TARGETED KNOCKDOWN OF G PROTEIN SUBUNITS SELECTIVELY PREVENTS RECEPTOR-MEDIATED MODULATION OF EFFECTORS AND REVEALS COMPLEX CHANGES IN NON-TARGETED SIGNALING PROTEINS

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Running Title: RNAi Silencing of G Protein Subunits

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Heterotrimeric G protein signaling specificity has been attributed to select combinations of Ga, β, and γ subunits, their interactions with other signaling proteins, and their localization in the cell. With few exceptions, the G protein subunit combinations that exist in vivo and the significance of these specific combinations are largely unknown. We have begun to approach these problems in HeLa cells by (1) determining the concentrations of Ga and Gβ subunits, (2) examining receptor-dependent activities of two effector systems (adenylyl cyclase and phospholipase Cβ), and (3) systematically silencing each of the Ga and Gβ subunits by using siRNA1 while quantifying resultant changes in effector function and the concentrations of other relevant proteins in the network.

HeLa cells express equimolar amounts of total Ga and Gβ subunits. The most prevalent Ga proteins were one member of each Ga subfamily (Gαs, Gαi3, Gαt1, and Gα13). We substantially abrogated expression of most of the Ga and Gβ proteins expressed in these cells – singly and some combinations. As expected, agonist-dependent activation of adenylyl cyclase or phospholipase Cβ was specifically eliminated following the silencing of Gαs or Gαq/11, respectively. We also confirmed that Gβ subunits are necessary for stable accumulation of Gα proteins in vivo. Gβ subunits demonstrated little isoform specificity for receptor-dependent modulation of effector activity. We observed compensatory changes in G protein accumulation following silencing of individual genes, as well as an apparent reciprocal relationship between the expression of certain Gαq and Gαi subfamily members. These findings provide a foundation for understanding the mechanisms that regulate the adaptability and remarkable resilience of G protein signaling networks.

Signal-transducing heterotrimeric G proteins are associated with the inner face of the plasma membrane – positioned as middle-men for activation by membrane-spanning, heptahelical receptors and regulation of a variety of intracellular effectors. Interactions among these proteins are controlled by agonist-induced changes of receptor conformation and nucleotide-driven conformational changes of the α subunits of the G proteins (Gα). A ligand-bound receptor catalyzes the exchange of GDP for GTP on a cognate Gα, and as a result, the (at least partial) dissociation of Gα from a complex of Gβ and γ subunits. These activated subunits are then capable of modulating the functional properties of effector proteins (e.g., adenylyl cyclases and phospholipases). The intrinsic GTPase activity of Gα serves as a molecular timer, returning the protein to the GDP-bound state and allowing reformation of the inactive heterotrimer.

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1 The abbreviations used are: RNAi, RNA interference; siRNA, small-interfering RNA; INE, isoproterenol; PGE1, prostaglandin E1; IP, total inositol-phosphate; AC, adenylyl cyclase; PLC β, phospholipase C β; GPCR, G Protein Coupled Receptor
Much remains to be learned about the specificity of G protein signaling in vivo, the relative importance of isoforms of G protein subunits with apparently redundant functions, and the qualitative and quantitative significance of the fact that many hundreds of G protein heterotrimers can be assembled from the collection of G protein α, β, and γ subunits that are expressed in single cells. There is evidence for exquisite specificity of signaling through certain pathways. For example, intranuclear injection of antisense oligonucleotides against specific G protein subunits revealed that the M4-muscarinic receptor-mediated inhibition of L-type Ca²⁺ channels requires Goα, Gβ1, and Gγ4, whereas similar inhibition initiated by somatostatin receptors requires Goζb, Gβ3, and Gγ3 subunits [1-3]. The generality of these and related studies has not been examined, and there has been little effort to monitor and understand the compensatory mechanisms that such perturbations may set in motion. Furthermore, studies performed in vitro do not reveal such demanding specificity, and mechanisms of such phenomena are not known.

Herein we describe a more comprehensive attempt to examine these issues. We have sought information about the expression of most G protein subunits in a clonal human cell line (HeLa), and we have examined the functional and compensatory effects of siRNA-mediated silencing of the expression of genes encoding members of the Goα, Goζ, Goq, and Gβ subfamilies of G protein subunits.

EXPERIMENTAL PROCEDURES

Reagents — All reagents were purchased from Sigma-Aldrich unless noted otherwise.

Mammalian Cell Culture — HeLa cells (from ATCC) were cultured at 37°C in Dulbecco’s modified Eagle’s medium (DMEM) with high glucose (Invitrogen) supplemented with 10% fetal bovine serum under an atmosphere of 95% air/5% CO₂. Cells were cultured in the same lot of serum and passed twice weekly by trypsinization. Fresh cultures were established from the same frozen stock after 10 passages.

Transient RNAi Transfection — Single-stranded 21-mer oligonucleotides (containing 19 ribonucleotides and 2 3’-deoxythymidine residues) targeting the open reading frames (ORFs) of selected proteins were designed using the Dharmacon siRNA Design Center. Candidate sequences were subjected to further BLAST analysis against the human genome database (NCBI) and selected for further study if no more than 14 contiguous bases were identical to coding regions of known human gene sequences. Single-stranded oligonucleotides were annealed as described [4]. The sequences of the RNAi sense strands and the targeted sites in the ORFs are shown in Table I. Transient transfections of RNAi duplexes were accomplished using Oligofectamine transfection reagent (Invitrogen) according to manufacturer’s instructions. Briefly, HeLa cells were seeded at 44 x 10⁴/100 mm dish 24 hr before transfection. The cells were transfected using 19 µl Oligofectamine and a total of 190 pmol of RNAi duplex(es) in a final volume of 3.8 ml. Two days later the cells were trypsinized, counted, plated at 55 x 10⁴/100 mm dish, and subjected to a second transfection. One day after the second transfection the cells were trypsinized, pooled, and distributed to 12-well plates. Three days after the second transfection, the cells were used for experiments or harvested for immunoblot analyses and determinations of protein concentration.

cAMP Accumulation — cAMP accumulation assays were conducted using cells seeded into 12-well plates (7-10 x 10⁴ cells/well) and incubated with 10 µCi/ml [2,8-3²H]-adenine (1 mCi/ml, PerkinElmer LifeSciences) for 24 hr in culture medium. Cells were incubated with cyclic nucleotide phosphodiesterase inhibitors (100 µM Ro-20-1724 and 500 µM 3-isobutyl-1-methylxanthine) for 5 min at 30°C prior to the initiation of ligand-stimulated cAMP synthesis. Experiments were terminated by addition of ice-cold 5 mM ATP, 0.2 mM cAMP, and 5% TCA containing [3²P]-cAMP (to monitor recovery during cAMP purification). Radiolabeled cAMP was separated from other labeled compounds by successive Dowex (AG 50W-X4, BioRad) and alumina chromatography as described [5].

Total Inositol Phosphate Accumulation — Total inositol phosphate (IP) accumulation was determined by incubating HeLa cells for 48 hr with 10 µCi/ml myo-[2-³²H(N)]-inositol (1 mCi/ml, PerkinElmer LifeSciences) in inositol-free DMEM (Specialty Media; Phillipsburg, NJ) supplemented
with 5% FBS. Cells were incubated for 20 min at 37°C with bicarbonate-free DMEM/20 mM Na HEPES (pH 7.4) containing 20 mM LiCl prior to the initiation of ligand-dependent IP synthesis. Reactions were terminated by addition of ice-cold 10 mM formic acid to precipitate protein. The soluble lysate was applied to a 1-ml Dowex AG 1-X8 column, which was washed successively with 15 ml of 10 mM formic acid containing 10 mM myo-inositol and 15 ml of 5 mM Na-tetraborate, 60 mM ammonium formate, prior to elution with 2.0 ml of 1.0 M ammonium formate, 0.1 M formic acid. Total [3H]-IP was determined by scintillation counting and normalized to the amount of acid-precipitated protein. Protein was quantified as described by Bradford (BioRad) [6].

Radioligand Binding — β2-adrenergic receptor concentrations were estimated for cells seeded in 6-well plates using 2 nM [125I]-CYP (PerkinElmer LifeSciences) in the presence or absence of the β2-adrenergic receptor-selective antagonist ICI-118,551 (10 μM) or the non-selective β-adrenergic receptor antagonist timolol (10 μM). Each measurement was conducted in triplicate. Ligand-binding reactions were quenched in ice-cold 50 mM Tris-HCl (pH 7.4), 10 mM MgCl2, and 100 mM NaCl and immediately filtered through GF/C Whatman filters. Radioactivity was quantified by scintillation counting. Duplicate 6-well plates were cultured to permit determinations of cell number and total protein content.

Recombinant Ga and Gβ Proteins — Recombinant bovine Gαsshort, Gαi1 (rat), Gαi2 (rat), and Gαi3 (rat) were purified after expression in E. coli as described [7]; the Gαi proteins were coexpressed with protein N-myristoyl transferase and were thus myristoylated at their amino termini. Sf9 cells were coinfected with baculoviruses encoding human Gαsshort, mouse Gαi1, or mouse Gαi3 and baculoviruses encoding Gβ1 and His6-Gγ2, and the G proteins were isolated as described [8]. Purified mouse Gαq and Gα12 were gifts from G. Tall and P. Sternweis, respectively (U.T. Southwestern). Recombinant bovine Gβ1 and human Gβ2, Gβ3, and Gβ4 proteins were provided by S. Gibson (U.T. Southwestern). Protein concentrations were determined using an amido black staining assay [9]. Purity was >90% for all proteins as assessed by Coomassie Blue stained SDS-PAGE gels.

Sample Preparation for Immunoblotting — Total cellular lysates were prepared by rinsing cells with phosphate-buffered saline and harvesting in SDS-PAGE lysis buffer (62.5 mM Tris-HCl, pH 6.8, 1.25% (w/v) SDS, 12.5% (v/v) glycerol, 0.2% (w/v) bromphenol blue, 25 mM dithiothreitol, and 1.25% (v/v) β- mercaptoethanol). The lysates were centrifuged for 1 hr (100,000 x g, 4°C, Beckman TLA 45 rotor) to pellet viscous DNA.

Plasma membrane fractions from HeLa cells were prepared essentially as described [10]. Briefly, cells were disrupted by nitrogen cavitation (600 p.s.i. for 30 min). Nuclei and unbroken cells were removed by centrifugation for 5 min (600 x g, 4°C). The resulting supernatant fraction was layered onto a 23% and 43% sucrose step gradient and centrifuged (100,000 x g, 1 hr, 4°C, Beckman SW 41 rotor). The enriched plasma membranes were collected at the gradient interfaces and recovered by centrifugation (200,000 x g, 30 min, 4°C, Beckman TLA 100.3 rotor). The membranes were suspended in 20 mM Na HEPES (pH 7.4)/150 mM NaCl, flash frozen in liquid N2, and stored at -80°C. Protein concentrations for total lysates and membranes were determined with the amido black protein assay [9] using BSA as standard.

Immunoblotting and Antibodies — In preparation for immunoblotting, total cell lysates (5-30 μg) or plasma membranes (5-20 μg) were solubilized in SDS-PAGE gel buffer [11]. For detecting adenylyl cyclase proteins, plasma membranes (50-100 μg) were treated with 1% SDS, 0.2 mM DTT, and 5 mM N-ethylmaleimide (5 min at 80°C) prior to the addition of SDS-PAGE buffer.

Antibody specificities, dilutions, and sources are shown in Table I. Following incubation with primary antibody, immunoblots were washed with phosphate-buffered saline containing 0.1% Tween-20. Secondary anti-rabbit or anti-mouse antibodies conjugated to horse radish peroxidase were diluted in wash buffer and incubated with the blots for 1 hr. The blots were subsequently washed and immunoreactive bands were detected by enhanced chemiluminescence (PerkinElmer Life Sciences).

Densitometry — Immunoblots were scanned into Adobe Photoshop 5.0, and the pixel
intensities (arbitrary units) of the immunoreactive bands were quantified. For quantitative immunoblots, endogenous HeLa cell protein levels were interpolated from the pixel intensities of the known amounts of purified Gα or Gβ subunit standards loaded on the same gel. For relative protein determinations, the pixel intensity of immunoreactive bands in samples prepared from RNAi-transfected cells was normalized to the corresponding immunoreactive band intensity identified in an equal amount of lysate prepared from mock-transfected cells.

Quantitative Real-Time PCR — Relative quantification of mRNA levels was achieved using quantitative RT-PCR. Single component amplification reactions were conducted using the relative standard curve or comparative C\text{t} methods and TaqMan Gene Expression Assays (Applied Biosystems) according to manufacturer’s instructions. Briefly, a TaqMan gene expression probe specific for amplifying a particular Gα or Gβ subunit cDNA sequence was mixed together with a One-Step TaqMan RT-PCR master mix and 100 ng total RNA per reaction. Total RNA was isolated from HeLa cells using the RNAqueous-4PCR kit (Ambion) and quantified spectrophotometrically (OD\text{260}). Fluorescence data were collected following each amplification cycle using the ABI PRISM 7500 Sequence Detection System (Applied Biosystems). C\text{t} values were calculated from threshold values set to manual.

Data Analysis — Differences between measured values (mean ± S.D.) were determined using a one-tailed T-test or single analysis of variance (p < 0.05, InStat, Prism).

RESULTS

Endogenous G Protein Subunits in HeLa Cells — Ten proteins were identified as products of the sixteen mammalian Gα genes by both RT-PCR (data not shown) and immunoblotting (see control lanes in Fig. 1A): Gαz (long and short splice variants), Gαq, Gα11, Gαi1, Gαi2, Gαi3, Gαo, Gα12, and Gα13. All positive RT-PCR signals were confirmed by cDNA sequencing, and antibody specificity was verified by target-specific RNAi-mediated silencing of antigens (also shown in Fig. 1A). Transcripts of two additional Gα genes, Gαz and Gα16, were identified by RT-PCR, but the corresponding proteins were not detected despite multiple attempts to blot 100 µg of enriched plasma membrane fractions with sensitive antibodies (detection limit of < 1 ng of purified protein). No mRNA or protein was detected for Gα14. We did not address possible expression of Gα subunits that are primarily found in specialized sensory tissues (Gαolf, Gαt (1 and 2), and Gαg).

Of the five Gβ genes, we detected expression of four in HeLa cells: Gβ1, Gβ2, Gβ4, and Gβ5; we found no evidence for expression of Gβ3. Because Gβ5 subunits appear to form selective heterodimers with RGS proteins of the R7 family (which contain Gγ-like domains) {12} and there is little credible evidence for the existence in vivo of heterodimers containing Gβ5 and Gγ subunits, we focused our efforts on Gβ1, Gβ2, and Gβ4 subunits (Fig. 1B).

We also examined the expression of Gγ subunits in HeLa cells. Perhaps surprisingly, RT-PCR analysis positively identified transcripts of all 12 Gγ genes, including Gγ1, whose expression is largely restricted to the retina {13}. Because of the lack of specific and sensitive antisera to most Gγ proteins, we have limited our studies to date to Gα and Gβ.

Prior to examining the effects of silencing individual G protein subunits, we quantified the amounts of Gα and Gβ proteins in total lysates from control cells (Fig. 1C). Quantitative immunoblotting revealed that 80% of the entire Gα subunit protein pool was accounted for by one member of each of the four subfamilies of Gα proteins: Gαi3, Gα11, Gα13, and the sum of long and short splice variants of Gαz. Gβ1 and Gβ2 were expressed at similar levels and together accounted for 80% of the total Gβ subunit pool; Gβ4 comprised the remaining 20%. There was no significant difference between the total amount of Gβ protein detected with a pan Gβ antibody (Gβ1-4) and the sum of Gβ subunits calculated from data obtained using isoformal-specific Gβ antibodies. Transformation of the data in Fig. 1C to molar quantities reveals that the sum of the Gα subunits (22 ± 2 pmol/mg lysate) closely approximates that of the Gβ proteins (21 ± 3
pmol/mg lysate), consistent with the notion that most or all of these subunits exists as oligomers.

**Knockdown of G Protein Subunits** – Immunoblots showed remarkable (more than 90%) knockdown of endogenous protein expression for each Gα and Gβ subunit (Fig. 1A,B) after silencing by siRNAs, either individually or in combination. Greater than 90% knockdown of Gα subunits was deemed necessary to test changes in receptor-modulated effector responses, since the reported stoichiometry of G protein to effector can, in some instances, exceed 10:1 [14]. Thus, multiple RNAi duplexes (range: two to ten) were designed and generated towards each target; in general, most duplexes silenced target protein expression more than 75%.

In several instances (particularly with Gβ subunits) treatment with RNAi oligonucleotides not only silenced the intended target but also caused cell death. Interestingly, our most lethal RNAi duplex targeted green fluorescent protein (GFP) using a sequence with no significant (>12 nucleotide) overlap with the open reading frame of any known human gene, indicating that cell death may be caused by mechanisms unrelated to specific mRNA sequence recognition by RISC complexes. We rigorously avoided using siRNA’s that had adverse effects on cell growth or viability.

**Knockdown of Gαs: Anticipated Effect on Ligand-Stimulated Activation of Adenylyl Cyclases** — Gαs proteins mediate activation of adenylyl cyclases by so-called Gs-coupled GPCRs. As anticipated, knockdown of Gαs completely abrogated ligand-dependent increases in cAMP synthesis (Fig. 2A,B) stimulated by either INE, a β-adrenergic receptor agonist, or PGE1, an EP prostanoid receptor agonist. A combination of RT-PCR, radioactive ligand-binding assays, and RNAi-mediated silencing of cell surface receptors (see Table I) demonstrated that INE stimulation of cAMP synthesis occurred exclusively via the β2-adrenergic receptor, while PGE1 signaling was mediated via two EP receptor isoforms, EP2 and EP4 (data not shown). We found no evidence of expression of β1- or β3-adrenergic receptors or EP1 or EP3 prostanoid receptors in HeLa cells. The functional effects of loss of Gαs were specific. Thus, silencing of Gαs did not prevent UK 14,304-dependent inhibition of cAMP synthesis or stimulation of phospholipase Cβ activity by histamine (data not shown). These data confirm the functionality and specificity of Gαs-mediated activation of adenylyl cyclases using RNAi-mediated gene suppression and illustrate the utility of this approach to study G protein signaling in a population of intact cells.

**Silencing of Gαq and Gα11: Anticipated Effects on Activation of Phospholipase Cβ** — Gαq and Gα11 (along with Gα14 and Gα16) mediate cell-surface receptor activation of PLCβ, which catalyzes the synthesis of inositol-(1,4,5)-trisphosphate from phosphatidylinositol-(4,5)-bisphosphate. Inositol phosphate (IP) accumulation in HeLa cells was increased following activation of the H1-histamine receptor by agonist. The simultaneous knockdown of Gαq and Gα11 (denoted as Gαq/11) eliminated histamine-stimulated IP accumulation (Fig. 2C). Interestingly, the silencing of either Gαq or Gα11 individually decreased histamine-dependent IP accumulation by ~50%, despite the fact that concentrations of Gα11 in HeLa cells exceed those of Gαq by 10-fold (Fig. 1C). The amount of [3H] incorporated into acid-precipitable material was unchanged in cells lacking Gαq/11, suggesting that phosphatidylinositol kinase and phosphatase activities were unaffected. Silencing Gαq/11 modestly (but significantly) increased ligand-dependent cAMP responses (~30%) without affecting UK 14,304-mediated inhibition of adenylyl cyclase (data not shown).

**Knockdown of Gαi/o: Incomplete Effects on Inhibition of Adenylyl Cyclase** — Gαi/o subunits transduce signals from cell surface receptors to a variety of intracellular pathways (see [15] for review), including inhibition of adenylyl cyclase activity. cAMP synthesis in HeLa cells can be inhibited by α2a- and α2b-adrenergic receptors. Despite silencing each of the Gα1-3 subunits, individually or in combinations of two, we observed no significant change in the ability of the synthetic α2-adrenergic receptor agonist UK-14,304 to inhibit INE- (data not shown) or forskolin-stimulated cAMP synthesis (Fig. 2D). We observed a 60% reduction in the magnitude of such inhibition only when all three Gαi subunits were silenced (denoted Gαi-3) (Fig. 2D). Thus,

2 S.K. Gibson, unpublished observations
α2-adrenergic receptors displayed no absolute specificity for G\(\alpha\)1-3 isoforms for the inhibition of INE or forskolin-stimulated cAMP synthesis.

Interestingly, knockdown of G\(\alpha\)1 did specifically interfere with UK 14,304-mediated inhibition of PGE1-stimulated cAMP synthesis (Fig. 2E), suggesting that a PGE1-stimulated adenylyl cyclase may preferentially localize with or couple to this G protein. It is most puzzling, therefore, that simultaneous silencing of G\(\alpha\)1-3 subunits did not abrogate inhibition of PGE1-activated cAMP synthesis by UK 14,304. Residual inhibition of adenylyl cyclase activity in all experiments was eliminated by treatment of cells with pertussis toxin, suggesting that G\(\alpha\)1 subunit expression may have been insufficiently silenced and/or that G\(\alpha\)o subunits, in the absence of G\(\alpha\)1-3 subunits, may have contributed to the inhibition of cAMP synthesis [16]. Individual silencing of G\(\alpha\)o (both a and b splice variants) had no effect on UK-14,304-mediated inhibition of cAMP synthesis. Recall that G\(\alpha\)z was not detected in these cells.

**Silencing of G\(\alpha\) Subunits: Alterations in Accumulation of Other G\(\alpha\) Proteins** — Silencing of individual G protein \(\alpha\) subunits routinely resulted in loss of more than 90% of that protein, detected by immunoblotting (Table II, highlighted in yellow). Quantification of non-targeted G\(\alpha\) proteins revealed that the concentrations of G\(\alpha\)3 and G\(\alpha\)11 (Table II) and G\(\alpha\)13 (data not shown) were not significantly altered by such perturbations. However, we observed modest (~2-fold) increases in concentrations of G\(\alpha\)2 and G\(\alpha\)3 (Table II: highlighted in green) and increased G\(\alpha\)2 protein after silencing of G\(\alpha\)3. Thus, loss of the major isoform of G\(\alpha\)z resulted in increased accumulation of the other two isoforms of G\(\alpha\). Increased accumulation of G\(\alpha\)1 protein was associated with a dramatic increase in detectable mRNA for this protein (Fig. 3A). The level of G\(\alpha\)o protein also increased modestly, but significantly, after combined knockdown of all three G\(\alpha\) subunits (Table II). These data indicate that reductions in the concentrations of G\(\alpha\)1 isoforms are associated with compensatory changes in the levels of other proteins within the G\(\alpha\) subfamily.

Unexpectedly, we detected substantially increased concentrations of G\(\alpha\)1 and G\(\alpha\)o protein following the knockdown of either G\(\alpha\)q, G\(\alpha\)11, or both of these proteins (denoted as G\(\alpha\)q/11) (Fig. 3B and Table II, highlighted in green). These changes in protein concentrations were accompanied by increases in mRNA for both G\(\alpha\)1 and G\(\alpha\)o (Fig. 3A). Taken together with the fact that the absolute amounts of G\(\alpha\)11 and G\(\alpha\)q are so different (and observed effects are thus less likely to be caused by alterations of ratios of G\(\alpha\) to G\(\beta\)\(\gamma\)), these data suggest that G\(\alpha\)q/11 proteins regulate (directly or indirectly) the rate of synthesis of G\(\alpha\)1 and G\(\alpha\)o. Although knockdown of G\(\alpha\)q and G\(\alpha\)11 increased accumulation of G\(\alpha\)1 and G\(\alpha\)o, the reciprocal was not generally true. However, a modest increase in G\(\alpha\)q protein level was observed following knockdown of G\(\alpha\)3 (Fig. 3B and Table II, highlighted in green).

Amounts of G\(\alpha\)12 mRNA were also increased about 4-fold in cells lacking G\(\alpha\)q/11 protein (data not shown), but the effects on protein concentrations were not examined because of lot variability of the anti-G\(\alpha\)12 antibody.

G\(\alpha\)1 stands out in examination of all of the effects of G\(\alpha\) protein knockdowns on G protein expression. Although apparently a minor player in HeLa cells in terms of protein concentration (Fig. 1C), levels of G\(\alpha\)1 message and protein increased significantly when expression of either G\(\alpha\)s, G\(\alpha\)q, G\(\alpha\)11, G\(\alpha\)2, or G\(\alpha\)3 was compromised (Fig. 3A,B and Table II).

We also examined the effects of silencing G\(\alpha\) protein expression on G\(\beta\) subunits (Fig. 3C and Table II). Interesting and in some cases specific effects were observed, although they were not predictable by consideration of the relative concentrations of the proteins involved or other facts known to us. Thus silencing of G\(\alpha\)s, G\(\alpha\)11, or individual G\(\alpha\) isoforms (G\(\alpha\)2 and G\(\alpha\)3) significantly decreased accumulation of G\(\beta\)4, the least prevalent G\(\beta\) subunit. Knockdown of G\(\alpha\)s also decreased levels of G\(\beta\)2, whereas silencing G\(\alpha\)2 decreased levels of G\(\beta\)1 substantially, while having only modest effects on other G\(\beta\) proteins. The simultaneous knockdown of the three G\(\alpha\)i proteins (denoted as G\(\alpha\)i1-3) decreased the total immunodetectable G\(\beta\) subunit pool by more than 50%. Decreased accumulation of G\(\beta\) protein was
consistent with the existence of isoform-specific
effects on accumulation of Gα with this perturbation. These Gβ correlates with the increase in Gα mRNA; this is especially true of Gβ in unperturbed HeLa cells.

Knockdown of Single and Paired Gβ Isoforms: Alterations in Expression of Other G Protein Subunits — Gβ subunits were targeted for silencing in attempts to observe coordinated disappearance of combinations of Gα and Gβ subunits that might be prevalent in vivo, isoform-specific functions of Gβ subunits, and regulation of Gβ subunit expression. siRNA-mediated targeting of Gβ1, Gβ2, or Gβ4 resulted in loss of more than 90% of immunodetectable protein (Fig. 1B and Table II, highlighted in yellow). No dramatic changes were observed in non-targeted Gβ proteins or mRNA (not shown) following the silencing of individual Gβ proteins, although Gβ1 and Gβ2 levels appeared to rise modestly following silencing of Gβ4. No Gβ3 protein was detected after Gβ1, Gβ2, or Gβ4 knockdown. Regulation of expression of individual Gβ isoforms thus appears to occur largely independently of other individual isoforms.

Because Gα proteins are stabilized by association with Gβγ dimers {17}, we hypothesized that loss of a single Gβ subunit might reduce the level of cognate Gα proteins. Since three Gβ subunits must “service” ten Gα proteins, overlapping relationships are mandatory. Silencing of Gβ1 resulted in reduced accumulation of Gαs and Gαi3; knockdown of Gβ2 decreased Gαs, Gαi1, and Gαi3; and silencing Gβ4 diminished Gαq and Gαo, while increasing Gαi1 and Gαi2 (Fig. 4A and/or Table II). Reduced accumulation of Gα proteins was not well correlated with changes in mRNA levels (Fig. 4B and not shown), consistent with the notion that Gβγ complexes promote stability of Gα. In examining these data, Gαi1 again stands out. Silencing of any Gβ provoked an increase in Gαi1 mRNA; this is especially true of Gβ4 and correlates with the increase in Gαi1 protein seen with this perturbation. These Gβ subunit-specific effects on accumulation of Gα protein are consistent with the existence of isoform-specific combinations of Gα and Gβ subunits in unperturbed HeLa cells.

Silencing of individual Gβ isoforms did not decrease concentrations of Gαi2, Gαi11, and Gαi13, suggesting that Gαs, Gαi1, Gαi3, and Gαo are less stable than Gαi2, Gαi11, and Gαi13 in the absence of Gβ or that the latter group more readily redistributes among other Gβ subunits. To shed further light on these questions, we evaluated the effects of simultaneous silencing of multiple Gβ subunits.

Simultaneous knockdown of Gβ1 and Gβ2 was accomplished with either of two distinct RNAi duplexes, targeting site 865 (denoted as Gβ1/2 [a]) or 866 (denoted as Gβ1/2 [b]), both of which contain 19-nucleotide identities with the Gβ1 and Gβ2 open reading frames. Concomitant silencing of Gβ1 and Gβ2 eliminated nearly all Gαi family protein, in contrast with a recent study {18} in which Gαi subfamily protein was maintained in a cultured mouse J774A.1 macrophage cell line following the knockdown of Gβ1 and Gβ2.

Surprisingly, Gαs protein expression was maintained at 50% of control values despite the loss of Gβ1/2 (Fig. 4C, Table II), as were Gαq, Gαi11, Gαi13, and Gαo. Interestingly, each Gα subfamily was represented despite the knockdown of 80% of the initial Gβ pool. Sustained expression of some Gα proteins in our Gβ1/2 ablated cells could not be attributed to greater stability of these free Gα subunits because expression of nearly all Gα subunits was lost following the silencing of all Gβ isoforms (Fig. 4A and Table II). Decreased Gα protein concentrations following knockdown of multiple Gβ subunits occurred independently of changes in levels of Gα subunit mRNA (Fig. 4B), likely indicating that Gβ subunits are requisite for the stability of virtually all Gα proteins.

The retention of some Gα subunits in cells lacking Gβ1/2 protein was likely due to formation of new associations with increased concentrations of Gβ4 (Fig. 1B, Table II, Fig. 5). No Gβ3 protein was detected in cells where Gβ1 and Gβ2 were eliminated. Increased Gβ4 protein was detected 48-72 hrs after transfection with siRNA directed against Gβ1/2 until a new steady-state level of Gβ4 protein was established (compare the relative Gβ4 level 72 hr after transfection [Fig. 5C] with
that after 144 hr (Table II). Interestingly, there was no detectable increase in Gβ4 mRNA during the same time period.

Knockdown of Single and Multiple Gβ Subunits: Functional Effects — Silencing of Gβ1 (by either of two RNAi duplexes) substantially reduced PGE1-dependent activation of cAMP synthesis by 70% (Fig. 6A). Targeted knockdown of Gβ2 and/or Gβ4, on the other hand, had little effect on PGE1-dependent activity. The data indicate that at least one of the EP2 and EP4 prostanoid receptors primarily stimulates cAMP synthesis via Gαs associated with Gβ1 in HeLa cells. This interesting example of specificity is perhaps reminiscent of the observation (above) that Gαi1 was specifically required for α2-adrenergic receptor-mediated inhibition of PGE1-(but not INE- or forskolin-) stimulated adenylyl cyclase activity.

By contrast INE-stimulated cAMP synthesis was mostly unaffected following the silencing of Gβ1, Gβ2, or Gβ4 (Fig. 6B). Radioligand binding studies suggest that possible loss of INE-stimulated activity in cells lacking Gβ1 may have been offset, at least in part, by a compensatory increase in the β2-adrenergic receptor density: control = 23,200 ± 10,200; knockdown of Gβ1 = 53,600 ± 17,100, Gβ2 = 29,200 ± 4,300, and Gβ4 = 28,200 ± 10,100 (receptors/cell for n=3 independent experiments measured in triplicate). Cells lacking both Gβ1 and Gβ2 showed no difference in receptor density (26,500 ± 12,100) compared to control cells.

Knockdown of individual Gβ subunits did not affect histamine-dependent activation of PLCβ (Fig. 6C), and little requirement for specific Gβ isoforms was observed for α2-adrenergic receptor-dependent inhibition of ligand- or forskolin-activated cAMP synthesis (Fig. 6D, E and data not shown).

We then examined the effects of simultaneous elimination of Gβ1 and Gβ2 on ligand-dependent effector activity; these proteins account for 80% of the total cellular Gβ in unperturbed cells. Histamine-dependent accumulation of IP was unaffected (Fig. 6C), whereas α2-adrenergic receptor-mediated inhibition of ligand-activated cAMP synthesis was reduced by at least 90% (Fig. 6D,E). These data indicate that Gβ4 could fully sustain both the stability and function of the Gαq subfamily of Gα proteins but not the Gαi subfamily, whose members largely disappear under this condition. Coincidental silencing of all three Gβ subunits largely abrogated ligand-dependent accumulation of cAMP and IP. No Gβ3 protein was detected in cells lacking Gβ1, Gβ2, and Gβ4.

Very curiously, silencing of both Gβ1 and Gβ2 did not cause the loss of PGE1-dependent cAMP synthesis that occurred following the knockdown of only Gβ1 (Fig. 6A). It also did not impair INE-stimulated adenylyl cyclase activity, which is not unanticipated since Gαs concentrations are reasonably well maintained under this circumstance. Instead, PGE1- and INE-dependent activities were augmented nearly 2-fold in cells transfected with one siRNA duplex directed at both Gβ1 and Gβ2 (Gβ1/2 [b]), but not the other (Gβ1/2 [a]). Immunoblots revealed that Gαs protein concentrations were 45% lower in cells transfected with Gβ1/2 [a] than in those transfected with Gβ1/2 [b]. The difference may be attributed to a modestly better Gβ subunit silencing efficiency of the Gβ1/2 [a] RNAi duplex compared to the Gβ1/2 [b] RNAi duplex (data not shown). No significant differences in relative protein expression were observed for any other Gα subunit (data not shown).

We addressed a number of mechanistic possibilities that might account for the augmented cAMP response seen after silencing Gβ1/2 with the Gβ1/2[b] siRNA. Of interest, forskolin, a direct activator of adenylyl cyclases, caused a 3-5-fold greater cAMP response in cells lacking Gβ1/2 (regardless of which Gβ1/2 RNAi duplex was transfected) compared to control cells (Fig. 7). No significant difference was noted in the concentration of forskolin required to elicit a half-maximal response (control EC_{50} = 110 ± 60 µM and Gβ1/2 EC_{50} = 60 ± 20 µM). Cells lacking Gβ1/2 that were exposed to a maximally effective concentration of forskolin demonstrated up to a 10-fold greater rate of cAMP accumulation than control cells (Fig. 7 inset).

This forskolin-sensitized cAMP response could not be attributed to increased β2-adrenergic receptor density or decreased phosphodiesterase activity (data not shown). Differences in basal
and maximal cAMP responses (assayed with a mixture of INE, PGE1, and forskolin at saturating concentrations), in cells lacking Gβ1/Gβ2 expression and cells transiently transfected with a constitutively active mutant of Gαs (Gαs Q227L) {19}, were not consistent with augmented cAMP activity being due to an increased pool of free (unassociated with Gβγ) Gαs subunits.

We also explored whether decreased expression of Gαi isoforms in cells lacking Gβ1 and Gβ2 contributed to the augmented cAMP response. Concomitant knockdown of all three Gαi subunits (Gαi1-3) did not increase the cAMP response; instead, agonist-dependent and forskolin-stimulated adenylyl cyclase was modestly reduced by 20% (not shown). Individually silencing Gαi1 or Gαi3 did not significantly affect INE- or PGE1-stimulated cAMP synthesis; however, forskolin-dependent adenylyl cyclase activity was increased 90% in cells lacking Gαi3 expression. Interestingly, silencing of Gαi2 augmented PGE1-, INE-, and forskolin-dependent cAMP synthesis by 50%, 70%, and 200%, respectively (data not shown). However, silencing Gαi2 concomitantly with Gβ1/2 caused additive effects on forskolin-dependent cAMP responses (data not shown), consistent with independence of the mechanisms involved. Thus it is unlikely that loss of Gαi contributed significantly to the augmented adenylyl cyclase activity in cells lacking Gβ1 and Gβ2 expression.

Lastly, we examined whether the lack of Gβ1/2 affected concentrations of adenylyl cyclase proteins. There are nine known genes that encode adenylyl cyclases; they are all activated by Gαs but they are differentially regulated by Gαi, Gβγ, Ca2+, Ca2+-binding proteins, and/or phosphorylation {for review see 20}. RT-PCR analyses revealed that HeLa cells express AC types I, III, VI, VII, and IX; there was no evidence of AC types II, IV, V, or VIII using a variety of RT-PCR primers and total RNA isolated from control cells or cells lacking Gβ1/2.

However, immunoblots of enriched plasma membranes (Fig. 8) revealed that concentrations of AC type VI (glycosylated plus non-glycosylated) and AC type III were increased 250% and 60%, respectively, in fractions prepared from cells lacking Gβ1/2 compared to controls. Lack of reliable antibodies precluded examination of AC types I, III, and IX. No changes in mRNA levels were observed for any of the five AC isotypes expressed in HeLa cells (data not shown). Increased concentrations of AC types VI and III, in conjunction with the fact that increased accumulation of Gβ4 permits retention of significant amounts of Gα, provide an explanation for the robust forskolin- and ligand-stimulated cAMP responses in cells lacking Gβ1 and Gβ2.

Knockdown of Gγ Subunits — HeLa cells express all 12 Gγ isoforms. We sought to silence the expression of all 12 endogenous Gγ subunits using siRNA while monitoring the effects on ligand-dependent cAMP responses. The efficacy and specificity of RNAi duplexes targeting Gγ subunits were determined by co-transfection of siRNAs with expression plasmids encoding Gγ-GFP fusion proteins (Table I). Silencing of individual Gγ subunits reduced PGE1-stimulated cAMP synthesis for 7 of the 9 Gγ subunits tested, and the sum of the individual percentage decreases was significantly greater than 100% (data not shown). Further examination of Gγ contribution to signaling specificity will require specific and sensitive antibodies to all 12 Gγ proteins.

**DISCUSSION**

This study brings appreciation of the fact that concentrations of G protein subunits are highly inter-related by a variety of mechanisms and that these effects can extend as well to cognate receptors and effectors. It also sounds a clear warning that those who undertake other than short-term perturbations of such networks must be willing to search broadly for the consequences of their actions, focusing on the proteins themselves and not just on more easily measured mRNA transcripts. Our best example of these two conclusions: simultaneous knockdown of Gβ1 and Gβ2 in HeLa cells results in a compensatory, non-transcriptionally mediated increase in Gβ4; this is sufficient to stabilize an adequate (but reduced) amount of Gαs, which can activate substantially increased levels of at least two isoforms of adenylyl cyclase. The result is an enhanced response to agonist when severe impairment was anticipated. This perturbation also resulted in loss
of essentially all G\(\alpha\) protein, despite a large increase in mRNA for G\(\alpha\)11. Substantial changes in G\(\gamma\) subunits certainly occurred as well, but were not measured. How much further were these changes felt in these normal-appearing viable cells?

Concentrations of G\(\alpha\) and G\(\beta\) in HeLa Cells — We know of no other study where there has been reasonably comprehensive quantification of G protein \(\alpha\) and \(\beta\) subunits in a homogeneous cell population. HeLa cells contain very similar total amounts of G\(\alpha\) and G\(\beta\) proteins, suggesting that all subunits are present largely as heterotrimeric. Stabilization of G\(\alpha\) by \(\beta\gamma\) is likely a major mechanism for maintenance of stoichiometric equivalence between G\(\alpha\) and G\(\beta\gamma\), since silencing of the G\(\beta\) subunits present in these cells results in very substantial loss of all G\(\alpha\) proteins.

Four G\(\alpha\) subunits (G\(\alpha\)s, G\(\alpha\)11, G\(\alpha\)i3, and G\(\alpha\)13), each representing one of the four G\(\alpha\)-subfamilies, were the most prevalent G\(\alpha\) proteins and together accounted for >80% of the total G\(\alpha\) pool. Contrary to the prevalent notion that levels of G\(\alpha\)i proteins usually exceed that of either G\(\alpha\)s or G\(\alpha\)11, HeLa cells contain comparable amounts of G\(\alpha\)s, G\(\alpha\)11, and total G\(\alpha\)i1-3, with somewhat lower amounts of G\(\alpha\)12/13. The ratios of the amounts of the family members are at least to some extent cell-type or tissue specific; the amounts of G\(\alpha\)i proteins in brain are very high.

Functions of G\(\alpha\) Proteins: Implications for Signaling Specificity — Certain tenets of G protein signaling, such as the specific requirement for G\(\alpha\)s to activate adenylyl cyclase, G\(\alpha\)q/11 to stimulate PLC\(\beta\), and G\(\beta\gamma\) to stabilize G\(\alpha\) were verified herein. The effects of silencing G\(\alpha\)s on agonist-dependent signaling were straightforward: one gene and one functional response.

By comparison, activation of PLC\(\beta\) through H1-histamine receptors is more complex. Two G\(\alpha\) proteins, G\(\alpha\)q and G\(\alpha\)11, are coupled to the same type of receptor. Of interest, silencing of G\(\alpha\)q or G\(\alpha\)11 caused indistinguishable phenotypes – loss of half of histamine-stimulated PLC\(\beta\) activity – despite the fact that concentrations of G\(\alpha\)11 exceed those of G\(\alpha\)q by ten-fold. Since biochemical studies indicate that recombinant G\(\alpha\)q and G\(\alpha\)11 are equally effective at stimulating PLC\(\beta\) \((21)\), it is possible that specific localization or organization could be invoked to explain this observation. For example, perhaps only a fraction of the highly expressed G\(\alpha\)11 in HeLa cells is accessible to H1 receptors and/or PLC\(\beta\).

No specificity was detected among the G\(\alpha\)i proteins when \(\alpha\)2-adrenergic receptor-mediated inhibition of INE- or forskolin-stimulated adenylyl cyclase activity was examined, but silencing of G\(\alpha\)11 did specifically interfere with \(\alpha\)2 receptor-mediated inhibition of PGE1-stimulated adenylyl cyclase. Speculatively related: silencing of G\(\beta\)1 interfered with stimulation of cAMP accumulation by PGE1 but not INE, and we observed no effect of this perturbation on other signaling pathways. There appears to be preferential communication between at least one EP-receptor, G\(\alpha\)s, G\(\alpha\)11, and G\(\beta\)1, although the mechanism for these apparent relationships are obscure.

Knockdown of G\(\beta\) Isoforms Affects G\(\alpha\) Protein Stability — Concomitant knockdown of all three G\(\beta\) subunits expressed in HeLa cells resulted in near complete loss of each G\(\alpha\) without affecting G\(\alpha\) mRNA levels (except for an increase in G\(\alpha\)11). Thus, G\(\beta\gamma\) subunits stabilize endogenous G\(\alpha\) proteins in intact cells. A nearly G protein-free and viable cell is produced, but little GPCR-mediated signaling remains.

Our data demonstrating the loss of G\(\alpha\) protein in cells lacking G\(\beta\) subunits are in contrast to a recently published study demonstrating apparent stability of free G\(\alpha\) subunits in a mouse J774A.1 macrophage cell line \(\{(18)\}\). The authors hypothesized and subsequently demonstrated that G\(\alpha\)i proteins modified with both myristate and palmitate were more stable than G\(\alpha\) subunits carrying only one lipid modification. We speculate that the differences observed between the two cell types may be due to the existence of proteins that control the fate of free \(i.e\) unassociated with G\(\beta\gamma\) G\(\alpha\) subunits or that the stoichiometry of palmitoylation of the G\(\alpha\)i proteins may differ substantially in the two cells.

We found modest amounts of G\(\alpha\)11 and G\(\alpha\)13 remain after silencing of G\(\beta\)1, G\(\beta\)2, and G\(\beta\)4. We speculate that this may be a reflection of the intrinsic rates of nucleotide exchange that characterize these G\(\alpha\) proteins. Their relatively
slower rates of nucleotide exchange minimizes time spent in the nucleotide-free state, which is unstable. Other explanations are possible, including high intrinsic affinity for residual Gβγ or interactions with other proteins.

Unanticipated Functional Effects of Silencing Gβ Isoforms — No requirement for a specific Gβ isoform was revealed for β2-adrenergic, H1-histaminergic, or α2-adrenergic receptor-dependent modulation of effector activities. However, as noted above, some degree of functional specificity was exhibited for Gβ1 coupling of prostanoid receptors to adenylyl cyclase. It was thus surprising that prostanoid-dependent activation of cAMP synthesis was re-established when Gβ2 was silenced along with Gβ1. There are a number of possibilities that could explain the retention of PGE1-mediated signaling under this circumstance. The simplest of these involves the increase in adenylyl cyclase concentrations noted after silencing of Gβ1 and Gβ2. Alternatively, severe perturbation of the system, such as the knockdown of Gβ1 and Gβ2, may have disrupted and redistributed prostanoid-dependent adenylyl cyclase signaling components from specialized plasma membrane compartments [22].

Our study also revealed some interesting features about Gβ4, the least well studied of the conventional Gβ subunits. The amino acid sequence of Gβ4 is more than 90% identical to those of Gβ1 and Gβ2, it is ubiquitously expressed [23], and it behaves similarly to Gβ1 and Gβ2 in vitro [23,24]. We show that endogenous Gβ4 has the ability to maintain GPCR-dependent signaling by interacting functionally with and supporting the coupling of Gαs, Gαq, and Gα11 to effectors in cells that lack Gβ1 and Gβ2.

There appear to be differences in the regulation of expression of Gβ4 compared with that of Gβ1 and Gβ2. Gβ4 was the only Gβ subunit whose protein expression was significantly reduced (50% or more) following knockdown of more than one Gα subfamily. With the exception of Gαi2, loss of Gβ4 protein was correlated with decreased Gβ4 mRNA, indicating that Gαs, Gαi3, and Gα11 each, in some manner, regulated expression of Gβ4 mRNA. Moreover, elimination of the functionally important (but not prevalent) Gβ4 increased accumulation of Gβ1 and Gβ2, while only the elimination of both Gβ1 and Gβ2 had a significant effect on Gβ4.

Alterations of Non-Targeted Proteins — Knockdown of a specific G protein subunit often resulted in increased accumulation of a member of the same subfamily. Thus, loss of the prevalent Gαi3 doubled the levels of Gα1 and Gα2, while loss of Gαi2 doubled Gα1. Silencing of all Gαi1-3 proteins impacted the accumulation of Gαo. The relationships between expression of Gβ1, Gβ2, and Gβ4 isoforms is noted above. Some of these effects appeared to be mediated transcriptionally; some did not. The exception is Gαq and Gα11, where compensatory increases were not observed following loss of either protein.

Less expected was observation of an apparently reciprocal relationship between expression of members of the Gαq and Gαi subfamilies. Loss of Gαq or Gα11 caused increased accumulation of Gαi1 and Gαo; loss of Gαi3 resulted in increased accumulation of Gαq. Significance is not clear.

It is also notable that levels of Gαi1 and its mRNA increased following the knockdown of almost every Gα and Gβ subunit, which is suggestive of some as yet unknown common mechanism. Little is known about the specific signaling pathways affected by Gαi1. It is widely expressed, particularly in brain, but there has been no obvious phenotype associated with Gαi1 knockout mice [25].

The impact of Gβ knockdowns went beyond G protein family members. We documented changes in the amounts of β-adrenergic receptors with the knockdown of Gβ1 and increased levels of adenylyl cyclase when Gβ1 and Gβ2 were silenced simultaneously. Increased β2-adrenergic receptor expression correlated with a 2- to 3-fold increase in mRNA for the receptor in cells lacking Gβ1, whereas, AC type VI protein appeared to increase independently of changes in mRNA. We do not know if the changes in adenylyl cyclases are a direct result of loss of Gβ1 and Gβ2, increase in Gβ4, partial loss of Gαs, or other factors; however, knockdown of Gαs increased adenylyl cyclase (types III and VI) expression 60-75%, suggesting that Gαs likely
contributes to the regulation of adenylyl cyclase expression.

We cannot comment on the generality of the specific network interactions that are revealed by these knockdown experiments in HeLa cells. Our analyses revealed many changes in non-targeted G protein subunit expression and specific network interactions are likely to be dependent on the specific cell type, the presence of different regulatory mechanisms, and relative stoichiometries of G protein subunits. Future studies using different cells and tissues will reveal the generality of such relationships.

It is clear from this study that G protein signaling pathways in HeLa cells are elastic and resilient in their ability to maintain robust regulation of effectors in the face of change. The mechanisms used to achieve this are varied and complex. It will be a daunting challenge to understand how and to what extent the concentrations and activities of the many interacting components of such networks are regulated to resist and respond to the forces that impinge upon them.

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Figure Legends

Figure 1: Identification of G protein subunits in HeLa cells.  A) Western blots of endogenous Gα subunits in total lysates of unperturbed HeLa cells. Proteins detected by immunoblotting are indicated at the left of each panel. Antisera specificity was verified by blotting total lysates of cells where expression of individual subunits was silenced by RNA interference (shown across the top of each panel). Positions of the long (52 kD) and short (46 kD) Gα splice variants, as well as immunodetectable bands corresponding to other Gα subunits, are indicated by ticks. Gαq (42 kD) and Gαi1 (40 kD) are each shown migrating below a non-specific immunoreactive band. Equal amounts (30 µg) of total lysates were loaded in each lane of gels used for Western immunoblotting of Gα subunits. Gα12 antiserum did not cross-react with purified Gα13 standard (not shown). B) Western blots of endogenous Gβ subunits in total lysates of unperturbed HeLa cells. Gβ subunits targeted for RNAi silencing are shown across the top of each panel, and proteins identified by Western blotting are indicated at the left. Gβ1 migrated as a 36 kD protein whereas Gβ2 and Gβ4 migrated as 35 kD. Thirty µg of each total lysate was loaded per lane for individual Gβ isoform examination by Western blotting and 5 µg/lane of each total lysate was loaded for Western blotting examination of total Gβ1-4 subunit expression. C) Results of quantitative Western blotting for Gα and Gβ subunits in unperturbed HeLa total cell lysates. Five to 30 µg/lane of each lysate preparation was separated by SDS-PAGE along with purified Gα or Gβ subunit standards (1-10 ng range). Western blots and densitometry analysis were conducted as described under Experimental Procedures. Each bar represents the mean ± standard deviation of ten different total cell lysates. Gα protein amounts are represented by open bars and Gβ proteins by closed bars.

Figure 2: Effects of silencing Gαs, Gαi1-3, or Gαq/11 on ligand-modulated effector responses. cAMP responses in control cells (closed square) and cells lacking Gαs (closed triangle) were examined following exposure to either A) 10 µM isoproterenol (INE) or B) 10 µM prostaglandin E1 (PGE1) for the indicated times. Each data point represents the average ± the range of duplicate measurements. The figure is representative of at least three independent experiments. C) Total inositol-phosphate (IP) accumulation in whole cells lacking Gαq (closed triangle), Gα11 (inverted closed triangle), or Gαq and Gα11 (Gαq/11, closed diamond). Total IP responses were examined in cells exposed to 100 µM histamine for the indicated times. Each point represents the average of duplicate measurements of the amount of [3H]-IP (cpm) recovered from Dowex column chromatography normalized to total protein content ± the range. The data are representative of at least three independent experiments. D) Inhibition of cAMP synthesis in cells lacking Gαi subunits. [3H]-cAMP production in cells was monitored over time following the addition of either D) 200 µM forskolin or forskolin (200 µM) and the synthetic α2-adrenergic agonist UK 14,304 (10 µM) or E) 10 µM prostaglandin E1 (PGE1) in the presence and absence of 10 µM UK 14,304. Data are presented as the percent of forskolin-stimulated cAMP synthesis that was inhibited by UK 14,304 following 5 min of ligand treatment. Each bar represents the mean percent inhibition ± S.D. from three independent experiments.

Figure 3: Relative mRNA and protein expression of G protein subunits following select Gα silencing. A) Quantitative PCR reactions were conducted using total RNA isolated from control cells and cells lacking Gαs, Gαq, Gα11, Gαi1, Gαi2, Gαi3, or Gαo. Relative amounts of Gα subunit mRNA expression (Gαs [black], Gαq [pale blue], Gα11 [orange], Gα13 [purple], Gαi1 [pink], Gαi2 [pale green], Gαi3 [yellow], and Gαo [blue]) are shown for cells lacking selected Gα protein expression (shown across the bottom). Fold differences were calculated using the ΔΔCt method. Ct values for each probe were corrected for the amount of endogenous 18S rRNA in each sample and normalized using Ct values determined for each probe in unperturbed cell total RNA. Each bar represents the mean ± S.D. from three separate total RNA preparations. B) Western blots of Gαo, Gαi1, and Gαq following

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knockdown of select Gα subunits. Gα proteins targeted for RNAi silencing are shown across the top of each panel and proteins identified by Western blot are indicated to the left of each panel. Migration of Gαq, indicated by ticks, is below a non-specific band. The data are representative of at least three separate experiments. C) Relative Gβ mRNA levels following knockdown of Gα subunits. The relative Gβ subunit mRNA expression (Gβ1 [pale red], Gβ2 [green], and Gβ4 [brown]) is shown using the same total RNA preparations described in A). Each bar represents the average ± S.D. of three separate preparations.

Figure 4: Protein and relative mRNA expression of Gα subunits following silencing of Gβ isoforms. A) Western blots of Gαz, Gα11, Gα13, Gαι1, Gαι2, and Gαι3 using total lysates prepared from unperturbed cells and cells lacking Gβ1, Gβ2, Gβ4, Gβ1/2, or Gβ1/2/4. The data are representative of four independent preparations for each condition. B) Relative Gα mRNA expression in cells lacking Gβ protein expression. Relative Gα mRNA amounts (Gαz [black], Gα11 [orange], Gα13 [purple], Gαι1 [pink], Gαι2 [pale green], and Gαι3 [yellow]) were determined as described previously using total RNA prepared from cells lacking Gβ1, Gβ2, Gβ4, Gβ1/2, and Gβ1/2/4. Each bar represents the average ± range of relative mRNA levels calculated from two independent preparations. C) Amount of Gα and Gβ subunit protein expression in total lysates prepared from cells lacking Gβ1/2 expression. Total cell lysates were prepared from Gβ1/2 RNAi-transfected cells and quantitative Western blots were obtained for each Gα and Gβ subunit as described previously. Each bar represents the average ± S.D. from six independent preparations.

Figure 5: Relative Gβ mRNA and protein expression as a function of time following concomitant knockdown of Gβ1 and Gβ2 (Gβ1/2[a]). Relative mRNA levels for A) Gβ1, B) Gβ2, and C) Gβ4 are represented by dashed lines ([open square] for control and [open triangle] for Gβ1/2 silenced preparations) and relative protein expression is represented by solid lines ([closed square] for control and [closed triangle] for Gβ1/2 silenced preparations). Each point is normalized against mRNA or protein expression in 1 hr mock-transfected cells and represents the average ± range of two independent preparations.

Figure 6: Effects of silencing Gβ subunit protein expression on ligand-modulated effector activity. A) Prostaglandin E1 (10 μM PGE1) and B) isoproterenol (10 μM INE) stimulation of cAMP response. Ligand-dependent cAMP responses were examined in cells lacking an individual Gβ subunit (Gβ1, Gβ2, or Gβ4), two Gβ subunits (Gβ1/2), or three Gβ subunits (Gβ1/2/4). Each bar corresponds to the relative cAMP response in cells lacking Gβ subunit expression compared to control cell activity following a 2 min exposure to ligand and represents the mean ± standard deviation of at least eight independent experiments. C) Histamine (100 μM) dependent total inositol-phosphate (IP) accumulation. Each bar corresponds to the relative total IP response in cells lacking Gβ expression compared to control cell activity following a 15 min exposure to histamine and represents the mean ± S.D. of three independent experiments. D) and E) Inhibition of cAMP activity in cells lacking Gβ subunit expression. The percent UK 14,304-mediated inhibition of either D) prostaglandin E1 (10 μM PGE1) or E) isoproterenol (10 μM INE) -stimulated activity was determined as described previously for cells lacking Gα subunit expression. Each bar represents the average ± the range of two independent experiments.

Figure 7: Forskolin dose response for cAMP accumulation in cells lacking Gβ1/2 protein expression (closed triangle) and unperturbed cells (closed square). (inset) cAMP accumulation monitored as a function of time using water soluble forskolin (1 mM). Each point represents the average ± the range of duplicate measurements. The data are representative of at least two independent experiments.
Figure 8: Adenylyl cyclase protein expression in plasma membranes prepared from cells lacking Gβ1/2 subunits. One hundred µg/lane of enriched plasma membranes were blotted for A) type VI or B) type III adenylyl cyclase. Glycosylated adenylyl cyclase bands migrated as large smears with apparent MWs slightly larger than 150,000. Glycosylation of both type VI and III adenylyl cyclases was confirmed by observing increased mobility following enzymatic removal of glycosyl groups with PNGase F (data not shown). Non-glycosylated type III adenylyl cyclase could not be detected, whereas non-glycosylated type VI adenylyl cyclase co-migrated with a 100 kD standard (Precision Plus Protein Standards, BioRad). Antibody specificity was verified following isotype-specific siRNA-mediated knockdown of select adenylyl cyclases. No cross-silencing effects were observed with either isotype-specific RNAi duplex. Data are representative of three independent preparations.

Table I: Reagents used in the present study to silence or identify proteins.

Table II: Relative Gα and Gβ protein expression following select G protein subunit silencing. G protein subunits targeted for siRNA silencing are shown across the top of the table and proteins identified by Western blotting are indicated at the left. Western blots and densitometry analyses were conducted as described under Experimental Procedures. Each value corresponds to the pixel intensity of a particular antigen identified in siRNA-transfected total cell lysates normalized against the pixel intensity obtained for the same antigen in unperturbed control cell lysates loaded on the same gel. Data are represented as the mean ± standard deviation for a minimum of three separate total lysate preparations. Expression data were analyzed using single-sided ANOVA (p < 0.05) comparison of log (base 2) transformation of relative antigen expression for a particular perturbation against log transformation of the variance of protein expression for the same antigen determined from multiple control lysate preparations (range 5-12). Effects of siRNA transfection directed at the antigen are highlighted in yellow. Significant increases in non-targeted protein expression following a knockdown of G protein subunits are highlighted in green, and significant decreases are highlighted in red.
Figure 1A

RNAi Target: Control Gαs Gαq Gα11 Gαq/11

Gαs
Long
Short

Gα11

Gαq

RNAi Target: Control Gαi1 Gαi2 Gαi3 Gαi1-3

Gαi1

Gαi2

Gαi3

RNAi Target: Control Gαo

Gαo
Figure 1C

Endogenous G Protein Expression

Amount of Subunit (ng/mg total lysate)
Figure 2

A. INE

B. PGE1

C. Total Dose-Response Kinetics

D. Fsk Activated

E. PGE1 Activated

RNAi Target: Control, Goα1, Goα2, Goα12, Goα13, Goα12/3

Fsk A Kinase activation (% inhibition)

PGE1 A Kinase activation (% inhibition)
Figure 3A

The figure shows the relative Goq mRNA levels normalized to control for various RNAi targets: Gαs, Gαq, Gα11, Gα11, Gα2, Gα3, Gαo. The data is represented in a bar graph with error bars indicating the variability.
Figure 3B

RNAi Target: Control, Gαs, Gαq, Gα11, Gαq/11

Gαo

Gα11

Gαq

Control, Gα1, Gα2, Gα3
Figure 3C

Relative Gβ mRNA Levels (Normalized to Control)

RNAi Target: Gas, Gαq, Gα11, Gai1, Gai2, Gai3, Gai0

Gβ1
Gβ2
Gβ4
Figure 4A
Figure 4B

![Graph showing relative mRNA levels for different RNAi targets and Gα subunits.](image-url)
Figure 5
Figure 6

A.

B.

C.

D.

E.
Figure 7
Figure 8

A

RNAi Target:
Glycosylated-AC6
Non-Glycosylated AC6

B

RNAi Target:
Glycosylated-AC3

Control AC6 G812

Control AC3 G812
| Target | Name (Sequence) | Detection | Antibody Specificity (dilution factor) | Source/Citation |
|--------|----------------|-----------|----------------------------------------|-----------------|
| Gαs   | Gαs (715-733) CGAUUGACUGCACAUUC | AP 584 Gαs | (1:100) | 1 |
| Gαi1  | Gαi1 (41-59) AGCGAGAUAGAUGAUCGA | Anti-Gαi1 Gαi1 | (1:500) | Upstate Biotech. Clone 7H7 |
| Gαi2  | Gαi2 (681-699) CUGAGCUGCCUAGACUUG | Anti-Gαi2 Gαi2 | (1:2,000) | EMD Biosci. #371727 |
| Gαi3  | Gαi3 (383-401) CGGUAAUGCAGAGAUCUU | C-260 Gαi3> Gαo> Gαi1,2 | (1:10,000) | 2 |
| Gαi1 and Gαi3 | GGAGAUCUGAAAGCGAUCUG | See above for Gαi1 and Gαi3 |
| Gαo   | Gαo (525-544) CUCGGAACCAGGGUCAAA | 3C2 Gαo | (1:2,000) | 3 |
| Gαz   | Not Tested | P-961 Gαz | (1:4,000) | 4 |
| Gαq   | Gαq (231-249) GCUGGUGUAUCAGAACAUUC | W-082 Gαq | (1:1,000) | 5 |
| Gα11  | Gα11 (231-249) GCUGGUGUAUCAGAACAUUC | B-825 Gα11 | (1:1,000) | 6 |
| Gα12  | Not Tested | Anti-Gα12 Gα12 | (1:400) | EMD Biosci. #371778 |
| Gα13  | Gα13 (772-790) GAAGAUGACUGACAAUC | B-860 Gα13 | (1:5,000) | 7 |
| Gα16  | Not Tested | B-861 Gα16 | (1:1,000) | 8 |
| Gβ    | Not Tested | B-600 Gβ1=Gβ2=Gβ3=Gβ4 | (1:10,000) | 2 |
| Gβ1   | Gβ1 (351-379) GAAUAACAUUGCUCAAUU Gβ1 (855-873) CCUGGUGUGUACGACGAC | U-49 Gβ1>>Gβ2, Gβ4 | (1:5,000) | 9 |
| Gβ2   | Gβ2 (516-534) ACUGGGUACCUGUGCUUGU | J-887 Gβ2 | (1:1,000) | 10 |
| Gβ3   | Not Tested | U-5563 Gβ3 | (1:250) | Generated for this study |
| Gβ4   | Gβ4 (855-873) GUUGGUGGUGUACGACUGAC | Anti-Gβ4 Gβ4 | (1:200) | Santa Cruz #sc-382 |
| Gβ1/2 | Gβ1/2 (a) (865-883) UACGACGACUCAACUGCA Gβ1/2 (b) (866-884) ACUGGACGUCAACUCAACUGCA | See above for Gβ1 and Gβ2 |
| Gγ3   | Gγ3 (16-34) CCGGUAAACACUCAUGA |  |  |
| Gγ4   | Gγ4 (7-25) GAGGGCAUGUGUCAAAAC |  |  |
| Gγ5   | Gγ5 (119-137) UGCAGAUGCUAAACUGA |  |  |
| Gγ7   | Gγ7 (61-79) GCCGGGAUUGAGCGCAUCA |  |  |
| Gγ8   | Gγ8 (98-116) CAGCGGAACUCUGGCUU |  |  |
| Gγ9   | Gγ9 (72-90) CACAAAGAUAUUCCGAAUU |  |  |
|     |     |     |     |
|-----|-----|-----|-----|
| Gly10 | Gly10 (61-79) | GCUGCGUGGAGAGGAUCA |     |
| Gly11 | Gly11 (210-219) | AGGCACUGUGUUAUUUCA |     |
| Gly12 | Gly12 (64-82) | AGAUUAGAGCCCUAUUG |     |

**Effector**

| A Cyclase type III | AC3 (1791-1809) | AGAGUCCGCCCAAGUAGUA | Anti-ACIII | ACIII (1:200) | Santa Cruz #sc-588 |
|--------------------|------------------|----------------------|------------|--------------|-------------------|
| A Cyclase type VI  | AC6 (588-606) | GGUGUGUAACCGGCAUAGC | Anti-ACV/VI | ACV/VI (1:200) | Santa Cruz #sc-590 |

**Receptor**

| β2-adrenergic | B2-AR (549-567) | CUGCUAUGGCGGAGACCC | [[125]I]-CYP | Radiolabel competed with 118,551 | PerkinElmer LifeSciences |
| EP2            | EP2 (619-637) | CUCAUUGUCUGGUGCUAG |     |     |     |
| EP4            | EP4 (928-946) | CCAGAUAUGGCGGCAUCC |     |     |     |

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Table II: Relative Protein Expression

| NAi | Gαs | Gαq | Gα11 | GαI1 | Gα2 | GαI2 | Gα3 | GαO | Gβ1 | Gβ2 | Gβ4 | Gαq-1 | GαI-3 | Gβ1/2 | Gβ1-4 |
|-----|-----|-----|------|------|-----|------|-----|-----|-----|-----|-----|-------|-------|-------|-------|
|     |     |     |      |      |     |      |     |     |     |     |     |       |       |       |       |
| Blot|     |     |      |      |     |      |     |     |     |     |     |       |       |       |       |
| Gαs | 0.06 ± 0.06 | 1.1 ± 0.2 | 1.1 ± 0.4 | 1.1 ± 0.1 | 1.6 ± 0.1 | 1.2 ± 0.3 | 1.1 ± 0.2 | 0.6 ± 0.1 | 0.6 ± 0.0 | 1.1 ± 0.0 | 1.2 ± 0.2 | 1.2 ± 0.0 | 0.5 ± 0.2 | 0.03 ± 0.0 |
| Gαq | 0.8 ± 0.1 | 0.09 ± 0.02 | 0.6 ± 0.1 | 1.3 ± 0.5 | 0.7 ± 0.2 | 1.9 ± 0.3 | 0.8 ± 0.2 | 0.8 ± 0.2 | 0.6 ± 0.4 | 0.6 ± 0.1 | 0.08 ± 0.02 | 1.0 ± 0.3 | 0.8 ± 0.2 | 0.2 ± 0.1 |
| Gα11 | 0.9 ± 0.05 | 1.0 ± 0.2 | 0.01 ± 0.01 | 1.0 ± 0.1 | 1.2 ± 0.2 | 1.4 ± 0.3 | 0.8 ± 0.1 | 1.1 ± 0.3 | 1.0 ± 0.2 | 1.2 ± 0.4 | 0.0 ± 0.0 | 0.8 ± 0.2 | 0.9 ± 0.2 | 0.3 ± 0.1 |
| GαI1 | 2.4 ± 0.6 | 3.2 ± 1.0 | 2.8 ± 1.0 | 0.13 ± 0.13 | 1.7 ± 0.4 | 2.3 ± 1.0 | 0.6 ± 0.2 | 1.1 ± 0.5 | 0.4 ± 0.4 | 2.6 ± 1.3 | 2.6 ± 1.5 | 0.15 ± 0.07 | 0.07 ± 0.07 | 0.0 ± 0.0 |
| Gα2 | 1.1 ± 0.1 | 0.8 ± 0.05 | 0.8 ± 0.6 | 1.4 ± 0.6 | 0.1 ± 0.1 | 2.0 ± 0.4 | 1.4 ± 0.5 | 1.4 ± 0.8 | 1.0 ± 0.2 | 1.7 ± 0.4 | 0.8 ± 0.5 | 0.02 ± 0.04 | 0.07 ± 0.06 | 0.03 ± 0.05 |
| GαI2 | 1.0 ± 0.3 | 1.0 ± 0.2 | 0.9 ± 0.2 | 0.9 ± 0.2 | 0.8 ± 0.1 | 0.04 ± 0.04 | 1.0 ± 0.05 | 0.5 ± 0.1 | 0.5 ± 0.1 | 1.0 ± 0.3 | 0.9 ± 0.1 | 0.0 ± 0.02 | 0.07 ± 0.05 | 0.01 ± 0.01 |
| Gα3 | 1.4 ± 0.4 | 2.7 ± 2.1 | 2.7 ± 1.6 | 0.9 ± 0.4 | 1.2 ± 0.4 | 1.0 ± 0.2 | 0.03 ± 0.05 | 1.7 ± 0.9 | 1.3 ± 0.6 | 0.3 ± 0.1 | 0.0 ± 0.04 | 0.07 ± 0.07 | 0.0 ± 0.04 | 0.0 ± 0.04 |
| Gβ1 | 1.3 ± 0.3 | 1.4 ± 0.2 | 1.2 ± 0.1 | 0.7 ± 0.2 | 0.2 ± 0.04 | 0.7 ± 0.1 | 1.0 ± 0.01 | 0.03 ± 0.02 | 1.2 ± 0.1 | 1.5 ± 0.1 | 1.5 ± 0.3 | 0.2 ± 0.02 | 0.0 ± 0.0 | 0.0 ± 0.0 |
| Gβ2 | 1.5 ± 0.06 | 1.5 ± 0.1 | 1.5 ± 0.3 | 0.9 ± 0.3 | 0.7 ± 0.1 | 0.9 ± 0.3 | 1.1 ± 0.5 | 1.2 ± 0.4 | 0.01 ± 0.01 | 2.2 ± 0.7 | 1.5 ± 0.1 | 0.3 ± 0.1 | 0.02 ± 0.05 | 0.02 ± 0.03 |
| Gβ4 | 1.3 ± 0.0 | 0.9 ± 0.0 | 0.9 ± 0.1 | 0.7 ± 0.2 | 0.5 ± 0.1 | 0.2 ± 0.06 | 0.8 ± 0.1 | 1.4 ± 0.2 | 1.2 ± 0.3 | 0.02 ± 0.02 | 0.7 ± 0.3 | 0.3 ± 0.1 | 2.1 ± 0.7 | 0.09 ± 0.01 |
| Gβ1-4 | 0.8 ± 0.2 | 1.1 ± 0.2 | 1.0 ± 0.2 | 0.8 ± 0.1 | 0.6 ± 0.1 | 0.9 ± 0.1 | 1.0 ± 0.2 | 0.7 ± 0.1 | 0.6 ± 0.1 | 1.3 ± 0.0 | 1.0 ± 0.2 | 0.4 ± 0.2 | 0.3 ± 0.1 | 0.04 ± 0.04 |
Targeted knockdown of G protein subunits selectively prevents receptor-mediated modulation of effectors and reveals complex changes in non-targeted signaling proteins

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