Lyso phosphatidic acid (LPA) utilizes a heterotrimeric guanine nucleotide regulatory (G) protein-coupled receptor to activate the mitogen-activated protein kinase pathway and induce mitogenesis in fibroblasts and other cells. A single cell assay system was used to examine the functional interaction of the LPA receptor with G proteins in intact mouse fibroblasts, by measuring LPA-stimulated induction of the immediate-early gene, c-fos, as read out by a stably expressed fos-lacZ reporter gene. Pretreatment of these cells with pertussis toxin at 100 ng/ml almost completely abolished LPA-stimulated c-fos induction. Western blotting revealed that two pertussis toxin (PTX)-sensitive G proteins, G$_{i2}$ and G$_{i3}$, were present in membranes prepared from these cells, and Northern blotting confirmed the absence of message for other PTX-sensitive subunits. Microinjection of an $\alpha_i$/G$_{i2}$-specific antibody into living cells decreased LPA-stimulated induction of c-fos by 60%, whereas introduction of antibodies to either $\alpha_q$ or $\alpha_{i10}$, a subtype not present in these cells but used as a control, decreased LPA-stimulated c-fos induction by only 19%. In contrast, the $\alpha_{i1}$/G$_{i2}$-specific antibody had no effect on insulin-induced c-fos expression, which is thought to utilize a G protein-independent mechanism of signaling. In addition, cellular expression of an epitope-tagged PTX-resistant mutant of G$_{i2}$, but not PTX-resistant G$_{i3}$, restored LPA-stimulated c-fos induction in cells in which endogenous G protein $\alpha$ subunits were uncoupled from the receptor by pretreatment with PTX. Together, these results provide conclusive in vivo evidence that G$_{i2}$ is the PTX-sensitive G protein $\alpha$ subunit which mediates LPA-stimulated c-fos induction and perhaps mitogenesis in these cells.

Lysophosphatidic acid (LPA) is a water-soluble phospholipid that is a normal constituent of mammalian serum. It is released from activated platelets (1) and is rapidly generated by growth factor-stimulated fibroblasts (2). LPA has been shown to activate a variety of second messenger pathways, including stimulation of phospholipases C and D and inhibition of adenyl cyclase (3, 4), and elicits a diverse range of physiological responses including smooth muscle contraction (5), remodeling of the actin cytoskeleton (6), and mitogenesis (3).

LPA is thought to interact with a specific cell surface receptor identified as a 38–40-kDa protein by photoaffinity labeling (7) and, more conclusively, by membrane binding studies (8). Several lines of evidence suggest that this LPA receptor couples to downstream effector molecules through the activation of a guanine nucleotide regulatory (G) protein. For example, pertussis toxin (PTX) abolishes the LPA-induced inhibition of adenyl cyclase (3) and the ability of guanine nucleotides to regulate LPA binding in rat brain and Swiss 3T3 cell membranes (8). In addition, guanine nucleotides enhance LPA-induced inositol trisphosphate formation in permeabilized cells (9). Finally, LPA has been shown to stimulate high affinity GTPase activity and cholera toxin-catalyzed ADP-ribosylation of G$_i$ in membranes prepared from Rat-1 fibroblasts (10).

LPA stimulates mitogenesis through the activation of p21ras (11, 12) and the subsequent activation of the mitogen-activated protein (MAP) kinase pathway (13). However, in contrast to the activation of p21ras that occurs in response to ligands of receptor tyrosine kinases, such as insulin or epidermal growth factor (reviewed in Ref. 14), the LPA-induced activation of p21ras is abolished by pretreatment with PTX (11, 12). It also appears to be largely independent of the phospholipase C-mediated calcium mobilization induced by LPA, since PTX had no effect on this pathway (3). These findings suggest that a G protein of the G$_i$ family, or G$_o$, regulates the activation of p21ras by LPA. G$_o$ is limited in tissue distribution, being found predominately in brain, where it has been shown to be involved in regulating ion channels (15). In contrast, G$_i$ proteins are expressed ubiquitously and are the most abundant G proteins found in many cell types.

The identity of the G$_i$ protein that couples the LPA receptor to the mitogenic response is unknown. One candidate is G$_{i2}$, since mutationally activated G$_{i2}$ mimics the G$_i$-mediated inhibition of cAMP accumulation caused by LPA (16). In addition, expression of the gip2 oncogene, which encodes a constitutively active form of G$_{i2}$, has been shown to induce neoplastic transformation in Rat-1 cells, but not in NIH 3T3 or Swiss 3T3 cells (17, 18). However, LPA-induced activation of G$_{i2}$ has not been tested directly.

The agonist-stimulated induction of the immediate-early genes, such as c-fos, precedes the progression of cells through the cell cycle. Therefore, agonist-stimulated induction of c-fos can be used as a marker for mitogenesis. In this study we used a functional assay to examine receptor-G protein interactions in mouse fibroblasts where the induction of c-fos was read out by a stably expressed fos-lacZ reporter gene. Inhibitory anti-
bodies directed against specific G protein α subunits were microinjected into living cells. We assessed the potential inhibitory effect of these antibodies on the LPA-stimulated induction of c-fos, in order to investigate which of the G subtypes mediates LPA-stimulated mitogenesis. In addition, using transfection of PTX-resistant mutants of specific G protein α subunits, we examined whether cellular expression of a single α subunit was sufficient to restore LPA-stimulated signaling in cells in which the LPA receptor was uncoupled from its cognate G proteins by pretreatment with PTX.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—Fibroblasts derived from fos-lacZ mice (19) were kindly provided by Dr. Tom Curran, St. Jude Children’s Research Hospital, Memphis, TN. The cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, 2 mM glutamine, and antibiotics (100 units/ml penicillin/100 μg/ml streptomycin) and were incubated in a humidified atmosphere of air with 5% CO₂ at 37 °C. Mouse fos-lacZ fibroblasts stably transfected with the human insulin receptor (see below) were cultured in the above medium which was also supplemented with 0.6 mg/ml G418. Mouse fos-lacZ fibroblasts stably coexpressing the insulin receptor and the PTX-resistant mutant of Gαs (supplied in medium containing all of the above supplements plus 0.3 mg/ml hygromycin B. All media reagents except for hygromycin B (Boehringer Mannheim) were obtained from Life Technologies, Inc.

**Generation of Stable Cell Lines**—A stably transfected derivative of the fos-lacZ cell line was created that overexpressed the human insulin receptor and resulted in insulin-stimulated nuclear c-fos expression. The human insulin receptor cDNA in the SRα expression vector (20) was kindly provided by Dr. Richard Roth, Stanford University, Palo Alto, CA. fos-lacZ fibroblasts were transfected with a 10:1 mixture of the plasmid containing the human insulin receptor cDNA (10 μg) and pSV2neo (1 μg; American Type Culture Collection, number 37184, Rockville, MD) by the calcium phosphate method (21). Briefly, subconfluent cells (50–70% confluence) growing in 60-mm dishes were incubated for 16 h with the DNA/CaCl₂ mixture, washed twice briefly with Hanks’ balanced salt solution, and then incubated with standard growth medium. Twenty-four h later the cells were split 1:20 and cultured in the presence of 0.6 mg/ml G418. After 7 days, colonies that appeared to originate from single cells were isolated from the dishes, expanded, and further subcloned by an additional 1:20 passage. Following separation of colonies that originated from a responsive line were screened by examining insulin-stimulated β-galactosidase (β-gal) activity in single cells. Five candidate cell lines were obtained which differed mainly in the percentage of cell nuclei staining positive for β-gal in response to 10 nM insulin. Of these, the cell line designated 12B had the highest percentage of cells staining positive for β-gal. The microinjection experiments described here were conducted using cells in which fewer than 1% of the cell nuclei showed β-gal staining in the serum-deprived state, and 75–85% of the cells exhibited β-gal staining 110 min after stimulation with 10 nM insulin. The β-gal staining was localized to the cell nucleus, and the pattern of staining was identical to that observed in immunofluorescence studies utilizing anti-c-fos or anti-β-gal antibodies (data not shown). Another line derived from line 12B was generated that stably overexpressed the PTX-resistant mutant of Gαs, termed GαSat/S. Briefly, cells were co-transfected with a 10:1 ratio of plasmid containing the cDNA encoding GαSat/C-S in the pRc/CMV expression vector (22) and a hygromycin plasmid to allow for selection. Cells were selected for stable transfection with 300 μg/ml hygromycin B by techniques identical to those described above. Hygromycin-resistant lines were screened for overexpression of GαSat by Western blotting following separation of membrane proteins by high resolution urea SDS-PAGE (23).

**Antibodies to G Protein α Subunits**—Affinity purified rabbit polyclonal antibodies to G protein α subunits were generously provided by Dr. Allen Spiegel (National Institutes of Health, Bethesda, MD); the preparation and specificity of these antibodies has been described in detail (24, 25). Briefly, antibody AS was raised to a synthetic decapetide (KKNLKGDCGF) corresponding to the carboxy-terminal region of transducin α; its specificity is αs/αi/αo > αo > αs > αi. Only a single amino acid difference exists between the human and rat sequences for peptide EC. In the case of antibodies AS and EC, the peptide antigen was derived from the region of the protein thought to interact with the receptor (27, 28). Antiserum AR was raised to a carboxy-terminal decapetide of Gαo. This antibody does not cross-react with other known α subunits, and the αo species has been found in mouse hepatocytes and in BHK cells transiently transfected with an expression vector containing Gαo by using an antibody conjugate to a synthetic decapetide (LDRIAGPNYY) corresponding to internal sequence 159–168 in Gαo, and was purchased from Calbiochem-Novabiochem Corp. (San Diego, CA). In some experiments, a GαoN200 subunit selective rabbit antisemurum, number 982, purchased from Calbiochem, was used for immunoblotting. Antibody to the α subunit of Gαs was purchased from Upstate Biotechnology Inc. (Lake Placid, NY) and was used for immunoblotting only.

**Membrane Preparation and Western Blotting**—Two confluent T-15 flask of mouse fibroblasts were briefly washed twice with ice-cold phosphate-buffered saline without calcium and magnesium (PBS/−, pH 7.2), collected by scraping into ice-cold PBS/−, pooled, and centrifuged at 1,000 × g for 5 min at 4 °C. The cell pellet was resuspended in ice-cold containing 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (Sigma) and 20 μg/ml leupeptin (Sigma) in 20 mM Tris-Cl (pH 7.4), homogenized by 20 strokes in a Teflon-glass tissue grinder (Thomas Scientific, Philadelphia, PA) and centrifuged at 500 × g for 5 min at 4 °C to remove nuclei and unbroken cells. The supernatant was set aside at 4 °C and the pellet was subjected to a second round of homogenization and centrifugation. The combined supernatants were centrifuged at 100,000 × g for 30 min at 4 °C. The resulting pellet was resuspended in lysis buffer and taken as the membrane fraction. Protein concentration was measured by a dye binding method (Bio-Rad). Membranes from bovine brain were kindly provided by W. Koch (Duke University, Durham, NC). Membrane pro- teins, or, in some cases, whole cell lysates were subjected to either electrophoresis on 12% SDS-polyacrylamide gels according to Laemmli (30), or to high resolution urea SDS-PAGE and transferred to nitrocel- lulose (Schleicher & Schuell, Keene, NH) for immunoblotting according to the procedure in the Bio-Rad Immun-Blot assay kit. Individual membranes were incubated with anti-Gi antibodies at the following concentrations: AS, 3.6 μg/ml; EC, 4.3 μg/ml; AR, 2.8 μg/ml; and anti-Gαi2, and Gαi3 at a 1:1,000 dilution. Proteins were visualized using a horseradish peroxidase-conjugated goat anti-rabbit IgG (Bio-Rad) at a 1:1,500 dilution. Immunoblotting with the EE monoclonal antibody (Berkley Antibody Co., Berkely, CA), to detect expression of the tran- siently transfected GEEC-S construct, which is the epitope-tagged and PTX-resistant analog of Gαsat (31), was carried out with a 1:1,000 dilution of the primary antibody, and a 1:1,000 dilution of the horserad- ish peroxidase-conjugated goat anti-mouse IgG (Bio-Rad). Any changes to these protocols indicated in the figure legends.

**Isolation of RNA and Northern Analysis**—Total RNA was isolated from mouse fibroblasts or frozen mouse or rat brains by a modification (32) of the single step acid-guanidinium thiocyanate/phenol/chloroform extraction (33). The RNA was separated on 2.2% agarose gel, transferred to a Nytran membrane (Schleicher & Schuell), and for Northern blot analysis as described previously (34). The full-length coding rat cDNAs for Gαsat and Gαi3 were excised from the bacterial expression vector NtpT7-5 by complete digestion with XbaI and partial digestion with NcoI. The 1.13- and 1.29-kb fragments, corresponding to Gαsat and Gαi3, respectively, were gel purified and concentrated using the GeneClean kit according to the manufacturers instruc- tions (Bio101 Inc., La Jolla, CA). The cDNA probes were labeled with [α-32P]dCTP using the Random Primers DNA labeling system (Life Technologies) and unincorporated nucleotide was removed by chroma- tography on a NucTrap column (Strategene, La Jolla, CA). Filters were hybridized at 42 °C with denatured probes at 2 × 10⁶ cpm/ml for 45 h, then washed and subjected to autoradiography at -70 °C. The blots were then stripped in 0.1% SSPE, 0.1% SDS as described previously (35), or to 0.1× SSPE, 1.1× PEG 8000, 2 × 10⁶ cpm/ml of chicken α-tubulin to ensure equal RNA loading.

**Microinjection**—Approximately 48 h prior to the microinjection experiments, cells were seeded from trypsinized stock flasks onto single round sterile glass coverslips (Carolina Biological Supply, Burlington, 2 A. M. Spiegel, personal communication.
NC in 35-mm dishes at a density sufficient to achieve approximately 50–60% confluence 24 h later. Twenty-four h after seeding, cells were washed three times with Hanks’ balanced salt solution and incubated for an additional 24 h in Dulbecco’s modified Eagle’s medium containing 2 mM glutamine, antibiotics, and 1% bovine serum albumin (BSA; crystalline, AR, and lyophilized, Sigma) to induce quiescence. Just prior to microinjection, fresh aliquots of affinity purified G protein α subunit antibodies or buffer alone (50 μM HEPES, 40 mM NaCl, pH 7.3) were mixed with a pure rabbit IgG marker antibody and centrifuged at 100,000 × g for 40 min at 4 °C. The antibodies were present in the injection needle at the following concentrations: AS, 3.2 mg/ml; EC, 3.9 mg/ml; AR, 2.5 mg/ml; monoclonal antibody 5-bromo-4-chloro-3-indoyl-D-galactoside (Life Technologies) in PBS containing 4.6 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 4.2 mM MgCl₂, 0.02% Nonidet P-40 (v/v), and 0.1 mg/ml sodium deoxycholate (Sigma), which yields an insoluble blue reaction product (36). The cells were washed three times with PBS–, blocked for 30 min at 25 °C with 5% (v/v) goat serum (Life Technologies) and 0.5% (v/v) BSA (Fraction VI Sigma) in PBS–, then incubated with a fluorescein-conjugated sheep anti-rabbit IgG (Cappel) at 1:500 in blocking solution for 60 min at 37 °C to identify the injected cells. The cells were then washed three times with 0.2% (v/v) Tween 20 (Sigma) and 0.5% BSA in PBS–, one time with PBS–, and the coverslips mounted on glass slides with a 17% (w/v) solution of Airvol polyvinyl alcohol (Air Products & Chemicals, Inc., Allentown, PA) and 33% (v/v) glycerol in PBS–. Immunofluorescence microscopy and light microscopy, using a Zeiss Axiohot microscope, was used to visualize the injected cells and nuclear β-gal staining, respectively. Images were recorded using T-Max 400 film (Kodak). These agonist concentrations and exposure times were found to produce maximal induction of c-fos in pilot experiments. Percentage of inhibition was calculated by first determining the number of fluorescent (injected) cells that stained positive for β-gal divided by the total number of injected cells, and dividing the number of positive staining buffer-injected cells were counted and divided by the total number of cells injected. These two ratios were divided, multiplied by 100, and subtracted from 100 to arrive at the percent inhibition. In pilot experiments, we determined that the number of buffer-injected cells staining positive for β-gal was similar to the number of un.injected cells staining positive. Also, the mechanical act of injection by itself did not induce detectable c-fos expression.

Transient Transfection Protocol—c-fos-lacZ wild-type mouse fibroblasts were seeded at a density of 3 × 10⁵ cells/well into individual wells of 24-well plates that contained glass coverslips (12 mm diameter), and allowed to attach overnight. The following day, the cells were transfected with either 1 μg of pcSV2CAT or GαEC→S DNA per well by the calcium phosphate method. 15 h later the coverslips were washed briefly, first with PBS–and then with PBS– containing 0.5% EDTA and 0.5 μM EGTA, then incubated overnight in Dulbecco’s modified Eagle’s medium containing 5% fetal calf serum. The next morning the media was replaced with Dulbecco’s modified Eagle’s medium containing 0.5% BSA and the cells incubated an additional 24–30 h to induce quiescence. The appropriate wells were treated with 200 ng/ml PTX (List Biological, Campbell, CA) for the final 16 h of the starvation period. The cells were stimulated with 2.1 μM LPA for 110 min, then fixed and stained for β-gal as described previously. Cells were then washed four times with PBS–, incubated for 60 min at room temperature in blocking solution consisting of 0.1% BSA/PBS–, and then incubated for 60 min at 37 °C with a 1:50 dilution of the EE monoclonal antibody, a 1:100 dilution of an anti-CAT Ab (5 Prime – 3 Prime, Inc., Boulder, CO). The coverslips were washed twice in wash buffer (0.2% Triton, 0.1% BSA, PBS–) and then incubated 60 min with a 1:750 dilution of fluorescein isothiocyanate-conjugated goat anti-mouse IgG in the case of EE, or a 1:100 dilution of rhodamine-conjugated goat anti-rabbit IgG to visualize the CAT protein. The coverslips were washed three times in wash solution, one time in PBS–, then mounted as described above. Positive transfectants on the coverslips were identified by immunofluorescence microscopy, and scored as positive or negative for β-gal staining by light microscopy. The cells used in these experiments had a higher percentage of cells staining positive for β-gal in the unstimulated state than those used in the microinjection experiments, possibly because a quality transfection required a relatively lower initial seeding density. The lower cell density and the lack of cell-cell contact at the time of starvation may have increased the time necessary to induce quiescence. To verify that the GαEC→S protein expressed in these cells was resistant to PTX treatment, ribosylated and non-ribosylated α subunits were separated from each other by high resolution gel electrophoresis. Briefly, 100-mm dishes of cells that were seeded at a density of 3.5 × 10⁴ cells/dish were transfected with 20 μg of DNA or treated with the transfection mixture alone, as described previously. Some dishes were treated with 100 ng/ml PTX for 16–20 h prior to harvesting the cells for membrane preparation, or, alternatively, whole cell lysates were prepared by scraping the cells directly into 2 × sample buffer. Membrane proteins or lysates were resolved by high resolution urea SDS-PAGE, transferred to nitrocellulose, and probed with antibodies to the epitope tag and/or GαEC as described above.

RESULTS

Effect of Pertussis Toxin on LPA- and Insulin-stimulated induction of c-fos—Although PTX has been shown to inhibit agonist-stimulated activation of Ras and MAP kinase (11, 12), we first verified that PTX exerted a similar inhibitory effect on LPA-stimulated induction of c-fos. As shown in Fig. 1a, a 24-h period of quiescence was sufficient to eliminate detectable β-gal staining in these fibroblasts; fewer than 1% of the cells showed positive staining at the end of this time. The addition of 2.1 μM LPA for 110 min resulted in approximately 60–75% of the cells showing positive staining for β-gal (Fig. 1b). When the cells were pre-treated for 16 h with PTX (100 ng/ml), the LPA-induced β-gal staining was almost completely eliminated (Fig. 1d), although PTX by itself had no effect on the β-gal staining in quiescent cells (Fig. 1c). These cells also stably expressed the human insulin receptor and stimulation with 70 nM insulin for 110 min resulted in positive β-gal staining in 75–85% of the cells (Fig. 1e). The insulin-stimulated induction of c-fos was completely unaffected by PTX (Fig. 1f), and thus served as a negative control for the microinjection experiments.

Identification of Gα Subunits in Mouse Fibroblast Membranes—The α subunits of the Gα and Gβ γ family contain sites susceptible to ADP-ribosylation catalyzed by PTX and are therefore candidates for mediating the inhibitory effect of PTX.
on LPA-induced c-fos expression. To determine which α subunits were present in the mouse fibroblasts used in these studies, we used a panel of α1-specific antibodies to probe cell extracts by Western analysis. Antibody AS has been shown to have equal specificity for α11 and α12. As shown in Fig. 2, this antibody recognized a single protein in these cells (lane 1). Because this band could represent α11 or α12, or both, we utilized another antibody which is monospecific for α11. This antibody failed to cross-react with any protein in equal amounts of fibroblast membranes (lane 4); however, it did detect a protein in equal amounts of membranes prepared from bovine brain (lane 6). In addition, when as much as 100 μg of membrane protein was separated using high resolution urea SDS-PAGE, which resolves G\textsubscript{a11} and G\textsubscript{a12} and the subsequent blot probed with the AS antibody, only a single band was detected (data not shown). Antibody EC has a high degree of specificity for α11, although to some extent it will react with α12. As shown in lane 2, a single band of immunoreactivity was detected using this antibody. Antibody AR, which recognizes only G\textsubscript{a12}, failed to detect any proteins in mouse fibroblast membranes (lane 3). The G\textsubscript{a12}-selective antibody used did not recognize any protein in the mouse fibroblasts (lane 5), while reacting strongly with an equivalent amount of protein from bovine brain (lane 7).

Although G\textsubscript{a0} and G\textsubscript{a11} were not detected by Western analysis, in order to ensure that the lack of immunological detection of these species was not due to its presence at levels less than the limits of detection, we prepared RNA from these cells to determine if there was detectable message for G\textsubscript{a0} and G\textsubscript{a11}. Using full-length coding cDNAs as probes, a band of approximately 4.5 kb corresponding to G\textsubscript{a0} was detected in both rat and mouse brain which we used as a positive control, but not in the equivalent amount of RNA prepared from mouse fibroblasts (data not shown). Similarly, the G\textsubscript{a11} cDNA hybridized to a message of approximately 3.5 kb in both rat and mouse brain, but not mouse fibroblasts (data not shown). The size of the message detected in both rat and mouse brain using the G\textsubscript{a0} and G\textsubscript{a11} probes was consistent with a previously published report using rat brain RNA (37). Therefore, the combined evidence obtained by both Northern and Western analysis suggests that mouse fibroblasts contain little, if any, G\textsubscript{a0} and G\textsubscript{a11} mRNA or protein, and suggest that the predominant α subunits present in the fibroblasts used in these studies are G\textsubscript{a11} and G\textsubscript{a12}.

Effect of Cellular Microinjection of G\textsubscript{a0} Antibodies on LPA-induced β-gal Activity—The mechanical act of scraping adherent cells has been proposed to initiate a wounding response, which includes the induction of c-fos (38, 39). However, microinjection per se did not induce β-gal activity in this study (data not shown). Injections were made into the cytoplasm of quiescent fibroblasts with the affinity-purified antibodies AS, EC, and AR, described above. Each of the antibody solutions contained a pure rabbit marker IgG as did a solution of injection buffer alone which served as a standardizing control. As depicted in Fig. 3, panels a, c, e, g, and i show injected cells that were detected by immunofluorescence staining. The identical fields of cells demonstrating immunofluorescence and β-gal staining are shown. Bar, 25 μm.

The overall inhibitory effect of each of the antibodies on LPA-induced β-gal activity was quantitated and is summarized in Table I. Antibody AS decreased the number of positive responses by an average of 57.5 ± 0.5% (± S.E., n = 3). Small but detectable decreases of about 19.1 ± 9.8% and 19.1 ± 9.9%, respectively, were observed for both antibodies EC and AR. As can be seen in Figs. 1 and 3, the intensity of β-gal staining was somewhat variable among individual cells. Our system of scor-
showing cross-reactivity with the equivalent amount of membrane protein was loaded in each lane. The percent inhibition was calculated as described under "Experimental Procedures." The inhibition data shown are the mean ± S.E. from three separate experiments.

### Table I

| Antibody | Agonist | Total number of injected cells | Number of injected cells staining positive for β-gal | % Inhibition |
|----------|---------|--------------------------------|-----------------------------------------------------|--------------|
| AS       | LPA     | 436                            | 113                                                 | 57.5 ± 0.5   |
| EC       | LPA     | 390                            | 188                                                 | 19.1 ± 9.8   |
| AR       | LPA     | 449                            | 225                                                 | 19.1 ± 9.9   |
| Buffer   | LPA     | 339                            | 243                                                 | NA           |
| AS       | Insulin | 255                            | 194                                                 | 0            |
| Buffer   | Insulin | 286                            | 218                                                 | NA           |

* NA, not applicable.

Expression of PTX-resistant $G_{a2}$ but not $G_{a3}$ Restores LPA Signaling—Although the G protein $\alpha$ subunit antibodies used for microinjection showed reasonably good specificity in recognizing their corresponding protein in immunoblotting, we considered that there could be significant nonspecific inhibitory effects of these antibodies when used in the *in vivo* studies described here. In light of this possibility, we utilized an approach that has previously been used successfully to examine PTX-sensitive signaling pathways in *in vivo* (22, 40, 41), and introduced PTX-resistant $\alpha$ subunits into these cells by transfection, where the contribution of endogenous G protein $\alpha$ subunits could be eliminated by treatment of the cells with PTX. First, we attempted to create cell lines that stably overexpressed the PTX-resistant mutants of $G_{a2}$ or $G_{a3}$, as well as their wild-type counterparts, with the reasoning that signaling in cells expressing these mutants could be systematically restored and assayed following treatment of the cells with PTX. Transient transfection of these constructs would yield only a small population of cells that expressed the PTX-resistant protein, and would not be amenable to the single cell assay system described here, because the specific cells that expressed the protein could not be identified. One hundred clones resistant to hygromycin were expanded and assayed for overexpression by Western blot analysis following separation of membrane proteins using high resolution urea SDS-PAGE. A single PTX-resistant $G_{a3}$ clone (designated 12B/48) demonstrated elevated levels of immunoreactivity to the $G_{a3}$ antibody, EC, compared to the wild-type cells (Fig. 4A, upper band in each lane). The high resolution gel system separated the 41-kDa $G_{a3}$ and the 40-kDa $G_{a2}$ subunits; and as shown in Fig. 4A, antibody EC shows considerable cross-reactivity with the $G_{a3}$ species. This cross-reactivity, although stronger than expected, nonetheless provided a convenient internal control demonstrating that the PTX-resistant form of membrane protein was isolated in each lane, based on similar levels of $\alpha_{i2}$ immunoreactivity. Additional blots with this clone using the $G_{a2}$-specific AS antibody following separation on high resolution gels, confirmed that the levels of $G_{a2}$ in this clone were similar to those in control cells (data not shown). Experiments using this stable PTX-resistant cell line were then carried out to determine if the $G_{a3}$ species of $\alpha$ subunit could restore LPA-induction of c-fos in cells that were pretreated overnight with PTX; however, LPA did not induce c-fos in these cells (data not shown). We were unsuccessful in isolating a clone that overexpressed either the wild-type or the PTX-resistant mutant of the $G_{a2}$. This suggests that the levels of expression of this protein may be very highly regulated in the cells used in this study.

Based on the above finding, that the PTX-resistant form of $G_{a3}$ did not restore the LPA induction of c-fos, and given the...
inability to test the PTX-resistant form of Gai2 using stable expression, we devised a transient transfection protocol that took advantage of earlier work (31) whereby a monoclonal antibody epitope was introduced into an internal site of the PTX-resistant form of Gai2. This approach allowed us to transfect and analyze a population of cells in which transfected Gai2 could be identified against a cellular background of endogenous G proteins. Epitope-tagged Gai2C-S was readily detected in lysates prepared from transfected cells by immunoblotting using the EE antibody (Fig. 4B, lane 1), and migrated at the same position as a protein detected by blotting with the AS antibody, which reacts with Gai2 in these cells (Fig. 4B, lane 3). The EE antibody was highly specific for the EE epitope; the antibody did not react with any protein in an equivalent amount of lysate from wild-type cells (Fig. 4B, lane 2). To confirm that the transfected construct resulted in the expression of a protein that was resistant to PTX in these cells, we utilized high resolution urea SDS-PAGE to separate ribosylated Gai2 from unmodified Gai2. PTX-catalyzed ADP-ribosylation results in a reduced migration rate of Gai2 through this gel system, as is demonstrated in Fig. 4C. In membranes prepared from untransfected PTX-treated cells, a single band of slower migrating immunoreactivity is detected (lane 2) compared to untransfected cells that were not exposed to PTX (lane 1). In contrast, in an equal amount of membrane protein prepared from transfected cells, a single band appears in the lane prepared from non-PTX-treated cells (lane 3), while PTX treatment results in two bands of immunoreactivity (lane 4); the upper band corresponds to endogenous ribosylated Gai2, and the lower, faster migrating band corresponds to the PTX-resistant form of Gai2. These results also demonstrate that introduction of the EE epitope into the Gai2 cDNA did not interfere with membrane localization of the expressed protein.

To investigate whether G protein heterotrimers containing only Gai2 are capable of coupling the LPA receptor to the induction of c-fos, we conducted experiments on c overslipped plates that were transiently transfected with either the epitope-tagged and PTX-resistant Gai2 construct or with a CAT plasmid. Following transfection, the cells were starved in the absence or presence of PTX and then stimulated with LPA. At each condition tested, the coverslips were examined by immunofluorescence microscopy following staining with an antibody to the EE epitope or to CAT (Fig. 5A), to visualize positive transfected cells. Each transfected cell was also examined by light microscopy to determine whether c-fos had been induced. We used the expressed CAT protein as a marker that enabled us, in parallel experiments, to quantitate the extent of PTX-resistant form of Gai2 using stable expression, we devised a transient transfection protocol that took advantage of earlier work (31) whereby a monoclonal antibody epitope was introduced into an internal site of the PTX-resistant form of Gai2. This approach allowed us to transfect and analyze a population of cells in which transfected Gai2 could be identified against a cellular background of endogenous G proteins. Epitope-tagged Gai2C-S was readily detected in lysates prepared from transfected cells by immunoblotting using the EE antibody (Fig. 4B, lane 1), and migrated at the same position as a protein detected by blotting with the AS antibody, which reacts with Gai2 in these cells (Fig. 4B, lane 3). The EE antibody was highly specific for the EE epitope; the antibody did not react with any protein in an equivalent amount of lysate from wild-type cells (Fig. 4B, lane 2). To confirm that the transfected construct resulted in the expression of a protein that was resistant to PTX in these cells, we utilized high resolution urea SDS-PAGE to separate ribosylated Gai2 from unmodified Gai2. PTX-catalyzed ADP-ribosylation results in a reduced migration rate of Gai2 through this gel system, as is demonstrated in Fig. 4C. In membranes prepared from untransfected PTX-treated cells, a single band of slower migrating immunoreactivity is detected (lane 2) compared to untransfected cells that were not exposed to PTX (lane 1). In contrast, in an equal amount of membrane protein prepared from transfected cells, a single band appears in the lane prepared from non-PTX-treated cells (lane 3), while PTX treatment results in two bands of immunoreactivity (lane 4); the upper band corresponds to endogenous ribosylated Gai2, and the lower, faster migrating band corresponds to the PTX-resistant form of Gai2. These results also demonstrate that introduction of the EE epitope into the Gai2 cDNA did not interfere with membrane localization of the expressed protein.

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**DISCUSSION**

In this study we investigated the functional interaction of the LPA receptor with G proteins in intact mouse fibroblasts. Because LPA-induced mitogenesis in mouse fibroblasts can be blocked by PTX, we undertook this investigation to determine which of the PTX-sensitive G protein subtypes was involved in this response. Western blotting revealed that two PTX-sensitive G protein subunits, Gai2 and Gai3, were present in membranes prepared from the mouse fibroblasts used in this study. These data are consistent with those reported for Balb3T3 mouse fibroblasts (26). The absence of message for other PTX-sensitive G proteins, namely Gai1 and Gai0, was confirmed by Northern analysis.

Microinjection of specific G protein a subunit antibodies suggested that the LPA receptor selectively activated Gai2, based on the approximate 60% inhibition of LPA-stimulated c-fos induction seen following microinjection of a Gai2 specific antibody. In light of the ability of PTX to almost completely abolish LPA-stimulated c-fos induction, the reason for the incomplete ablation of c-fos induction in all of the cells injected with the antibody is not clear. It is conceivable that variable amounts of antibody were delivered to individual cells, and that a small number of unbound Gai2 molecules would be capable of eliciting a detectable response. Another possible explanation is related to the experimental protocol used in this study. The time lag between the injection of antibody into the cells and the subsequent addition of agonist was somewhat variable, i.e. cells injected first were exposed to antibody for up to 40 min while those injected last were exposed for 10 min. The injected antibody may require longer than 10 min to bind the G protein with full saturation. Levels of inhibition greater than that seen with the Gai2 antibody are possible in these cells; for example, injection of an inhibitory Ras antibody blocked both LPA- and insulin-stimulated c-fos expression by approximately 80%.

The inability of the Gai2 antibody to block insulin-induced c-fos expression serves as a negative control, since PTX is generally ineffective at blocking insulin-stimulated c-fos induction and/or mitogenesis (present data and Refs. 42 and 43). We were unsuccessful in our attempt to restore the LPA response by microinjecting purified GDP-bound Gai2 or Gai3 protein into PTX-treated wild-type cells. This approach may have been unsuccessful because the subunits could have been modified by PTX immediately upon delivery to the cell, or possibly because the protein was not localized to the proper site within the cell or was not present in sufficient amounts.

We cannot rule out the possibility that there could be appreciable nonspecific effects of the antibodies when used in the microinjection experiments. Indeed, our results using high resolution gel electrophoresis to separate Gai2 and Gai3 indicate that antibody EC detects not only Gai3, but also Gai2, by immunoblotting, as has been described (25). Therefore, it is possible that part or all of the 19% inhibition of LPA-stimulated c-fos we observed with antibody EC may reflect an effect on receptor-Gai2 coupling, rather than a general nonspecific effect on the signaling pathway. Results from other studies suggest that antibody EC is capable of inhibiting Gai3-mediated signaling. For example, antibody EC inhibited 5-hydroxytryptamine1A-stimulated phospholipase C activity and attenuated receptor-mediated inhibition of adenyl cyclase to a much greater extent than did the AS antibody in HeLa cells, which contain at least 10 times more Gai3 protein than Gai2 (44, 45). In Chinese hamster ovary cells, antibody AS and antibody EC partially inhibited, to a similar extent, a2-adrenergic-mediated inhibition of adenyl cyclase, and in combination completely

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3 J. K. Chuprun and P. J. Blackshear, unpublished observations.
reversed the inhibition (46). Despite these observations, it may remain difficult to unequivocally assess the relative roles of the G\textsubscript{i} proteins in mediating specific signaling pathways by the use of antibodies, especially in cells that contain more than one G\textsubscript{i} species. Given the potential limitation of the antibody approach, we

**FIG. 5.** Cellular expression of the PTX-resistant mutant of G\textsubscript{ai2} restores LPA induction of c-fos in PTX-treated cells. A, coverslip-plated cells in 24-well plates were transfected with either a constitutively active CAT construct or the epitope-tagged PTX-resistant mutant of G\textsubscript{ai2}, and approximately 41 h after the cells were first exposed to the DNA mixture, the coverslips were transferred to new wells of a 24-well plate and incubated 24–30 h in starving media to induce quiescence. PTX was added to the appropriate wells (panels c, g, k, and o) for the final 16 h of starvation. Cells were then incubated for 110 min either in the absence (panels a, c, i, and k) or presence (panels e, g, m, and o) of 2.1 \( \mu \)M LPA, then fixed and stained for expression of the CAT protein or epitope-tagged G\textsubscript{ai2}C\textsuperscript{S} protein and in situ \( \beta \)-gal activity. Positive transfected cells expressing CAT (panels a, c, i, and k) or positive transfected cells expressing the epitope-tagged G\textsubscript{ai2}C\textsuperscript{S} protein (panels i, k, m, and o) were identified by immunofluorescence microscopy and then were scored as positive or negative for \( \beta \)-gal staining by light microscopy. In the \( \beta \)-gal panels, arrows identify the positive staining nuclei of the corresponding transfected cells that are observed in the immunofluorescence panels. Identical fields of cells demonstrating immunofluorescence and \( \beta \)-gal staining are shown. Bar, 25 \( \mu \)M. B, for each coverslip, cells identified by immunofluorescence as expressing either the CAT (open bars) or the G\textsubscript{ai2}C\textsuperscript{S} (shaded bars) protein were scored as positive or negative for nuclear \( \beta \)-gal staining. The data shown are the percentage of transfected cells staining positive for \( \beta \)-gal, based on counting every transfected cell on each coverslip, from two separate experiments. The total number of cells counted at each experimental condition was: untreated: CAT = 873, EEtag = 1320; PTX-treated: CAT = 652, EEtag = 839; LPA-treated: CAT = 936, EEtag = 997; PTX+LPA-treated: CAT = 1083, EEtag = 755.
conducted experiments in cells that either stably or transiently expressed PTX-resistant \( G_{a2} \) or \( G_{a12} \) proteins, and examined whether expression of either of these mutant proteins restored LPA signaling following pretreatment of the cells with PTX, which eliminated the contribution of endogenous G protein \( \alpha \) subunits. The PTX-resistant proteins lack the critical carboxy-terminal cysteine required for modification catalyzed by PTX, and have been demonstrated to remain functional in cells treated with PTX (22, 40, 41). Our studies revealed that G protein heterotrimers containing only mutant \( G_{a12} \) protein were sufficient to restore the activation of \( c-fos \) by LPA, and that expression of mutant \( G_{a12} \) did not. The PTX-resistant \( G_{a3} \) cDNA that was overexpressed in these cells encodes a functional protein, because in separate experiments, transient transfection of this construct restored 5-hydroxytryptamine \( G_{\alpha} \)-stimulated Na/H exchange in fibroblasts pretreated with PTX.\(^4\)

LaMorte et al. (26) have demonstrated that pretreatment of Balb3T3 cells with PTX decreased serum-stimulated DNA synthesis by approximately 30%. They proposed that \( G_{o2} \) mediates serum-stimulated mitogenesis, since an antibody specific for \( G_{o2} \) when microinjected into these cells, decreased serum-stimulated DNA synthesis by 37%. Total inhibition of serum-stimulated DNA synthesis would be somewhat surprising, since other ligands in serum, such as platelet-derived growth factor, can activate the MAP kinase pathway without the mediation of G proteins (47). However, the inhibitory effects seen by LaMorte et al. (26) using either PTX or \( G_{o2} \) antibody may also reflect inhibition of the LPA effect, since LPA is a component of serum.

Previous data have suggested that LPA-stimulated mitogenesis is independent of the PTX-insensitive \( G_{o} \)-mediated calcium mobilization (3). The simplest explanation, based on the results of this study, is that \( G_{o2} \) is the only G protein that is necessary for restoration of \( c-fos \) inducibility, although our studies were not designed to test the contribution of non-PTX-sensitive G proteins. These results may be in contrast to thornin-stimulated DNA synthesis in fibroblasts, which requires both \( G_{o2} \) and \( G_{o3} \) (48).

The functional strategy used here to examine receptor-G protein coupling relied on the expression of \( c-fos \), a nuclear protein. Previous reports have proposed a role for \( G_{o} \) or \( G_{o12} \)-like proteins in post-receptor events, such as mitosis, based on the ability of insulin or epidermal growth factor to induce a redistribution of \( G_{o} \) subunits from the plasma membrane to perinuclear sites (49), or based on the observation that G protein \( \alpha \) subunits have been detected in the nuclei of certain cells (50). We believe that the mechanism studied here, in which G protein antibodies inhibited signaling, was due to an inability of the receptor to activate its cognate G protein, because the antibodies used were raised against COOH-terminal peptides located in a region of the G protein thought to interact with receptors (27, 28). Likewise, expression of defined PTX-resistant \( \alpha \) subunits restored signaling, presumably because the \( \alpha \) subunit recombines with free \( \beta\gamma \) subunits in the membrane, and interacts with the receptor as the holoprotein. It was on the basis of this proposed mechanism that we conducted our immunoblotting studies with membrane protein, and disregarded cytoplasmic and nuclear proteins. However, epitope-tagged G proteins like the one used here may be important reagents for examining possible alternative or additional mechanisms of action for G proteins in mitogenesis.

The principal finding of this study, that LPA interacts selectively with \( G_{o12} \) to induce \( c-fos \), is supported by the following additional evidence: 1) mutationally activated \( \alpha_{o2} \) mimics LPA-mediated inhibition of forskolin- or prostaglandin E\(_2\)-stimulated adenylyl cyclase (16), and 2) expression of \( \alpha_{o2} \)-inhibited \( \alpha_{o2} \) alters normal growth control in some cell types but not in others (17, 18). \( G_{o2} \) also couples several other well known receptors to downstream effector molecules. For example, \( G_{o2} \) has been reported to mediate \( \alpha_{o2} \)-adrenergic inhibition of adenylyl cyclase in platelet membranes (25) and Rat-1 fibroblasts (51). In contrast, not all receptors sensitive to PTX proceed through \( G_{o2} \). For example, \( G_{o2} \) can couple angiotensin II receptors to inhibition of adenylyl cyclase in rat hepatocytes (52), and also regulates multiple effector enzymes in Chinese hamster ovary cells stimulated by m2-muscarinic agonists (22).

Recent experiments have revealed that the principal stimulatory agent of MAP kinase by G protein-coupled receptors is the \( \beta \gamma \) heterodimer (53–55). To determine whether the \( \beta \gamma \) complex was involved in the pathway studied here, we microinjected a \( \beta\gamma \)-sequostering peptide into cells and assessed the effect on LPA-stimulated induction of \( c-fos \). We were unable to detect any inhibitory effect of the \( \beta\gamma \) binding peptide on LPA-stimulated \( c-fos \) expression. Possible explanations for this result are that we were not able to completely titrate the activating molecules with the protein, or that LPA induction of \( c-fos \) is not mediated by the \( \beta\gamma \) subunits, but perhaps by \( G_{o1} \) or some other unknown mechanism. In support of this possibility are results from experiments in Rat-1 cells in which the activated \( \alpha_{o2} \) subunit itself has been shown to induce neoplastic transformation (17) and constitutively activate the MAP kinase cascade (56). These findings may reveal differing regulatory mechanisms utilized by different cells used to control proliferation.

In summary, we have demonstrated here by two complementary approaches that of the PTX-sensitive G protein \( \alpha \) subunits, heterotrimers containing only the \( \alpha_{o2} \) subunit are sufficient for the activation of an LPA-stimulated signaling pathway which results in the transcription of the \( c-fos \) gene. Although several reports which are based on the results of experiments using broken cell preparations have raised questions about the assumption that different \( \alpha \) subunits mediate specific signaling processes (57–59), the results from this study support the proposal that specific G protein subtypes mediate specific signaling events.

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