G_{i2}-mediated Activation of the MAP Kinase Cascade

Ann M. Pace,* Michel Faure,[†] and Henry R. Bourne[‡]

Departments of Cellular and Molecular Pharmacology and Medicine, and the Cardiovascular Research Institute, University of California, San Francisco, California 94143

Submitted June 20, 1995; Accepted September 18, 1995
Monitoring Editor: Joseph Schlessinger

The G_i class of heterotrimeric G proteins has been implicated in transmitting mitogenic signals from a variety of seven-transmembrane domain receptors. In addition, the α subunit of G_{i2} (α_{i2}) is oncogenic when mutated to a constitutively active form (*gip2*). The mechanism by which G_{i2} stimulates cellular proliferation is unknown, but is believed to involve activation of the mitogen-activated protein kinase (MAPK) signaling cascade. To study G_{i2} activation of the cascade, we transiently expressed a mutant, pertussis toxin (PTX)-resistant α_{i2} in Chinese hamster ovary cells. After PTX treatment of these cells, G_i-coupled receptors specifically activated PTX-resistant G_{i2} without activating other G_i proteins. Receptor-mediated activation of G_{i2} led to activation of MAPK and its upstream activator, MAPK/ERK-activating kinase (MEK). Activation of MAPK and MEK by G_{i2} was blocked by expression of a dominant-negative mutant of Ras. G_{i2} activation did not, however, detectably increase the proportion of Ras protein in the GTP-bound form. Additional experiments suggest that G_{i2} stimulates the MAPK pathway, at least in part, by mechanisms that involve release of its $\beta\gamma$ subunit, as well as activation of phosphatidylinositol-3 kinase.

INTRODUCTION

Pertussis toxin (PTX)¹ inhibits the mitogenic responses of cultured cells to certain hormones and growth factors acting on G protein-coupled receptors (reviewed in Seuwen and Pouyssegur, 1990). PTX exerts this effect by catalyzing ADP-ribosylation of α subunits in the G_i subclass of G proteins, a modification that prevents G_i proteins from interacting with receptors. The G_i family contains seven PTX-sensitive α subunits,

three of which, α_{i1} , α_{i2} , and α_{i3} , are broadly expressed. Inhibition of mitogenesis by PTX therefore implies that one or more of the G_i family members can stimulate cell proliferation, but does not identify the specific subtypes that may be involved.

A variety of G_i-coupled receptors stimulate PTX-sensitive activation of mitogen-activated protein kinase (MAPK, also called ERK, for extracellular signal-regulated kinase) (L'Allemain *et al.*, 1991; Alblas *et al.*, 1993; Howe and Marshall, 1993; Winitz *et al.*, 1993; Honda *et al.*, 1994; Flordellis *et al.*, 1995), a component of the MAPK signal transduction cascade. The MAPK cascade, which seems to be required for mitogenesis, is composed of a series of kinases that propagate a signal by phosphorylating and activating their downstream kinase substrates (reviewed in Marshall, 1994). The cascade begins with a serine/threonine kinase, either Raf or MEK kinase (MEKK), which phosphorylates and activates the dual-specificity kinase MEK (MAPK/ERK-activating kinase); MEK in turn phosphorylates and activates the serine/threonine kinase MAPK. Activation of the GTPase Ras can lead to activation of Raf and MEKK (reviewed in Hall, 1994), thus triggering the cascade. G_i-coupled receptors activate MAPK by both Ras-dependent (Alblas *et al.*, 1993;

* Present address: Department of Biological Sciences, Stanford University, Stanford, CA 94305.

[†] Present address: Sugen, Inc., 515 Galveston Dr., Redwood City, CA 94063.

[‡] Corresponding author: S-1212, Box 0450, University of California Medical Center, San Francisco, CA 94143.

¹ Abbreviations used: A1R, A1 adenosine receptor; bFGF, basic fibroblast growth factor; bt-MAPK, bacterially-expressed MAPK; β ARK, β -adrenoceptor kinase; CHO-K1, Chinese hamster ovary cells; EE, "glu-glu" epitope; HA, hemagglutinin epitope; hCG, human chorionic gonadotropin; M1R, M1 muscarinic acetylcholine receptor; MAPK, mitogen-activated protein kinase; MBP, myelin basic protein; MEK, MAPK/ERK-activating kinase; MEK*, constitutively active Δ N3-S218E-S222D; MEKK, MEK kinase; PIA, R-N⁶-(2-phenylisopropyl)-adenosine; PI3K, phosphatidylinositol-3 kinase; PLC, phospholipase C; PTX, pertussis toxin.

van Corven *et al.*, 1993; Winitz *et al.*, 1993) and Ras-independent mechanisms (Honda *et al.*, 1994; Flordellis *et al.*, 1995). Furthermore, release of $\beta\gamma$ subunits seems to be important for G_i -mediated activation of Ras and MAPK (Crespo *et al.*, 1994; Faure *et al.*, 1994; Koch *et al.*, 1994a), although the steps linking $\beta\gamma$ to activation of the cascade are unknown. Thus, although the ability of G_i -coupled receptors to activate the MAPK cascade is well-documented, two important questions remain: Which of the G_i family members are responsible? How do G_i proteins activate the cascade?

To begin to address these questions, we have created a system in which one particular G_i , G_{i2} , can be activated by receptor specifically, without activation of any other G_i subtype. We chose G_{i2} because of its role in mitogenic signal transduction: mutationally activated α_{i2} (the *gip2* oncogene; Lyons *et al.*, 1990) neoplastically transforms Rat-1 cells in culture (Pace *et al.*, 1991; Gupta *et al.*, 1992b) and enhances the growth of NIH-3T3 and Swiss-3T3 cells (Hermouet *et al.*, 1991; Gupta *et al.*, 1992b). Furthermore, microinjection of antibodies specific for α_{i2} substantially inhibits serum-stimulated DNA synthesis in BALB/c 3T3 cells (LaMorte *et al.*, 1992). To activate specifically G_{i2} by receptor, we transfected Chinese hamster ovary (CHO-K1) cells with a G_i -coupled receptor and a mutant α_{i2} cDNA in which cysteine 352, the site of PTX modification, is replaced by serine. PTX treatment of these cells inactivated endogenous G_i proteins, allowing the transfected G_i -coupled receptor to activate only the mutant, toxin-insensitive G_{i2} . We report that activation of G_{i2} in CHO-K1 cells leads to activation of MAPK and MEK, but does not lead to Ras activation. Unexpectedly, given the failure of G_{i2} to activate Ras, expression of a dominant-negative mutant of Ras (N17ras) potently blocked G_{i2} -mediated activation of MAPK and MEK. We present further data suggesting that activation of MAPK by G_{i2} is at least partially dependent on release of $\beta\gamma$, and may involve activation of phosphatidylinositol 3-kinase (PI3K). The latter finding is consistent with previous data implicating roles for PI3K in mitogenesis (Fantl *et al.*, 1992; Valius and Kazlauskas, 1993), and in activation of the MAPK cascade (Cross *et al.*, 1994; Ferby *et al.*, 1994; Sakanaka *et al.*, 1994; Welsh *et al.*, 1994).

MATERIALS AND METHODS

Materials

The following antibodies were used: Anti-glu-glu monoclonal antibody (Onyx Pharmaceuticals, Richmond, CA); anti-HA monoclonal antibody (12CA5) (Berkeley Antibody, Richmond, CA); polyclonal antibodies against p42^{mapk} (C-14), p44^{mapk} (C-16), A-Raf (C-10), B-Raf (C-19), Raf-1 (C-12 and C-20), and MEKK (C-22) (Santa Cruz Biotechnology, Santa Cruz, CA); polyclonal antibodies against MEK1 and MEK2 (MEK1,2) (Transduction Labs, Lexington, KY); and anti-rat IgG (Cappel, Durham, NC). Anti-Ras monoclonal antibody Y13-259 was a gift from W.J. Fantl, Chiron, Emeryville, CA.

The following reagents were used: PTX (List Biologicals, Campbell, CA); basic fibroblast growth factor (bFGF) (Calbiochem, La Jolla, CA); wortmannin, R-N⁶-(2-phenylisopropyl)-adenosine (PIA), myelin basic protein (MBP), carbachol, and Narit A-activated charcoal (Sigma, St. Louis, MO). Human chorionic gonadotropin (hCG) was obtained from the National Hormone and Pituitary Program, Baltimore, MD. Cell culture supplies and protein A agarose were obtained from Life Technologies (Gaithersburg, MD).

Plasmids

The "glu-glu" sequence EYMPTE (abbreviated EE), a modification of an epitope identified by Grussenmeyer *et al.* (1985), was incorporated into mouse α_{i2} cDNA to replace codons 166 to 172 in a location designated site II (Wilson and Bourne, 1995). The oligonucleotide *gacctggagcgcatagcacaggaggagtagtacc-gacagagcaggatgtgctgcggaccgtgtg* was used to mutate mouse α_{i2} cDNA by oligonucleotide-directed mutagenesis. The mutant DNA was verified by dideoxy-sequencing and subcloned as a *HindIII*/*EcoRV* fragment into the expression vector pcDNA3 (Invitrogen, San Diego, CA) to create α_{i2} EE'. Rat α_{i2} cDNA in which codon 352 had been mutated from cysteine to serine (plasmid pRT2016-2A; provided by Ron Taussig, University of Texas Southwestern Medical Center, Dallas, TX) was subcloned into pcDNA3 as an *EcoRI* fragment, after which a *BglIII*/*NotI* fragment encompassing the mutation was removed and ligated into α_{i2} EE' in place of the analogous fragment, to create α_{i2} that is both epitope-tagged and mutant at codon 352 (α_{i2} EECS). We also replaced the *BglIII*/*NotI* fragment α_{i2} EE' with a wild-type rat *BglIII*/*NotI* fragment, to make α_{i2} EE, the control construct for α_{i2} EECS. Sources of HA-tagged p44^{mapk} and HA-tagged p45^{MEK} (pHA-MAPK1 and pHA-MAPKK) are described by Faure and Bourne (1995). Other plasmids were kindly provided by the following sources: A₁ adenosine receptor, P.D. Garcia, University of California, San Francisco, CA (Garcia and Myers, 1994); lutropin receptor, D.L. Segaloff, University of Iowa, Iowa City, IA (McFarland *et al.*, 1989); M1 muscarinic receptor, W. Sadée, University of California, San Francisco, CA (Maeda *et al.*, 1990); dominant negative Ras (N17ras) and constitutively-active H-ras^{val12} (v-ras), W.J. Fantl; constitutively active MEK1 (MAPKK1), Δ N3-S218E-S222D, N.G. Ahn, University of Colorado, Boulder, CO (Mansour *et al.*, 1994); constitutively active, N-terminally truncated Raf (v-raf), A.M. MacNicol, University of Chicago, Urbana, IL; constitutively active, membrane-targeted Raf (rafCAAX), D. Stokoe, Onyx Pharmaceuticals (Stokoe *et al.*, 1994); pRK- β ARK1-(495-689), R.J. Lefkowitz, Duke University, Durham, NC (Koch *et al.*, 1994b).

Cell Culture and DNA Transfection

CHO-K1 cells were maintained in minimal essential medium α without nucleosides/10% fetal calf serum. DNA was transiently transfected using adenovirus and DEAE-dextran according to the method of Forsayeth and Garcia (1994). Cells to be transfected were resuspended directly into the transfection medium, 1 ml per 10⁶ cells, and seeded into dishes or wells as described for the individual assays. Following a 2.5- to 3-h incubation at 37°C, the transfected cells were washed with 10% dimethyl sulfoxide/phosphate-buffered saline and fed with growth medium. Transfection efficiencies were determined by transfection of the β -galactosidase-encoding plasmid pON249 and assayed as described (Forsayeth and Garcia, 1994). Expression was consistently detected in at least 90% of the transfected cells.

Western Blotting

For determining expression of α_{i2} EE and α_{i2} EECS, approximately 10⁶ transiently transfected cells were lysed in Laemmli sample buffer, boiled, separated by 12% SDS-PAGE, and transferred to Immobilon-P membranes (Millipore, Bedford, MA). Membranes were blocked for 1 h in blocking buffer (5% nonfat dry milk, 50 mM

Tris-HCl, pH 7.5, 150 mM NaCl, 0.05% Tween 20, and 0.01% sodium azide), incubated overnight in blocking buffer containing 2–4 $\mu\text{g/ml}$ anti-glu-glu monoclonal antibody, then incubated for 1 h in blocking buffer (minus azide) with horseradish peroxidase-linked anti-mouse Ig (Amersham, Arlington Heights, IL) and processed for chemiluminescence (Dupont NEN, Boston, MA). All other immunoblotting was carried out as follows. Cells were washed with cold phosphate-buffered saline, incubated for 30 min on ice with lysis buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1% Triton X-100, 5 mM EDTA, 40 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 1 mM dithiothreitol, 2 $\mu\text{g/ml}$ leupeptin, 2 $\mu\text{g/ml}$ aprotinin, 1 $\mu\text{g/ml}$ pepstatin, and 0.1 $\mu\text{g/ml}$ phenylmethylsulfonyl fluoride), scraped into microcentrifuge tubes, and centrifuged at $14,000 \times g$ at 5°C for 8 min. The supernatant was mixed with sample buffer, and processed as described above. HA-tagged p42^{mapk} and HA-p44^{mapk} were detected with 1 $\mu\text{g/ml}$ monoclonal antibody 12CA5. Antibodies against endogenous p42^{mapk}, p44^{mapk}, A-Raf, B-Raf, Raf-1, and MEKK were used at a concentration of 1 $\mu\text{g/ml}$.

cAMP Accumulation

Cells were transfected as described above in 24-well plates at a density of 5×10^5 cells/well, and after 24 h placed in serum-free medium containing 5 $\mu\text{Ci/ml}$ [³H]adenine (23 Ci/mmol, Amersham) to label and induce quiescence. Two days after transfection, the cells were pretreated for 4 h with PTX (100 ng/ml) and stimulated with appropriate agonists. cAMP accumulation was determined as previously described (Wong *et al.*, 1991).

Measurement of MAPK Activity

Cells were transfected as described above in 12-well plates at 10^6 cells/well, placed in serum-free medium after 24 h, and assayed after 48 h. After PTX (100 ng/ml for 4 h) and wortmannin (15 min) pretreatment as needed, cells were stimulated with appropriate agonists and lysed on ice as described for Western blotting. Insoluble material was removed by microcentrifugation and supernatants were precleared with protein A agarose for 30 min. For immunoprecipitation of MAPK, precleared lysates were incubated at 5°C for 1–2 h with 25 μl protein A agarose (50% slurry with lysis buffer) and 1–5 $\mu\text{g/ml}$ of either 12CA5, anti-p42^{mapk}, or anti-p44^{mapk} antibodies. Immunoprecipitates were washed twice with lysis buffer and twice with kinase buffer (40 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid, pH 7.5, 5 mM magnesium acetate, 1 mM EGTA, 1 mM dithiothreitol, 0.2 mM sodium orthovanadate). MAPK activity was measured by performing an *in vitro* kinase reaction with immunoprecipitated MAPK, using MBP (12.5 $\mu\text{g}/55 \mu\text{l}$ reaction) as described (Faure *et al.*, 1994), except that reactions contained 1 μCi of [^γ-³²P]ATP (supplied as 30 Ci/mmol, Dupont NEN) and were carried out at 30°C. Radioactivity incorporated into MBP was determined by scintillation counting as described by Faure *et al.* (1994).

Measurement of MEK Activity

Cells were transfected as described above in 6-well plates at 2×10^6 cells/well. From this point the procedure was identical to that described above for measurement of MAPK activity, with the following exceptions: 15 $\mu\text{g/ml}$ anti-MEK1,2 antibodies or 3 $\mu\text{g/ml}$ 12CA5 were used for immunoprecipitation of endogenous or HA-p45^{MEK}, respectively; instead of MBP, the reaction (55 μl) contained 1 μg of bacterially expressed MAPK (bt-MAPK), a glutathione S-transferase fusion protein of p44^{mapk} purified from bacteria (Faure and Bourne, 1995); 5 μCi of [^γ-³²P]ATP was used per reaction. The reactions were stopped by the addition of 15 μl 5× Laemmli sample buffer; 30 μl of each reaction was subjected to 12% SDS-PAGE; the gels were dried and autoradiographed.

Analysis of GTP- and GDP-bound Ras

Cells were transfected as described above in 10-cm plates at 7×10^6 cells/plate, transferred to serum-free medium after 24 h, and then labeled overnight with 250 $\mu\text{Ci/ml}$ [³²P]-orthophosphoric acid (8500–9120 Ci/mmol, Dupont NEN) in phosphate-free, serum-free medium. Two days after transfection, the method of Satoh *et al.* (1990), with modifications as noted below, was used for measuring relative amounts of GDP and GTP bound to Ras. After treatment with PTX and agonists for specified times, cells were washed twice with cold Tris-buffered saline and lysed on ice for 30 min with immunoprecipitation buffer (400 $\mu\text{l}/\text{plate}$) containing Y13–259 anti-Ras monoclonal antibody. Lysates were transferred to microcentrifuge tubes and centrifuged at $14,000 \times g$ for 8 min to pellet insoluble material. After removing free guanine nucleotides from the lysates with activated charcoal, Ras immunocomplexes were precipitated by a 1-h incubation with 30 μl of a 50% slurry of anti-rat IgG precoupled to protein A agarose beads. Immunoprecipitates were washed, and radioactive GDP and GTP were eluted and analyzed by thin layer chromatography as described (Satoh *et al.*, 1990). Results were quantified by PhosphorImager analysis (Molecular Dynamics, Sunnyvale, CA). Percent GTP-bound Ras was calculated as $100 \times \text{GTP}/[\text{GTP} + (1.5 \text{ GDP})]$.

RESULTS

A G_i-coupled Receptor Specifically Activates PTX-insensitive G₁₂

The cDNA construct $\alpha_{12}\text{EECS}$ encodes a mutant α_{12} in which cysteine 352 has been replaced by serine, and which has the epitope EEYMPTE (EE) incorporated into an internal region designated site II (Wilson and Bourne, 1995). Incorporation of EE into site II of the α subunits of G_q, G_z, and G_s was previously shown to leave the signaling functions of these proteins intact (Wilson and Bourne, 1995). Cysteine 352 is the site of ADP-ribosylation by PTX; mutation of analogous cysteines of α_o and α_{13} render those α subunits insensitive to the inhibitory effect of PTX (Taussig *et al.*, 1992; Hunt *et al.*, 1994). To test whether this construct, $\alpha_{12}\text{EECS}$, encodes a functional protein, we assessed its ability to interact with receptor (which implies interaction with $\beta\gamma$) and with a known effector, adenylyl cyclase, which should be inhibited by active α_{12} . $\alpha_{12}\text{EECS}$ cDNA, or a control construct ($\alpha_{12}\text{EE}$, which contains the epitope but lacks the cysteine to serine mutation), was transiently transfected into CHO-K1 cells along with cDNAs encoding the G_i-coupled A1 adenosine receptor (A1R) and the G_s-coupled lutropin receptor (which is responsive to hCG). Western blots using a monoclonal antibody recognizing the EE epitope showed that the two α_{12} constructs were expressed at similar levels (our unpublished data). In cells expressing $\alpha_{12}\text{EE}$, PIA (an A1R agonist) inhibited hCG-stimulated cAMP accumulation in a PTX-sensitive manner (Figure 1). In cells expressing $\alpha_{12}\text{EECS}$, however, PIA-induced inhibition of cAMP accumulation was insensitive to PTX. Because PIA-induced signaling was completely PTX-sensitive in the absence of $\alpha_{12}\text{EECS}$, any PIA-mediated signaling occurring in PTX-treated cells expressing $\alpha_{12}\text{EECS}$ must be due to

Figure 1. Inhibition of cAMP accumulation by G_{12} . CHO-K1 cells were transfected with cDNAs encoding the A1R, LHR, and either α_{12} EE or α_{12} EECS (100 ng of each per 10^6 cells). Cells were placed in serum-free medium containing [3 H]adenine 24 h after transfection and assayed 48 h after transfection. After a 4-h pretreatment $\pm$ PTX, cells were treated with agonists for 30 min at 37°C, as indicated in the figure, and then assayed for cAMP content as described in MATERIALS AND METHODS. Treatment medium contained 1 mM isobutylmethylxanthine with or without hCG (50 ng/ml) and PIA (10 μ M). Bars represent the mean $\pm$ SD of triplicate determinations. Similar results were obtained in two additional experiments.

activation of the mutant, PTX-insensitive G_{12} . These results demonstrate that the A1R can activate G_{12} composed of exogenous α_{12} EECS and endogenous $\beta\gamma$, and that PTX prevents all other G_i proteins from coupling with the A1R.

G_{12} -mediated Activation of MAPK

We used this system to investigate the ability of G_{12} to activate components of the MAPK signal transduction cascade, beginning with MAPK itself. Treatment with PIA increased the activities of both the 42- and 44-kDa isoforms of MAPK in cells expressing the A1R and either α_{12} EE or α_{12} EECS (Figure 2, A and B), as measured by the ability of the immunoprecipitated kinases to phosphorylate myelin basic protein *in vitro*. PTX inhibited PIA-induced activation of MAPK in cells transfected with α_{12} EE by 70 to 100%, but inhibited MAPK activation only 30% in cells transfected with α_{12} EECS. Because, in the presence of PTX, the A1R activates α_{12} EECS only (Figure 1), these results indicate that activation of G_{12} leads to activation of MAPK. As expected for a growth

factor acting on a receptor tyrosine kinase, bFGF stimulation of MAPK was not significantly affected by PTX (Figure 2B). Similar results were obtained in assays measuring PIA activation of a transiently expressed hemagglutinin epitope (HA)-tagged MAPK isoform (see below).

G_{12} -mediated Activation of MEK

We then asked whether activation of G_{12} leads to activation of MEK, an activator of MAPK. PIA treatment increased HA-tagged p45^{MEK} (HA-p45^{MEK}) activity in cells transfected with A1R and either of the α_{12} constructs (Figure 3), as reflected by increased autophosphorylation of immunoprecipitated HA-p45^{MEK}, and increased phosphorylation of its substrate, recombinant bt-MAPK. PTX almost completely blocked activation of HA-p45^{MEK} by PIA in cells expressing α_{12} EE, but only partially blocked its activation in cells expressing α_{12} EECS. As expected, PTX had little effect on bFGF stimulation of HA-p45^{MEK}. G_{12} activation led to stimulation of endogenous MEK as well (see below). These results demonstrate that activation of G_{12} leads to activation of MEK.

Measurement of Raf/MEKK Activity

Western blotting revealed that CHO-K1 cells express the three Raf isoforms (A-Raf, B-Raf, and Raf-1) and MEKK (our unpublished data), protein kinases known to activate MEK. We tested for MEK activator activity by immunoprecipitating each of these kinases from detergent lysates of cells or from detergent extracts of particulate fractions, and performing *in vitro* kinase reactions using kinase-inactive MEK as a substrate. We could not reliably detect stimulation of any of these MEK activators by either G_{12} or bFGF, although serum treatment caused a mild but reproducible activation of Raf-1 (our unpublished results). Because we are not sure whether the failure to observe Raf/MEKK activity was due to a technical problem or truly represents a lack of stimulation of these kinases, we cannot draw conclusions regarding G_{12} activation of Raf or MEKK.

Involvement of Ras in G_{12} -mediated Activation of MAPK

We also asked whether G_{12} activation of MAPK involves Ras, which has been shown to bind Raf, leading to its activation (reviewed in Hall, 1994). The dominant-negative Ras mutant N17ras, in which serine 17 is changed to asparagine (Feig and Cooper, 1988), when co-expressed with the A1R and α_{12} EECS, blocked PIA-induced activation of both endogenous MAPK (Figure 4A) and endogenous MEK (Figure 4B) in cells pretreated with PTX. N17ras also blocked activation of MAPK and MEK

Figure 2. Activation of MAPK in response to G₁₂ stimulation. cDNAs encoding the A1R and either α_{12} EE or α_{12} EECS were transfected into CHO-K1 cells (100 ng of each per 10⁶ cells). Cells were placed in serum-free medium 24 h after transfection, and stimulated 48 h after transfection. Pretreatment $\pm$ PTX was followed by incubation at 37°C with serum-free medium alone or including PIA for 7 min (A and B) or bFGF for 10 min (B). Endogenous MAPK activity was measured as described in MATERIALS AND METHODS. (A) Activity of endogenous p42^{mapk}. (B) Activity of endogenous p44^{mapk}. Similar results were obtained in at least six other experiments for each MAPK isoform.

by bFGF, as expected for a mitogen acting through a receptor tyrosine kinase (Figure 4A and our unpub-

lished observations). The inhibitory effects of N17ras were consistent with it acting at the level of Ras or upstream of Ras, in that it did not block MAPK activation by constitutively active mutants of Raf (*v-raf* and *raf-CAAX*) or MEK (Δ N3-S218E-S222D, designated MEK*) (Figure 4A), nor did it affect the expression of co-transfected DNA (our unpublished observation).

To confirm the involvement of Ras in G₁₂-mediated activation of MAPK, we tested whether activation of G₁₂ leads to activation of Ras. Surprisingly, in cells transfected with A1R and α_{12} EECS and pretreated with PTX, PIA did not increase the fraction of Ras bound to GTP (Figure 4, C and D). Identical results were obtained with shorter and longer PIA incubation times (our unpublished results). bFGF treatment, however, increased Ras_{GTP} $\sim$ 2.6-fold, from 9% to 23%, indicating that the assay was able to detect Ras activation. Given the high efficiency of transfection for these experiments, and the fact that PIA-induced activation of endogenous MAPK and MEK was easily detected in similarly transfected cells, the lack of Ras activation in response to PIA cannot be due to poor expression of the A1R or α_{12} EECS. Therefore, even though G₁₂-mediated activation of MEK and MAPK was blocked by N17ras, activation of G₁₂ did not lead to a detectable activation of Ras.

Figure 3. Activation of HA-tagged p45^{MEK} by G₁₂. cDNAs encoding the A1R, HA-p45^{MEK}, and either α_{12} EE, α_{12} EECS, or pcDNA3 (control) were transfected into CHO-K1 cells (100 ng of each construct per 10⁶ cells). Cells were placed in serum-free medium 24 h after transfection and assayed 48 h after transfection. After pretreatment $\pm$ PTX, cells were incubated at 37°C with either serum-free medium (basal), PIA (2.5 min), or bFGF (5 min). HA-p45^{MEK} activity was measured as described in MATERIALS AND METHODS. The upper band represents phosphorylation of HA-p45^{MEK}; the lower band represents phosphorylation of bt-MAPK. Results are representative of five different experiments.

Figure 4. Effects of N17ras on activation of MAPK and MEK by G_{12} (A and B), and lack of activation of Ras by G_{12} (C and D). (A) CHO-K1 cells were transfected with 100 ng each of A1R and α_{12} EECS cDNA, 200 ng of either vector or Ras construct, and 400 ng of either vector, v-*raf*, rafCAAX, or MEK* per 10^6 cells. Cells were placed in serum-free medium 24 h after transfection and assayed 48 h after transfection. All cells were pretreated with PTX, then treated with serum-free medium for 10 min (basal, v-*raf*, rafCAAX, and MEK*), or stimulated with either PIA (7 min) or bFGF (10 min). p44^{mapk} activity was measured as described in MATERIALS AND METHODS. Similar results were obtained in six other experiments. (B) CHO-K1 cells were transfected with 100 ng each of A1R and α_{12} EECS cDNA, and 200 ng of either Ras construct per 10^6 cells. Cells were placed in serum-free medium 24 h after transfection and assayed 48 h after transfection. All cells were pretreated with

PTX, then incubated with serum-free medium (basal) or PIA for 2.5 min. Endogenous p45^{MEK} activity was measured as described in MATERIALS AND METHODS. Results are representative of three separate experiments. (C and D) CHO-K1 cells were transfected with A1R and α_{12} EECS cDNAs (100 ng of each per 10^6 cells). The next day, the cells were placed in serum-free medium for 24 h, and within that time they were labeled overnight with [32 P]orthophosphoric acid. Two days after transfection, the cells were pretreated with PTX and then stimulated with the indicated agonists for 4 min. The proportion of Ras bound to [32 P]GTP versus [32 P]GDP was determined as described in MATERIALS AND METHODS. (C) Autoradiograph of a thin layer chromatography showing radioactive GDP and GTP bound to Ras. (D) Quantitation of fraction of GTP-bound Ras by PhosphorImager analysis. Shown is the average of three determinations with bars indicating the range of responses.

A Role for $\beta\gamma$ in G_{12} -mediated Activation of MAPK

In light of recent evidence that G protein $\beta\gamma$ subunits can lead to activation of MAPK (Crespo *et al.*, 1994; Faure *et al.*, 1994), we asked whether $\beta\gamma$ released from G_{12} is responsible for MAPK activation. To do so, we transiently expressed a carboxyl terminal fragment of the β -adrenoceptor kinase, β ARK1-(495–689), which binds $\beta\gamma$ in vitro and antagonizes $\beta\gamma$ -mediated signaling in intact cells (Koch *et al.*, 1994b). Expression of the β ARK fragment was previously shown to decrease G_i -coupled receptor activation of Ras and MAPK in Rat-1 fibroblasts and COS-7 cells (Koch *et al.*, 1994a). In CHO-K1 cells transfected with A1R, α_{12} EECS, and HA-tagged p44^{mapk} (HA-p44^{mapk}), and pretreated with PTX, co-transfection of β ARK1-(495–689) decreased PIA stimulation of HA-p44^{mapk} (Figure 5A). Inhibition by β ARK1-(495–689) increased with increasing amounts of transfected β ARK1-(495–689) DNA, to a maximum of approximately 50%. To test

for specificity of β ARK1-(495–689) action, we performed the same experiment with the M1 muscarinic receptor (M1R); M1R activation of MAPK should be less sensitive to a $\beta\gamma$ antagonist, because it is primarily mediated by the α subunit of G_q (Faure *et al.*, 1994). β ARK1-(495–689) failed to inhibit stimulation of HA-p44^{mapk} by the M1R (Figure 5B), consistent with the theory that it acts by antagonizing $\beta\gamma$, rather than by a nonspecific mechanism. β ARK1-(495–689) had no effect on expression of α_{12} EECS and HA-p44^{mapk} (our unpublished results). G_{12} therefore appears to activate MAPK in CHO-K1 cells, at least in part, by releasing $\beta\gamma$.

Involvement of PI3K in G_{12} -mediated Activation of MAPK

G protein $\beta\gamma$ subunits can modulate the activities of several effector molecules, including ion channels, phospholipases, adenylyl cyclase, and PI3K (reviewed

Figure 5. Effect of β ARK1-(495-689) on activation of HA-tagged MAPK by G₁₂. A1R, α_{12} EECS, and HA-p44^{mapk} cDNAs (100 ng of each per 10⁶ cells) (A), or M1R cDNA (200 ng per 10⁶ cells) and HA-p44^{mapk} cDNA (100 ng per 10⁶ cells) (B) were transfected into CHO-K1 cells with the indicated amounts of β ARK1-(495-689) cDNA. Transfected cells were placed in serum-free medium 24 h after transfection and stimulated 48 h after transfection. All cells were pretreated with PTX. (A) Cells were stimulated with serum-free medium (basal) or PIA for 7 min and assayed for activity of HA-p44^{MAPK} as described in MATERIALS AND METHODS. Three additional experiments gave similar results, with an average maximal inhibition of 46%. (B) Cells were treated with serum-free medium or 200 mM carbachol for 7 min and assayed for activity of HA-p44^{MAPK}. Similar results were obtained in two to four experiments.

in Clapham and Neer, 1993; Stephens *et al.*, 1994; Thomason *et al.*, 1994). Of these, activation of adenylyl cyclase, phospholipase C (PLC), and PI3K have been associated with the activation of MAPK (Cross *et al.*, 1994; Faure *et al.*, 1994; Ferby *et al.*, 1994; Sakanaka *et al.*, 1994; VanRenterghem *et al.*, 1994; Welsh *et al.*, 1994) and are therefore potential mediators of $\beta\gamma$ -induced activation of MAPK. $\beta\gamma$ activation of adenylyl cyclase can be ruled out as the mechanism of G₁₂-mediated activation of MAPK in CHO-K1 cells, however, because G₁₂ inhibits adenylyl cyclase (see above), and because raising cAMP levels inhibits MAPK activity in these cells (our unpublished data); nor can $\beta\gamma$ activation of PLC account for G₁₂-mediated activation of MAPK in these cells, because activation of G₁₂ did not increase inositol phosphate levels, a measure of PLC activity (our unpublished data). We therefore asked whether PI3K is involved in G₁₂-mediated activation of MAPK. Wortmannin, a potent inhibitor of PI3K (Arcaro and Wymann, 1993; Thelen *et al.*, 1994), inhibited G₁₂ activation of MAPK with an IC₅₀ of approximately 80 nM; maximal inhibition was ~40% (Figure 6). Wortmannin also partially blocked activation of MAPK by bFGF (our unpublished result), consistent with evidence that FGF activates PI3K (Stephens *et al.*, 1993). In contrast, wortmannin did not inhibit MAPK activity stimulated by mutationally activated MEK or v-ras (our unpublished results), indicating that its ef-

Figure 6. Effect of wortmannin on G₁₂ activation of HA-tagged MAPK. A1R, α_{12} EECS, and HA-p44^{mapk} cDNAs were transfected (100 ng of each per 10⁶ cells) into CHO-K1 cells. Transfected cells were placed in serum-free medium 24 h after transfection and stimulated 48 h after transfection. All cells were pretreated with PTX, then incubated for 15 min with wortmannin at the indicated concentrations before stimulation with PIA for 7 min. HA-p44^{mapk} was assayed as described in MATERIALS AND METHODS. Each point represents the average of four or five determinations; bars represent the standard deviations.

fects were not due to a nonspecific mechanism. These findings suggest that G_{12} activates the MAPK pathway at least in part by stimulating PI3K.

DISCUSSION

We have devised a system to specifically activate by receptor one particular G_i subtype, G_{12} , and we have used this system to investigate G_{12} -mediated activation of the MAPK cascade. Although activation of the MAPK cascade by G_i -coupled receptors is well documented, this approach has allowed us to characterize activation of the pathway at the level of a specific G_i , information that cannot be ascertained from standard receptor studies.

G_{12} Activates the MAPK Cascade

Like wild-type α_{12} , the epitope-tagged, cysteine 352 to serine mutant of α_{12} (α_{12} EECS) can be activated by a G_i -coupled receptor and can inhibit adenylyl cyclase. PIA-induced inhibition of cAMP accumulation, which is completely blocked by PTX, was restored by expression of α_{12} EECS (Figure 1), indicating that, in the presence of PTX, mutant G_{12} is the only G_i activated. Furthermore, the extent of cAMP reduction mediated by G_{12} alone was similar to that mediated by PIA in the absence of PTX, indicating that, with respect to adenylyl cyclase inhibition, G_{12} is functionally equivalent to other endogenous G_i proteins that couple to the A1R in CHO-K1 cells.

Activation of PTX-insensitive G_{12} by the A1R led to activation of MAPK and MEK (Figures 2 and 3). These findings agree with and extend previous reports based on cells transformed by mutationally activated α_{12} (Gupta *et al.*, 1992a; Gardner *et al.*, 1993), in which it could not be discerned whether the elevated MAPK and MEK activities were a direct result of signaling by α_{12} , or occurred as a secondary effect (for instance, due to activation of an autocrine pathway). In cells expressing α_{12} EECS, PIA-mediated activation of both MAPK and MEK was partially blocked by PTX, indicating that activation of PTX-resistant G_{12} alone did not suffice for maximal A1R-mediated activation of the cascade, although it did suffice for inhibition of adenylyl cyclase. This finding suggests, although other explanations are possible, that in the absence of PTX, the A1R activates not only G_{12} but also another G_i , and that this G_i furnishes an additional signal to activate the MAPK cascade. Because CHO cells have been reported to express only the G_{12} and G_{13} subtypes of the G_i family (Dell'Acqua *et al.*, 1993), this would imply that in the absence of PTX, G_{13} augments the effect of G_{12} on the MAPK pathway, either quantitatively or qualitatively. The possibility of differences in signaling between G_{12} and G_{13} is intriguing,

and can be addressed by studies using a PTX-insensitive α_{13} mutant.

Involvement of Ras

Activation of G_{12} in CHO-K1 cells did not lead to a detectable activation of Ras, despite the fact that bFGF treatment caused a two- to threefold stimulation (Figure 4, C and D). This result agrees with results of other experiments with G_i -coupled receptors: the platelet-activating factor receptor and the α_{2B} and α_{2D} adrenergic receptors, all of which mediate PTX-sensitive activation of MAPK, also failed to activate Ras in CHO cells (Honda *et al.*, 1994; Flordellis *et al.*, 1995). G_i -coupled receptors that have been shown to activate Ras (the LPA, thrombin, M2 muscarinic, and α_{2A} adrenergic receptors) all did so in cell types other than CHO (Rat-1, CCL39, or COS-7; Alblas *et al.*, 1993; van Corven *et al.*, 1993; Winitz *et al.*, 1993; Koch *et al.*, 1994a). Although other explanations cannot be ruled out, the data suggests two testable possibilities. One is that G_i proteins can stimulate the MAPK cascade by different mechanisms in different cell types, Ras-dependent or Ras-independent. If so, G_i proteins in CHO cells utilize a Ras-independent pathway to activate MAPK. A second (nonexclusive) possibility is that different G_i subtypes inherently activate the cascade by different mechanisms, so that the ability of a G_i -coupled receptor to activate Ras depends on the G_i subtypes that it activates. The fact that PTX partially blocked MAPK activation by an A1R agonist in α_{12} EECS-expressing CHO-K1 cells (see above) is in keeping with this idea. A third possibility is that G_{12} caused a weak but physiologically significant activation of Ras, however, the fact that bFGF and G_{12} activated MAPK and MEK to similar extents predicts that they would likewise activate Ras to similar extents.

In view of the failure to detect G_{12} activation of Ras, we do not know why N17ras inhibited G_{12} -mediated activation of MAPK and MEK (Figure 4, A and B). N17ras is believed to exert its dominant negative effect by sequestering Ras guanine nucleotide exchange factors, thereby preventing Ras activation. Its effect in CHO-K1 cells was somewhat specific, because it did not block activation of MAPK by mutationally activated raf or MEK. One possible explanation is that G_{12} activation of the MAPK cascade requires a basal level of Ras_{GTP}, and that N17ras lowered this level below a required threshold. Precedents for this type of signaling have been described. For instance, $\beta\gamma$ subunit activation of type II adenylyl cyclase (AC-II) takes place only when AC-II has been "primed" by the GTP-bound α subunit of G_s (Tang and Gilman, 1991). Perhaps a threshold level of Ras_{GTP} is required to "prime" an effector activated by G_{12} . Another possibility is that Ras exchange factors perform a second function leading to MAPK activation, in addition to

Ras GDP/GTP exchange, and that N17ras disrupted this second role. Answers to these questions await more detailed analysis of the activities of Ras and its exchange factors.

Involvement of $\beta\gamma$

The ability of β ARK1-(495–689) to inhibit G₁₂-dependent activation of MAPK (Figure 5A) strongly suggests that the $\beta\gamma$ subunit of this G protein mediates, at least in part, PIA-induced activation of the MAPK pathway. This result agrees with reports that $\beta\gamma$ subunits can stimulate the MAPK pathway (Crespo *et al.*, 1994; Faure *et al.*, 1994). The same β ARK fragment inhibits stimulation of MAPK by LPA in Rat-1 cells, and the α_2 adrenergic and M2 muscarinic receptors in COS-7 cells (Koch *et al.*, 1994a). In both sets of experiments, β ARK1-(495–689) exerted only a partial inhibition of MAPK activation. Although the reason for these partial effects is unknown, one possibility is that α_1 -in our experiments, α_{12} -acts in parallel with $\beta\gamma$ to stimulate the MAPK pathway. We attempted to address this hypothesis by transient expression of a mutationally activated α_{12} subunit in CHO-K1 cells; elevated MAPK activity was not observed (our unpublished result). Interpretation of such an experiment is difficult, however, because it is possible that constitutive activation of the MAPK pathway could elicit some type of negative feedback regulation. This model will therefore best be tested with a reagent (or a mutant) that blocks the ability of α_{12} to interact with effectors but specifically allows release of $\beta\gamma$ in response to receptor stimulation. If α_{12} plays no role in G₁₂-mediated activation of MAPK (except to release $\beta\gamma$), how would neoplastic transformation by the *gip2* oncogene be explained? One possibility is that mutationally activated α_{12} generates a proliferative signal by mediating a prolonged release of $\beta\gamma$.

Linking G₁₂ to the MAPK Cascade

To address the question of how G₁₂ activates the MAPK cascade, we considered three $\beta\gamma$ -activated effectors that have been implicated in triggering the cascade: adenylyl cyclase, PLC, and PI3K. Both adenylyl cyclase and PLC can be ruled out as mediators of G₁₂ activation of the cascade, at least in CHO-K1 cells, because in these cells G₁₂ inhibits adenylyl cyclase and fails to activate PLC (Figure 1 and our unpublished observation).

Partial inhibition of G₁₂-mediated MAPK activation by low concentrations of wortmannin (Figure 6) suggests that PI3K could play an important role in linking G₁₂ to the cascade. This finding agrees with previous evidence that G protein $\beta\gamma$ subunits can activate PI3K activity (Stephens *et al.*, 1994; Thomason *et al.*, 1994), and raises the possibility that $\beta\gamma$ released from G₁₂ may act to stimulate PI3K in CHO-K1 cells. In theory,

testing the effect of wortmannin and β ARK1-(495–689) in combination on G₁₂ activation of MAPK would indicate whether $\beta\gamma$'s effect on MAPK depended on PI3K activity. In practice, however, this experiment would not be interpretable, because the efficiency with which either inhibitor alone disrupts its putative target is unknown. Instead, more direct biochemical experiments are necessary to determine whether activation of G₁₂ does in fact activate PI3K activity in CHO-K1 cells, whether this activity is stimulated by $\beta\gamma$, and whether it contributes to activation of the MAPK cascade.

In summary, we have begun to characterize the mechanism by which G₁₂ activates the MAPK cascade. Similar experiments with other PTX-insensitive G_i subtypes and with a variety of cell lines should delineate the roles of different G proteins in activating this pathway, and may help to explain discrepancies in the pathways activated by various G_i-coupled receptors.

ACKNOWLEDGMENTS

We thank A.L. DeFranco, D.O. Morgan, P.T. Wilson, T. Iiri, P.B. Wedegaertner, D.W. Markby, J.M. Morales, E.R. Neptune, and W.J. Fantl for useful discussions and/or critical reading of the manuscript; P.D. Garcia, W.J. Fantl, T.A. Voyno-Yasenetskaya, and P.T. Wilson for technical help; R. Taussig, P.D. Garcia, D. Segaloff, W. Sadee, J. Pouyssegur, W.J. Fantl, N.G. Ahn, A.M. MacNicol, D. Stokoe, and R.J. Lefkowitz for plasmids; and W.J. Fantl for monoclonal antibody Y13-259. This work was supported by National Institutes of Health training grant GM-07175-17 and an Achievement Reward for College Scientists (to A.M.P.), a fellowship from the Centre National de la Recherche Scientifique (to M.F.), and grants (to H.R.B.) from the NIH (GM-27800 and CA-54427) and the March of Dimes.

REFERENCES

- Alblas, J., van Corven, E.J., Hordijk, P.L., Milligan, G., and Moolenaar, W.H. (1993). G_i-mediated activation of the p21^{ras}-mitogen-activated protein kinase pathway by α_2 -adrenergic receptors in fibroblasts. *J. Biol. Chem.* 268, 22235–22238.
- Arcaro, A., and Wymann, M.P. (1993). Wortmannin is a potent phosphatidylinositol 3-kinase inhibitor: the role of phosphatidylinositol 3,4,5-trisphosphate in neutrophil responses. *Biochem. J.* 296, 297–301.
- Clapham, D.E., and Neer, E.J. (1993). New roles for G-protein $\beta\gamma$ dimers in transmembrane signalling. *Nature* 365, 403–406.
- Crespo, P., Xu, N., Simonds, W.F., and Gutkind, J.S. (1994). Ras-dependent activation of MAP kinase pathway mediated by G-protein $\beta\gamma$ subunits. *Nature* 369, 418–420.
- Cross, D.A.E., Alessi, D.R., Vandenheede, J.R., McDowell, H.E., Hundal, H.S., and Cohen, P. (1994). The inhibition of glycogen synthase kinase-3 by insulin or insulin-like growth factor 1 in the rat skeletal muscle cell line L6 is blocked by wortmannin, but not by rapamycin: evidence that wortmannin blocks activation of the mitogen-activated protein kinase pathway in L6 cells between Ras and Raf. *Biochem. J.* 303, 21–26.
- Dell'Acqua, M.L., Carroll, R.C., and Peralta, E.G. (1993). Transfected m2 muscarinic acetylcholine receptors couple to G_{o12} and G_{o13} in Chinese hamster ovary cells. *J. Biol. Chem.* 268, 5676–5685.

- Fantl, W.J., Escobedo, J.A., Martin, G.A., Turck, C.W., del Rosario, M., McCormick, F., and Williams, L.T. (1992). Distinct phosphotyrosines on a growth factor receptor bind to specific molecules that mediate different signaling pathways. *Cell* 69, 413–423.
- Faure, M., and Bourne, H.R. (1995). Differential effects of cAMP on the MAP kinase cascade: evidence for a cAMP-insensitive step that can bypass Raf-1. *Mol. Biol. Cell* 6, 1025–1035.
- Faure, M., Voyno-Yasenetskaya, T.A., and Bourne, H.R. (1994). cAMP and $\beta\gamma$ subunits of heterotrimeric G proteins stimulate the mitogen-activated protein kinase pathway in COS-7 cells. *J. Biol. Chem.* 269, 7851–7854.
- Feig, L.A., and Cooper, G.M. (1988). Inhibition of NIH 3T3 cell proliferation by a mutant *ras* protein with preferential affinity for GDP. *Mol. Cell. Biol.* 8, 3235–3243.
- Ferby, I.M., Waga, I., Sakanaka, C., Kume, K., and Shimizu, T. (1994). Wortmannin inhibits mitogen-activated protein kinase activation induced by platelet-activating factor in guinea pig neutrophils. *J. Biol. Chem.* 269, 30485–30488.
- Flordellis, C.S., Berguerand, M., Gouache, P., Barbu, V., Gavras, H., Handy, D.E., Béréziat, G., and Masliah, J. (1995). $\alpha 2$ Adrenergic receptor subtypes expressed in Chinese hamster ovary cells activate differentially mitogen-activated protein kinase by a $p21^{ras}$ independent pathway. *J. Biol. Chem.* 270, 3491–3494.
- Forsayeth, J.R., and Garcia, P.D. (1994). Adenovirus-mediated transfection of cultured cells. *BioTechniques* 17, 354–359.
- Garcia, P.D., and Myers, R.M. (1994). Pituitary cell line GH₃ expresses two somatostatin receptor subtypes that inhibit adenylyl cyclase: functional expression of rat somatostatin receptor subtypes 1 and 2 in human embryonic kidney 293 cells. *Mol. Pharmacol.* 45, 402–409.
- Gardner, A.M., Vaillancourt, R.R., and Johnson, G.L. (1993). Activation of mitogen-activated protein kinase/extracellular signal-regulated kinase by G protein and tyrosine kinase oncoproteins. *J. Biol. Chem.* 268, 17896–17901.
- Grussenmeyer, T., Scheidtmann, K.H., Hutchinson, M.A., Eckhart, W., and Walter, G. (1985). Complexes of polyoma virus medium T antigen and cellular proteins. *Proc. Natl. Acad. Sci. USA* 82, 7952–7954.
- Gupta, S.K., Gallego, C., Johnson, G.L., and Heasley, L.E. (1992a). MAP kinase is constitutively activated in *gip2*- and *src*-transformed Rat1a fibroblasts. *J. Biol. Chem.* 267, 7987–7990.
- Gupta, S.K., Gallego, C., Lowndes, J.M., Pleiman, C.M., Sable, C., Eisfelder, B.J., and Johnson, G.L. (1992b). Analysis of the fibroblast transformation potential of GTPase-deficient *gip2* oncogenes. *Mol. Cell. Biol.* 12, 190–197.
- Hall, A. (1994). A biochemical function for Ras: at last. *Science* 264, 1413–1414.
- Hermouet, S., Merendino, J.J., Jr., Gutkind, J.S., and Spiegel, A.M. (1991). Activating and inactivating mutations of the α subunit of G_{12} protein have opposite effects on proliferation of NIH 3T3 cells. *Proc. Natl. Acad. Sci. USA* 88, 10455–10459.
- Honda, Z., Takano, T., Gotoh, Y., Nishida, E., Ito, K., and Shimizu, T. (1994). Transfected platelet-activating factor receptor activates mitogen-activated protein (MAP) kinase and MAP kinase kinase in Chinese hamster ovary cells. *J. Biol. Chem.* 269, 2307–2315.
- Howe, L.H., and Marshall, C.J. (1993). Lysophosphatidic acid stimulates mitogen-activated protein kinase activation via a G-protein-coupled pathway requiring $p21^{ras}$ and $p74^{raf-1}$. *J. Biol. Chem.* 268, 20717–20720.
- Hunt, T.W., Carroll, R.C., and Peralta, E.G. (1994). Heterotrimeric G proteins containing $G_{\alpha 3}$ regulate multiple effector enzymes in the same cell. *J. Biol. Chem.* 269, 29565–29570.
- Koch, W.J., Hawes, B.E., Allen, L.F., and Lefkowitz, R.J. (1994a). Direct evidence that G_i -coupled receptor stimulation of mitogen-activated protein kinase is mediated by $G_{\beta\gamma}$ activation of $p21^{ras}$. *Proc. Natl. Acad. Sci. USA* 91, 12706–12710.
- Koch, W.J., Hawes, B.E., Inglese, J., Luttrell, L.M., and Lefkowitz, R.J. (1994b). Cellular expression of the carboxyl terminus of a G protein-coupled receptor kinase attenuates $G_{\beta\gamma}$ -mediated signaling. *J. Biol. Chem.* 269, 6193–6197.
- L'Allemain, G., Pouyssegur, J., and Weber, M.J. (1991). p42/mitogen-activated protein kinase as a converging target for different growth factor signaling pathways: use of pertussis toxin as a discrimination factor. *Cell Regul.* 2, 675–684.
- LaMorte, V.J., Goldsmith, P.K., Spiegel, A.M., Meinkoth, J.L., and Feramisco, J.R. (1992). Inhibition of DNA synthesis in living cells by microinjection of G_{12} antibodies. *J. Biol. Chem.* 267, 691–694.
- Lyons, J., Landis, C.A., Harsh, G., Vallar, L., Grünwald, K., Feichtinger, H., Duh, Q.Y., Clark, O.H., Kawasaki, E., Bourne, H.R., and McCormick, F. (1990). Two G protein oncogenes in human endocrine tumors. *Science* 249, 655–659.
- Maeda, S., Lamah, J., Mallet, W.G., Philip, M., Ramachandran, J., and Sadée, W. (1990). Internalization of the Hm1 muscarinic cholinergic receptor involves the third cytoplasmic loop. *FEBS Lett.* 269, 386–388.
- Mansour, S.J., Matten, W.T., Hermann, A.S., Candia, J.M., Rong, S., Fukasawa, K., Vande Woude, G.F., and Ahn, N.G. (1994). Transformation of mammalian cells by constitutively active MAP kinase kinase. *Science* 265, 966–970.
- Marshall, C.J. (1994). MAP kinase kinase kinase, MAP kinase kinase and MAP kinase. *Curr. Opin. Genet. Dev.* 4, 82–89.
- McFarland, K.C., Sprengel, R., Phillips, H.S., Köhler, M., Rosemblyt, N., Nikolics, K., Segaloff, D.L., and Seeburg, P.H. (1989). Lutropin-choriogonadotropin receptor: an unusual member of the G protein-coupled receptor family. *Science* 245, 494–499.
- Pace, A.M., Wong, Y.H., and Bourne, H.R. (1991). A mutant α subunit of G_{12} induces neoplastic transformation of Rat-1 cells. *Proc. Natl. Acad. Sci. USA* 88, 7031–7035.
- Sakanaka, C., Ferby, I., Waga, I., Bito, H., and Shimizu, T. (1994). On the mechanism of cytosolic phospholipase A_2 activation in CHO cells carrying somatostatin receptor: wortmannin-sensitive pathway to activate mitogen-activated protein kinase. *Biochem. Biophys. Res. Commun.* 205, 18–23.
- Satoh, T., Endo, M., Nakafuku, M., Nakamura, S., and Kaziro, Y. (1990). Platelet-derived growth factor stimulates formation of active $p21^{ras}$: GTP complex in Swiss mouse 3T3 cells. *Proc. Natl. Acad. Sci. USA* 87, 5993–5997.
- Seuwen, K., and Pouyssegur, J. (1990). Serotonin as a growth factor. *Biochem. Pharmacol.* 39, 985–990.
- Stephens, L., Smrcka, A., Cooke, F.T., Jackson, T.R., Sternweis, P.C., and Hawkins, P.T. (1994). A novel phosphoinositide 3 kinase activity in myeloid-derived cells is activated by G protein $\beta\gamma$ subunits. *Cell* 77, 83–93.
- Stephens, L.R., Jackson, T.R., and Hawkins, P.T. (1993). Agonist-stimulated synthesis of phosphatidylinositol (3,4,5)-trisphosphate: a new intracellular signalling system? *Biochim. Biophys. Acta* 1179, 27–75.
- Stokoe, D., Macdonald, S.G., Cadwallader, K., Symons, M., and Hancock, J.F. (1994). Activation of Raf as a result of recruitment to the plasma membrane. *Science* 264, 1463–1467.
- Tang, W.-J., and Gilman, A.G. (1991). Type-specific regulation of adenylyl cyclase by G protein $\beta\gamma$ subunits. *Science* 254, 1500–1503.

- Taussig, R., Sanchez, S., Rifo, M., Gilman, A.G., and Belardetti, F. (1992). Inhibition of the omega-conotoxin-sensitive current by distinct G proteins. *Neuron* 8, 799–809.
- Thelen, M., Wymann, M.P., and Langen, H. (1994). Wortmannin binds specifically to 1-phosphatidylinositol 3-kinase while inhibiting guanine nucleotide-binding protein-coupled receptor signaling in neutrophil leukocytes. *Proc. Natl. Acad. Sci. USA* 91, 4960–4964.
- Thomason, P.A., James, S.R., Casey, P.J., and Downes, C.P. (1994). A G-protein $\beta\gamma$ -subunit-responsive phosphoinositide 3-kinase activity in human platelet cytosol. *J. Biol. Chem.* 269, 16525–16528.
- Valius, M., and Kazlauskas, A. (1993). Phospholipase C- γ 1 and phosphatidylinositol 3 kinase are the downstream mediators of the PDGF receptor's mitogenic signal. *Cell* 73, 321–334.
- van Corven, E.J., Hordijk, P.L., Medema, R.H., Bos, J.L., and Moolenaar, W.H. (1993). Pertussis toxin-sensitive activation of p21^{ras} by G protein-coupled receptor agonists in fibroblasts. *Proc. Natl. Acad. Sci. USA* 90, 1257–1261.
- VanRenterghem, B., Browning, M.D., and Maller, J.L. (1994). Regulation of mitogen-activated protein kinases A and C in a cell-free system. *J. Biol. Chem.* 269, 24666–24672.
- Welsh, G.I., Foulstone, E.J., Young, S.W., Tavaré, J.M., and Proud, C.G. (1994). Wortmannin inhibits the effects of insulin and serum on the activities of glycogen synthase kinase-3 and mitogen-activated protein kinase. *Biochem. J.* 303, 15–20.
- Wilson, P.T., and Bourne, H.R. (1995). Fatty acylation of α_z . *J. Biol. Chem.* 270, 9667–9675.
- Winitz, S., Russell, M., Qian, N., Gardner, A., Dwyer, L., and Johnson, G.L. (1993). Involvement of Ras and Raf in the G_i-coupled acetylcholine muscarinic m2 receptor activation of mitogen-activated protein (MAP) kinase kinase and MAP kinase. *J. Biol. Chem.* 268, 19196–19199.
- Wong, Y.H., Federman, A., Pace, A.M., Zachary, I., Evans, T., Pouyssegur, J., and Bourne, H.R. (1991). Mutant α subunits of G₁₂ inhibit cyclic AMP accumulation. *Nature* 351, 63–65.