Silencing the expression of multiple Gβ-subunits eliminates signaling mediated by all four families of G proteins

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The Gβγ-subunit complex derived from heterotrimeric G proteins can act to regulate the function of a variety of protein targets. We established lentiviral-based RNA interference in J774A.1 mouse macrophages to characterize the role of Gβ in G protein-coupled receptor signaling. The expression of Gβ1 and Gβ2, the major subtypes present in J774A.1 cells, was eliminated by sequential treatment with small hairpin RNA expressing lentivirus. These βγ complex-deficient cells lost the ability to respond to G protein-mediated signals. Chemotaxis and the phosphorylation of Akt in response to C5a were both blocked. Similarly, C5a-mediated actin polymerization, C5a- and UTP-stimulated intracellular calcium mobilization, and the stimulation of cAMP formation by isoproterenol were all eliminated in the absence of the Gβ-subunits. In addition, stabilization and membrane localization of several Gα- and Gγ-subunit proteins was strongly effected. Furthermore, in DNA microarray analysis, regulation of gene expression stimulated by prostaglandin E2 and UTP was not observed in cells lacking Gβ-subunits. In contrast, phagocytotic activity, serum-dependent cell growth and the patterns of gene expression induced by stimulating the Toll receptors with LPS were similar in wild-type cells and small hairpin RNA-containing cells. Thus, ablation of the Gβ-subunits destabilized Gα- and Gγ-subunits and effectively eliminated G protein-mediated signaling responses. Unrelated ligand regulated pathways remained intact. These cells provide a system that can be used to study signaling in the absence of most G protein-mediated functions.

cell signaling | RNA interference

Upon interaction of G protein-coupled receptors (GPCRs) with their cognate ligands, heterotrimeric G proteins are activated and dissociate into a GTP-bound α-subunit and a βγ dimer, and both of these can regulate specific downstream effector molecules (1, 2). The effector molecules regulated by Gβγ in mammals include certain isoforms of adenylyl cyclase, phospholipase C-β (PLC-β), phosphatidylinositol 3-kinase, p21-activated kinase, and GPCR kinases (3–7). In addition, Gβγ has been shown to modulate the activity of various ion channels (8, 9). The Gβγ-subunits are always found in the dimeric form, and the interaction of the Gβ protein with the Gγ protein appears to be required for their mutual stability (10). The Gγ-subunit contains a CAAX motif at its carboxyl terminus that directs lipid modification and is required for membrane localization of the Gβγ dimer (11).

The function of the Gβ molecule has mainly been studied in cell-free systems or on the cellular level. These studies have helped to define the nature of Gβ interactions with other molecules (12). A genetic approach to the study of the question of Gβ function was conducted in *Dictyostelium*, which contains a single Gβ-subunit (13). The Gβ-null mutants were severely defective in development, chemotaxis, and phagocytosis. Genetic analysis has also been useful in establishing some of the functions of the Gβ1-subunit in *Caenorhabditis elegans*. Thus, for example, Zwaal *et al.* (14) showed that expression of Gβ1 was important for orientation of the early cell division axis in the first larval stage. However, it is difficult to study the roles of the Gβ family members in mammalian systems by using genetic techniques because mammalian cells express multiple Gβ subtypes that can have similar or overlapping functions (15). However, gene silencing techniques employing double-stranded small interfering RNA (siRNA) can be used to observe the effects of specific target protein depletion on cellular function (16, 17). Furthermore, lentiviral vectors containing a selection marker, e.g., an antibiotic resistance gene, have been shown to act as efficient means for delivering siRNA (15, 18). They insert into the cellular genome and can be used to generate cell lines that express a specific siRNA as a hairpin structure (shRNA). In the present study, we demonstrate the effects of eliminating individual Gβ isoforms, or all Gβ isoforms, by lentivirus-delivered shRNAs. We conclude that the Gβ-subunits modulate all of the GPCR-related cellular responses that we measured. Interestingly, the elimination of a functional G protein-signaling system leaves other signal transduction pathways intact.

Materials and Methods

Virus Generation. Most lentiviral vectors were constructed and used for virus generation as described (15, 19). To make pL-UGIH, puromycin-resistance gene sequences were replaced with hygromycin-resistance gene sequences in pL-UGIP.

Adenovirus-expressing recombinant Gαq proteins were generated through homologous recombination between a linearized transfer vector pAD-Track CMV and the adenoviral backbone vector pAD-easy1 as described (20). The adenoviral vector contained either the wild-type Gαq cDNA (GqW) or the myristylation mutant Gαq cDNA (GqAG) with a Glu-Glu (EE) epitope in the internal recognition site (171–176 residues) (21). Expression of recombinant proteins in the virus-infected cells was verified by immunoblotting with anti-EE antibody (Novus Biologicals).

Western Blotting. Western blotting was performed with the following antibodies. Anti-phospho Akt antibody (raised against pSer473-Akt) (Cell Signaling Technology), anti-tubulin antibody (Oncogene), antibodies for Gαi2 and Gαq (kind gift from Susanne Mumby, University of Texas Southwestern Medical Center, Dallas), anti-Gγ12 antibody (kind gift from Tomiko Asano, Aichi Human Service Center, Aichi, Japan) (22), anti-C5a receptor (Pharmingen), and other anti-G protein antibodies (Santa Cruz Biotechnology) were used to detect their corresponding proteins. Peroxidase-conjugated goat anti-mouse and anti-rabbit antibodies (Amersham Pharmacia) were used, and then the proteins were detected by using an enhanced chemiluminescence (ECL) reagent (Amersham Pharmacia).

Abbreviations: GPCR, G protein-coupled receptor; PLC, phospholipase C; siRNA, small interfering RNA; shRNA, small hairpin RNA; PGE_2, prostaglandin E2; $[Ca^{2+}]_i$, intracellular Ca^{2+}.

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Measurement of Intracellular Ca^{2+} Mobilization and cAMP Assay. Cells (2×10^4 per well) were grown in 96-well black wall/clear bottom plates (Corning) overnight. After incubation with 1 μM fura-2/AM in Hanks' balanced salt solution (HBSS) for 30 min, cells were washed with HBSS twice. The ratio of fluorescence emission at 510 nm after excitation at 340 nm and 380 nm, caused by intracellular Ca^{2+} mobilization, was determined by using the Flexstation, a fluorometric imaging plate reader (Molecular Devices). cAMP generation was measured by using a HitHunter cAMP assay kit as described by the manufacturer (Applied Biosystems).

Chemotaxis and Actin Polymerization. The *in vitro* cell migration assay was performed as described (15). J774A.1 cells were plated on poly{L-lysine)-coated cover slips and incubated overnight in culture medium. After 6-h serum starvation, cells were stimulated with C5a for 2 min. They were fixed by using 4% paraformaldehyde in PBS for 30 min. Cells were permeabilized with 0.1% Triton X-100, stained with 0.5 unit of Texas red-conjugated phalloidin (Molecular Probes), and analyzed by confocal microscopy (Leica).

Cellular Fractionation. Cells were harvested with hypotonic buffer (20 mM Hepes, pH 7.5/3 mM $MgCl_2$/10 mM KCl) containing protease inhibitors, homogenized by passing through a 26-gauge needle 10 times. Lysates were centrifuged at $1,000 \times g$ for 10 min to remove unbroken cells and nuclei. The cleared lysates were subject to ultracentrifugation at $100,000 \times g$ for 1 h. The supernatants were kept as the cytosolic fractions. The precipitates which were kept as the membrane fraction were washed with same buffer twice and sonicated in a half volume of RIPA buffer containing protease inhibitors. Thirty micrograms of each fraction was then loaded on SDS/PAGE, followed by Western blotting with appropriate antibodies.

DNA Microarray. After growth in media containing 0.5% FBS for 18 h, cells were stimulated with agonist for 1 or 4 h, and then harvested with Trizol (Invitrogen). Total RNAs from the cells were used for microarray analysis. The 16,000 mouse oligonucleotide arrays were inkjet-printed by Agilent Technologies. These arrays include 13,536 70mers (Operon Technologies Inc.) and 2,304 65mers (Sigma-Genosys). The aminoallyl method was used for the preparation of fluorescently labeled target samples. All procedures and data analysis methods are described on the web site of the Alliance for Cellular Signaling (www.signaling-gateway.org/data/ProtocolLinks.html).

Results

Establishment of Cells Lacking Gβ1 and Gβ2 Proteins. Transfection of J774A.1 cells with plasmid-based expression vectors is very inefficient (15). However, pseudotyped lentivirus, which efficiently infects these cells and can be integrated into chromosomes, is a good carrier for delivery and sustained expression of genes (18). We constructed lentiviral vectors with cassettes that allow the expression of GFP and antibiotic selection markers to identify and enrich the fraction of transduced cells. We have shown in previous studies that Gβ1 and Gβ2 are the major abundant Gβ proteins in J774A.1 cells; the other isotypes were not detected in immunoblot analysis (15). A very low level of Gβ4 mRNA was found in RT-PCR experiments, but no Gβ4 protein was detected by anti-Gβ4 antibody. To establish cell lines expressing two siRNA genes, two cycles of infection and selection were performed. In the first cycle, cells were infected with a virus containing the Gβ1 shRNA that we previously demonstrated to be effective (15) and an expression cassette for puromycin resistance. The cells were then selected by growth in puromycin. In the second cycle, puromycin-resistant cells were infected with a virus containing the Gβ2 shRNA expression construct and a gene expressing hygromycin resistance. They were then grown and selected with hygromycin. The specific gene silencing activity of shRNA in the J774A.1 cells was confirmed by quantitative RT-PCR with specific primers and immunoblotting for endogenous proteins with isotype-specific antibodies. The mRNA level of each gene, normalized with GAPDH mRNA, was decreased by >90% in the doubly infected cells (Fig. 1 *A* and *B*). Consistent with RT-PCR data, significant decreases and inability to detect levels of Gβ1 and Gβ2 proteins were observed in Western blots (Fig. 1*C*). Nonetheless, when we transferred the cells to new plates and cultured at high density, wild-type and Gβ knockdown cells showed similar growth rates. At low plating densities the Gβ depleted cells showed slightly lower viability (data not shown).

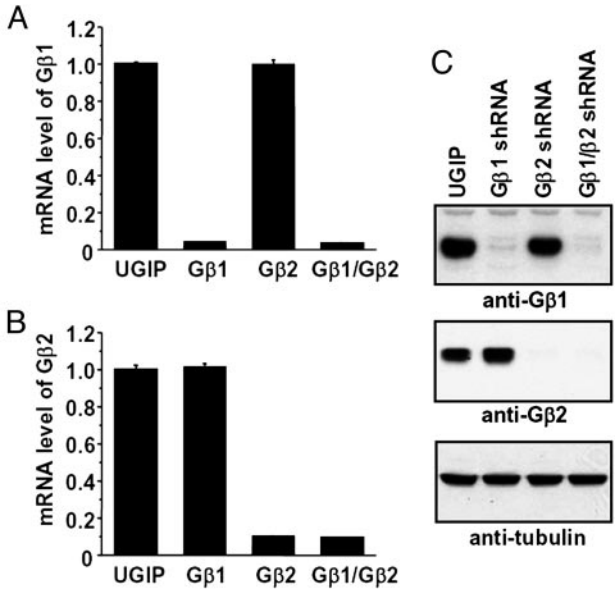

Fig. 1. shRNA-mediated inhibition of endogenous Gβ expression in J774A.1 mouse macrophages. (*A* and *B*) After lentivirus infection and puromycin selection for Gβ1 and Gβ2 or two cycles of infection and selection with puromycin and hygromycin for Gβ1/Gβ2, mRNA levels of Gβ-subunits were analyzed by TaqMan RT-PCR. mRNA levels were normalized to GAPDH mRNA. (*C*) Western blotting using subtype-specific antibodies. Protein expression was normalized with tubulin levels in total cell extracts.

Intracellular Ca^{2+} ($[Ca^{2+}]_i$) Transients and cAMP Generation Were Abolished in Cells Depleted of Gβ-Subunits. Increase of cytoplasmic Ca^{2+} concentration by GPCRs is known to be triggered by at least two different signaling pathways initiated by components of heterotrimeric G proteins, the Gαq- and G$\beta\gamma$-subunits. Upon treatment with chemokines or with C5a, G$\beta\gamma$-subunits released from the GTP-bound forms of Gαi increase cytoplasmic Ca^{2+} concentration by activating PLC-β2 or PLC-β3 (23). C5a-induced $[Ca^{2+}]_i$ increases in a pertussis toxin-sensitive manner (Fig. 2*A* and previous reports). Gβ2 shRNA-containing cells showed a weak response (peaking at approximately half the level of control cells and of Gβ1 shRNA-containing cells), suggesting that Gβ2 may be the major transducer of C5a receptor signaling. When we treated Gβ1 and Gβ2 shRNA-containing cells with C5a, no $[Ca^{2+}]_i$ release response was observed (Fig. 2*A*).

The P2Y receptors, a family of nucleotide receptors, respond to ligand binding by increasing $[Ca^{2+}]_i$ release. They are thought to activate Gαq which in turn directly stimulates PLC-β activation (24). Compared with the C5a-dependent $[Ca^{2+}]_i$ response, UTP, a ligand interacting with P2Y receptors stimulated more potent and sustained responses in J774A.1 cells. The $[Ca^{2+}]_i$ responses were slightly down-regulated in the cells containing single shRNA directed against Gβ1 alone or against Gβ2 alone. However, cells

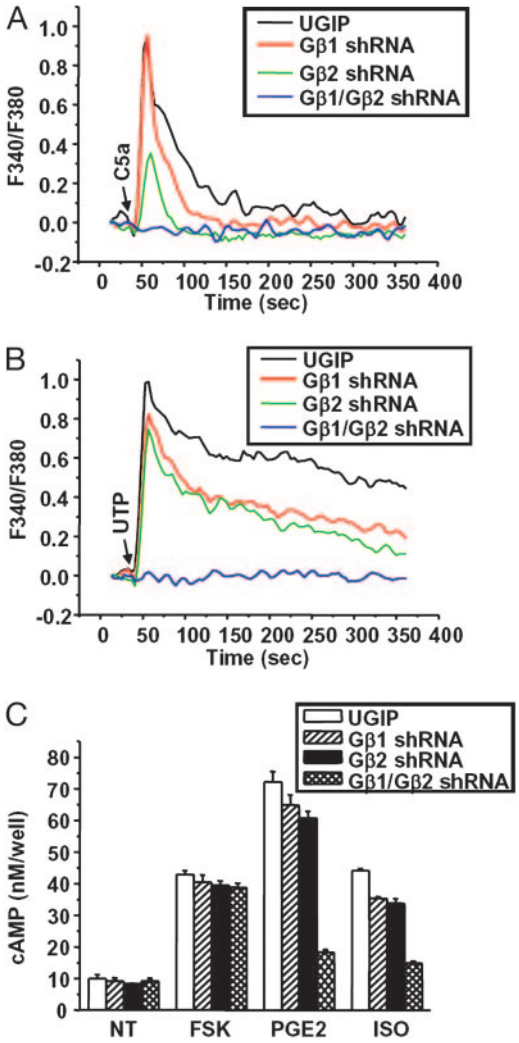

Fig. 2. Ca^{2+} mobilization and cAMP production. Arrows indicate the time points that stimulants were added. (*A* and *B*) The effect of Gβ shRNA on $[Ca^{2+}]_i$ changes stimulated by GPCR ligands. Cells were stimulated with 20 nM C5a (*A*) or 10 μM UTP (*B*), and $[Ca^{2+}]_i$ was monitored fluorometrically by using Fura-2/AM as described in *Materials and Methods*. Black, pL-UGIP (control); red, Gβ1 shRNA; green, Gβ2 shRNA; blue, Gβ1/Gβ2 shRNA. (*C*) cAMP production in the cells containing shRNA. Cells were stimulated with 10 μM forskolin, 10 μM PGE2, or 1 μM isoproterenol for 10 min, and cAMP production was measured as described in *Materials and Methods*. Relative light units from experiment were converted to cAMP concentration by using standard curve per the manufacturer's description.

depleted of both Gβ1 and Gβ2 by the presence of both shRNAs showed no detectable $[Ca^{2+}]_i$ response to UTP exposure (Fig. 2*B*). There is a large body of evidence indicating that different $\beta\gamma$ combinations can substitute for each other in activating PLC (25). The loss of the $[Ca^{2+}]_i$ response in doubly depleted cells suggests that the level of $\beta\gamma$ has been reduced by the absence of both Gβ1 and Gβ2, so that no effective response normally stimulated by a GPCR through a heterotrimeric complex of α and $\beta\gamma$ can be mounted. To exclude the possibility that the lack of ligand-stimulated $[Ca^{2+}]_i$ release in the absence of Gβ was due to shortage of intracellular Ca^{2+}, we evaluated the integrity of the Ca^{2+} stores. Thapsigargin induced similar $[Ca^{2+}]_i$ increases in all cell types (data not shown), indicating that Ca^{2+} stores were not influenced by shRNA or by absence of Gβ-subunits.

Gαs-mediated signaling modulates immune cell responses by regulating downstream signaling events and chemokine production (26, 27). According to gene expression analysis data, J774A.1 cells express several types of prostaglandin E2 (PGE_2) receptors and β2-adrenergic receptors, which activate Gαs (data not shown). To examine the effect of loss of G$\beta\gamma$ on Gαs-mediated signaling, we measured cAMP production stimulated with PGE_2 and isoproterenol. Both agonists induced cAMP generation in wild-type cells and single shRNA-containing cells, but not in the cells containing both shRNAs of Gβ1 and Gβ2 (Fig. 2*C*). On the other hand, forskolin, which directly activates adenylyl cyclases, induced cAMP production in all cell types. Taken together, the data suggest that Gβ-subunits are also essential for Gαs-mediated signaling.

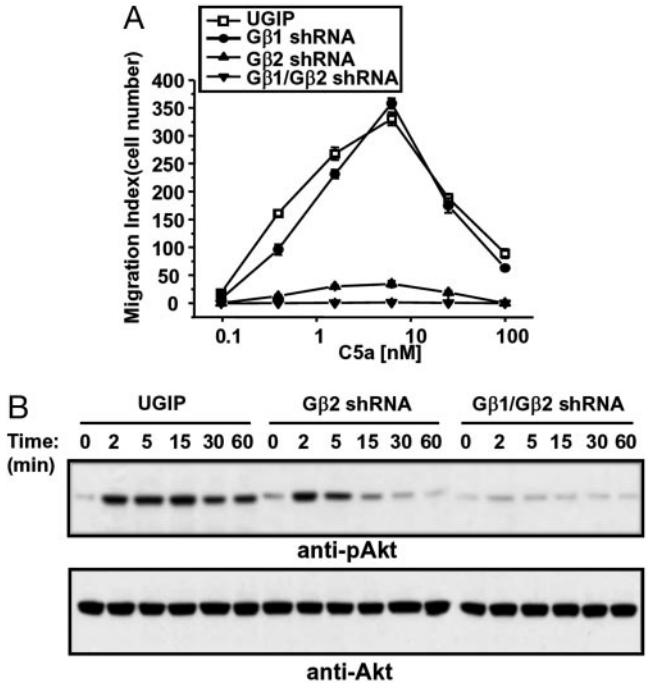

Fig. 3. Effects of Gβ silencing on cell responses to chemokines. (*A*) Chemotaxis of J774A.1 cells toward C5a. Chemotaxis was analyzed by using transwell migration, as described in *Materials and Methods*. The data are expressed as the mean ± SE of three independent experiments. Open square, UGIP (control); filled circle, Gβ1 shRNA; filled triangle, Gβ2 shRNA; inverted filled triangle, Gβ1/Gβ2 shRNA. (*B*) C5a-stimulated phosphorylation of Akt. After incubation with media containing 1% FBS for 12 h, cells were stimulated with C5a for the indicated times. Cell lysates were subjected to immunoblotting with anti-phospho Akt antibody. Immunoblots were stripped and reprobed with anti-Akt antibody.

Migration Activity Is Impaired in Cells Lacking Gβ Proteins. Migration of immune cells to most chemokines depends on members of the pertussis toxin-sensitive Gαi signal transducing gene family (28). The activated receptor releases the G$\beta\gamma$-subunits from the Gαi heterotrimer, and they in turn activate a variety of proteins that are directly involved in initiating chemotaxis (29). We used Boyden chamber assays to examine migration activity of the cells to C5a, whose receptor is expressed in J774A.1. As shown in our previous report (15), control and Gβ1 shRNA-containing cells migrated to C5a in a dose-dependent manner. However, manyfold fewer migrating Gβ2 shRNA-expressing cells were observed as a result of C5a stimulation. For the cells containing both shRNAs that simultaneously eliminated Gβ1 and Gβ2, we could not detect any chemotaxis toward C5a (Fig. 3*A*). This result further supports the conclusion that all signaling pathways involved in migration to C5a were impaired in the absence of Gβ proteins.

Phosphatidyl inositol 3-kinase γ (PI3Kγ) plays a major role in cell

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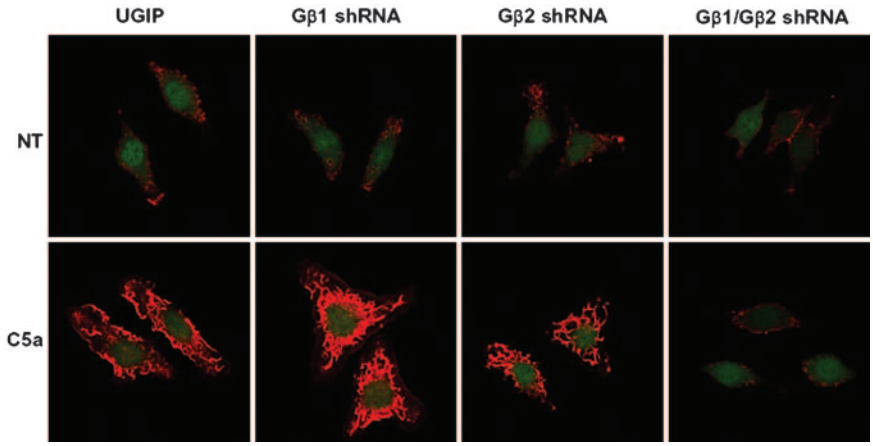

Fig. 4. Effects of Gβ silencing on actin polymerization by C5a. Cells were treated with C5a for 2 min, fixed with 4% paraformaldehyde, and stained with Texas red-conjugated phalloidin. C5a induced actin polymerization in control and the cells containing either Gβ1 or Gβ2 shRNA. However, actin polymerization was not detected in the cells containing both Gβ1 and Gβ2 shRNA.

migration by interacting with G$\beta\gamma$-subunits (30, 31). PI3K produces phosphatidyl inositol-3,4,5-tris-phosphate, which induces membrane translocation and phosphorylation of Akt. We examined Akt phosphorylation, an event reflective of PI3K activation. The Akt phosphorylation on Ser-473 by C5a was observed after 2 min of stimulation and was sustained for >30 min in C5a-treated control cells and Gβ1 shRNA-containing cells. In Gβ2 shRNA-containing cells, phosphorylation occurred after 2 min but disappeared within 15 min, implying that Gβ2 is important for extended Akt phosphorylation. However, Akt phosphorylation was not detected at all in the cells containing both shRNAs (Fig. 3*B*).

C5a Cannot Increase Actin Polymerization in Cells Depleted of Gβ-Subunits. Polymerization of actin filaments is an indispensable intracellular event for cell migration in cells responding to chemokines. Rho GTPases are implicated in this process (32). Rho is implicated in the actin polymerization and formation of focal adhesion complexes, resulting in actomyosin assembly and the contractile tension necessary for movement (33). Gα12/13 mediates the Rho activation signal from chemokine receptors by stimulating guanine nucleotide exchange factor, p115Rho-GEF (34). To determine the effect of Gβ depletion on Gα12/13-mediated signaling by chemokine receptors, we stimulated the cells with C5a. Actin polymerization occurred in control cells and single shRNA containing cells stimulated with uniform C5a for 2 min (Fig. 4). However, polymerized actin filaments were not observed in cells containing shRNA directed against both Gβ1 and Gβ2. We observed cells for 30 min in 5-min intervals to exclude delayed responses to stimulation, but no actin polymerization was detected (data not shown).

Gβ-Subunits Affected the Expression of Gα and Gγ. It is well known that the individual members of the G protein heterotrimers are necessary for the stability of their binding partners (10, 15). According to Western blotting and FACS analysis with anti-C5a receptor antibody, expression levels of the receptor were not changed in control and shRNA-containing cells, and membrane localization was maintained (Fig. 5*A*), indicating that the absence of

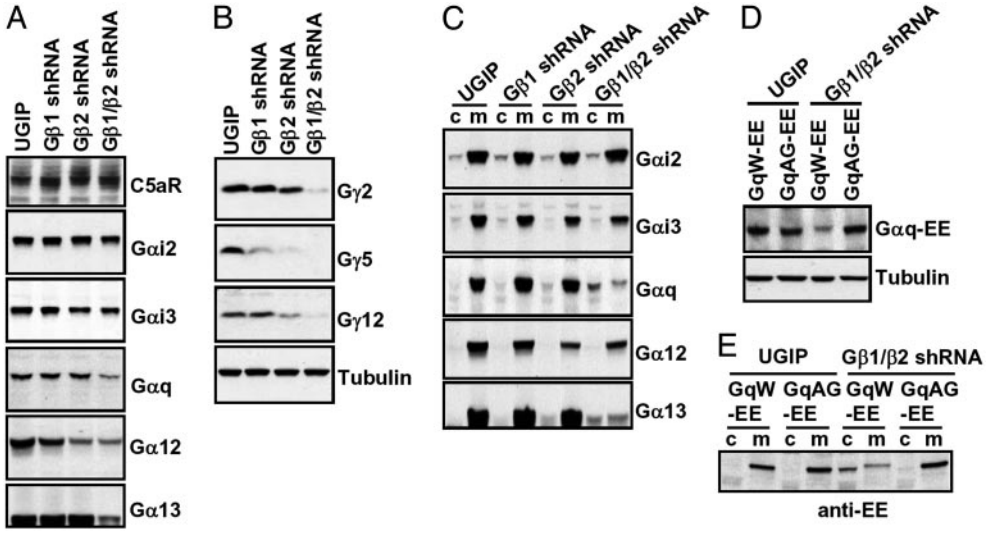

Fig. 5. Expression of C5a receptors, Gα, and Gγ in the absence of Gβ-subunits. (*A* and *B*) Cells were lysed with lysis buffer containing 0.1% SDS, and expression of each protein was analyzed with specific antibodies. Protein expression was normalized to tubulin levels in total cell extracts. (*C*) The distribution of Gα-subunits between cytosol (c) and membrane (m) was analyzed by Western blotting. (*D* and *E*) The effect of myristylation on expression and localization of Gαq. Adenovirus containing EE-tagged forms of wild-type Gαq and GαqAG (myristylation form) were transduced into J774A.1 cells. The cells were harvested, and the expression of EE-tagged Gαq was detected with anti-EE.

Gβ-subunit proteins does not affect expression of GPCRs. To examine the stability of Gα- and Gγ-subunits in the absence of Gβ subunits, we performed quantitative RT-PCR and Western blot analysis. The mRNA levels corresponding to all examined proteins were not affected in the cells depleted of Gβ subunits, compared with control cells (data not shown). Protein levels of Gαi2 and Gαi3 were not changed significantly, whereas Gαq, Gα12, and Gα13 were considerably down-regulated in cells lacking Gβ1 and Gβ2 (Fig. 5*A*). Gα12 was also down-regulated in the absence of Gβ2 alone. Expression of the Gγ2-, Gγ5-, and Gγ12-subunits was investigated by using specific antibodies. All Gγ-subunits tested were significantly diminished in cells depleted of both Gβ1 and Gβ2, indicating that Gβ affected the generation or stability of Gγ-subunit protein (Fig. 5*B*).

Interaction with Gβγ-subunits and Gα lipid modification myristylation and palmitylation are required for the membrane localization of Gα-subunits (21, 35–37), but there is some controversy about the relative role of Gβγ in the localization of Gα-subunits. For example, release of Gβγ from the membrane did not prevent retention of Gαo in the growth cone of nerve cells (38). Several Gα-subunits can be localized in the Golgi membrane in the absence of Gβγ-subunits (39). It has been suggested that double lipid modification is sufficient to maintain Gα at the plasma membrane; however, in the absence of interaction with Gβγ-subunits, the Gα subunit protein may not be effective. In this study, cells were separated into cytosol and membrane to identify subcellular localization of Gα-subunits. As shown in Fig. 5*C*, the majority of Gα-subunits in mock-treated cells were detected in membrane fractions. In the double knockdown cell, the membrane localization of Gαi2, Gαi3, and a portion of the Gα12 protein was not changed. On the other hand, a large fraction of the Gαq and Gα13 protein was absent and residual amounts were detected in the cytosol fractions of the Gβ-depleted cells. Interestingly, Gαi family members have dual lipidation (myristylation and palmitylation), but Gαq and Gα13 lack a myristylation site and have only a palmitylation residue. To determine the effect of lipid modification on the expression of Gα-subunits in the Gβγ-deficient cells, we followed the work of Evanko *et al.* (21) and constructed recombinant Gαq (GαqAG) containing dual lipidation sites by substituting Ala with Gly in N-terminal region and using an adenoviral vector to mediate gene expression. After adenovirus infection into control cells and Gβ-deficient cells, subcellular fractionation was performed. The expression of GαqAG was not changed in both cells, although wild-type Gαq was down-regulated in cells lacking Gβ-subunits (Fig. 5*D*). Furthermore, GαqAG was detected in the membrane fraction but not in cytosol fraction, which is similar to its expression pattern in control cells (Fig. 5*E*). The results further support the notion that dual lipid modification, myristylation in addition to palmitylation, even in the absence of Gβγ, is sufficient for membrane localization of the Gαq-subunits and perhaps for the Gαi-subunits as well. Once bound to the membrane, the Gα-subunit proteins can remain in a stable form. The Gβγ-subunits also play a critical role in stabilizing and directing the singly modified Gα-subunits to a suitable membrane region for interaction with the GPCR (36, 37). The exception not covered by this rule is Gα12. Although its level is markedly decreased, the residual protein does remain associated with the membrane fraction. However, our fractionation procedure does not allow us to distinguish between plasma membrane, Golgi, or endoplasmic reticulum. Gα12 could interact with other components and be stabilized in the Golgi fraction.

Gβ-Subunits Were Not Involved in Phagocytotic Activity or Growth of J774A.1 Cells. In studies of *Dictyostelium discoidium*, deletion of Gβ resulted in impaired phagocytosis and chemotaxis (14). Using opsonized zymosan particles with Ig, we examined phagocytotic activity of the cells. Contrary to the results in *Dictyostelium*, the activity was similar in all cells (data not shown).

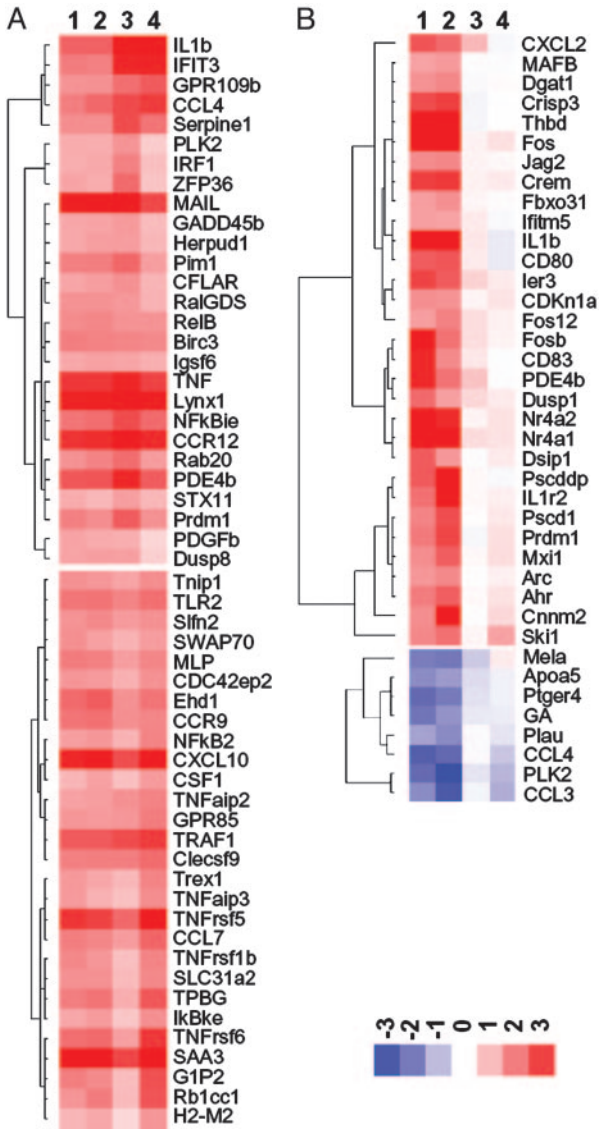

Fig. 6. Hierarchically clustered dendrograms of expression changes induced by LPS or PGE_2. Clustering was achieved by using the CLUSTER and TREEVIEW programs (http://rana.lbl.gov/EisenSoftware.htm). Each row represents a gene, and each column represents a particular sample (1, wild type 1 h; 2, wild type 4 h; 3, Gβ1/β2 shRNA-harboring cells 1 h; 4, Gβ1/β2 shRNA-harboring cells 4 h). The expression level for each gene relative to the expression level of that gene in the 0-h sample was provided by a red–blue color scale shown in log_2 scale. (*A*) LPS-regulated gene expression changes. Note that the pattern of regulation observed in wild type was also present in Gβ1/β2 shRNA-harboring cells. (*B*) PGE_2 regulated gene expression changes. Note that the regulation observed in wild type was completely absent in Gβ1/β2 shRNA-harboring cells.

Although phagocytosis depends on actin polymerization and rearrangement, immune cells recognize opsonins such as Ig and complement that bind to foreign bodies through cognate receptors. These receptors presumably triggered signaling pathways for phagocytosis without activating heterotrimeric G proteins.

Gene Expression Induced by PGE_2 and UTP Was Blocked in the Absence of Gβ-Subunits. Extracellular stimulants may induce various cellular responses by regulating specific gene expression. To observe the effect of the absence of Gβ on ligand-dependent gene regulation, we examined the gene expression in J774A.1 cells stimulated with LPS (100 ng/ml LPS and 100 pM LPS-binding peptide), 10 μM

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PGE_2, and 10 μM UTP by using microarrays that monitored the expression of ≈11,000 transcripts. By the criterion of a ≥2-fold change in expression in all experiments, exposure of the cells to LPS for 1 h resulted in the up-regulation of >100 transcripts in control cells. LPS treatment of cells lacking Gβs for 1 h led to a similar pattern of gene expression as in the control cells. The expression of >200 genes was changed in response to LPS exposure for 4 h in cells lacking Gβs. Although the overall pattern of gene expression was similar to that of the control cells, there were subtle differences in the changes in transcript levels induced by LPS treatment in the cells lacking Gβ protein (Fig. 6*A* and data not shown). On the other hand, PGE_2 regulated the expression of 60 genes after 1 h of treatment and 180 genes after 4 h (Fig. 6*B*). However, there was no change in transcript level in the cells depleted of Gβ-subunits. Thus, whereas Gβ-subunits are not necessary to mount the Toll receptor response pattern, the presence of Gβ protein is absolutely required for the PGE_2 response. There is evidence to suggest that some of the subsequent steps in LPS-stimulated gene expression can be modulated by GPCR signaling. Thus, for example, PGE_2 receptors are up-regulated by LPS treatment and, when activated, attenuate LPS-induced gene expression in macrophage cells (26, 40). Extended treatment with LPS may recruit G protein-signaling pathways and modify later gene expression. A more complete analysis of the effects of the loss of Gβ-subunit protein and the subsequent interruption of G protein-mediated signaling on ligand induced responses is required.

Discussion

J774A.1 cells primarily express the Gβ1 and Gβ2 isoforms. We have previously shown that "knockdown" of gene expression of Gβ2 can lead to migration defects to some chemokines (15). The loss of both Gβ1- and Gβ2-subunits leaves the cells with essentially no Gβ. Because Gγ is stable only in a complex with Gβ, the loss of Gβ resulted in the collateral loss of the Gγ-subunits. Interestingly the Gα-subunit proteins that did not contain multiple lipid modification residues and thus did not reach the membrane were also destabilized. It is not clear whether the membrane-associated Gα-subunits have any alternative activity, but it would be surprising if they could be activated by GPCRs. On the other hand, there might be other exchange factors that could activate the residual Gα-subunits. At present, there is no evidence to support this conjecture. In fact, all of the data suggest that, although GPCRs remain intact, the absence of $\beta\gamma$-subunits results in the specific inactivation of all of the tested G protein gene family-mediated signaling pathways. The Gαs pathway includes gene activation and cAMP generation by PGE_2 and isoproterenol stimulation. The Gαi pathway includes chemotaxis to C5a. The Gαq pathway is responsible for Ca^{2+} release in response to UTP, and the Gα13 pathway is critical for actin polymerization.

We do not have a comprehensive picture of how the cells adapt to the absence of the G protein-mediated pathways. However, it is clear that, when not stressed, they behave relatively normally. They can grow at rates similar to those of parent cells, can generate complex responses to ligands such as LPS, and can organize complex cellular movements and shape changes required for phagocytosis. On the other hand, the transcriptional regulation as well as the elevation of cAMP levels mediated by the application of isoproterenol or PGE_2, which utilizes the Gs pathway, were completely eliminated.

The data with the Gβ1/Gβ2 double knockdowns demonstrate how the individual components of a protein complex can depend on protein folding and assembly for both their stability and function. In addition, the data show that, in the absence of a functioning G protein-mediated signaling system, cells can grow and other signaling pathways can function. By using a variety of ligand addition protocols (as described by the Alliance for Cell Signaling), it may be possible to use the Gβ1/Gβ2-depleted cells in transcript analysis experiments to determine the nature of signal pathway interaction.

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