Molecular Biology of the Cell
Vol. 11, 1129–1142, April 2000

Activation of B-Raf and Regulation of the Mitogen-activated Protein Kinase Pathway by the G_o α chain

Valeria Antonelli,* Francesca Bernasconi,* Yung H. Wong,† and Lucia Vallar*‡

*Department of Pharmacology, University of Milan, Consiglio Nazionale delle Ricerche, Cellular and Molecular Pharmacology Center, and Department for Biological and Technological Research, Scientific Institute San Raffaele, 20132 Milan, Italy; and †Department of Biology and Biotechnology Research Institute, Hong Kong University of Science and Technology, Clear Water Bay, Hong Kong, China

Submitted August 9, 1999; Revised December 20, 1999; Accepted February 3, 2000
Monitoring Editor: Tony Hunter

Many receptors coupled to the pertussis toxin-sensitive $G_{i/o}$ proteins stimulate the mitogen-activated protein kinase (MAPK) pathway. The role of the α chains of these G proteins in MAPK activation is poorly understood. We investigated the ability of $G\alpha_o$ to regulate MAPK activity by transient expression of the activated mutant $G\alpha_o$-Q205L in Chinese hamster ovary cells. $G\alpha_o$-Q205L was not sufficient to activate MAPK but greatly enhanced the response to the epidermal growth factor (EGF) receptor. This effect was not associated with changes in the state of tyrosine phosphorylation of the EGF receptor. $G\alpha_o$-Q205L also potentiated MAPK stimulation by activated Ras. In Chinese hamster ovary cells, EGF receptors activate B-Raf but not Raf-1 or A-Raf. We found that expression of activated $G\alpha_o$ stimulated B-Raf activity independently of the activation of the EGF receptor or Ras. Inactivation of protein kinase C and inhibition of phosphatidylinositol-3 kinase abolished both B-Raf activation and EGF receptor-dependent MAPK stimulation by $G\alpha_o$. Moreover, $G\alpha_o$-Q205L failed to affect MAPK activation by fibroblast growth factor receptors, which stimulate Raf-1 and A-Raf but not B-Raf activity. These results suggest that $G\alpha_o$ can regulate the MAPK pathway by activating B-Raf through a mechanism that requires a concomitant signal from tyrosine kinase receptors or Ras to efficiently stimulate MAPK activity. Further experiments showed that receptor-mediated activation of $G\alpha_o$ caused a B-Raf response similar to that observed after expression of the mutant subunit. The finding that $G\alpha_o$ induces Ras-independent and protein kinase C- and phosphatidylinositol-3 kinase-dependent activation of B-Raf and conditionally stimulates MAPK activity provides direct evidence for intracellular signals connecting this G protein subunit to the MAPK pathway.

INTRODUCTION

The mitogen-activated protein kinase (MAPK) pathway plays a central role in the stimulation of cell growth by cell surface receptors (Marshall, 1994; Cobb and Goldsmith, 1995). Both tyrosine kinase receptors and G protein-coupled receptors lead to the activation of the serine/threonine kinases known as p44 MAPK and p42 MAPK or extracellular signal-regulated kinases 1 and 2, which phosphorylate and regulate a large array of substrates, including nuclear transcription factors that control genes essential for cell proliferation (Davis, 1993). A well-characterized signaling pathway links tyrosine kinase receptors to MAPK activation. Growth factor-induced tyrosine phosphorylation of these receptors, and the subsequent recruitment of the adaptor molecules Shc and Grb2 bring to the plasma membrane the Sos protein, which acts as a guanine nucleotide exchange factor for Ras (Boguski and McCormick, 1993; Schlessinger, 1994). Ras activation is followed by a kinase cascade in which one or more of the proteins referred as Raf-1, A-Raf, and B-Raf phosphorylate and activate the MAPK/extracellular signal-regulated kinase kinases (MEK), which in turn phosphorylate and activate p44 and p42 MAPK (Marshall, 1994; Cobb and Goldsmith, 1995; Marais and Marshall, 1996;

‡ Corresponding author. E-mail address: Vallar.Lucia@hsr.it.
Abbreviations used: CHO, Chinese hamster ovary; CLAP, chymostatin, leupeptin, antipain, and pepstatin; EGF, epidermal growth factor; FGF, fibroblast growth factor; GST, glutathione *S*-transferase; HA, hemagglutinin; MAPK, mitogen-activated protein kinase; MBP, myelin basic protein; MEK, MAPK/extracellular signal-regulated kinase kinase; PDGF, platelet-derived growth factor; PI3K, phosphatidylinositol-3 kinase; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; PTX, pertussis toxin.

Campbell *et al.*, 1998). G protein-coupled receptors appear to initiate a variety of pathways that mediate Ras-dependent or Ras-independent stimulation of the MAPK cascade (van Biesen *et al.*, 1996b; Gutkind, 1998; Luttrell *et al.*, 1999). These pathways, which involve various G protein subunits and downstream signaling molecules, have been only partially elucidated.

A large group of G protein-coupled receptors induce mitogenic responses by activating members of the pertussis toxin (PTX)-sensitive family of G proteins (Pouyssegur and Seuwen, 1992; van Biesen *et al.*, 1996b). This family includes four G proteins potentially involved in mitogenic signaling pathways: G_{i1}, G_{i2}, G_{i3}, and G_o (Neer, 1995). Microinjection of inhibitory antibodies has shown that both G_{i2} and G_o heterotrimers can exert a positive effect on cell proliferation (LaMorte *et al.*, 1992; Baffy *et al.*, 1994). Evidence for a mitogenic action of the α chains of these G proteins comes from studies using constitutively activated mutants. Transfection of activated $G\alpha_{i2}$ causes transformation of Rat-1 fibroblasts and enhances the proliferation of other cell lines (Hermouet *et al.*, 1991; Pace *et al.*, 1991; Gupta *et al.*, 1992). Expression of activated $G\alpha_o$ can induce transformation of NIH 3T3 cells (Kroll *et al.*, 1992). Furthermore, mutations that activate $G\alpha_{i2}$ have been identified in a limited subset of human tumors (Lyons *et al.*, 1990). The ability of $G_{i/o}$-coupled receptors to activate the MAPK pathway is well documented. A variety of these receptors have been shown to stimulate MAPK activity in fibroblasts and other cell types (van Biesen *et al.*, 1996b; Gutkind, 1998). Several lines of evidence indicate that the $G\beta\gamma$ complex plays an important role in PTX-sensitive MAPK activation (van Biesen *et al.*, 1996b; Gutkind, 1998). Cells expressing free $G\beta\gamma$ heterodimers have revealed that these subunits initiate a phosphatidylinositol-3 kinase (PI3K)- and Src-dependent pathway that leads to tyrosine phosphorylation of Shc, formation of a Shc–Grb2 complex, and Ras-dependent activation of the MAPK cascade (van Biesen *et al.*, 1996b; Gutkind, 1998). Although both G_{i2} and G_o appear to be involved in the MAPK responses induced by receptors coupled to PTX-sensitive G proteins (Pace *et al.*, 1995; van Biesen *et al.*, 1996a), a role of $G\alpha_{i2}$ and $G\alpha_o$ in regulation of the MAPK pathway has not been clearly established. In fact, prolonged expression of activated $G\alpha_{i2}$ is accompanied by increased MAPK activity in Rat-1 fibroblasts, but stable or transient transfection of the same subunit in other cell types does not induce MAPK activation (Gallego *et al.*, 1992; van Biesen *et al.*, 1996b; Gutkind, 1998). It has been proposed that in Chinese hamster ovary (CHO) cells the muscarinic M_1 receptor, which couples to both G_q and G_o, activates a $G\alpha_o$-dependent pathway leading to MAPK stimulation (van Biesen *et al.*, 1996a). However, direct evidence for regulation of the MAPK pathway by this $G\alpha$ subunit has not been provided yet.

Here we report that expression of the constitutively activated mutant $G\alpha_o$-Q205L (Kroll *et al.*, 1992; Wong *et al.*, 1992) in CHO cells is not sufficient to induce MAPK activation but strongly potentiates the stimulatory effects of the epidermal growth factor (EGF) receptor and Ras. We show that $G\alpha_o$ can regulate the activity of the B-Raf kinase through a Ras-independent and protein kinase C (PKC)- and PI3K-dependent mechanism. Our results suggest that B-Raf regulation mediates, at least in part, the action of $G\alpha_o$ on the MAPK pathway.

MATERIALS AND METHODS

Materials

The $G\alpha_o$ antiserum was generated by immunization of rabbits with a synthetic peptide corresponding to the C-terminal sequence ANNLRGCGLY (Baffy *et al.*, 1994). Mouse monoclonal antibodies against phosphotyrosine and human EGF receptor (LA1) were from Upstate Biotechnology (Lake Placid, NY); the anti-hemagglutinin (HA) mouse monoclonal antibody 12CA5 was from Boehringer Mannheim (Mannheim, Germany); the anti-phospho-Akt (Ser-473) mouse monoclonal antibody 4E2 was from New England BioLabs (Beverly, MA); and rabbit polyclonal antibodies against p42 MAPK (C-14), Raf-1 (C-20), A-Raf (C-20), B-Raf (C-19), and human EGF receptor (1005) were from Santa Cruz Biotechnology (Santa Cruz, CA). ATP, myelin basic protein (MBP), phorbol 12-myristate 13-acetate (PMA), LY 294002, and dopamine were purchased from Sigma (St. Louis, MO). Human EGF was from Boehringer Mannheim; wortmannin, GF 109203X, and human fibroblast growth factor (FGF) were from Calbiochem (La Jolla, CA), and PTX was from List Biological Laboratories (Campbell, CA). Recombinant glutathione *S*-transferase (GST)-p42 MAPK and GST-MEK1 were from Upstate Biotechnology; protein A-Sepharose CL-4B and GammaBind G-Sepharose were from Amersham Pharmacia Biotech (Uppsala, Sweden); and [γ-^{32}P]ATP was from DuPont New England Nuclear (Boston, MA). All other chemicals were reagent grade.

DNA Constructs

The cDNAs encoding wild-type $G\alpha$, $G\beta$, and $G\gamma$ subunits, constitutively activated $G\alpha$ chains ($G\alpha_o$-Q205L, $G\alpha_{i2}$-Q205L, $G\alpha_{i3}$-Q204L, and $G\alpha_q$-R183C) (Conklin *et al.*, 1992; Wong *et al.*, 1992), and the human dopaminergic D_{2L} receptor (Wong *et al.*, 1992) were in the pcDNAI expression vector. L. Beguinot (Scientific Institute San Raffaele, Milan, Italy) provided the pCO11 plasmid containing humam EGF receptor cDNA. HA-p44 MAPK (Meloche *et al.*, 1992a) in pcDNAI and pRSV vectors containing cDNAs for the activated mutant Ras-Q61L and the dominant negative mutant N17-Ras were gifts from E.P. Sturani and R. Zippel (University of Milan, Milan, Italy).

Cell Culture, DNA Transfection, and Preparation of Lysates

CHO cells were maintained in culture in Dulbecco's modified Eagle's medium-Ham's F-12 (1:1) supplemented with 10% FCS. Transient transfections were performed using the Transfectam reagent (Promega, Madison, WI). Cells were grown to 60–70% confluence in 6- or 10-cm dishes, washed with serum-free medium, and incubated in the same medium (1.5 ml per 6-cm dish and 4 ml per 10-cm dish) containing DNA:Transfectam (1 μg:2 μl) complexes. In all experiments, the total amount of transfected DNA was kept constant by addition of empty vector. After 5 h at 37°C, dishes were washed and incubated in 10% FCS medium for 24 h. Transfection efficiencies were determined by transfection of a plasmid encoding a mutant green fluorescent protein (pEGFP-N1; Clontech, Cambridge, United Kingdom). Expression was detected by fluorescence microscopy and was consistently observed in ~35% of the transfected cells. For the various assays, cells were serum starved for 18 h in medium containing 0.5% FCS and, where indicated, pretreated with the appropriate agents and/or incubated in serum-free medium in the absence or presence of EGF or dopamine. The cells were then washed once with ice-cold Ca^{2+}- and Mg^{2+}-free PBS and incubated on ice for 20 min with the lysis buffer specified for each assay (0.6 ml per 6-cm dish and 1 ml per 10-cm dish). Cell lysates were cleared by centrifugation at 13,000 rpm for 15 min at 4°C.

Measurement of MAPK Activity

The activities of endogenous p42 MAPK and HA-p44 MAPK were determined by an immune complex kinase assay. Cells in 6-cm

dishes were lysed in buffer containing 50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 5 mM EDTA, 40 mM $Na_4P_2O_7$, 200 μM Na_3VO_4, 1% Triton X-100, 1 mM dithiothreitol, 1 mM PMSF, and 10 μg/ml chymostatin, leupeptin, antipain, and pepstatin (CLAP). Lysates were incubated at 4°C with 1 μg of anti-p42 MAPK antibodies for 1 h or with 3 μg of anti-HA antibodies for 2 h. Immune complexes were collected by incubation with 50 μl of protein A-Sepharose (50% slurry) for 1 h at 4°C, centrifuged (2 min at 3000 rpm at 4°C), and washed three times in lysis buffer and twice in kinase buffer (40 mM HEPES-NaOH, pH 7.5, 10 mM $MgCl_2$, 1 mM EGTA, 200 μM Na_3VO_4, 2 mM dithiothreitol, 1 mM PMSF, 10 μg/ml CLAP, and 3 mM benzamidine). The final pellets were resuspendend in 50 μl of kinase buffer supplemented with 250 μg/ml MBP as a substrate. The kinase reaction was started by addition of 5 μl of 500 μM ATP containing 4 μCi of [γ-^{32}P]ATP, incubated at 30°C for 30 min, and stopped with 5 μl of 88% formic acid. After a brief centrifugation, 35 μl of the supernatant fraction were spotted on squares of Whatman (Maidstone, United Kingdom) P81 paper. Free [γ-^{32}P]ATP was eluted by four washes in 150 mM phosphoric acid, and the amount of radioactivity incorporated into MBP was measured by scintillation counting. Blank kinase reactions, carried out after incubation of antibodies with lysis buffer and protein A-Sepharose, were always performed. The radioactivity of these reactions (~500 cpm) was subtracted from the result of each sample.

Measurement of Raf Activity

The ability of Raf-1, A-Raf, and B-Raf to activate MEK was measured by an immune complex "coupled" assay in which recombinant GST-MEK1 activates recombinant GST-p42 MAPK (Alessi *et al.*, 1995). Cells in 6- or 10-cm dishes were lysed in buffer containing 20 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 10 mM EGTA, 50 mM NaF, 40 mM $Na_4P_2O_7$, 200 μM Na_3VO_4, 1% Triton X-100, 20 mM *N*-octyl-β-D-glucopyranoside, 1 mM PMSF, 10 μg/ml CLAP, and 20 μg/ml aprotinin. Lysates were incubated with antibodies against Raf-1, A-Raf, or B-Raf (1.5 μg/ml) for 2 h at 4°C. Immune complexes were collected with protein A-Sepharose (50 μl of 50% slurry) for 1 h at 4°C, centrifuged, and washed three times in lysis buffer without PMSF and twice in washing buffer (50 mM Tris-HCl, pH 7.5, 0.1 mM EGTA, 0.5 mM Na_3VO_4, and 0.1% 2-mercaptoethanol). For the first step of the kinase reaction, pellets were resuspended in 30 μl of kinase buffer (30 mM Tris-HCl, pH 7.5, 10 mM magnesium acetate, 0.1 mM EGTA, 0.1% 2-mercaptoethanol, 0.03% Brij 35, 20 mM *N*-octyl-β-D-glucopyranoside, and 200 μM ATP) supplemented with 3 μg/ml GST-MEK1 and 30 μg/ml GST-p42 MAPK. After 30 min at 30°C, 15 μl of the supernatant were mixed with 15 μl of washing buffer containing 1 mg/ml BSA. Activation of GST-p42 MAPK was assayed by incubating 10 μl of this mixture with 40 μl of kinase buffer (50 mM Tris-HCl, pH 7.4, 12.5 mM magnesium acetate, 0.1 mM EGTA, 50 μM ATP, and 2 μCi [γ-^{32}P]ATP) supplemented with 400 μg/ml MBP. The reaction (15 min at 30°C) was terminated by spotting 40 μl on squares of Whatman P81 paper, and radioactivity incorporated into MBP was determined as described for p42 MAPK and HA-p44 MAPK assays. The values of blank reactions performed after incubation of antibodies with lysis buffer and protein A-Sepharose (~4000 cpm) were subtracted from the result of each sample. Preliminary experiments showed that no activity was associated with protein A-Sepharose incubated with lysates in the absence of antibodies. The activity detected in reactions performed without GST-MEK1 was ~15% (for Raf-1 and A-Raf) and <0.01% (for B-Raf) of that observed in the presence of the recombinant kinase. The presence of comparable amounts of Raf in the immunoprecipitates used in the kinase assay was verified by immunoblot analysis of the protein A-Sepharose pellets as described below.

Analysis of Protein Expression and Tyrosine Phosphorylation

Expression and tyrosine phosphorylation of transfected EGF receptors were evaluated by immunoprecipitation and subsequent immunoblotting. Cells in 10-cm dishes were lysed in buffer containing 20 mM Tris-HCl, pH 7.5, 100 mM NaCl, 5 mM EDTA, 40 mM $Na_4P_2O_7$, 1 mM Na_3VO_4, 1% Triton X-100, 10% glycerol, 1 mM PMSF, and 10 μg/ml CLAP. Lysates were precleared with protein G-Sepharose (40 μl of 50% slurry) for 45 min at 4°C and incubated with 5 μg of anti-EGF receptor LA1 antibody overnight at 4°C. Immune complexes were collected with protein G-Sepharose (60 μl of 50% slurry) for 2 h at 4°C, centrifuged, and washed three times in lysis buffer. Two equal parts of each immunoprecipitate were separated by 7.5% SDS-PAGE and transferred to nitrocellulose filters (Schleicher & Schuell, Keene, NH). For detection of the EGF receptor, filters were blocked for 1 h in Blotto (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.05% Tween 20, 0.01% sodium azide, and 5% nonfat dry milk), incubated overnight at 4°C in Blotto containing 0.4 μg/ml anti-EGF receptor 1005 antibody, incubated for 2 h in Blotto with horseradish peroxidase-conjugated secondary antibodies (Bio-Rad, Hercules, CA), and finally processed for enhanced chemiluminescence (Amersham Pharmacia Biotech). For analysis of tyrosine phosphorylation, filters were kept for 2 h in BSA blocking solution (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.02% sodium azide, and 5% BSA) and incubated in the same solution with anti-phosphotyrosine antibodies (1 μg/ml) overnight at 4°C. Detection was performed with ^{125}I-labeled secondary antibodies (Amersham Pharmacia Biotech). Preliminary experiments showed that both the LA1 and 1005 antibodies used in these studies recognize equally well the nonphosphorylated and phosphorylated forms of the EGF receptor. For detection of Akt phosphorylation, cells were lysed in buffer containing 20 mM Tris-HCl, pH 7.5, 100 mM NaCl, 5 mM EDTA, 20 mM NaF, 40 mM $Na_4P_2O_7$, 1 mM Na_3VO_4, 1% Triton X-100, 10% glycerol, 1 mM PMSF, 10 μg/ml CLAP, and 20 μg/ml aprotinin. Lysates (150 μg of protein) were separated by 7.5% SDS-PAGE and immunoblotted using anti-phospho-Akt antibodies. The expression of transfected $G\alpha_o$ and HA-p44 MAPK was assessed by immunoblotting of cell lysates (100 μg of protein) prepared as described for MAPK assays and separated by 10% SDS PAGE. Immunoblot analysis of Raf proteins was performed on immunoprecipitates prepared as described for kinase assays and resolved by 7.5% SDS-PAGE. Filters were incubated with $G\alpha_o$ antiserum (1:200), anti-HA antibody (2.5 μg/ml), anti-phospho-Akt antibody (0.5 μg/ml), or anti-Raf-1, A-Raf, and B-Raf antibodies (0.3 μg/ml) overnight at 4°C in Blotto. The $G\alpha_o$ antiserum was detected with ^{125}I-labeled protein A (Amersham Pharmacia Biotech), and all other antibodies were detected with horseradish peroxidase-conjugated secondary antibodies and enhanced chemiluminescence.

RESULTS

Activated $G\alpha_o$ Potentiates EGF Receptor-dependent MAPK Stimulation

Transient expression of wild-type or mutationally activated $G\alpha_o$ in CHO cells did not lead to a significant activation of endogenous p42 MAPK (Figure 1A) or cotransfected HA-p44 MAPK (Figure 1B). Previous studies have detected stimulation of the MAPK pathway by certain PTX-insensitive $G\alpha$ chains only in the presence of growth factors acting through tyrosine kinase receptors (De Vivo and Iyengar, 1994; Voyno-Yasenetskaya *et al.*, 1994). The responses induced by various receptors coupled to $G_{i/o}$ proteins suggest that specific subunits of these heterotrimers can also mediate signals that potentiate tyrosine kinase receptor-dependent MAPK activation (Meloche *et al.*, 1992b; Vouret-Craviari *et al.*, 1993; Fujitani and Bertrand, 1997). We therefore investigated the

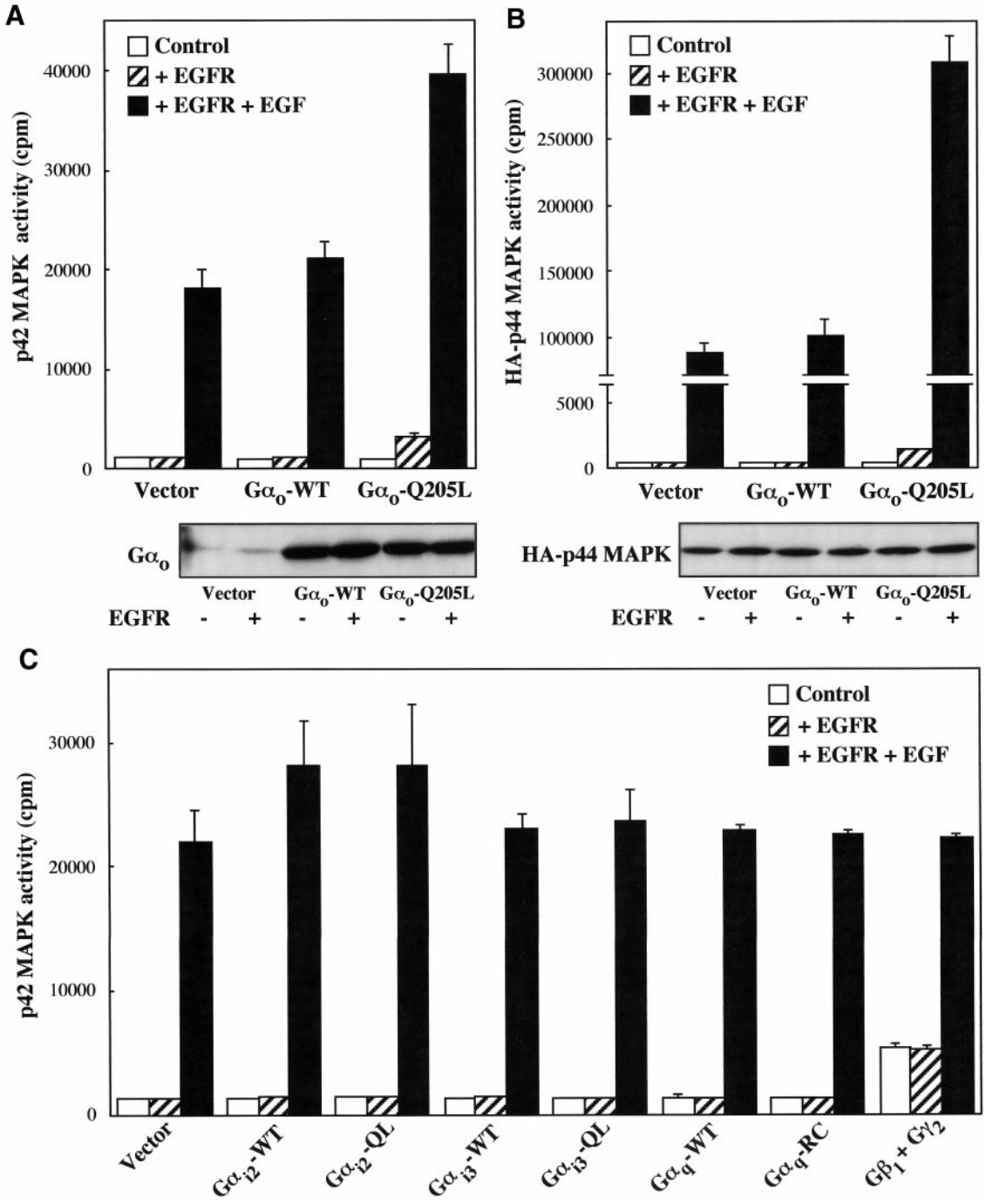

Figure 1. Effect of activated $G\alpha_o$ on MAPK activity. CHO cells transfected in 6-cm dishes were placed for 18 h in 0.5% FCS medium and incubated for 5 min in the absence or presence of 100 ng/ml EGF. MAPK activity was determined as described in MATERIALS AND METHODS. (A) Endogenous p42 MAPK activity in cells transfected with 1.8 μg of $G\alpha_o$-wild-type (WT) or $G\alpha_o$-Q205L and, where indicated, 1.8 μg of EGF receptor (EGFR). The data represent means ± SE of two to four independent experiments performed in triplicate. (B) HA-p44 MAPK activity in cells transfected with 1.4 μg of HA-p44 MAPK, 1.4 μg of $G\alpha_o$-WT or $G\alpha_o$-Q205L and, where indicated, 1.4 μg of EGFR. The data are means ± SE of two independent experiments performed in duplicate. (C) Endogenous p42 MAPK activity in cells transfected with 1.8 μg of wild-type (WT) or activated (QL and RC) $G\alpha_{i2}$, $G\alpha_{i3}$, and $G\alpha_q$ subunits, 1.4 μg of $G\beta_1$ and $G\gamma_2$ chains, and, where indicated, 1.8 μg of EGFR. Means ± SE from two independent experiments performed in duplicate are given. When nontransfected CHO cells were incubated with 100 ng/ml EGF for 5 min, the values of endogenous p42 MAPK activity in the absence and presence of the growth factor were 4097 ± 429 and 4278 ± 620 cpm, respectively (n = 4). Immunoblots show the expression of wild-type or activated $G\alpha_o$ (A) and HA-p44 MAPK (B) in cells transfected with different cDNAs. Similar results were obtained in two additional experiments.

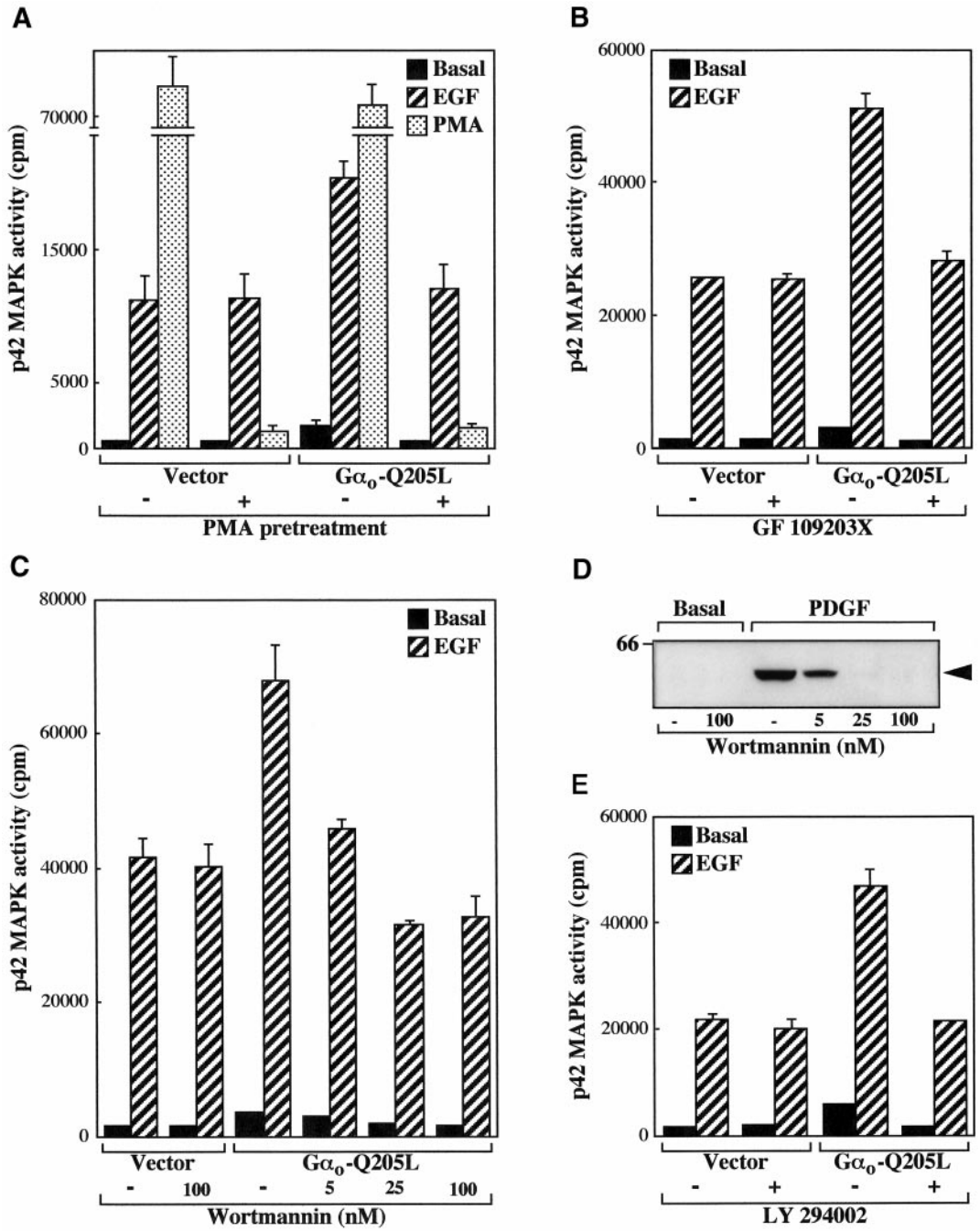

Figure 2. MAPK stimulation by activated $G\alpha_o$ involves PKC and PI3K. (A–C and E) CHO cells in 6-cm dishes were transfected with 1.8 μg of EGFR and 1.8 μg of either empty vector or $G\alpha_o$-Q205L and assayed for endogenous p42 MAPK activity as described in the legend to Figure 1. (A) Effect of PMA pretreatment on MAPK activity. Cells were incubated for 18 h in 0.5% FCS medium containing 1 μM PMA before stimulation with 100 ng/ml EGF or 1 μM PMA for 5 min. The data represent means ± SE of two independent experiments performed in duplicate. (B, C, and E) Effects of PKC or PI3K inhibitors on MAPK activity. Cells were serum starved for 18 h and incubated for 15 min with 1 μM GF 109203X (B), the indicated concentrations of wortmannin (C), or 20 μM LY 294002 (E) before stimulation with 100 ng/ml EGF for 5 min. The data are means ± SE of four to nine observations. Additional assays showed that GF 109203X completely abolished the increase in p42 MAPK activity induced by short exposure to PMA. (D) Effect of wortmannin on Akt phosphorylation. Nontransfected CHO cells grown in 6-cm dishes were serum starved for 18 h, incubated with the indicated concentrations of wortmannin for 15 min, and stimulated with 20 ng/ml PDGF for 10 min. Phosphorylated Akt was detected by immunoblot analysis of cell lysates with specific antibodies as described in MATERIALS AND METHODS. The molecular mass (kilodaltons) of a marker protein is shown to the left, and the position of phosphorylated Akt is indicated by an arrow. Data are representative of two similar experiments.

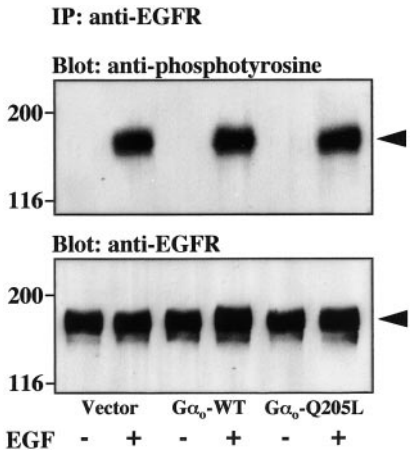

Figure 3. Effect of activated $G\alpha_o$ on EGF receptor tyrosine phosphorylation. CHO cells in 10-cm dishes were transfected with 5 μg of EGFR and 5 μg of empty vector, $G\alpha_o$-WT, or $G\alpha_o$-Q205L, serum starved for 18 h, and stimulated with 100 ng/ml EGF for 5 min. Immunoprecipitation (IP) with anti-EGFR antibodies and immunoblot analysis of the immunoprecipitates with anti-phosphotyrosine or anti-EGFR antibodies were performed as described in MATERIALS AND METHODS. The molecular masses (kilodaltons) of marker proteins are shown to the left, and the position of the EGFR is indicated by arrows. Data are representative of three similar experiments.

ability of $G\alpha_o$ to stimulate endogenous p42 MAPK in CHO cells transiently expressing the EGF receptor. In nontransfected CHO cells, EGF had no significant effect on p42 MAPK activity (see legend to Figure 1). In the presence of EGF receptors, basal p42 MAPK activity was not significantly different from that detected in control cells and increased ~15-fold upon stimulation with 100 ng/ml EGF for 5 min (Figure 1A). Coexpression of $G\alpha_o$-Q205L induced a modest elevation of unstimulated p42 MAPK activity and a twofold enhancement of the response induced by EGF. In contrast, no significant changes in the p42 MAPK response to the EGF receptor were observed in cells coexpressing wild-type $G\alpha_o$. These results could not be accounted for by variable levels of expression of the $G\alpha_o$ subunit (Figure 1A) or the EGF receptor (see Figure 3) in cells transfected with different cDNA combinations. When coexpressed with the EGF receptor, activated $G\alpha_o$ significantly increased unstimulated activity and greatly amplified the effect of EGF also in HA-p44 MAPK assays (Figure 1B). These responses were not accompanied by detectable variations of HA-p44 MAPK expression. As shown in Figure 1C, measurements of p42 MAPK activity revealed that the stimulatory effect of $G\alpha_o$-Q205L on the MAPK pathway was not mimicked by mutationally activated $G\alpha_{i2}$, $G\alpha_{i3}$, or $G\alpha_q$ subunits. Expression of $G\beta_1\gamma_2$ chains induced per se a fourfold activation of p42 MAPK, as expected from previous results obtained in CHO cells and other systems (Hawes *et al.*, 1995; van Biesen *et al.*, 1996b; Gutkind, 1998), but failed to potentiate the response to the EGF receptor (Figure 1C). Similar results were obtained when the effects of activated $G\alpha_{i2}$, $G\alpha_{i3}$, and $G\alpha_q$ subunits and $G\beta_1\gamma_2$ complexes were tested in HA-p44 MAPK assays (our unpublished results).

Regulation of the MAPK Pathway by $G\alpha_o$ Involves PKC and PI3K

It has been reported that MAPK activation by some receptors capable of coupling to the G_o protein is dependent on PKC activity (van Biesen *et al.*, 1996a; Wylie *et al.*, 1999). We therefore investigated the role of PKC in the regulation of the MAPK pathway mediated by $G\alpha_o$. Cells transfected with the EGF receptor in the absence or presence of $G\alpha_o$-Q205L were incubated with PMA for 18 h to down-regulate the expression of phorbol ester-sensitive PKC isozymes. As shown in Figure 2A, this procedure abolished the stimulation of p42 MAPK activity induced by short exposure to PMA. PKC down-regulation did not affect the p42 MAPK response to EGF in cells transfected with the EGF receptor but completely inhibited the enhancement of the stimulatory effect of the growth factor observed after coexpression of $G\alpha_o$-Q205L. Pretreatment with PMA also prevented the increase in unstimulated p42 MAPK activity observed in cells expressing activated $G\alpha_o$. Entirely comparable results were obtained using the PKC inhibitor GF 109203X (Toullec *et al.*, 1991) (Figure 2B).

Activation of PI3K has been identified as a major signaling pathway leading to MAPK stimulation (Toker and Cantley, 1997). Upon activation of $G_{i/o}$-coupled receptors, PI3K is believed to mediate stimulation of MAPK activity by the G protein $\beta\gamma$ subunits (van Biesen *et al.*, 1996b; Gutkind, 1998). To determine whether the action of $G\alpha_o$ on MAPK also involves any of the PI3K isoforms, we used the inhibitors wortmannin (Ui *et al.*, 1995) and LY 294002 (Vlahos *et al.*, 1994). As shown in Figure 2C and previously described in other systems (Wennstrom and Downward, 1999), no significant effect of 100 nM wortmannin was detected in cells transfected with the EGF receptor. In contrast, a concentration of wortmannin as low as 5 nM efficiently reduced the high p42 MAPK activity displayed by cells coexpressing $G\alpha_o$-Q205L. Complete inhibition was achieved in the presence of 25 nM wortmannin. We compared this effect with the inhibition of Akt phosphorylation, a well-established PI3K-dependent event (Toker and Cantley, 1997). In our experiments, endogenous platelet-derived growth factor (PDGF) receptors, but not transfected EGF receptors or endogenous FGF receptors (our unpublished results), induced a detectable phosphorylation of Akt. As shown in Figure 2D, the concentrations of wortmannin that inhibited $G\alpha_o$-mediated p42 MAPK activation also blocked Akt phosphorylation by PDGF. As shown in Figure 2E, LY 294002 mimicked the effect of wortmannin on p42 MAPK activity. Taken together, these results indicate that the $G\alpha_o$ subunit potentiates MAPK activation by the EGF receptor through a pathway that involves both PKC and PI3K.

$G\alpha_o$ Potentiates Ras Activation of the MAPK Pathway

The effect of $G\alpha_o$ on MAPK activity implies that this subunit is able to modulate the function of at least one component of the signaling pathway connecting the EGF receptor to MAPK. We first checked whether, under the conditions used in MAPK assays, $G\alpha_o$ affects the state of tyrosine phosphorylation of the EGF receptor itself. As shown in Figure 3, immunoprecipitation of lysates from transfected cells with anti-EGF receptor antibodies followed by immunoblot anal-

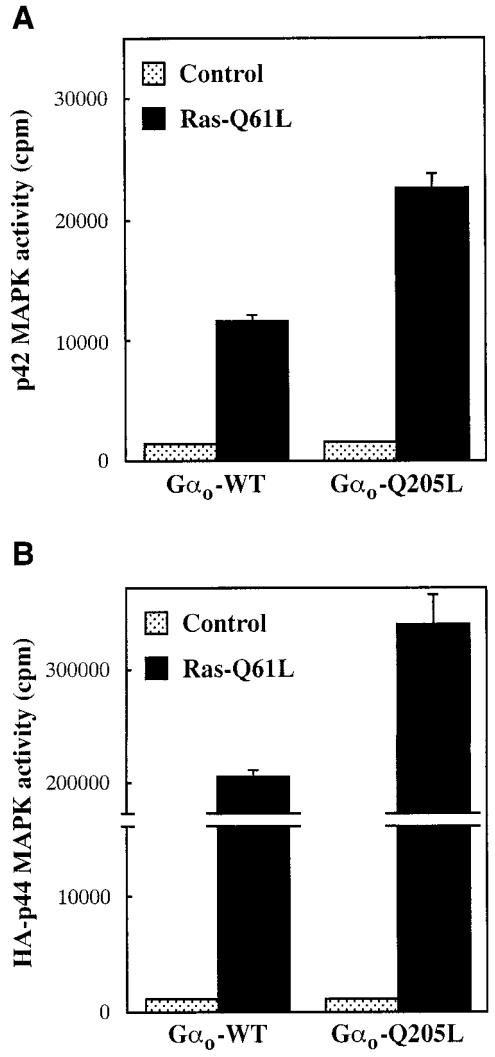

Figure 4. Effect of activated $G\alpha_o$ on Ras-stimulated MAPK activity. CHO cells transfected in 6-cm dishes were serum starved for 18 h and assayed for MAPK activity as described in the legend to Figure 1. (A) Endogenous p42 MAPK activity in cells transfected with $G\alpha_o$-WT or $G\alpha_o$-Q205L (1.8 μg) and either empty vector or Ras-Q61L (2.2 μg). (B) HA-p44 MAPK activity in cells transfected with HA-p44 MAPK (1.4 μg), $G\alpha_o$-WT or $G\alpha_o$-Q205L (1.4 μg), and either empty vector or Ras-Q61L (1.4 μg). Data represent means ± SE of two separate experiments performed in duplicate. Expression of Ras-Q61L in the absence of exogenous $G\alpha_o$ gave results similar to those obtained in cells transfected with the wild-type subunit.

ysis with anti-phoshotyrosine antibodies failed to reveal a significant effect of $G\alpha_o$-Q205L on basal or EGF-induced tyrosine phosphorylation of the EGF receptor. To identify possible sites of action of $G\alpha_o$ downstream of the EGF receptor, we asked whether $G\alpha_o$-Q205L can potentiate the stimulation of the MAPK pathway induced by constitutively activated Ras. In control cells, expression of activated Ras increased the activity of endogenous p42 MAPK (Figure 4A) and cotransfected HA-p44 MAPK (Figure 4B) ~8- and 200-fold, respectively. The responses of both forms of MAPK to Ras were markedly enhanced by coexpression of $G\alpha_o$-Q205L. These results indicate that $G\alpha_o$ potentiates EGF receptor-dependent activation of the MAPK pathway at the level or downstream of Ras.

$G\alpha_o$ Activates B-Raf by a Ras-independent and PKC- and PI3K-dependent Mechanism

The components of the MAPK cascade located immediately downstream of Ras are the Raf kinases, Raf-1, A-Raf, and B-Raf. The central role of Ras in activation of these kinases is well established (Marais and Marshall, 1996; Campbell *et al.*, 1998). However, increasing evidence indicates that additional signals converge on the Raf proteins and, at least in some cases, cooperate with Ras in regulating their function (Marais and Marshall, 1996; van Biesen *et al.*, 1996b; Marais *et al.*, 1997; Vossler *et al.*, 1997; Campbell et *al.*, 1998; York *et al.*, 1998). We therefore asked whether $G\alpha_o$ can affect EGF receptor-dependent activation of the Raf kinases. As shown in Figure 5A, CHO cells were found to express Raf-1, A-Raf, and two B-Raf isoforms migrating with approximate molecular masses of 92 and 66 kDa. We first determined which of these kinases are activated after stimulation of EGF receptors. The activity of each type of Raf was measured by a coupled assay in which the kinase immunoprecipitated from cell lysates is used to activate sequentially recombinant forms of MEK and MAPK. As shown in Figure 5B, CHO cells transfected with the EGF receptor displayed no activation of Raf-1 and A-Raf but an ~40% increase in B-Raf activity upon exposure to EGF. It should be noted that in these assays the basal activity of B-Raf was much higher than those of Raf-1 and A-Raf. This finding most probably reflects not only different levels of expression of the various Raf proteins in CHO cells but also the elevated rate of basal activity of B-Raf, which has been demonstrated by previous work (Marais *et al.*, 1997). However, the lack of effect of EGF on Raf-1 and A-Raf could not be explained by a failure to detect increases in the relatively low activities of these kinases, because our assays easily revealed stimulation of Raf-1 by transfected dopaminergic D_2 receptors (our unpublished results) and stimulation of both Raf-1 and A-Raf by endogenous FGF receptors (Figure 6A). We next examined the responses of the various Raf kinases to $G\alpha_o$. The experiments presented in Figure 5C showed that activated but not wild-type $G\alpha_o$ was able to stimulate B-Raf activity. Parallel measurements of the activities of Raf-1 and A-Raf revealed no significant effect of $G\alpha_o$-Q205L on these kinases (our unpublished results). In sharp contrast with the results obtained in MAPK assays, B-Raf activation by $G\alpha_o$ was completely independent of the EGF receptor. $G\alpha_o$-Q205L caused an ~50% increase in basal B-Raf activity both in the absence and in the presence of EGF receptors and enhanced the stimulation induced by EGF in a simply additive manner.

As shown in Figure 5D, the activation of B-Raf induced by $G\alpha_o$-Q205L was not inhibited when endogenous Ras function was blocked by coexpression of the dominant negative mutant N17-Ras. In contrast, prolonged PMA pretreatment and exposure to GF 109203X, wortmannin, and LY 294002, which prevent the ability of $G\alpha_o$-Q205L to potentiate EGF receptor-dependent MAPK stimulation, also abolished the effect of the mutant subunit on B-Raf (Figure 5D). Taken altogether, our findings indicate that

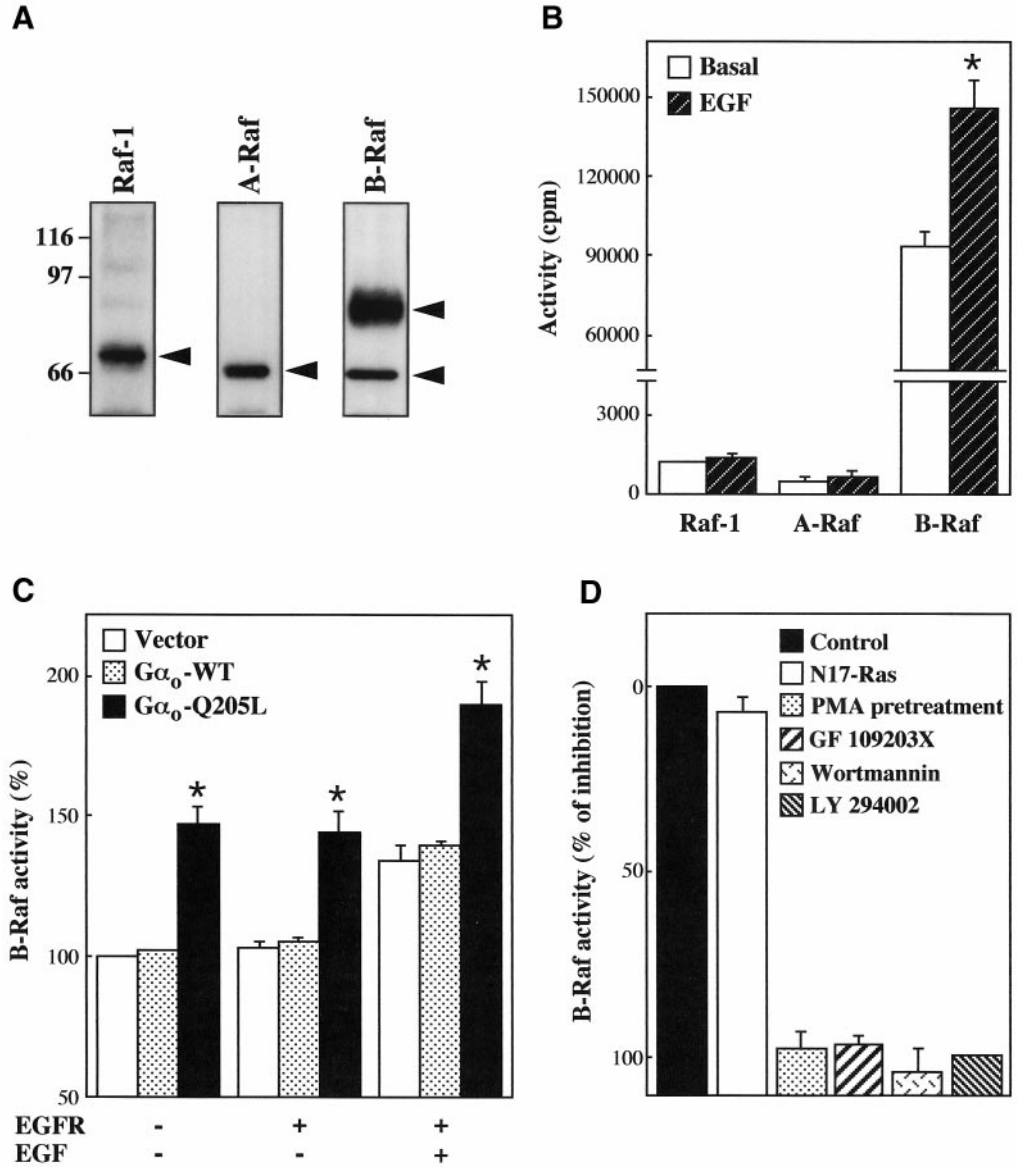

Figure 5. Activated $G\alpha_o$ stimulates B-Raf activity. (A) Expression of Raf proteins in CHO cells. Raf-1, A-Raf, and B-Raf were immunoprecipitated from lysates of nontransfected cells and detected by immunoblotting with specific antibodies as described in MATERIALS AND METHODS. The molecular masses (kilodaltons) of marker proteins are shown to the left, and the positions of Raf-1, A-Raf, and B-Raf migration are indicated by arrows. (B–D) CHO cells transfected in 10-cm dishes were serum starved for 18 h and assayed for endogenous Raf-1, A-Raf, and B-Raf activities as described in MATERIALS AND METHODS. (B) Effect of EGFR stimulation on Raf activity. Cells were transfected with 5 μg of EGFR and incubated for 5 min in the absence or presence of 100 ng/ml EGF. Data represent means ± SE of two to three independent experiments performed in triplicate. *, Significantly higher than basal ($p < 0.01$ by unpaired *t* test). In similar experiments carried out with nontransfected CHO cells, basal B-Raf activity (106,370 ± 7071 cpm) was not significantly increased by EGF (102,468 ± 5748 cpm) ($n = 4$). (C) Effect of activated $G\alpha_o$ on B-Raf activity. Cells transfected with 5 μg of empty vector, $G\alpha_o$-WT or $G\alpha_o$-Q205L and, where indicated, 5 μg of EGFR were incubated for 5 min in the absence or presence of 100 ng/ml EGF. Values are means ± SE of three independent experiments performed in triplicate. EGF induced a significant increase in B-Raf activity in all transfected cells ($p < 0.01$ by unpaired *t* test). *, Significantly higher than values from cells transfected with vector or $G\alpha_o$-WT ($p < 0.01$ by unpaired *t* test). (D) Effects of N17-Ras expression, PMA pretreatment, and PKC or PI3K inhibitors on $G\alpha_o$-mediated B-Raf activation. Cells were transfected with 4 μg of empty vector or $G\alpha_o$-Q205L, and, where indicated, 8 μg of N17-Ras. PMA (1 μM), wortmannin (25 nM), GF 109203X (1 μM), and LY 294002 (20 μM) were used as described in the legend to Figure 2. Results are expressed as percentages of inhibition of the increase induced by $G\alpha_o$-Q205L and represent the mean ± SE of two experiments in duplicate determinations. Expression of N17-Ras, PMA pretreatment, and the various inhibitors did not modify B-Raf activity in cells transfected with vector. Additional assays showed that both PMA pretreatment and GF 109203X completely abolished the increase in B-Raf activity induced by 5-min stimulation with 1 μM PMA (~50%).

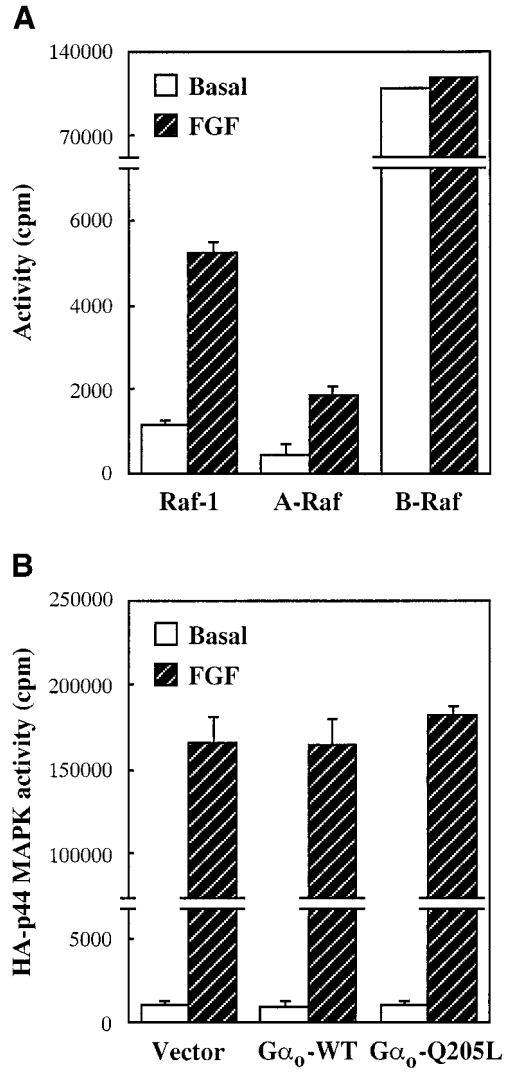

Figure 6. Activated $G\alpha_o$ does not affect MAPK stimulation by FGF receptors. (A) Effect of FGF on Raf activity in nontransfected CHO cells grown in 10-cm dishes. (B) Effect of $G\alpha_o$ on FGF-stimulated MAPK activity in CHO cells transfected in 6-cm dishes with 1.4 μg of HA-p44 MAPK and 1.4 μg of empty vector, $G\alpha_o$-WT, or $G\alpha_o$-Q205L. Cells were serum starved for 18 h before stimulation with 40 ng/ml FGF for 5 min. Raf-1, A-Raf, B-Raf, and HA-p44 MAPK activities were determined as described in the legends to Figures 1 and 5. Data are means ± SE for duplicate samples from two independent experiments.

$G\alpha_o$ regulates the activity of B-Raf by a Ras-independent and PKC- and PI3K-dependent mechanism that enables the kinase to activate MEK and MAPK in in vitro assays but not in intact cells. The simplest hypothesis suggested by the data is that $G\alpha_o$-activated B-Raf can efficiently couple to the downstream MAPK cascade when concomitantly reached by a regulatory signal provided by the EGF receptor or Ras. This model predicts that activated $G\alpha_o$ should fail to potentiate the stimulation of MAPK induced by B-Raf-independent pathways. We therefore tested the effect of $G\alpha_o$-Q205L on the MAPK response to endogenous FGF receptors, which stimulate Raf-1 and A-Raf but not B-Raf, activity (Figure 6A). As expected, expression of $G\alpha_o$-Q205L failed to affect activation of cotransfected HA-p44 MAPK by FGF (Figure 6B).

Activation of B-Raf by Receptors Coupled to G_o

To establish whether receptor-mediated activation of $G\alpha_o$ induces the B-Raf response observed after expression of the constitutively active subunit, we transiently transfected into CHO cells the dopaminergic D_2 receptor, which couples to both G_i and G_o proteins (Liu *et al.*, 1994; Watts *et al.*, 1998). Exposure of D_2 receptor-expressing cells to dopamine for 5 and 20 min stimulated the activity of B-Raf by ~70 and 60%, respectively (Figure 7A). This response was completely abolished by pretreatment with 100 ng/ml PTX for 16 h (our unpublished results). Because CHO cells express G_o as well as G_{i2} and G_{i3} (Dell'Acqua *et al.*, 1993; van Biesen *et al.*, 1996a), dopamine activation of B-Raf could be mediated by any of the subunits composing these heterotrimers. To determine the role of $G\alpha_o$ in the D_2 receptor response, we first investigated the ability of $G\alpha_{i2}$, $G\alpha_{i3}$, and $G\beta\gamma$ to activate B-Raf. As shown in Figure 7A, expression of either activated $G\alpha_{i2}$ and $G\alpha_{i3}$ subunits or $G\beta_1\gamma_2$ complexes failed to significantly increase B-Raf activity. We then asked whether activation of B-Raf by D_2 receptors is enhanced by cotransfection of wild-type $G\alpha_o$, which, by associating with endogenous $G\beta\gamma$ chains, is expected to form heterotrimers available for receptor coupling. As shown in Figure 7B, expression of wild-type $G\alpha_o$ markedly increased dopamine-induced stimulation of B-Raf activity. Consistent with an effect mediated, at least in part, by $G\alpha_o$, B-Raf activation by the D_2 receptor was not affected by coexpression of N17-Ras but was largely inhibited by PMA pretreatment and wortmannin (Figure 7C). We finally investigated whether stimulation of the kinase activity of B-Raf by receptor-activated $G\alpha_o$ has a positive effect on the MAPK pathway. In CHO cells, D_2 receptors caused a marked stimulation of endogenous p42 MAPK activity (Figure 8), which was totally blocked by pretreatment with PTX (our unpublished results). However, p42 MAPK displayed a more transient activation than B-Raf. The average 12-fold increase in p42 MAPK activity observed at 5 min was greatly reduced at 20 min (Figure 8). A complete dissociation between the effects of the D_2 receptor on B-Raf and MAPK became apparent in cells cotransfected with N17-Ras or pretreated with PMA. Coexpression of N17-Ras, which has no effect on B-Raf activation, efficiently inhibited the stimulation of p42 MAPK activity (Figure 8A). Conversely, PMA pretreatment, which largely prevents activation of B-Raf, did not significantly modify the p42 MAPK response (Figure 8B). These results indicate that, upon stimulation of the D_2 receptor, $G\alpha_o$-activated B-Raf does not induce a MAPK response. Cumulatively, the data are consistent with the conclusion that expression of mutationally activated $G\alpha_o$ and receptor-dependent activation of this subunit exert a similar effect on the B-Raf kinase.

DISCUSSION

Expression of mutationally activated $G\alpha$ chains or free $G\beta\gamma$ complexes in appropriate cell systems has greatly facilitated

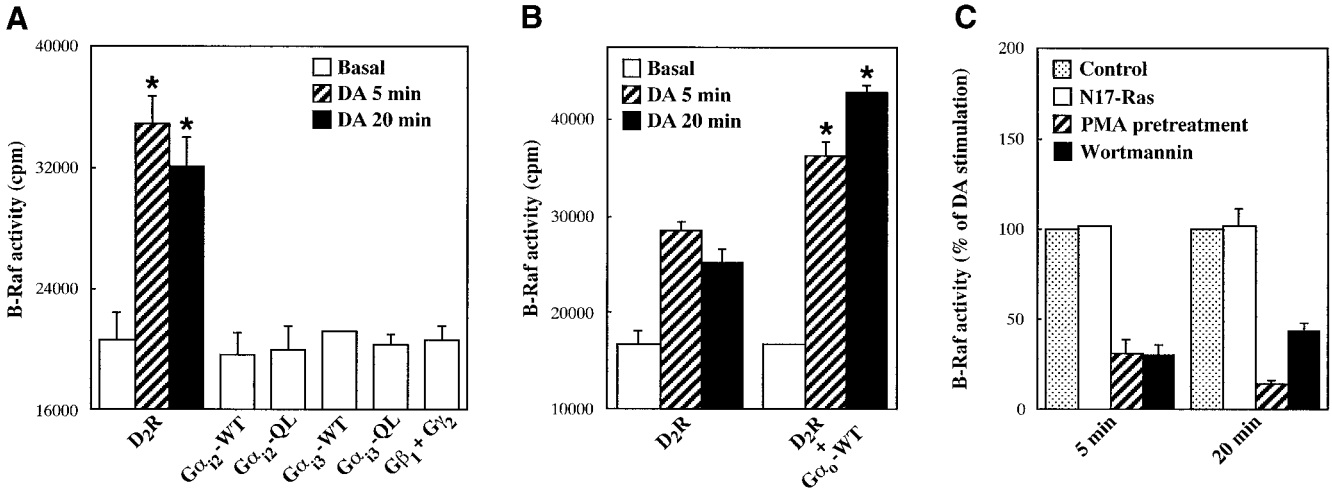

Figure 7. B-Raf activation by dopaminergic D_2 receptors. CHO cells transfected in 6-cm dishes were serum starved for 18 h and either left untreated or stimulated with 100 nM dopamine (DA) for 5 or 20 min. B-Raf activity was assayed as described in the legend to Figure 5. Data shown represent means ± SE of two independent experiments performed in duplicate. (A) Cells were transfected with 1.8 μg of dopaminergic D_2 receptor (D_2R), 1.8 μg of wild-type or activated $G\alpha_{i2}$ and $G\alpha_{i3}$ subunits, or 1.4 μg of $G\beta_1$ and $G\gamma_2$ chains. *, Significantly higher than basal ($p < 0.05$ by unpaired *t* test). (B) Cells were transfected with 1.8 μg of D_2R and, where indicated, 1.8 μg of $G\alpha_o$-WT. *, Significantly higher than values obtained in the absence of $G\alpha_o$-WT ($p < 0.01$ by unpaired *t* test). (C) Cells were transfected with 1.4 μg of D_2R and, where indicated, 2.8 μg of N17-Ras. PMA pretreatment and incubation with wortmannin were as described in the legend to Figure 5. Results are expressed as percentages of the increase induced by DA in control cells.

the understanding of the roles of individual G protein subunits in the regulation of intracellular signaling pathways. We have used this approach to investigate the effect of $G\alpha_o$ on the MAPK pathway. When expressed in CHO cells, activated $G\alpha_o$ is not sufficient to induce MAPK activation but substantially augments the stimulatory response to the EGF receptor. Previous studies have shown that other $G\alpha$ chains regulate the MAPK pathway in a similar manner. Expression of activated $G\alpha_{12}$ or $G\alpha_{13}$ significantly increases MAPK activity when Rat-1 cells are exposed to EGF (Voyno-Yasenetskaya *et al.*, 1994). Activated $G\alpha_q$, which in certain cell types acts on its own (van Biesen *et al.*, 1996b), requires the concomitant activation of PDGF receptors to stimulate MAPK activity in quiescent NIH 3T3 fibroblasts (De Vivo and Iyengar, 1994). However, the molecules and mechanisms involved in the interaction between these G protein subunits and the tyrosine kinase receptor signaling pathway have not been identified.

Receptors coupled to $G_{i/o}$ proteins can stimulate the MAPK pathway by inducing ligand-independent tyrosine phosphorylation of the EGF receptor (Hackel *et al.*, 1999; Luttrell *et al.*, 1999). The fact that the MAPK response to activated $G\alpha_o$ is clearly dependent on the presence of EGF argues against an involvement of this mechanism. Consistently, we found that expression of $G\alpha_o$-Q205L does not cause tyrosine phosphorylation of the EGF receptor. The finding that activated $G\alpha_o$ markedly increases MAPK activation by Ras further supports the conclusion that this subunit acts on a signaling molecule located downstream of the EGF receptor. A low "basal" activity of transfected EGF receptors therefore seems the most likely explanation of the modest activation of MAPK induced by $G\alpha_o$-Q205L in the absence of EGF.

We have identified the B-Raf kinase as a site for convergence of the signaling pathways initiated by the EGF receptor and $G\alpha_o$. There is increasing evidence for a differential regulation of the Raf family members by cell surface receptors (Reuter *et al.*, 1995; Knall *et al.*, 1996; Marais and Marshall, 1996; van Biesen *et al.*, 1996b; York *et al.*, 1998). Activation of the Raf kinases by growth factors varies among individual tyrosine kinase receptors and individual cell types (Reuter *et al.*, 1995; Marais and Marshall, 1996). We found that in CHO cells the EGF receptor induces activation of B-Raf without significantly affecting Raf-1 and A-Raf activities. The results of several studies have shown that EGF activates Raf-1 in various cell types, but not in Swiss 3T3 fibroblasts, and stimulates B-Raf activity in PC12 cells (Marais and Marshall, 1996). Regulation of the Raf kinase family by receptors coupled to $G_{i/o}$ proteins is less understood. Most work has concentrated on Raf-1 (van Biesen *et al.*, 1996b), but there is evidence that $G_{i/o}$-coupled receptors also activate B-Raf (Knall *et al.*, 1996). However, whether the subunits composing the $G_{i/o}$ heterotrimers differ in their ability to regulate specific Raf kinases has not been established. Here we show that activated $G\alpha_o$ selectively stimulates the activity of B-Raf in CHO cells. Interestingly, our experiments did not detect any significant effect of either $G\alpha_i$ or $G\beta\gamma$ subunits on this kinase.

The lack of correlation between B-Raf and MAPK activities observed in cells expressing activated $G\alpha_o$ clearly indicates that the effect of this subunit on B-Raf is sufficient to promote MEK and MAPK activation in in vitro assays but not in intact cells. Such a discrepancy cannot be explained by the fact that we used MEK1, rather than MEK2, to measure B-Raf activity. The two MEK kinases appear equally responsive to B-Raf activation (Marais *et al.*, 1997) and are ex-

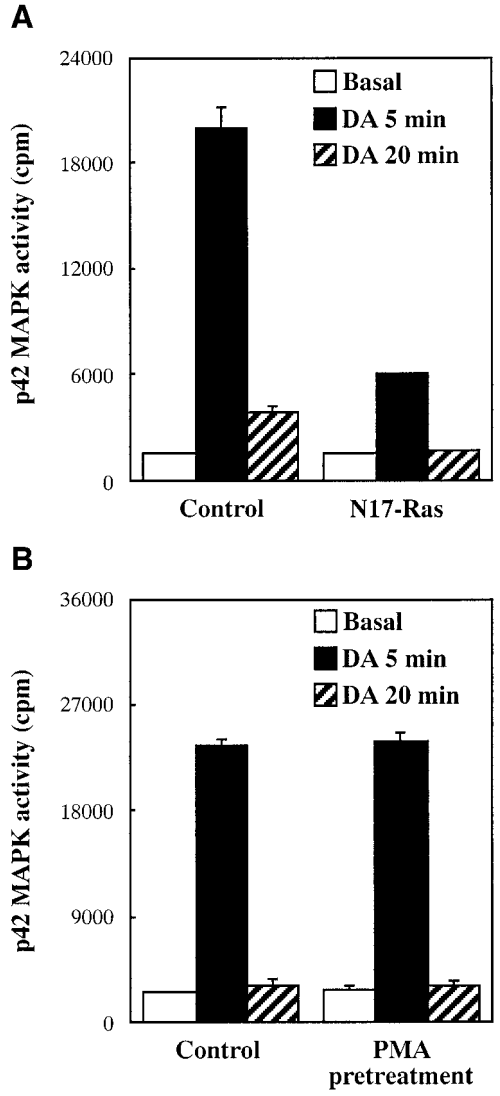

Figure 8. Effects of N17-Ras expression and PMA pretreatment on dopamine-stimulated MAPK activity. CHO cells transfected in 6-cm dishes were serum starved for 18 h and stimulated with 100 nM DA for 5 or 20 min. (A) Cells were transfected with 1.4 μg of D_2R and 2.8 μg of either empty vector or N17-Ras. (B) Cells were transfected with 1.4 μg of D_2R and pretreated with PMA as described in the legend to Figure 5. Endogenous p42 MAPK activity was measured as described in the legend to Figure 1. Data are means ± SE of triplicate determinations. Similar results were obtained in at least one additional experiment.

pressed in comparable amounts in CHO cells (Pace *et al.*, 1995; Xu *et al.*, 1997) (our unpublished results). In addition, the data obtained with the dopaminergic D_2 receptor seem to rule out the possibility that the "defective" response of B-Raf to $G\alpha_o$ is dependent on compensatory mechanisms or irrelevant effects that result from expression of the mutationally activated protein. It should also be considered that CHO cells contain at least two of the multiple B-Raf isoforms that have been identified (Barnier *et al.*, 1995). The antibodies used in our experiments immunoprecipitate preferentially the 92-kDa form (Vaillancourt *et al.*, 1994), and the results of kinase assays reflect mainly the activity of this protein. A differential activation of the B-Raf kinases has been suggested (Vossler *et al.*, 1997), and it is therefore possible that only the 92-kDa form is sensitive to the regulation observed in our studies. However, because in cell lysates the 92-kDa form is only slightly less abundant than the 66-kDa form (our unpublished results), it appears unlikely that the levels of expression of the protein can limit its ability to stimulate MAPK in intact cells. Several lines of evidence suggest, however, that B-Raf regulation is involved in the ability of activated $G\alpha_o$ to potentiate EGF receptor-dependent MAPK activation: 1) the enhancement of Ras-stimulated MAPK activity by $G\alpha_o$-Q205L is consistent with the involvement of a signaling molecule located downstream of Ras itself; 2) PKC inactivation and PI3K inhibition, which abolish B-Raf activation by $G\alpha_o$-Q205L, completely prevent the effect of the mutant subunit on MAPK; and 3) activated $G\alpha_o$ has no significant effect on the MAPK response induced by FGF, which does not activate the B-Raf kinase in CHO cells. Although we cannot exclude other possibilities, the simplest interpretation of our results is that $G\alpha_o$-activated B-Raf can efficiently stimulate the downstream MAPK cascade in the presence of a concomitant signal provided by the EGF receptor (see Figure 9).

Activation of B-Raf and the other members of the Raf family is a complex process involving mechanisms that have not been completely elucidated (Marais and Marshall, 1996; Morrison and Cutler, 1997; Campbell *et al.*, 1998). The ability of Ras to activate B-Raf is well established. The binding of Ras induces translocation of B-Raf to the plasma membrane and potently stimulates its kinase activity (Marais *et al.*, 1997). Recently, it has been reported that B-Raf is also directly activated by the small G proteins Rap1 and TC21 (Vossler *et al.*, 1997; York *et al.*, 1998; Rosario *et al.*, 1999). The results obtained here in cells expressing $G\alpha_o$-Q205L or the dopaminergic D_2 receptor indicate that $G\alpha_o$ regulates B-Raf activity by a Ras-independent mechanism that involves both PKC and PI3K. Previous studies have reported that PKC and PI3K can lead to B-Raf activation (Peraldi *et al.*, 1995; Reuter *et al.*, 1995; Knall *et al.*, 1996). The Ras-independent pathway connecting these signaling molecules to B-Raf remains to be defined. On the other hand, the fact that activated $G\alpha_o$ potentiates the action of Ras on the MAPK pathway would suggest that Ras activation is the EGF receptor-dependent signal that cooperates with the G protein subunit in regulating B-Raf function. We do not know how $G\alpha_o$ increases the kinase activity of B-Raf without allowing coupling of the kinase to its downstream effectors and how the tyrosine kinase receptor-Ras dependent pathway would promote the latter event. Mechanisms that influence the ability of B-Raf to activate MEK might involve subcellular localization, association with scaffolding proteins (Garrington and Johnson, 1999), or regulation by signaling molecules. Interestingly, signals that control the coupling of Raf-1 to the MAPK cascade have been recently reported. In NIH 3T3 cells exposed to serum, "adhesion deprivation" has no major effect on the in vitro activity of Raf-1 but strongly inhibits MAPK stimulation (Renshaw *et al.*, 1997). Similar results have been obtained in HEK-293 cells stimulated with agonists of G protein-coupled receptors after blockade of receptor inter-

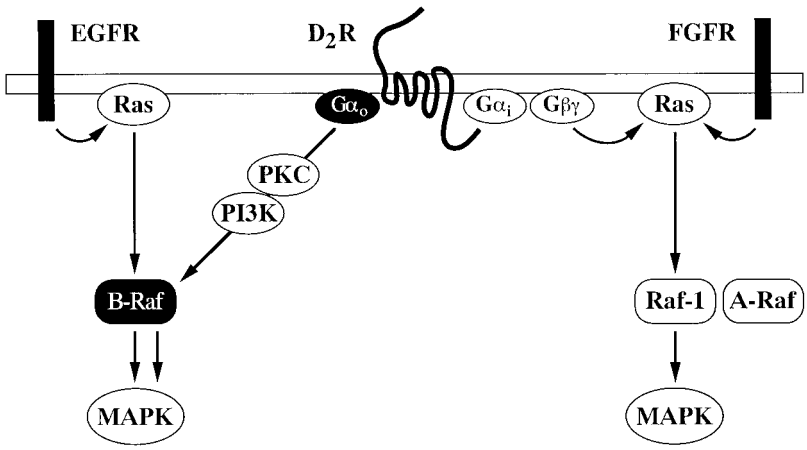

Figure 9. Model of signaling pathways regulating Raf and MAPK in CHO cells. The EGF receptor (EGFR) activates Ras and B-Raf, whereas the FGF receptor (FGFR) activates Ras, Raf-1, and A-Raf. Activated $G\alpha_o$ potentiates MAPK activation by Ras and EGFR but not FGFR. The G protein subunit stimulates the kinase activity of B-Raf by a Ras-independent and PKC- and PI3K-dependent mechanism, which is insufficient to promote MAPK activation. It is proposed that this mechanism can efficiently stimulate the MAPK pathway in the presence of a B-Raf-activating signal from Ras or the EGFR. The dopaminergic D_2 receptor (D_2R), which couples to both G_i and G_o, appears to stimulate MAPK through Ras activation of Raf-1. This effect is presumably mediated by G protein $\beta\gamma$ complexes and, possibly, $G\alpha_i$ subunits. Upon stimulation of this receptor, the Ras-independent and PKC- and PI3K-dependent activation of B-Raf induced by $G\alpha_o$ does not produce a MAPK response.

nalization (Daaka *et al.*, 1998). Finally, it should be mentioned that during the preparation of this manuscript two reports have shown that both $G\alpha_i$ and $G\alpha_o$ can interact with Rap1GAP (Jordan *et al.*, 1999; Mochizuki *et al.*, 1999). It has been proposed that Rap1GAP binds more avidly to the inactive form of $G\alpha_o$, and activation of the G protein subunit leads to the release of the GTPase-activating protein and the consequent inhibition of Rap1 function (Jordan *et al.*, 1999). In cells in which Rap1 exerts a positive effect on B-Raf and MAPK, such as the PC12 line, this mechanism causes increased MAPK activity in response to wild-type but not constitutively activated $G\alpha_o$ (Jordan *et al.*, 1999). Because other recent data suggest that Rap-1 induces B-Raf and MAPK activation also in CHO cells (Seidel *et al.*, 1999), it is difficult to imagine how inhibition of this small G protein can explain our present results. However, the effects of Rap1 on the MAPK pathway are rather complex and not completely understood (Bos and Zwartkruis, 1999), and further work will be needed to elucidate the role of Rap1GAP regulation in B-Raf and MAPK stimulation by $G\alpha_o$.

Different $G\alpha$ chains have a well-recognized role in the control of cell growth (Landis *et al.*, 1989; Vallar, 1996; Dhanasekaran *et al.*, 1998). The finding that $G\alpha_o$ can exert a positive effect on MAPK activity reinforces the idea that this subunit is involved in mitogenic signaling pathways (Kroll *et al.*, 1991; Kroll *et al.*, 1992; van Biesen *et al.*, 1996a). Our results can explain the increased proliferative response to serum previously observed in NIH 3T3 fibroblasts expressing $G\alpha_o$-Q205L (Kroll *et al.*, 1992), especially when taking into account that in these cells B-Raf has been identified as one of the major MEK activators (Reuter *et al.*, 1995). It appears conceivable that the effect of activated $G\alpha_o$ identified here also represents one of the mechanisms responsible for the synergistic effects of $G_{i/o}$-coupled receptors and tyrosine kinase receptors on MAPK and cell growth observed in several systems (Meloche *et al.*, 1992b; Pouyssegur and Seuwen, 1992; Vouret-Craviari *et al.*, 1993; Fujitani and Bertrand, 1997). Interestingly, a synergistic stimulation of MAPK activity by dopaminergic D_2 receptors and EGF receptors can be detected in CHO cells (our unpublished results). Our experiments indicate that activated $G\alpha_o$ requires the concomitant stimulation of the EGF receptor-Ras signaling pathway to regulate MAPK activity. However, previous results obtained in CHO cells expressing a PTX-insensitive mutant of $G\alpha_o$ have proposed that this subunit mediates Ras-independent and PKC-dependent MAPK activation by the muscarinic M_1 receptor (van Biesen *et al.*, 1996a). We cannot exclude the possibility that, in our experiments, desensitization or counter-regulation mechanisms prevent the detection of autonomous effects of $G\alpha_o$ on the MAPK pathway. However, it is also possible that upon activation of the muscarinic M_1 receptor, which couples to both G_o and G_q, other G protein subunits provide a Ras-independent signal that allows stimulation of the MAPK cascade by $G\alpha_o$-activated B-Raf. The existence of such "cross-talk" mechanisms would be one of the possible explanations of the fact that in CHO cells certain receptors capable of coupling to the G_o protein use a PKC-dependent pathway of MAPK activation (van Biesen *et al.*, 1996a; Wylie *et al.*, 1999), but others, like the dopaminergic D_2 receptor investigated here, are clearly unable to do so (Garnovskaya *et al.*, 1996; Hawes *et al.*, 1998) (see Figure 9). The ability of G protein subunits to regulate the MAPK pathway by a variety of signals (van Biesen *et al.*, 1996b; Gutkind, 1998), including transactivation of tyrosine kinase receptors (Hackel *et al.*, 1999; Luttrell *et al.*, 1999), suggests that there are several ways by which $G\alpha_o$-activated B-Raf might participate in the MAPK responses induced by G protein-coupled receptors in specific cell types.

The effector molecules and intracellular pathways involved in signal transduction mechanisms by $G\alpha_o$ are poorly understood. There is evidence that $G\alpha_o$ can directly inhibit a specific adenylyl cyclase isoform, which is mainly expressed in brain (Sunahara *et al.*, 1996). Other reported effects of the active form of $G\alpha_o$ include stimulation of polyphosphoinositide hydrolysis and PKC activity in *Xenopus* oocytes (Moriarty *et al.*, 1990; Kroll *et al.*, 1991) and activation of a Src-like kinase pathway in neurons (Diversé-Pierluissi *et al.*, 1997). Our results indicate that in CHO cells signaling from $G\alpha_o$ involves PKC, as previously suggested (van Biesen *et al.*, 1996a), as well as PI3K. The identities of the PKC and PI3K isoforms that participate in $G\alpha_o$-mediated signals and their mechanisms of regulation remain to be clarified. In CHO cells, $G\alpha_o$ uses PKC and PI3K to induce Ras-independent activation of B-Raf and conditional stimulation of MAPK. It appears most likely that these responses occur in a set of

selected cell types (van Biesen *et al.*, 1996a,b; Gutkind, 1998). $G\alpha_o$ is believed to play a most important role in the nervous system (Neer, 1995; Jiang *et al.*, 1998), where B-Raf is expressed at particularly high levels (Barnier *et al.*, 1995). Recent data have shown that, in PC12 cells overexpressing Rap1, constitutively active $G\alpha_o$ has a negative rather than a positive effect on the MAPK response to NGF (Jordan *et al.*, 1999). It will be of considerable interest to elucidate whether the signaling pathway identified here in CHO cells operates in other neuronal models and is therefore involved in intracellular networks that regulate not only growth but also differentiation and specialized cell functions.

ACKNOWLEDGMENTS

We thank A. Malgaroli, E.P. Sturani, and R. Zippel for helpful discussions and suggestions. This work was supported by grants from the Italian Association for Cancer Research and the Armenise-Harvard Foundation. Y.H.W. was supported by the Research Grants Council of Hong Kong (grant HKUST 653/96 M).

REFERENCES

Alessi, D.R., Cohen, P., Ashworth, A., Cowley, S., Leevers, S.J., and Marshall, C.J. (1995). Assay and expression of mitogen-activated protein kinase, MAP kinase kinase, and Raf. Methods Enzymol. *255*, 279–290.

Baffy, G., Yang, L., Raj, S., Manning, D.R., and Williamson, J.R. (1994). G protein coupling to the thrombin receptor in Chinese hamster lung fibroblasts. J. Biol. Chem. *269*, 8483–8487.

Barnier, J.V., Papin, C., Eychène, A., Lecoq, O., and Calothy, G. (1995). The mouse B-*raf* gene encodes multiple protein isoforms with tissue-specific expression. J. Biol. Chem. *270*, 23381–23389.

Boguski, M.S., and McCormick, F. (1993). Proteins regulating Ras and its relatives. Nature *366*, 643–654.

Bos, J.L., and Zwartkruis, F.J.T. (1999). Rhapsody in G proteins. Nature *400*, 820–821.

Campbell, S.L., Khosravi-Far, R., Rossman, K.L., Clark, G.J., and Der, C.J. (1998). Increasing complexity of Ras signaling. Oncogene *17*, 1395–1413.

Cobb, M.H., and Goldsmith, E.J. (1995). How MAP kinases are regulated. J. Biol. Chem. *270*, 14843–14846.

Conklin, B.R., Chabre, O., Wong, Y.H., Federman, A.D., and Bourne, H.R. (1992). Recombinant $G_q\alpha$ mutational activation and coupling to receptors and phospholipase C. J. Biol. Chem. *267*, 31–34.

Daaka, Y., Luttrell, L.M., Ahn, S., Della Rocca, G.J., Ferguson, S.S.G., Caron, M.G., and Lefkowitz, R.J. (1998). Essential role for G protein-coupled receptor endocytosis in the activation of mitogen-activated protein kinase. J. Biol. Chem. *273*, 685–688.

Davis, R.J. (1993). The mitogen-activated protein kinase signal transduction pathway. J. Biol. Chem. *268*, 14553–14556.

De Vivo, M., and Iyengar, R. (1994). Activated G_q-α potentiates platelet-derived growth factor-stimulated mitogenesis in confluent cell cultures. J. Biol. Chem. *269*, 19671–19674.

Dell'Acqua, M.L., Carroll, R.C., and Peralta, E.G. (1993). Transfected m2 muscarinic acetylcholine receptors couple to $G_{\alpha i2}$ and $G_{\alpha i3}$ in Chinese hamster ovary cells. J. Biol. Chem. *268*, 5676–5685.

Dhanasekaran, N., Tsim, S.T., Dermott, J.M., and Onesime, D. (1998). Regulation of cell proliferation by G proteins. Oncogene *17*, 1383–1394.

Diversé-Pierluissi, M., Remmers, A.E., Neubig, R.R., and Dunlap, K. (1997). Novel form of crosstalk between G protein and tyrosine kinase pathways. Proc. Natl. Acad. Sci. USA *94*, 5417–5421.

Fujitani, Y., and Bertrand, C. (1997). ET-1 cooperates with EGF to induce mitogenesis via a PTX-sensitive pathway in airway smooth muscle cells. Am. J. Physiol. *272*, 1492–1498.

Gallego, C., Gupta, S.K., Heasley, L.E., Qian, N.X., and Johnson, G.L. (1992). Mitogen-activated protein kinase activation resulting from selective oncogene expression in NIH 3T3 and Rat-1a cells. Proc. Natl. Acad. Sci. USA *89*, 7355–7359.

Garnovskaya, M.N., van Biesen, T., Hawe, B., Casanas Ramos, S., Lefkowitz, R.J., and Raymond, J.R. (1996). Ras-dependent activation of fibroblast mitogen-activated protein kinase by 5-HT1A receptor via a G protein $\beta\gamma$-subunit-initiated pathway. Biochemistry *35*, 13716–13722.

Garrington, T.P., and Johnson, G.L. (1999). Organization and regulation of mitogen-activated protein kinase signaling pathways. Curr. Opin. Cell Biol. *11*, 211–218.

Gupta, S.K., Gallego, C., Lowndes, J.M., Pleiman, C.M., Sable, C., Eisfelder, B.J., and Johnson, G.L. (1992). Analysis of the fibroblast transformation potential of GTPase-deficient *gip2* oncogenes. Mol. Cell. Biol. *12*, 190–197.

Gutkind, J.S. (1998). The pathways connecting G protein-coupled receptors to the nucleus through divergent mitogen-activated protein kinase cascades. J. Biol. Chem. *273*, 1839–1842.

Hackel, P.O., Zwick, E., Prenzel, N., and Ullrich, A. (1999). Epidermal growth factor receptors: critical mediators of multiple receptor pathways. Curr. Opin. Cell Biol. *11*, 184–189.

Hawes, B.E., Fried, S., Yao, X., Weig, B., and Graziano, M.P. (1998). Nociceptin (ORL-1) and μ-opioid receptors mediate mitogen-activated protein kinase activation in CHO cells through a G_i-coupled signaling pathway: evidence for distinct mechanisms of agonist-mediated desensitization. J. Neurochem. *71*, 1024–1033.

Hawes, B.E., van Biesen, T., Koch, W.J., Luttrell, L.M., and Lefkowitz, R.J. (1995). Distinct pathways of G_i- and G_q-mediated mitogen-activated protein kinase activation. J. Biol. Chem. *270*, 17148–17153.

Hermouet, S., Merendino, J.J., Jr., Gutkind, J.S., and Spiegel, A.M. (1991). Activating and inactivating mutations of the α subunit of G_{i2} protein have opposite effects on proliferation of NIH 3T3 cells. Proc. Natl. Acad. Sci. USA *88*, 10455–10459.

Jiang, M., Gold, M.S., Boulay, G., Spicher, K., Peyton, M., Brabet, P., Srinivasan, Y., Rudolph, U., Ellison, G., and Birnbaumer, L. (1998). Multiple neurological abnormalities in mice deficient in the G protein G_o. Proc. Natl. Acad. Sci. USA *95*, 3269–3274.

Jordan, J.D., Carey, K.D., Stork, P.J.S., and Iyengar, R. (1999). Modulation of Rap activity by direct interaction of $G\alpha_o$ with Rap1 GTPase-activating protein. J. Biol. Chem. *274*, 21507–21510.

Knall, C., Young, S., Nick, J.A., Buhl, A.M., Worthen, G.S., and Johnson, G.L. (1996). Interleukin-8 regulation of the Ras/Raf/mitogen-activated protein kinase pathway in human neutrophils. J. Biol. Chem. *271*, 2832–2838.

Kroll, S.D., Chen, J., De Vivo, M., Carty, D.J., Buku, A., Premont, R.T., and Iyengar, R. (1992). The $Q205LG_o$-α subunit expressed in NIH-3T3 cells induces transformation. J. Biol. Chem. *267*, 23183–23188.

Kroll, S.D., Omri, G., Landau, E.M., and Iyengar, R. (1991). Activated α subunit of G_o protein induces oocyte maturation. Proc. Natl. Acad. Sci. USA *88*, 5182–5186.

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_{i2} antibodies. J. Biol. Chem. *267*, 691–694.

Landis, C.A., Masters, S.B., Spada, A., Pace, A.M., Bourne, H.R., and Vallar, L. (1989). GTPase inhibiting mutations activate the α chain of G_s and stimulate adenylyl cyclase in human pituitary tumors. Nature *340*, 692–696.

Liu, Y.F., Jakobs, K.H., Rasenick, M.M., and Albert, P.R. (1994). G protein specificity in receptor-effector coupling. Analysis of the roles of G_o and G_{i2} in GH4C1 pituitary cells. J. Biol. Chem. *269*, 13880–13886.

Luttrell, L.M., Daaka, Y., and Lefkowitz, R.J. (1999). Regulation of tyrosine kinase cascades by G-protein-coupled receptors. Curr. Opin. Cell Biol. *11*, 177–183.

Lyons, J., *et al*. (1990). Two G protein oncogenes in human endocrine tumors. Science *249*, 655–659.

Marais, R., Light, Y., Paterson, H.F., Mason, C.S., and Marshall, C.J. (1997). Differential regulation of Raf-1, A-Raf, and B-Raf by oncogenic Ras and tyrosine kinases. J. Biol. Chem. *272*, 4378–4383.

Marais, R., and Marshall, C.J. (1996). Control of the ERK MAP kinase cascade by Ras and Raf. Cancer Surv. *27*, 101–125.

Marshall, C.J. (1994). MAP kinase kinase kinase, MAP kinase kinase and MAP kinase. Curr. Opin. Genet. Dev. *4*, 82–89.

Meloche, S., Pagès, G., and Pouysségur, J. (1992a). Functional expression and growth factor activation of an epitope-tagged p44 mitogen-activated protein kinase, $p44^{mapk}$. Mol. Biol. Cell *3*, 63–71.

Meloche, S., Seuwen, K., Pages, G., and Pouyssegur, J. (1992b). Biphasic and synergistic activation of p44mapk (ERK1) by growth factors: correlation between late phase activation and mitogenicity. Mol. Endocrinol. *6*, 845–854.

Moriarty, T.M., Padrell, E., Carty, D.J., Omri, G., Landau, E.M., and Iyengar, R. (1990). G_o protein as signal transducer in the pertussis toxin-sensitive phosphatidylinositol pathway. Nature *343*, 79–82.

Mochizuki, N., Ohba, Y., Kiyokawa, E., Kurata, T., Murakami, T., Ozaki, T., Kitabatake, A., Nagashima, K., and Matsuda, M. (1999). Activation of the ERK/MAPK pathway by an isoform of rap 1 Gap associated with $G\alpha_i$. Nature *400*, 891–894.

Morrison, D.K., and Cutler, R.E., Jr. (1997). The complexity of Raf-1 regulation. Curr. Opin. Cell Biol. *9*, 174–179.

Neer, E.J. (1995). Heterotrimeric G proteins: organizers of transmembrane signals. Cell *80*, 249–257.

Pace, A.M., Faure, M., and Bourne, H.R. (1995). G_{i2}-mediated activation of the MAP kinase cascade. Mol. Biol. Cell *6*, 1685–1695.

Pace, A.M., Wong, Y.H., and Bourne, H.R. (1991). A mutant α subunit of G_{i2} induces neoplastic transformation of Rat-1 cells. Proc. Natl. Acad. Sci. USA *88*, 7031–7035.

Peraldi, P., Frodin, M., Barnier, J.V., Calleja, V., Scimeca, J.C., Filloux, C., Calothy, G., Van Obberghen, E. (1995). Regulation of the MAP kinase cascade in PC12 cells: B-Raf activates MEK-1 (MAP kinase or ERK kinase) and is inhibited by cAMP. FEBS Lett. *357*, 290–296.

Pouyssegur, J., and Seuwen, K. (1992). Transmembrane receptors and intracellular pathways that control cell proliferation. Annu. Rev. Physiol. *54*, 195–210.

Renshaw, M.W., Ren, X.D., and Schwartz, M.A. (1997). Growth factor activation of MAP kinase requires cell adhesion. EMBO J. *16*, 5592–5599.

Reuter, C.W.M., Catling, A.D., Jelinek, T., and Weber, M.J. (1995). Biochemical analysis of MEK activation in NIH3T3 fibroblasts. J. Biol. Chem. *270*, 7644–7655.

Rosario, M., Paterson, H.F., and Marshall, C.J. (1999). Activation of the Raf/MAP kinase cascade by the Ras-related protein TC21 is required for the TC21-mediated transformation of NIH 3T3 cells. EMBO J. *18*, 1270–1279.

Schlessinger, J. (1994). SH2/SH3 signaling proteins. Curr. Opin. Genet. Dev. *4*, 25–30.

Seidel, M.G., Klinger, M., Freissmuth, M., and Holler, C. (1999). Activation of mitogen-activated protein kinase by the A_{2A}-adenosine receptor via a rap 1-dependent and via a $p21^{ras}$-dependent pathway. J. Biol. Chem. *274*, 25833–25841.

Sunahara, R.K., Dessauer, C.W., and Gilman, A.G. (1996). Complexity and diversity of mammalian adenylyl cyclases. Annu. Rev. Pharmacol. Toxicol. *36*, 461–480.

Toker, A., and Cantley, L.C. (1997). Signaling through the lipid products of phosphoinositide-3-OH kinase. Nature *387*, 673–676.

Toullec, D., Pianetti, P., Coste, H., Bellevergue, P., Grand-Perret, T., Ajakane, M., Baudet, V., Boissin, P., Boursier, E., and Loriolle, F. (1991). The bisindolylmaleimide GF 109203X is a potent and selective inhibitor of protein kinase C. J. Biol. Chem. *266*, 15771–15781.

Ui, M., Okada, T., Hazeki, K., and Hazeki, O. (1995). Wortmannin as a unique probe for an intracellular signaling protein, phosphoinositide 3-kinase. Trends Biochem. Sci. *20*, 303–307.

Vaillancourt, R.R., Gardner, A.M., and Johnson, G.L. (1994). B-Raf-dependent regulation of the MEK-1/mitogen-activated protein kinase pathway in PC12 cells and regulation by cyclic AMP. Mol. Cell. Biol. *14*, 6522–6530.

Vallar, L. (1996). Oncogenic role of heterotrimeric G proteins. Cancer Surv. *27*, 325–338.

van Biesen, T., Hawes, B.E., Raymond, J.R., Luttrell, L.M., Koch, W.J., and Lefkowitz, R.J. (1996a). G_o-protein α-subunits activate mitogen-activated protein kinase via a novel protein kinase C-dependent mechanism. J. Biol. Chem. *271*, 1266–1269.

van Biesen, T., Luttrell, L.M., Hawes, B.E., and Lefkowitz, R.J. (1996b). Mitogenic signaling via G protein-coupled receptors. Endocr. Rev. *17*, 698–714.

Vlahos, C.J., Matter, W.F., Hui, K.Y., and Brown, R.F. (1994). A specific inhibitor of phosphatidylinositol 3-kinase, 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (LY294002). J. Biol. Chem. *269*, 5241–5248.

Vossler, M.R., Yao, H., York, R.D., Pan, M.G., Rim, C.S., and Stork, P.J.S. (1997). cAMP activates MAP kinase and Elk-1 through a B-Raf- and Rap1-dependent pathway. Cell *89*, 73–82.

Vouret-Craviari, V., Van Obberghen-Schilling, E., Scimeca, J.C., Van Obberghen, E., and Pouyssegur, J. (1993). Differential activation of $p44^{mapk}$ (ERK1) by α-thrombin and thrombin-receptor peptide agonist. Biochem. J. *289*, 209–214.

Voyno-Yasenetskaya, T.A., Pace, A.M., and Bourne, H.R. (1994). Mutant α subunits of G_{12} and G_{13} proteins induce neoplastic transformation of Rat-1 fibroblasts. Oncogene *9*, 2559–2565.

Watts, V.J., Wiens, B.L., Cumbay, M.G., Vu, M.N., Neve, R.L., and Neve, K.A. (1998). Selective activation of $G\alpha_o$ by D_{2L} dopamine receptors in NS20Y neuroblastoma cells. J. Neurosci. *18*, 8692–8699.

Wennstrom, S., and Downward, J. (1999). Role of phosphoinositide 3-kinase in activation of ras and mitogen-activated protein kinase by epidermal growth factor. Mol. Cell. Biol. *19*, 4279–4288.

Wong, Y.H., Conklin, B.R., and Bourne, H.R. (1992). G_z-mediated hormonal inhibition of cyclic AMP accumulation. Science *255*, 339–342.

Wylie, P.G., Challiss, R.A.J., and Blank, J.L. (1999). Regulation of extracellular signal-regulated kinase and c-Jun N-terminal kinase by G-protein-linked muscarinic acetylcholine receptors. Biochem. J. *338*, 619–628.

Xu, S., Khoo, S., Dang, A., Witt, S., Do, V., Zhen, E., Schaefer, E.M., and Cobb, M.H. (1997). Differential regulation of mitogen-activated protein/ERK kinase (MEK)1 and MEK2 and activation by a Ras-independent mechanism. Mol. Endocrinol. *11*, 1618–1625.

York, R.D., Yao, H., Dillon, T., Ellig, C.L., Eckert, S.P., McCleskey, E.W., and Stork, P.J.S. (1998). Rap1 mediates sustained MAP kinase activation induced by nerve growth factor. Nature *392*, 622–626.