect kinetic assays indicated that L1 and S2 had similar rates of guanine nucleotide exchange and hydrolysis. In ligand binding assays of β-adrenergic receptors, similar binding constants of agonist for the high and low affinity states were observed in the membranes isolated from the L1 and S2 cell lines. Although the agonist appeared to promote a larger fraction of high affinity state in membranes from L1 cells, this difference likely results from the higher level

of $G_{s\alpha}$ in these membranes. We, thus, conclude from these studies that the long and short forms of $G_{s\alpha}$ are similarly competent in coupling to β-adrenergic receptors and to the adenylyl cyclase catalytic unit.

Although forskolin can serve as a direct activator of adenylyl cyclase, previous studies with variants of S49 cells indicated that the stimulation was dependent on the presence of a functional $G_{s\alpha}$ protein (39). Our results confirm these observations. In whole-cell assays, cAMP accumulation in response to forskolin was dramatically increased to S49 wild-type levels in $G_{s\alpha_L}$ and $G_{s\alpha_S}$ cell lines, compared with S49 *cyc*$^-$ cells.

Nukada *et al.* (19) reported that transfected S49 *cyc*$^-$ cells expressing low levels of $G_{s\alpha}$ showed a corresponding low response to various activators of adenylyl cyclase. In membrane-based assays, we also observed an apparent correlation between cyclase activity and the level of $G_{s\alpha}$ protein. However, whole-cell measurements of cAMP accumulation did not reflect this difference. This distinction may only reflect technical differences between the two assay procedures. Alternatively, whole-cell assays are intrinsically more complicated and, at these levels of $G_{s\alpha}$ expression, compensating mechanisms may exist within the intact cell that regulate cAMP metabolism in response to activators of adenylyl cyclase.

Previous studies with recombinant $G_{s\alpha}$ expressed in bacteria showed that the rate of dissociation of GDP was 2–2.5-fold more rapid for $G_{s\alpha_L}$ than for $G_{s\alpha_S}$ and that this difference was also reflected in reconstituted adenylyl cyclase assays (12, 13). However, these results may also reflect the relative efficiency of reconstitution of the bacterially produced proteins with other components of the adenylyl cyclase system. In our indirect assays of nucleotide exchange, we observed no difference in the GDP off rate for the long and short forms of $G_{s\alpha}$ (Fig. 6). It should be noted that a 2-fold differential would be at the detection limits of our assay and, thus, we cannot rule out such modest differences.

While our study was in progress, a related comparison of $G_{s\alpha_L}$ and $G_{s\alpha_S}$ was described by Jones *et al.* (15). These authors reported minor differences between the L1 and S2 forms of rat $G_{s\alpha}$; 1) $G_{s\alpha_{SS}}$ appeared to respond more rapidly to agonist-bound receptor, as inferred from a comparison of isoproterenol-stimulated adenylyl cyclase activation with receptor-independent adenylyl cyclase stimulation, and 2) $G_{s\alpha_L}$ either interacted more efficiently with adenylyl cyclase or had a slower rate of GTP hydrolysis than $G_{s\alpha_S}$. In contrast, we did not observe any differences between the short and long forms of $G_{s\alpha}$ in coupling adenylyl cyclase activity to agonist-bound receptors, nor did we observe a difference between the two forms in their ability to interact with adenylyl cyclase or in their rates of GTP hydrolysis (Fig. 7 and Table 1). We suggest that their observations may reflect methodological differences in the relative quantitation of the L1 and S2 proteins. Their quantification was based on the assumption that the long and short forms of $G_{s\alpha}$ were modified by cholera toxin in an equivalent manner, but, as shown in Fig. 3, cholera toxin appears to ADP-ribosylate $G_{s\alpha_S}$ more efficiently than $G_{s\alpha_L}$.

Receptor binding assays demonstrated that the interactions of the individual $G_{s\alpha}$ proteins with the β-adrenergic receptor were very similar. The calculated binding constants for the L1 and S2 membranes were the same within experimental error, in agreement with other studies in similar systems (15, 40, 41). However, we consistently observed a greater fraction of agonist-promoted high affinity state of the receptors with the L1 membranes, which we ascribe to the higher levels of $G_{s\alpha}$ in these membranes. This difference in high affinity state receptors correlates well with the 2-fold greater levels of adenylyl cyclase stimulation in steady state assays and with a previous report by Kent *et al.* (6), which indicated that the ability of an agonist to activate adenylyl cyclase parallels the fraction of high affinity state achieved in the presence of the agonist.

Recent studies have shown that the α subunits of G_s interact with additional effector systems other than adenylyl cyclase. $G_{s\alpha}$ was recently implicated in the regulation of dihydropyridine-sensitive Ca^{2+} channels in cardiac and skeletal muscle (42, 43). Using patch-clamp studies, recombinant L1 and S2 proteins produced in *E. coli* were observed to mediate the opening of Ca^{2+} channels in an equivalent manner (43). However, as observed for adenylyl cyclase stimulation, the recombinant $G_{s\alpha}$ proteins were less effective, on a molar basis, than isolated mammalian subunits in eliciting this response. G_s has also been implicated in β-adrenergic inhibition of Mg^{2+} uptake (44) and in β-adrenergic inhibition of Na^+ currents in cardiac myocytes (45). Perhaps the long and short isoforms of $G_{s\alpha}$ have differential roles in these other effector systems. However, at present the *raison d'etre* for the coexpression of the long and short forms of $G_{s\alpha}$ in many tissues remains unknown.

Acknowledgments

The authors would like to acknowledge the gift of pGEM2Gsar and pGEM2Gsar/sh from Dr. R. Reed of Johns Hopkins University. We are also indebted to Dr. G. Sathe and M. Gross of the Department of Molecular Genetics of SmithKline Beecham Pharmaceuticals for generously providing the synthetic oligonuleotides and the NS1 fusion construction used in this study.

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