0026-895X/04/6506-1427–1439$20.00
MOLECULAR PHARMACOLOGY
Copyright © 2004 The American Society for Pharmacology and Experimental Therapeutics
Mol Pharmacol 65:1427–1439, 2004

Vol. 65, No. 6
3075/1151864
Printed in U.S.A.

Signal Transducer and Activator of Transcription 3 Activation by the δ-Opioid Receptor via $G\alpha_{14}$ Involves Multiple Intermediates

Rico K. H. Lo and Yung H. Wong

Department of Biochemistry, Molecular Neuroscience Center, and Biotechnology Research Institute, Hong Kong University of Science and Technology, Clear Water Bay, Kowloon, Hong Kong, China

Received November 3, 2003; accepted February 26, 2004

This article is available online at http://molpharm.aspetjournals.org

ABSTRACT

The hematopoietic-specific $G\alpha_{14}$ links a variety of G protein-coupled receptors to phospholipase Cβ (PLCβ) stimulation. Recent studies reveal that several Gα subunits are capable of activating signal transducer and activator of transcription (STAT) proteins. In the present study, we investigated the mechanism by which $G\alpha_{14}$ mediates receptor-induced stimulation of STAT3. In human embryonic kidney 293 cells, coexpression of $G\alpha_{14}$ with δ-opioid receptor supported [D-Pen^2, D-Pen^5]enkephalin (DPDPE)-induced STAT3 phosphorylations at both Tyr^{705} and Ser^{727} in a pertussis toxin-insensitive manner. The constitutively active $G\alpha_{14}$QL mutant also induced STAT3 phosphorylations at these sites and promoted STAT3-dependent luciferase activity. Requirements for PLCβ, protein kinase C (PKC), and calmodulin-dependent kinase II (CaMKII) in $G\alpha_{14}$QL-induced STAT3 activation were demonstrated by their respective inhibitors as well as by coexpression of their dominant-negative mutants. Inhibition of c-Src and Janus kinase 2 and 3 activities abolished STAT3 activation induced by $G\alpha_{14}$QL, but no physical association between $G\alpha_{14}$QL and c-Src could be detected by coimmunoprecipitation. Various intermediates along the extracellular signal-regulated kinase signaling cascade were apparently required for $G\alpha_{14}$QL-induced STAT3 activation; they included Ras/Rac1, Raf-1, and mitogen-activated protein kinase kinase-1/2. In contrast, functional blockade of c-Jun N-terminal kinase, p38 mitogen-activated protein kinase, and phosphatidylinositol-3 kinase had no effect on $G\alpha_{14}$QL-induced responses. PLCβ, PKC, and CaMKII were shown to be involved in $G\alpha_{14}$QL-mediated c-Src phosphorylation. Similar results were obtained with human erythroleukemia cells upon DPDPE treatment. These results demonstrate for the first time that $G\alpha_{14}$ activation can lead to STAT3 stimulation via a complex signaling network involving multiple intermediates.

Guanine nucleotide-binding regulatory proteins (G proteins) are signal transducers for a large number of heptahelical receptors. $G\alpha_{14}$, a member of the G_q subfamily, is believed to be required for hematopoietic cell differentiation and function (Wilkie et al., 1991). $G\alpha_{14}$ is primarily expressed in hematopoietic cells including early myeloid cells, progenitor B cells, and bone marrow adherent cells (Wilkie et al., 1991). Like other G_q subfamily members, $G\alpha_{14}$ stimulates phospholipase Cβ (PLCβ) signaling cascades. Coexpression of $G\alpha_{14}$ with the parathyroid hormone (PTH)/parathyroid hormone-related peptide (PTHrP) receptor in rat aortic smooth muscle A10 cells permits the induction of intracellu-

This work was supported in part by grants from the Research Grants Council of Hong Kong (HKUST 6095/01M and HKUST 2/99C), the University Grants Committee (AoE/B-15/01), and the Hong Kong Jockey Club. Y.H.W. was a recipient of the Croucher Senior Research Fellowship.

ABBREVIATIONS: PLCβ, phospholipase Cβ; STAT, signal transducer and activator of transcription; DPDPE, [D-Pen^2,D-Pen^5]enkephalin; PKC, protein kinase C; CaMKII, calmodulin-dependent protein kinase II; ERK, extracellular signal-regulated kinase; IP_3, inositol 1,4,5-triphosphate; DOR, δ-opioid receptor; ORL_1 receptor, opioid receptor-like receptor; MAPK, mitogen-activated protein kinase; JNK, c-Jun N-terminal kinase; JAK, Janus kinase; GPCR, G protein-coupled receptor; MEK, mitogen-activated protein kinase kinase; PI3K, phosphatidylinositol-3 kinase; EGF, epidermal growth factor; TPR1, tetratricopeptide repeat 1; DN, dominant-negative; HEK, human embryonic kidney; PTX, pertussis toxin; PTH, parathyroid hormone; PTHrP, parathyroid hormone-related peptide; HEL, human erythroleukemia; DSP, dithiobis[succinimidylpropionate]; RIPA, radioimmunoprecipitation assay; U73122, 1-(6-[[17β-3-methoxyestra-1,3.5(10)-trien-17-yl]amino]hexyl)-1*H*pyrrole-2,5-dione; KN62, 1-[*N*,*O*-*bis*-(5-isoquinolinesulfonyl)-*N*-methyl-L-tyrosyl]-4-phenyl-piperazine; WHI-P131, 4-(4′-hydroxyphenyl)-amino-6,7-dimethoxyquinazoline; WHI-P258, 4-(phenyl)-amino-6,7-dimethoxyquinazoline; U0126, 1,4-diamino-2,3-dicyano-1,4-bis(2-aminophenylthio)butadiene; RLU, relative luminescent unit; DMSO, dimethyl sulfoxide; SB202190, 4-(4-fluorophenyl)-2-(4-hydroxyphenyl)-5-(4-pyridyl)-1*H*-imidazole; SB203580, 4-(4-fluorophenyl)-2-(4-methylsulfonylphenyl)-5-(4-pyridyl)imidazol; LY294002, 2-(4-morpholinyl)-8-phenyl-4*H*-1-benzopyran-4-one, U73343, 1-[6-((17β-3-methoxyestra-1,3,5(10)-trien-17-yl)amino)hexyl]-2,5-pyrrolidinedione; AG490, α-cyano-(3,4-dihydroxy)-*N*-benzylcinnamide; U0124, 1,4-diamino-2,3-dicyano-1,4-*bis*(methylthio)butadiene.

lar Ca^{2+} releases and inositol 1,4,5-triphosphate (IP_3) formation by PTHrP (Maeda et al., 1996). $G\alpha_{14}$ is rather promiscuous in terms of receptor coupling because it can interact productively with a variety of G_i- and G_s-coupled receptors, including the δ-opioid receptor (DOR), opioid receptor-like (ORL_1) receptor, and α_2- and β_2-adreoceptors (Ho et al., 2001). $G\alpha_{14}$ has also been reported to regulate mitogen-activated protein kinases (MAPKs). Agonist-bound ORL_1 receptor activates the c-Jun N-terminal kinase (JNK) via $G\alpha_{14}$ and subsequently stimulates c-Jun and activating transcription factor-2 (Chan and Wong, 2000). These findings indicate that $G\alpha_{14}$ may possess the ability to regulate gene transcription and thus modulate cell differentiation and other cellular events.

It has become widely accepted that Gα subunits play critical roles in cell growth, proliferation, and differentiation by activating the MAPK cascades. Apart from MAPKs, signal transducers and activators of transcription (STATs), a family of latent cytoplasmic transcription factor, have also been shown to play crucial roles in these cellular events (Hirano et al., 2000). The regulatory mechanisms for STAT3 activation are rather complex. In response to cytokines, growth factors, or interferons, activated Janus kinases (JAKs) phosphorylate and recruit cytoplasmic STATs. Phosphorylated STATs dimerize and translocate to the nucleus where they bind to specific gene-promoter sequences and induce DNA transcription. In addition to JAKs, the nonreceptor Src tyrosine kinase has been reported to induce the activation of STATs. v-Src can tyrosine-phosphorylate and constitutively activate STAT3, resulting in the induction of STAT3-mediated gene expression (Turkson et al., 1998). Members of MAPKs such as the extracellular signal-regulated kinase (ERK) are also capable of activating STATs. For instance, erythropoietin-induced STAT3 Ser^{727} phosphorylation is mediated by ERK signaling cascades (Haq et al., 2002). Taken together, STAT3 activation is modulated by complex mechanisms involving multiple signaling intermediates such as JAKs, Src kinase, and ERK.

Although STATs are considered to be primarily regulated by cytokines and growth factors, their activities can also be modulated by GPCRs. Angiotensin II type I receptor has been shown to activate the JAK2/STAT3 signaling pathway in neonatal rat cardiac myocytes (McWhinney et al., 1997). The precise mechanism by which GPCRs tap into the JAK/STAT pathway is unclear, but there is ample evidence to support the involvement of Gα subunits. In NIH-3T3 cells expressing a dominant-negative mutant of $G\alpha_{i2}$, Src kinase activity and STAT3 tyrosine phosphorylation are inhibited, resulting in a reduction of v-fms-induced proliferation (Corre et al., 1999). In contrast, constitutively active $G\alpha_o$ and $G\alpha_i$ mutants induce neoplastic transformation of NIH-3T3 cells via the activation of Src and STAT3 pathways (Ram et al., 2000). These studies imply that G_i subfamily members may possess the ability to activate STAT3, possibly via Src. Our recent demonstration that $G\alpha_{16}$ can mediate the stimulatory actions on STAT3 by the ORL_1, *N*-formyl-methionyl-leucyl-phenylalanine, and C5a receptors (Lo et al., 2003; Wu et al., 2003) further suggested that G_q subfamily members may similarly regulate the activities of STATs. In the present study, we sought to investigate whether activated $G\alpha_{14}$ can stimulate STAT3 activity through intermediate signaling molecules.

Materials and Methods

Reagents. The human $G\alpha_{14}$ and $G\alpha_{14}$QL cDNAs were obtained from Guthrie Research Institute (Sayre, PA). The cDNAs of STAT3 and its dominant-negative mutants STAT3Y705F and STAT3S727A were gifts from Dr. Nancy C. Reich (State University of New York, NY). The origins of plasmids encoding the DOR, dominant-negative mutants of PI3Kγ (PI3Kγ-DN), and c-Src (c-Src-DN), Ras and Rac1 (wild-type, dominant-negative mutants, and constitutively activated mutants) were as described previously (Kam et al., 2003). The PKCα and PKCε dominant-negative mutant cDNAs, PKCα-KR and PKCε-KR, were kindly obtained from Dr. Bernard Weinstein (Columbia University, New York, NY). The luciferase reporter gene pSTAT3-TA-luc was purchased from BD Biosciences Clontech (Palo Alto, CA). Human embryonic kidney 293 cells were obtained from the American Type Culture Collection (Manassas, VA). Human erythroleukemia (HEL) cells were purchased from German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany). Cell culture reagents, including LipofectAMINE Plus, were from Invitrogen (Carlsbad, CA). Antisera against $G\alpha_{14}$ and c-Src were purchased from Gramsch Laboratories (Schwabhausen, Germany) and Santa Cruz Biochemicals (Santa Cruz, CA), respectively, and other antibodies were supplied from Cell Signaling Technology Inc. (Beverly, MA). All kinase inhibitors and their negative analogs were ordered from Calbiochem (Darmstadt, Germany). Epidermal growth factor (EGF) and [D-Pen^2,D-Pen^5]enkephalin (DPDPE) were purchased from Sigma Chemical (St. Louis, MO). Pertussis toxin (PTX) was from List Biological Laboratories Inc.(Campbell, CA). Protein G-agarose and dithiobis[succinimidylpropionate] (DSP) cross-linker were obtained from Pierce Chemical (Rockford, IL). The luciferase substrate and its lysis buffer were purchased from Roche Diagnostics (Mannheim, Germany).

Cell Culture and Transfection. HEL cells were cultured in RPMI 1640 at 5% CO_2 and 37°C with 10% fetal bovine serum, 50 units/ml penicillin, and 50 μl/ml streptomycin. HEK 293 cells were grown in Eagle's minimum essential medium (growth medium) at 5% CO_2 and 37°C with 10% fetal bovine serum, 50 units/ml penicillin, and 50 μl/ml streptomycin. HEK 293 cells were seeded onto 96-well microtiter plates at 15,000 cells/well and were maintained in the growth medium for 24 h before transfection. Cells were transiently cotransfected with various cDNAs using LipofectAMINE Plus reagents. The transfection cocktails in serum-free Opti-MEM medium (100 μl/well; Invitrogen) contained 0.2 μl of both PLUS and LipofectAMINE reagents, 10 ng of G proteins, small GTPases or the control vector cDNAs, and 0.1 μg of pSTAT3-TA-luc. Three hours after transfection, 50 μl of Opti-MEM medium containing 30% fetal bovine serum was added into the wells and cultured for another 30 h.

Luciferase Assay. After 30 h of transfection, HEK 293 cells were serum-starved for 24 h or treated with a different kinase inhibitor overnight. Growth medium was then removed. 25 μl of lysis buffer from the luciferase assay kit (Roche Diagnostics) was added to the wells and then gently shaken on ice for 30 min. A further 25 μl of lysis buffer and 25 μl of luciferase substrate were added to each well to initiate the reaction. Luciferase activity was measured in a MicroLumatPlus LB96V luminometer (PerkinElmer Life and Analytical Sciences, Boston, MA).

Immunoblotting, Cross-Linking, and Immunoprecipitation Assays. HEK 293 cells were seeded on six-well plates at a density of 5×10^5 cells/well and maintained in growth medium the day before transfection. Cells were transiently cotransfected with various cDNAs by means of LipofectAMINE Plus reagents. Forty-eight hours later, the transfectants were serum-starved or treated with different kinase inhibitors overnight. Cells were lysed in 150 μl of lysis buffer (50 mM Tris-HCl, pH 7.5, 5 mM EDTA, 100 mM NaCl, 1% Triton X-100, 40 mM NaP_2O_7, 1 mM dithiothreitol, 200 μM Na_3VO_4, 0.7

μg/ml pepstatin, 4 μg/ml aprotinin, 100 μM phenylmethylsulfonyl fluoride, and 2 μg/ml leupeptin) and gently shaken on ice for 15 min. Supernatants were collected by centrifugation at 16,000*g* for 2 min. HEL cells were seeded at a density of 1×10^6 cells using serum-free medium with or without PTX (100 ng/ml) treatment overnight. Cells were incubated with different kinase inhibitors for 30 min and then treated with 100 nM DPDPE for another 20 min at 37°C. Subsequently, cells were lysed by the same lysis buffer. Clarified lysates (40 μg) were resolved on 12% SDS-polyacrylamide gels and transferred to a nitrocellulose membrane (GE Osmonics, Westborough, MA). STAT3, phospho-STAT3-Tyr^{705}, phospho-STAT3-Ser^{727}, ERK, phospho-ERK, JNK, phospho-JNK, p38 MAPK, phospho-p38 MAPK, c-Src, phospho-c-Src, Akt, and phospho-Akt proteins were detected by specific primary antisera and horseradish peroxidase-conjugated secondary antisera. The immunoblots were visualized by chemiluminescence with the enhanced chemiluminescence kit from Amersham Biosciences Inc. (Piscataway, NJ), and the images detected in X-ray films were quantified by densitometric scanning using the Eagle Eye II still video system (Stratagene, La Jolla, CA). For cross-linking, transfectants were rinsed with phosphate-buffered saline twice and then incubated in the same buffer plus 0.5 mM DSP for 15 min at room temperature. After that, cells were rinsed as above, incubated 5 min in quenching solution (50 mM glycine in phosphate-buffered saline, pH 7.4), and lysed in RIPA buffer (25 mM HEPES, 0.1% SDS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 1 mM dithiothreitol, 200 μM Na_3VO_4, 0.7 μg/ml pepstatin, 4 μg/ml aprotinin, 100 μM phenylmethylsulfonyl fluoride, and 2 μg/ml leupeptin). For immunoprecipitation, cell lysates were incubated with an anti-Src antiserum (4 μg/sample) at 4°C overnight and then followed by incubation with 30 μl of protein G Agarose (50% slurry) at 4°C for 2 h. Immunoprecipitates were washed with 400 μl of RIPA buffer and then resuspended in 50 μl of RIPA buffer and 10 μl of 6× sample buffer and boiled for 5 min. The c-Src and $G\alpha_{14}$ in the immunoprecipitates were detected by specific primary antisera and horseradish peroxidase-conjugated secondary antisera.

Results

Stimulation of STAT3 Induced by DPDPE-Activated DOR via $G\alpha_{14}$ in a Time-Dependent Manner. We have demonstrated previously that the G_i-linked DOR can efficiently stimulate PLCβ via $G\alpha_{14}$ in COS-7 cells (Ho et al., 2001). Hence, we began our investigation by examining the effects of DOR-mediated activation of $G\alpha_{14}$ on STAT3 activity. HEK 293 cells were transiently transfected with pcDNA1 (vector control), DOR, or DOR together with $G\alpha_{14}$. Transfectants were challenged with 100 nM DPDPE, a specific agonist of DOR, for different time periods up to 30 min. The phosphorylation status of STAT3 was then assessed by Western blot analysis using anti-phospho-STAT3 antisera, whereas total STAT3 expression was monitored by anti-STAT3 antiserum. As shown in Fig. 1A, STAT3 Tyr^{705} and Ser^{727} phosphorylations were unaffected by DPEPE in transfectants expressing the DOR alone compared with the controls (vector and basal). Yet a 30-min treatment of these cells with DPDPE significantly suppressed cAMP accumulation but had no effect on STAT3 phosphorylation (data not shown), implying that DOR could not mediate STAT3 phosphorylation via endogenous $G_{i/o}$ proteins. In cells coexpressing DOR and $G\alpha_{14}$, phosphorylation of STAT3 at Tyr^{705} and Ser^{727} was significantly stimulated upon treatment with DPDPE (Fig. 1A). These phosphorylations began at 5 min and were sustained up to 15 min, which then decreased to the basal level by 30 min (Fig. 1A). To confirm that DOR-stimulated STAT3 phosphorylations were indeed mediated by $G\alpha_{14}$, transfectants were preincubated with 100 ng/ml PTX to abolish signaling via the endogenous PTX-sensitive G proteins. As illustrated in Fig. 1A, the DPDPE-induced STAT3 Tyr^{705} and Ser^{727} phosphorylations were insensitive to PTX treatment. The effectiveness of PTX was confirmed by its ability to abolish DPDPE-induced inhibition of forskolin-stimulated cAMP accumulation (data not shown). Taken together, Tyr^{705} and Ser^{727} phosphorylations of STAT3 were induced by DPDPE-activated DOR via $G\alpha_{14}$ in a time-dependent manner.

Because $G\alpha_{14}$ is mainly expressed in hematopoietic cells, we sought to investigate whether STAT3 phosphorylation can be stimulated by DOR via $G\alpha_{14}$ in HEL cells. The presence of $G\alpha_{14}$ in HEL cells was confirmed by immunodetection with a $G\alpha_{14}$-specific antiserum (data not shown). We treated HEL cells with 100 nM DPDPE for various durations and then determined STAT3 phosphorylation status with anti-phospho-STAT3 antisera. As indicated in Fig. 1B, DPDPE significantly activated the phosphorylation of STAT3 at both Tyr^{705} and Ser^{727} residues compared with the untreated cells, whereas total STAT3 expression was unaffected. The optimal phosphorylation time of STAT3 Tyr^{705} and Ser^{727} induced by DPDPE was between 20 and 30 min (Fig. 1B). Because DOR can transduce signals through both PTX-sensitive and -insensitive G proteins, the potential involvement of G_i proteins in regulating DPDPE-induced STAT3 phosphorylation was eliminated by pretreating the HEL cells with PTX (100 ng/ml) overnight. As shown in Fig. 1B, DPDPE remained fully capable of mediating STAT3 Tyr^{705} and Ser^{727} phosphorylations in PTX-treated cells. These observations implied that DPDPE-induced STAT3 phosphorylation was induced by PTX-insensitive G proteins, such as $G\alpha_{14}$ and $G\alpha_{16}$. The identity of the PTX-insensitive G protein is unlikely to be G_q because DOR is incapable of interacting with G_q (Liu et al., 2003).

Stimulation of STAT3 Induced by Constitutively Active $G\alpha_{14}$QL. Next we examined the mechanism by which $G\alpha_{14}$ activates STAT3. The glutamine 205 to leucine (Q205L) mutation in $G\alpha_{14}$ results in a reduction of its intrinsic GTPase activity and produces a constitutively active phenotype (Chan and Wong, 2000). It mimics the ligand stimulation of GPCRs and is useful for mapping out the downstream signaling mechanism. To verify whether STAT3 could be stimulated by $G\alpha_{14}$QL in the absence of GPCR activation, HEK 293 cells were transiently transfected with pcDNA1 (vector control), $G\alpha_{14}$, or $G\alpha_{14}$QL cDNAs. As shown in Fig. 2A, the expression level of total STAT3 was unaffected in cells transfected with $G\alpha_{14}$ or $G\alpha_{14}$QL compared with the vector control. In contrast to the vector control or $G\alpha_{14}$ transfectants, $G\alpha_{14}$QL significantly induced STAT3 Tyr^{705} and Ser^{727} phosphorylations (Fig. 2A). Because phosphorylated STAT3 is capable of stimulating gene transcription, we sought to determine whether $G\alpha_{14}$QL could enhance STAT3-dependent transcriptional activity. We performed reporter gene assays by cotransfecting the cells with a STAT3-dependent luciferase construct (pSTAT3-TA-luc). As expected, $G\alpha_{14}$QL enhanced STAT3-dependent transcriptional activity significantly compared with both vector control and $G\alpha_{14}$ (Fig. 2A). The observed luciferase activity was positively correlated with increasing amounts of $G\alpha_{14}$QL cDNA used in the transfection (Fig. 2B). In contrast, the STAT3-dependent transcriptional activity of wild-type $G\alpha_{14}$ remained around the

basal level even at a cDNA concentration of 100 ng/ml (Fig. 2B). These results indicated that $G\alpha_{14}QL$ enhances STAT3-dependent transcriptional activity in a cDNA dose-dependent manner.

$G\alpha_{14}QL$-Induced STAT3 Stimulation Was Inhibited by STAT3 Dominant-Negative Mutants. To further confirm the phosphorylation and activation of STAT3 by $G\alpha_{14}QL$, we assessed the ability of tyrosine and serine dominant-negative mutants of STAT3 (STAT3Y705F and STAT3S727A) to suppress $G\alpha_{14}QL$-induced responses. HEK 293 cells were cotransfected with the cDNAs of pcDNA1, $G\alpha_{14}$, or $G\alpha_{14}QL$ in combination with wild-type STAT3 or its dominant-negative mutants. Western blot analysis with anti-STAT3 antiserum revealed increased STAT3 protein expression in cells transfected with wild-type or dominant-negative mutants of STAT3 (Fig. 2C). Basal and $G\alpha_{14}QL$-induced Tyr^{705} and Ser^{727} phosphorylations of STAT3 were elevated in STAT3-expressing cells compared with the corresponding controls (Fig. 2C). $G\alpha_{14}QL$-induced STAT3 Tyr^{705} phosphorylation, but not STAT3 Ser^{727} phosphorylation, was attenuated in STAT3Y705F-expressing cells (Fig. 2C). On the other hand, STAT3S727A reduced $G\alpha_{14}QL$-induced STAT3 Ser^{727} phosphorylation only and had no influence on STAT3 Tyr^{705} phosphorylation induced by $G\alpha_{14}QL$ (Fig. 2C). In either case, the magnitudes of the suppressed responses were similar to those observed with the control cells, in which only endogenous STAT3 proteins were present. These results implied that $G\alpha_{14}QL$ independently induced STAT3 Tyr^{705} and Ser^{727} phosphorylations. Despite the increased expression of STAT3 in wild-type STAT3 transfectants, $G\alpha_{14}QL$-induced luciferase activity was no different from that of the corresponding vector control and $G\alpha_{14}$ transfectants (Fig. 2C), presumably because other factors such as substrate availability became limiting. For cells coexpressing $G\alpha_{14}QL$ and either STAT3Y705F or STAT3S727A, the STAT3-dependent luciferase activities were significantly lower than that of wild-type STAT3-coexpressing cells (Fig. 2C). This might be explained by the fact that simultaneous phosphorylations at Tyr^{705} and Ser^{727} are required for maximal stimulation of STAT3.

The Involvement of PLCβ Signaling Pathway in $G\alpha_{14}QL$-Induced STAT3 Stimulation. As a member of the G_q subfamily, $G\alpha_{14}$ activates PLCβ, which hydrolyzes phosphatidylinositol-4,5-bisphosphate into diacylglycerol and IP_3. In A10 cells expressing PTH/PTHrP receptors, transient overexpression of $G\alpha_{14}$ conferred the ability of PTHrP to

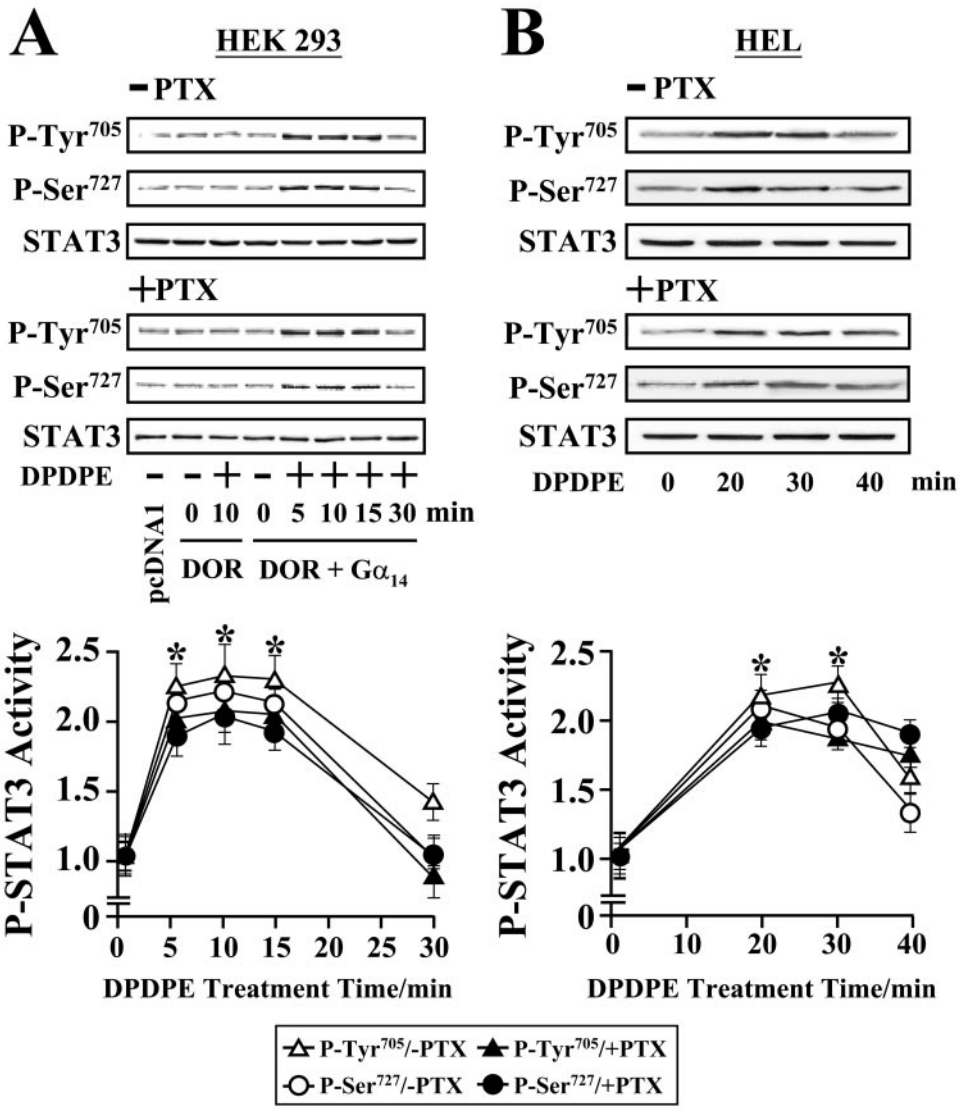

Fig. 1. STAT3 stimulation induced by DPDPE-activated DOR via $G\alpha_{14}$ in HEK 293 cells and HEL cells. A, HEK 293 cells were transiently transfected with pcDNA1, DOR, or DOR together with $G\alpha_{14}$. Two days after transfection, the transfectants were serum-starved with or without PTX treatment (100 ng/ml) overnight and then incubated with 100 nM DPDPE at indicated times. B, HEL cells were seeded at a density of 1×10^6 cells using serum-free medium with or without PTX (100 ng/ml) treatment overnight. Cells were incubated with 100 nM DPDPE for the indicated times at 37°C. For Western blot analysis, cell lysates were immunoblotted with anti-phospho-STAT3-Tyr^{705}, anti-phospho-STAT3-Ser^{727}, or anti-STAT3 antiserum. The immunoblots shown represent one of three sets; two other sets yielded similar results. Results of densitometric analyses are shown below the immunoblots; data shown represent the mean ± S.E.M. from three separate experiments. ⋆, $p < 0.05$, significantly higher than the control (zero time point) by Dunnett's t test.

increase intracellular Ca^{2+} and IP_3 formation (Maeda et al., 1996). These studies led us to examine whether PLCβ and its downstream effectors were required for $G\alpha_{14}$QL-induced STAT3 phosphorylation and activation. HEK 293 cells were thus treated with an inhibitor for either PLCβ (U73122) or CaMKII (KN62) at 10 μM. As illustrated in Fig. 3A, STAT3 Tyr^{705} and Ser^{727} phosphorylations induced by $G\alpha_{14}$QL were significantly attenuated by U73122 and KN62 compared with the corresponding controls. Likewise, for the reporter gene assays, U73122 or KN62 inhibited $G\alpha_{14}$QL-induced STAT3 activation (Fig. 3A). To further confirm these data, HEK 293 cells were incubated with 10 μM U73343 or KN92, the inactive analog of U73122 or CaMKII, respectively. As shown in Fig. 3B, $G\alpha_{14}$QL-induced STAT3 Tyr^{705} and Ser^{727} phosphorylations as well as STAT3 transcriptional activation were not suppressed by these inactive analogs, indicating that both PLCβ and CaMKII participated in the mediation of $G\alpha_{14}$QL-induced STAT3 phosphorylation and activation.

Next, we explored the role of PKC in $G\alpha_{14}$QL-induced STAT3 phosphorylation and activation. HEK 293 cells were treated with a PKC-selective inhibitor, calphostin C (100 nM). Inhibition of PKC by calphostin C suppressed the ability of $G\alpha_{14}$QL to induce STAT3 Tyr^{705} and Ser^{727} phosphorylations as well as the induction of STAT3-dependent luciferase activity (Fig. 4A). In addition to calphostin C, two PKC dominant-negative mutants, PKCα-KR and PKCϵ-KR, were tested. We chose these isoforms because PKCα and PKCϵ can be taken as representative members of conventional Ca^{2+}-dependent PKC and novel Ca^{2+}-independent PKC, respectively. As demonstrated in Fig. 4B, both PKCα-KR and PKCϵ-KR abolished STAT3 activation induced by $G\alpha_{14}$QL compared with the corresponding controls. Taken together, $G\alpha_{14}$QL-induced STAT3 phosphorylation and activation seemed to be mediated via a signaling pathway involving PLCβ, CaMKII, and PKC.

The Involvement of JAK2/3 and c-Src in $G\alpha_{14}$QL-Induced STAT3 Stimulation. There is ample evidence to support the notion that STAT activation by cytokines or growth factors is mediated via JAKs and Src family members. It is interesting that $G\alpha_s$ and $G\alpha_i$ can directly bind to the catalytic domain of c-Src and stimulate its kinase activity (Ma et al., 2000), thereby providing an avenue for GPCRs to regulate the JAK/STAT pathway. Indeed, G_i-coupled chemokine receptors have been shown to integrate G protein and JAK/STAT signals during chemotaxis (Soriano et al., 2003). These observations raised the likelihood that $G\alpha_{14}$QL-induced STAT3 phosphorylation and that JAKs and c-Src might mediate activation. To explore this possibility, transfectants were treated with selective inhibitors against JAK2 (AG490; 100 μM) or JAK3 (WHI-P131; 100 μg/ml). As shown

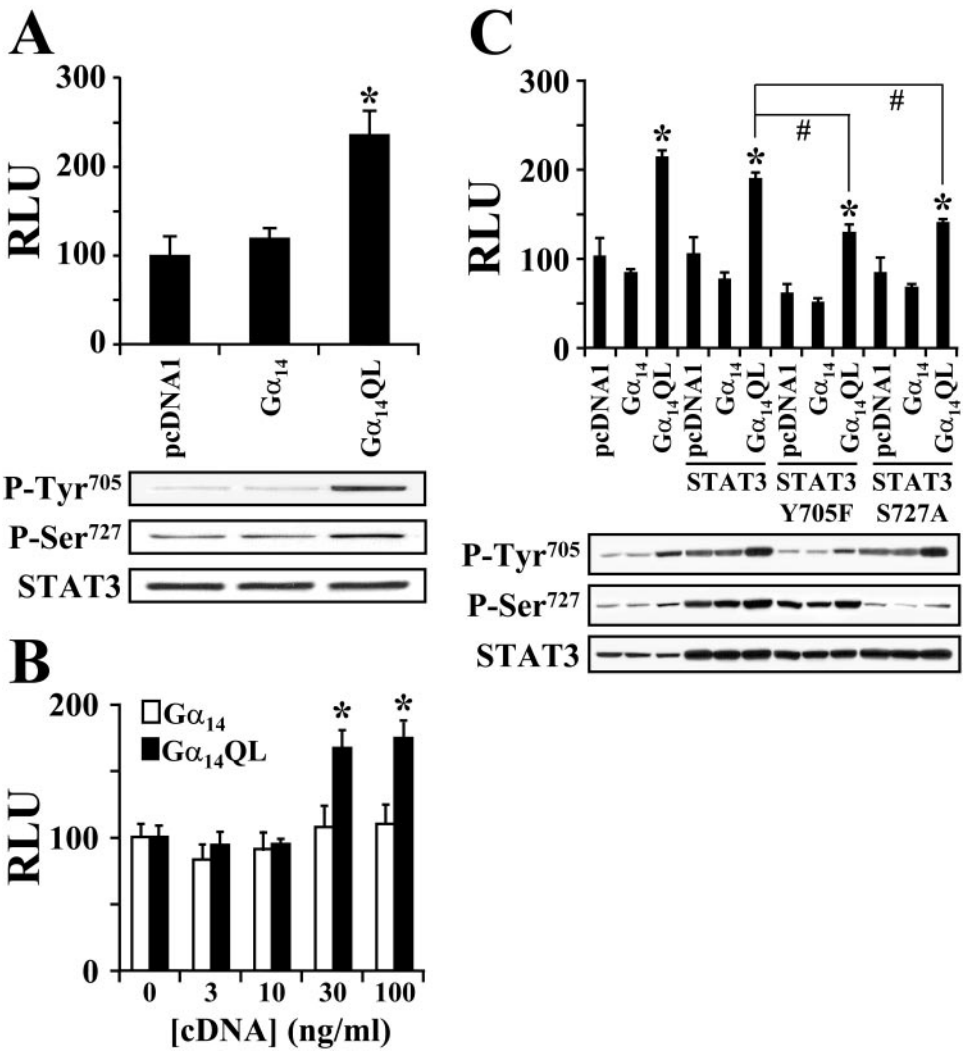

Fig. 2. STAT3 stimulation induced by constitutively active $G\alpha_{14}$QL. A, pSTAT3-TA-luc cDNA was transiently transfected into HEK 293 cells along with pcDNA1, $G\alpha_{14}$, or $G\alpha_{14}$QL. B, 1 μg/ml pSTAT3-TA-luc cDNA was transiently transfected into HEK 293 cells with varying concentrations of G protein cDNAs (up to 100 ng/ml cDNA and the total amount was corrected with pcDNA1). C, pSTAT3-TA-luc cDNA was transiently transfected into HEK 293 cells along with pcDNA1, $G\alpha_{14}$, or $G\alpha_{14}$QL in combination with STAT3, STAT3Y705F, or STAT3S727A. Cell lysates were immunoblotted with anti-phospho-STAT3-Tyr^{705} (top), anti-phospho-STAT3-Ser^{727} (middle) or anti-STAT3 (bottom) antiserum. The immunoblots shown represent one of three sets of immunoblots; two other sets yielded similar results. For reporter gene assays, cell lysates were used to measure luciferase activities. The luminescence emitted was quantified as the relative luminescent unit (RLU). RLUs were expressed as a percentage of the pcDNA1 control. Data shown represent the mean ± S.E.M. from four separate experiments performed in triplicate. ⋆, $p < 0.05$, significantly higher than the corresponding pcDNA1 control by Dunnett's t test. #, $p < 0.05$, significantly attenuated compared with those obtained with STAT3-overexpressing cells by Dunnett's t test.

in Fig. 3A, STAT3 Tyr^{705} and Ser^{727} phosphorylations induced by Gα_{14}QL were almost completely inhibited by AG490 and WHI-P131. Likewise, both AG490 and WHI-P131 attenuated the Gα_{14}QL-induced STAT3-dependent luciferase activity (Fig. 3A), suggesting that JAK2/3 were involved in the activation of STAT3 by Gα_{14}QL. In contrast, 100 μg/ml WHI-P258 (the inactive analog of WHI-P131) failed to affect the Gα_{14}QL-induced responses (Fig. 3B).

Next, we investigated the role of c-Src in Gα_{14}QL-induced STAT3 phosphorylation and activation by treating the transfectants with a specific c-Src inhibitor, PP2. As indicated in Fig. 5A, STAT3 Tyr^{705} and Ser^{727} phosphorylations in Gα_{14}QL-expressing cells were inhibited in the presence of PP2 (from 5 to 50 μM). PP2 had no effect on the endogenous STAT3 expression level (Fig. 5A, top) but reduced the amount of phosphorylated STAT3 at or greater than 10 μM, with the attenuation of Ser^{727} phosphorylation being more prominent. Furthermore, 25 μM PP2 attenuated STAT3-dependent luciferase activity in Gα_{14}QL-expressing cells compared with the corresponding controls (Fig. 5B). The specificity of PP2 was confirmed with the use of its inactive analog, PP3. Gα_{14}QL-induced STAT3 activation was not inhibited by 25 μM PP3 (Fig. 5B). To gain further insight into the involvement of c-Src in Gα_{14}QL-induced activation of STAT3, the effects of a c-Src dominant-negative mu-

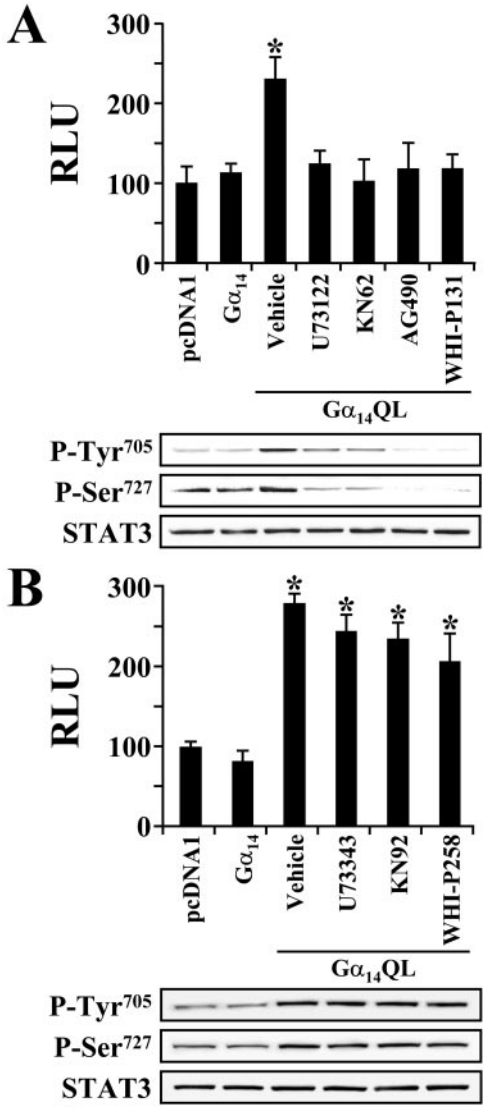

Fig. 3. The involvement of PLC-β, CaMKII, and JAK2/3 in Gα_{14}QL-induced STAT3 stimulation. pSTAT3-TA-luc cDNA was transiently transfected into HEK 293 cells along with pcDNA1, Gα_{14}, or Gα_{14}QL. A, transfectants were treated with 0.1% DMSO (v/v), 10 μM U73122, 10 μM KN62, 100 μM AG490, or 100 μg/ml JAK3 inhibitor (WHI-P131) using serum-free growth medium overnight. B, transfectants were treated with 0.1% DMSO (v/v), 10 μM U73343, 10 μM KN92, or 100 μg/ml JAK3 inhibitor negative control (WHI-P258). For reporter gene assays, STAT3-dependent luciferase activity was determined as described in Fig. 2. Data shown represent the mean ± S.E.M. from four separate experiments performed in triplicate. ⋆, $p < 0.05$, significantly increased compared with pcDNA1 control by Dunnett's t test. For Western blot analysis, cell lysates from the transfectants were immunoblotted with anti-phospho-STAT3-Tyr^{705} (top), anti-phospho-STAT3-Ser^{727} (middle), or anti-STAT3 (bottom) antiserum. The immunoblots shown represent one of three sets of immunoblots; two other sets yielded similar results.

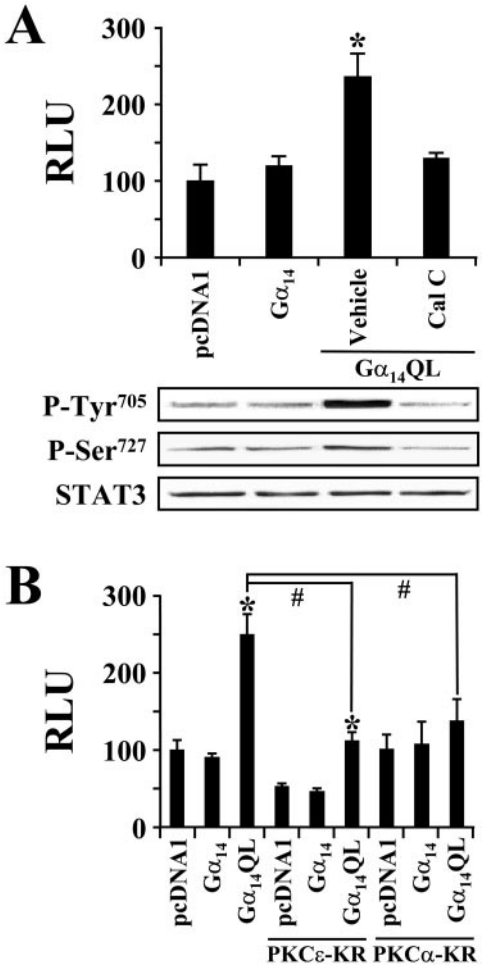

Fig. 4. The involvement of PKC in Gα_{14}QL-induced STAT3 stimulation. A, pSTAT3-TA-luc cDNA was transiently transfected into HEK 293 cells along with pcDNA1, Gα_{14}, or Gα_{14}QL. Transfectants were treated with 0.1% DMSO (v/v) or 100 nM calphostin C (Cal C) using serum-free growth medium overnight. B, pSTAT3-TA-luc cDNA was transiently transfected into HEK 293 cells along with pcDNA1, Gα_{14}, or Gα_{14}QL in combination with the dominant-negative mutants of PKCϵ (PKCϵ-KR) or PKCα (PKCα-KR). For reporter gene assays, STAT3-dependent luciferase activity was determined as described in Fig. 2. Data shown represent the mean ± S.E.M. from four separate experiments performed in triplicate. ⋆, $p < 0.05$, significantly higher than the corresponding pcDNA1 control by Dunnett's t test. #, $p < 0.05$, significantly attenuated by the dominant-negative mutants of PKC by Dunnett's t test. For Western blot analysis, cell lysates from the transfectants were immunoblotted with anti-phospho-STAT3-Tyr^{705} (top), anti-phospho-STAT3-Ser^{727} (middle), or anti-STAT3 (bottom) antiserum. The immunoblots shown represent one of three sets of immunoblots; two other sets yielded similar results.

tant (c-Src-DN) on STAT3 phosphorylation and activation were examined. As shown in Fig. 5C, STAT3 Tyr^{705} and Ser^{727} phosphorylations were attenuated in cells coexpressing c-Src-DN and $G\alpha_{14}QL$ compared with those expressing $G\alpha_{14}QL$ alone. Likewise, coexpression of c-Src-DN significantly inhibited the $G\alpha_{14}QL$-induced STAT3-dependent luciferase activity (Fig. 5C), further supporting the notion that $G\alpha_{14}QL$-induced STAT3 phosphorylation and activation were c-Src–dependent.

Because it has been demonstrated that activated $G\alpha_s$ and $G\alpha_i$ (but not $G\alpha_q$, $G\alpha_{12}$, and $G\beta\gamma$) can directly bind to and activate c-Src (Ma et al., 2000), perhaps $G\alpha_{14}QL$ can also associate with c-Src directly. To test this idea, we performed cross-linking and coimmunoprecipitation experiments using cells transfected with pcDNA1, $G\alpha_{14}$, or $G\alpha_{14}QL$. Transfectants were treated with the cross-linker DSP before immunoprecipitation with a c-Src antiserum. As shown in Fig. 5D (top), neither $G\alpha_{14}$ nor $G\alpha_{14}QL$ could be coimmunoprecipitated with c-Src despite clear evidence of c-Src being pulled down. The absence of $G\alpha_{14}$ immunoreactivity in the c-Src immunoprecipitates was not caused by a lack of expression of $G\alpha_{14}$ and $G\alpha_{14}QL$ in the transfectants, because both subunits were clearly detectable in the crude lysates by a $G\alpha_{14}$-specific antiserum (Fig. 5D, bottom). Equivalent levels of endogenous c-Src were detected in the immunoprecipitates and the crude lysates (Fig. 5D). These findings indicated that, like $G\alpha_q$, no direct physical association between $G\alpha_{14}QL$ and c-Src could be detected.

We next examined whether c-Src was downstream of PLCβ in response to stimulation by $G\alpha_{14}QL$. As shown in Fig. 5E, $G\alpha_{14}QL$ significantly induced the phosphorylation of c-Src compared with the vector- and $G\alpha_{14}$-transfected cells. $G\alpha_{14}QL$-transfected cells were incubated with 10 μM U73122, 100 nM calphostin C, or 10 μM KN62. $G\alpha_{14}QL$-induced c-Src phosphorylation was partially inhibited by U73122, calphostin C, and KN62, whereas the total expression level of c-Src was unaffected (Fig. 5E). These observations suggested that $G\alpha_{14}QL$-induced c-Src phosphorylation required PLCβ, PKC, and CaMKII.

The Involvement of ERK in $G\alpha_{14}QL$-Induced STAT3 Stimulation. G proteins are important upstream regulators

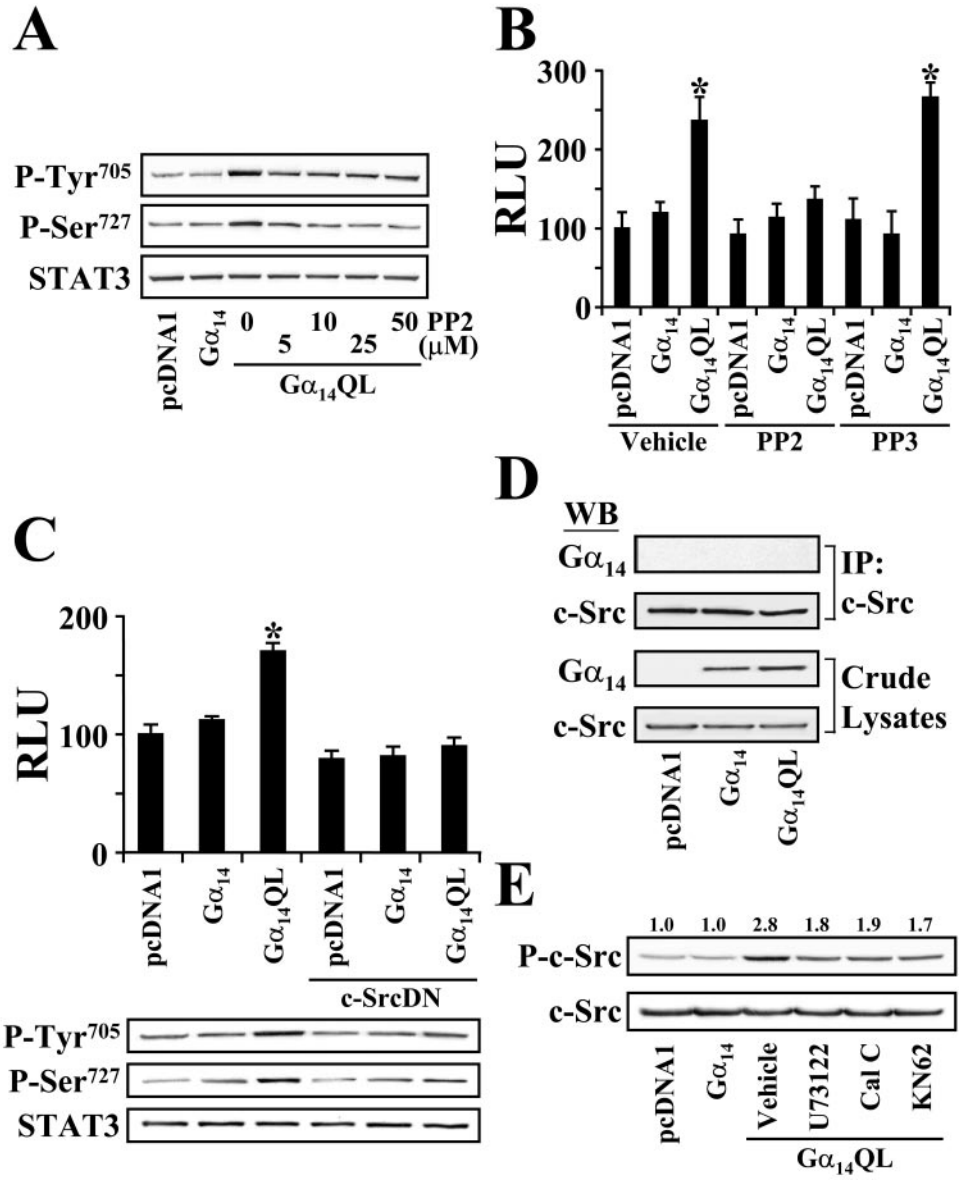

Fig. 5. The involvement of c-Src in $G\alpha_{14}QL$-induced STAT3 stimulation. HEK 293 cells were transiently transfected with pcDNA1, $G\alpha_{14}$ or $G\alpha_{14}QL$ cDNAs. A, transfectants were treated with indicated concentration of PP2. B, transfectants were treated with 0.1% DMSO (v/v), 25 μM PP2, or 25 μM PP3 overnight. C, HEK 293 cells were further cotransfected pcDNA1, $G\alpha_{14}$, or $G\alpha_{14}QL$ in combination with c-Src dominant-negative mutant c-Src-K295R/Y527F (c-SrcDN). For Western blot analysis, cell lysates from the transfectants were immunoblotted with anti-phospho-STAT3-Tyr^{705} (top), anti-phospho-STAT3-Ser^{727} (middle), or anti-STAT3 (bottom) antiserum. The immunoblots shown represent one of three sets of immunoblots; two other sets yielded similar results. For reporter gene assays, STAT3-dependent luciferase activity was determined as described in Fig. 2. Data shown represent the means ± S.E.M. from four separate experiments performed in triplicate. ⋆, $p < 0.05$, significantly higher than the corresponding pcDNA1 control by Dunnett's t test. D, after cross-linking, cell lysates from the transfectants were immunoprecipitated (IP) by c-Src antiserum (top). The immunoprecipitates were immunoblotted with $G\alpha_{14}$ or c-Src antiserum. Aliquots of crude lysates were used to detect $G\alpha_{14}$ and c-Src expression levels by Western blot analysis (bottom). E, transfectants were treated with 0.1% DMSO (v/v), 10 μM U73122, 100 nM calphostin C (Cal C), or 10 μM KN62 using serum-free growth medium overnight. Cell lysates from the transfectants were immunoblotted with anti-phospho-c-Src (top) or anti-c-Src (bottom). Numerical values shown above the immunoreactive bands represent the relative intensities of c-Src phosphorylation compared with the basal.

of numerous signal transduction pathways, including the MAPK cascades. The ERK, JNK, and p38 MAPK have all been shown to exert regulatory influence on STAT3 activity. Thus, we sought to determine whether MAPKs were required for $G\alpha_{14}$QL-induced STAT3 activation. We first established whether $G\alpha_{14}$QL can activate ERK, JNK, and p38 MAPK by using anti-phospho-MAPK antisera. All three subfamilies of MAPK became significantly phosphorylated in cells expressing $G\alpha_{14}$QL, in contrast to the corresponding control cells (Fig. 6A). Total amounts of endogenous ERK, JNK, and p38 MAPK were not affected by overexpressing $G\alpha_{14}$ or $G\alpha_{14}$QL (Fig. 6A). These observations indicated that $G\alpha_{14}$QL was capable of inducing the phosphorylation of ERK, JNK, and p38 MAPK.

To test whether one or more MAPKs were involved in $G\alpha_{14}$QL-induced STAT3 phosphorylation and activation, specific kinase inhibitors were used. Before determining the activation status of STAT3, cells transfected with pcDNA1, $G\alpha_{14}$, or $G\alpha_{14}$QL were pretreated with selective inhibitors (10 μM each) against Raf-1 and MEK1/2 (U0126), two upstream kinases in the ERK cascade. As shown in Fig. 6B, $G\alpha_{14}$QL-induced STAT3 Tyr^{705} and Ser^{727} phosphorylations were attenuated in the presence of either inhibitor, whereas total STAT3 expression was unaffected. Moreover, both inhibitors significantly suppressed the STAT3-dependent luciferase activity in $G\alpha_{14}$QL-expressing cells (Fig. 6B). The specificity of U0126 was verified with the use of its inactive analog, U0124. $G\alpha_{14}$QL-induced STAT3 responses were not inhibited by 10 μM U0124 (Fig. 6B). Our findings demonstrated that the ERK signaling cascade participated in $G\alpha_{14}$QL-induced STAT3 phosphorylation and activation. We then used specific kinase inhibitors to determine whether JNK and p38 MAPK were also involved. Our findings demonstrated that neither SB202190 nor SB203580 at 10 μM inhibited Tyr^{705} and Ser^{727} phosphorylations of STAT3 or STAT3-dependent luciferase activity in $G\alpha_{14}$QL-expressing cells (Fig. 6B), implying that both JNK and p38 MAPK were not required for $G\alpha_{14}$QL-induced STAT3 activation. In Fig. 6C, the control experiment indicated that $G\alpha_{14}$QL-induced JNK and p38 MAPK activations were completely suppressed by SB202190, whereas SB203580 only inhibited the activation of p38 MAPK. The lack of effect of JNK/p38 MAPK inhibitors further highlighted the importance of the ERK pathway for $G\alpha_{14}$QL-induced STAT3 activation.

The Involvement of Ras and Rac1 in $G\alpha_{14}$QL-Induced STAT3 Stimulation. A number of $G\alpha$ subunits have recently been reported to regulate MAPKs via the monomeric GTPases such as Ras and Rac1 (Woo and Kim, 2002). It has

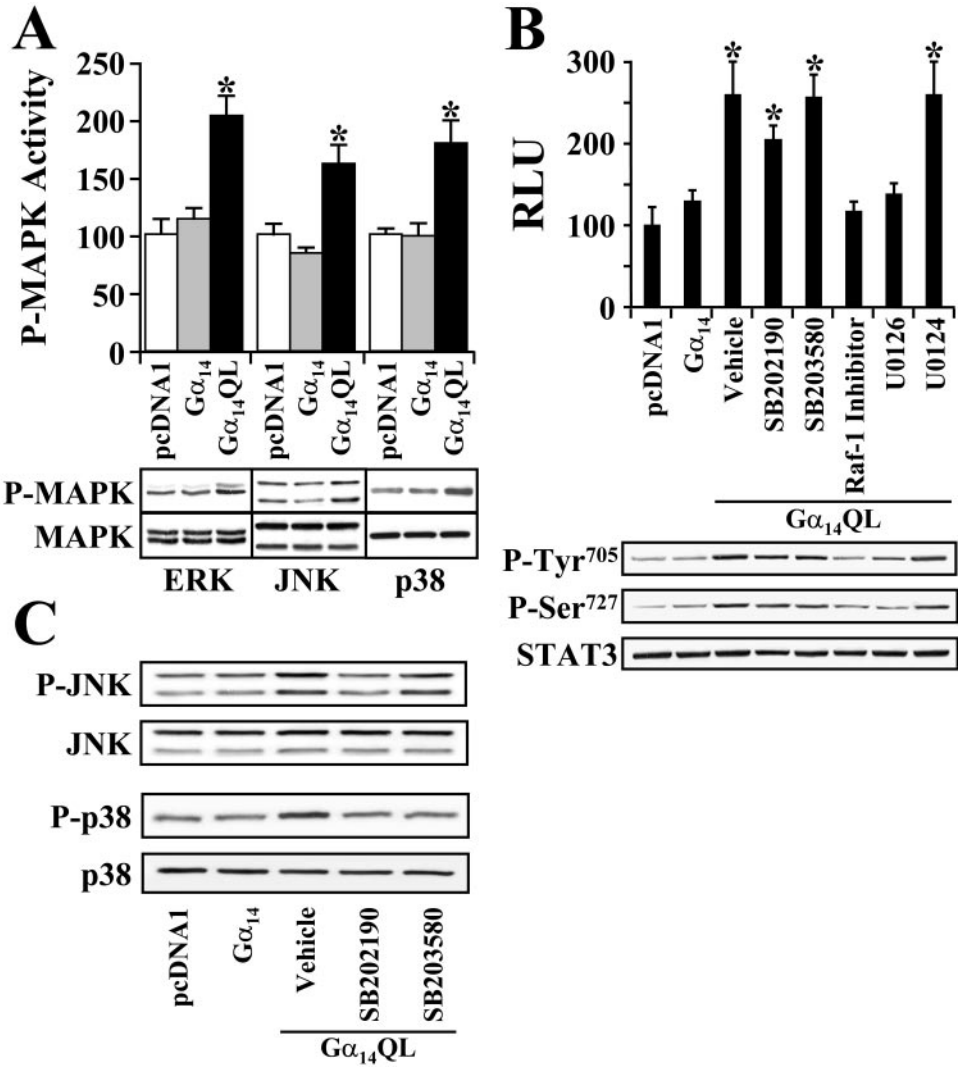

Fig. 6. The involvement of ERK signaling cascades in $G\alpha_{14}$QL-induced STAT3 stimulation. HEK 293 cells were transiently transfected with pcDNA1, $G\alpha_{14}$, or $G\alpha_{14}$QL. A, phosphorylations of ERK, JNK, and p38 MAPK (p38) in the cell lysates were determined by immunoblotting with anti–phospho-specific antisera against each of the three MAPKs (top). The result of the densitometry analysis is shown above the immunoblots; data shown represent the mean ± S.E.M. from three separate experiments. ⋆, $p < 0.05$, significantly higher than pcDNA1 control by Dunnett's t test. The bottom of each set of immunoblots represents total MAPKs present in the cell lysates. B, cells were treated with 0.1% DMSO (v/v), 10 μM SB202190, 10 μM SB203580, 10 μM Raf-1 kinase inhibitor, 10 μM U0126, or 10 μM U0124 using serum-free growth medium overnight. Cell lysates from the transfectants were immunoblotted with anti-phospho-STAT3-Tyr^{705} (top), anti-phospho-STAT3-Ser^{727} (middle), or anti-STAT3 (bottom) antiserum. C, cells were incubated with 0.1% DMSO (v/v), 10 μM SB202190, or 10 μM SB203580 using serum-free growth medium overnight. Phosphorylations of JNK and p38 MAPK (p38) in the cell lysates were determined by immunoblotting with anti–phospho-specific antisera against each of the two MAPKs. For reporter gene assays, STAT3-dependent luciferase activity was determined as described in Fig. 2. Data shown represent the mean ± S.E.M. from four separate experiments performed in triplicate. ⋆, $p < 0.05$, significantly higher than pcDNA1 control by Dunnett's t test. For Western blot analysis, data shown represent one of three sets of immunoblots; two other sets yielded similar results.

previously been demonstrated that Rac is necessary for GPCR-induced activation of JAK/STAT signaling (Pelletier et al., 2003). It was therefore of particular interest to study the role of Ras and Rac1 in $G\alpha_{14}$QL-induced STAT3 phosphorylation and activation. HEK 293 cells were overexpressed with the cDNAs of pcDNA1, $G\alpha_{14}$, or $G\alpha_{14}$QL in combination with various mutants of either Ras or Rac1. Transfectants were then assayed for STAT3 activity. If the monomeric GTPases were required for $G\alpha_{14}$QL-induced STAT3 activity, their constitutively active mutants should facilitate the response, whereas their dominant-negative mutants should suppress it. As depicted in Fig. 7B, STAT3 Tyr^{705} and Ser^{727} phosphorylations were increased in cells transfected with the constitutively activated mutant of Ras (RasG12V) in combination with either $G\alpha_{14}$ or $G\alpha_{14}$QL. RasG12V alone was capable of inducing the phosphorylations of STAT3 (data not shown). On the contrary, a dominant-negative mutant of Ras (RasS17N) significantly reduced STAT3 Tyr^{705} and Ser^{727} phosphorylations in $G\alpha_{14}$QL-expressing cells (Fig. 7B). Participation of Ras was further demonstrated with the reporter gene assay. RasG12V significantly stimulated STAT3-dependent luciferase activity in $G\alpha_{14}$-expressing cells compared with the vector or wild-type Ras transfectants (Fig. 7A). The dominant-negative RasS17N effectively abolished the $G\alpha_{14}$QL-induced STAT3 luciferase activity (Fig. 7A). Likewise, the constitutively activated mutant of Rac1 (Rac1G12V) stimulated STAT3 Tyr^{705} and Ser^{727} phosphorylations as well as STAT3-dependent luciferase activity when expressed alone (data not shown) or with $G\alpha_{14}$ (Fig. 7, A and B). The dominant-negative mutant of Rac1 (Rac1T17N) inhibited STAT3 Tyr^{705} and Ser^{727} phosphorylations as well as the STAT3-dependent luciferase activity in cells coexpressing $G\alpha_{14}$QL (Fig. 7, A and B). These results implied that both Ras and Rac1 were involved in $G\alpha_{14}$QL-induced STAT3 phosphorylation and activation.

Phosphoinositide-3 Kinase Is Not Required for $G\alpha_{14}$QL-Induced STAT3 Stimulation. Phosphoinositide-3 kinase (PI3K) family members control several cellular responses, including cell growth and survival. It has been demonstrated that the erythropoietin receptor activates MAPK via Ras/PI3K signaling in Chinese hamster ovary cells, whereas c-Src is capable of stimulating PI3K phosphorylation (Kumar et al., 2003). In addition, PI3K has been shown to play a critical role in the regulation of STAT3 Ser^{727} phosphorylation triggered by interleukin-2 (Fung et al., 2003). These established linkages between PI3K and the various signaling molecules (c-Src, Ras, and ERK) prompted us to examine the role of PI3K in $G\alpha_{14}$QL-induced STAT3 activation. We treated $G\alpha_{14}$QL-expressing cells with PI3K-specific inhibitors, 100 nM wortmannin or 10 μM LY294002. Neither wortmannin nor LY294002 had any inhibitory effect on $G\alpha_{14}$QL-induced STAT3 Tyr^{705} and Ser^{727} phosphorylations in $G\alpha_{14}$QL-expressing cells (Fig. 8A). Similar expression level of endogenous STAT3 was detected by STAT3 antiserum in these cells (Fig. 8A). In addition, $G\alpha_{14}$QL-induced STAT3-dependent luciferase activity was not inhibited by the PI3K inhibitors compared with the corresponding control (Fig. 8A). The role of PI3K in $G\alpha_{14}$QL-induced STAT3 phosphorylation and activation was further characterized by using a dominant-negative mutant of PI3Kγ (PI3KγDN). As illustrated in Fig. 8B, PI3KγDN did not affect STAT3 Tyr^{705} and Ser^{727} phosphorylations induced by $G\alpha_{14}$QL, and the dominant-negative mutant had no effect on the total amount of endogenous STAT3. Expression of PI3KγDN did not suppress the STAT3-dependent luciferase activity in $G\alpha_{14}$QL-expressing cells (Fig. 8B). Because Akt is a downstream effector of PI3K, we were able to demonstrate that wortman-

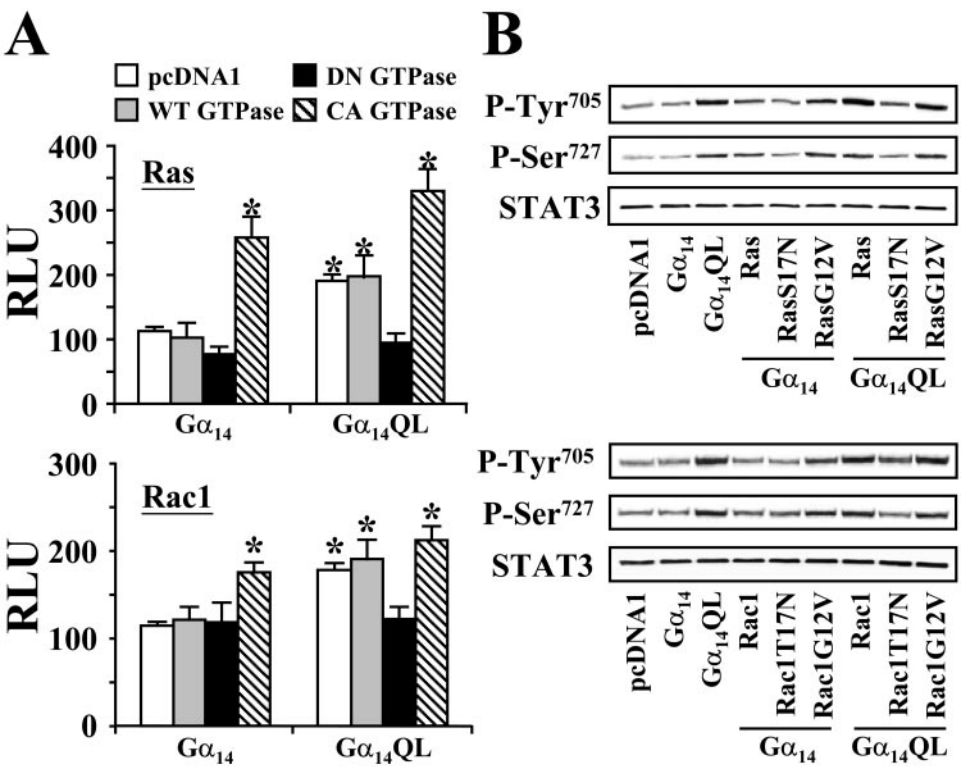

Fig. 7. The involvement of Ras and Rac1 in $G\alpha_{14}$QL-induced STAT3 stimulation. pSTAT3-TA-luc cDNA was transiently transfected into HEK 293 cells along with pcDNA1, $G\alpha_{14}$, or $G\alpha_{14}$QL in combination with the wild-type or mutant of Ras or Rac1. A, for reporter gene analysis, STAT3-dependent luciferase activity was determined as in the legend to Fig. 2. Data shown represent the mean ± S.E.M. from four separate experiments performed in triplicate. ⋆, $p < 0.05$, significantly higher than the pcDNA1 control by Dunnett's t test. B, for Western blot analysis, cell lysates from the transfectants were immunoblotted with anti-phospho-STAT3-Tyr^{705} (top), anti-phospho-STAT3-Ser^{727} (middle), or anti-STAT3 (bottom) antiserum. The immunoblots shown represent one of three sets of immunoblots; two other sets yielded similar results.

nin or LY294002 treatment completely inhibited EGF-induced Akt phosphorylation but not ERK phosphorylation, confirming that the activity of PI3K was indeed inhibited under these conditions (Fig. 8C). These studies collectively indicated that PI3K was not involved in $G\alpha_{14}$QL-induced STAT3 phosphorylation and activation in HEK 293 cells.

The Involvement of Various Signaling Intermediates in DPDPE-Induced STAT3 Phosphorylation in HEL Cells. Finally, we investigated the involvement of various signaling intermediates in DPDPE-induced STAT3 phosphorylation in HEL cells. HEL cells were pretreated with 100 ng/ml PTX overnight to eliminate the potential involvement of PTX-sensitive G_i proteins in regulating DPDPE-induced STAT3 phosphorylation. As already demonstrated in Fig. 1B, DPDPE was capable of inducing the phosphorylation of STAT3 at Tyr^{705} and Ser^{727} in PTX-treated HEL cells; however, the DPDPE-induced STAT3 phosphorylation was markedly inhibited by Raf-1 inhibitor, U73122, AG490, calphostin C, U0126, and PP2 (Fig. 9). These findings implied that DPDPE-induced STAT3 phosphorylation in HEL cells required the participation of Raf-1, PLCβ, JAK2, PKC, MEK1/2, and c-Src. Because the same signaling intermediates apparently were required for DPDPE-induced STAT3 phosphorylation in both HEK 293 and HEL cells, it further supported the notion that $G\alpha_{14}$ may well be the PTX-insensitive G protein mediating DPDPE-induced STAT3 activation in HEL cells.

Discussion

The restrictive expression pattern of $G\alpha_{14}$ suggests that it may subserve specialized functions, such as regulation of hematopoietic cell differentiation. Because hematopoietic-specific $G\alpha_{16}$ can regulate gene transcription (Yang et al., 2001; Lo et al., 2003), it is plausible that $G\alpha_{14}$ may possess a similar ability. First, we demonstrated that DPDPE-stimulated DOR was capable of inducing PTX-insensitive STAT3 phosphorylation in the presence of $G\alpha_{14}$. Second, expression of $G\alpha_{14}$QL, but not $G\alpha_{14}$, induced STAT3 Tyr^{705}/Ser^{727} phosphorylation and STAT3 transcriptional activation. Third, coexpression of STAT3 phosphorylation-resistant mutants revealed that $G\alpha_{14}$QL-induced STAT3 Tyr^{705} and Ser^{727} phosphorylation could occur independently. These observations illustrate that STAT3 activity can indeed be elicited by $G\alpha_{14}$ activation.

Many GPCRs have been shown to regulate STAT activities. Initial studies have implicated the involvement of JAK in angiotensin II AT1 receptor-induced STAT1/3 activation

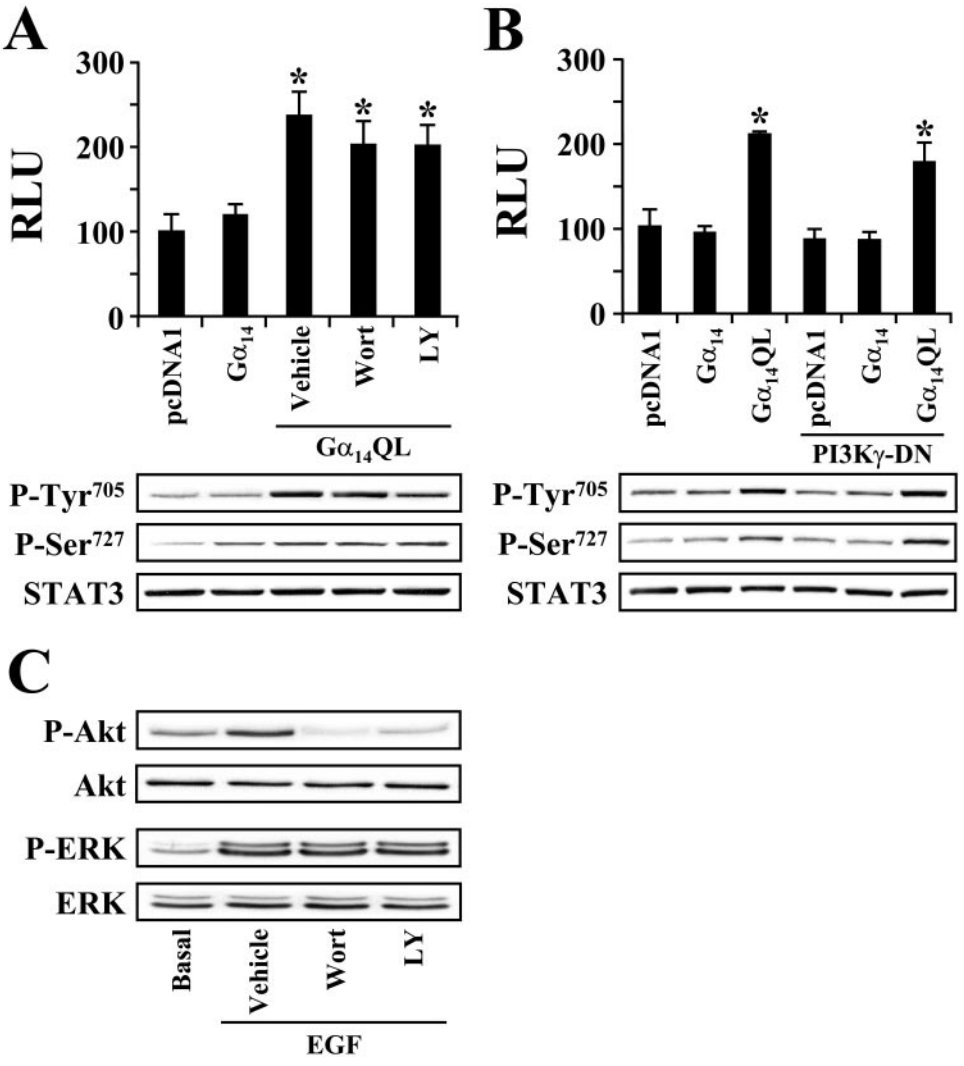

Fig. 8. No involvement of PI3K in $G\alpha_{14}$QL-induced STAT3 stimulation. A, pSTAT3-TA-luc cDNA was transiently transfected into HEK 293 cells along with pcDNA1, $G\alpha_{14}$, or $G\alpha_{14}$QL. Transfectants were treated with 0.1% DMSO (v/v), 100 nM wortmannin (Wort), or 10 μM LY294002 (LY) using serum-free growth medium overnight. B, pSTAT3-TA-luc cDNA was transiently transfected into HEK 293 cells along with pcDNA1, $G\alpha_{14}$, or $G\alpha_{14}$QL in combination with PI3Kγ dominant-negative mutant (PI3KγDN). For reporter gene assays, STAT3-dependent luciferase activity was determined as described in Fig. 2. Data shown represent the means ± S.E.M. from four separate experiments performed in triplicate. ⋆, $p < 0.05$, significantly higher than the corresponding pcDNA1 control by Dunnett's t test. For Western blot analysis, cell lysates from the transfectants were immunoblotted with anti-phospho-STAT3-Tyr^{705} (top), anti-phospho-STAT3-Ser^{727} (middle), or anti-STAT3 (bottom) antiserum. C, HEK 293 cells were treated with 0.1% DMSO (v/v), 100 nM wortmannin, or 10 μM LY294002 and then incubated with EGF for 5 min. Phospho-Akt, Akt, phospho-ERK, and ERK proteins were detected by specific primary antisera. The immunoblots shown represent one of three sets of immunoblots; two other sets yielded similar results.

(McWhinney et al., 1997). Our results also demonstrate that JAK2/3 is required for $G\alpha_{14}$QL-induced STAT3 activation. Recent studies suggest a possible involvement of c-Src. STAT3 activation induced by $G\alpha_{i2}$ and $G\alpha_{o}$ is Src-dependent (Corre et al., 1999; Ram et al., 2000), and JAKs can be directly tyrosine-phosphorylated by c-Src (Tsai et al., 2000). Our data indicate that $G\alpha_{14}$QL-induced STAT3 activation is inhibited by PP2 and c-Src-DN, implying that c-Src plays a role in $G\alpha_{14}$QL-induced STAT3 activation. Moreover, c-Src acts as a direct effector of G proteins because it can associate with activated $G\alpha_{s}$ or $G\alpha_{i}$, but not $G\alpha_{q}$, $G\alpha_{12}$, and $G\beta\gamma$ (Ma et al., 2000). Like $G\alpha_{q}$, $G\alpha_{14}$QL was unable to associate with c-Src in immunoprecipitation assays, suggesting that $G\alpha_{14}$QL-induced c-Src activation relies on mechanisms other than direct physical association. Hence, some adapter proteins or protein kinases may be involved in $G\alpha_{14}$QL-induced c-Src activation. An adaptor protein, tetratricopeptide repeat 1 (TPR1), has indeed been demonstrated to interact with $G\alpha_{16}$ and $G\alpha_{q}$ (Marty et al., 2003). Because extensive cross-talk between GPCRs and growth factor receptors is known to exist (Lowes et al., 2002), the Src/JAK/STAT3 pathway may represent yet another example of signaling cascades shared by two classes of receptors.

Our results also support a role for PLCβ cascade in $G\alpha_{14}$QL-induced STAT3 activation. PLCβ, CaMKII, and PKC-selective inhibitors, as well as coexpression of PKCα and PKCϵ dominant-negative mutants, significantly suppressed $G\alpha_{14}$QL-induced STAT3 activation. The fact that dominant-negative mutants of both PKCα and PKCϵ were effective might perhaps be explained by their binding to a common activator or effector. A previous study has shown that both PKCα and PKCϵ are required to mediate platelet-derived growth factor-induced ERK stimulation through the Grb2-associated binder-1 protein in vascular smooth muscle cells (Saito et al., 2002). It remains to be determined whether a similar mechanism applies to STAT3 activation. Although we did not investigate the contribution of atypical PKC in this signaling pathway, these PKC isoforms are unlikely to be required for $G\alpha_{14}$QL-induced STAT3 activation because their upstream activator, PI3K, did not participate in this signaling cascade (Fig. 8A). Apart from that, $G\alpha_{14}$QL was capable of inducing c-Src phosphorylation, which was partially suppressed by inhibitors against PLCβ, CaMKII, and PKC. Partial suppression of $G\alpha_{14}$QL-induced c-Src phosphorylation by these inhibitors implies that c-Src may play a secondary role in $G\alpha_{14}$QL-induced STAT3 activation. Nevertheless, these results provide clues as to how $G\alpha_{14}$QL can be indirectly linked to c-Src. In hypothalamic gonadotropin-releasing hormone neurons, gonadotropin-releasing hormone induces Src association with PKCα and PKCϵ, leading to Src-dependent transactivation of the EGF receptor (Shah et al., 2003). It has also been demonstrated in glomerular mesangial cells that c-Src can be activated via CaMKII (Wang et al., 2003). Hence, $G\alpha_{14}$QL can potentially stimulate the Src/JAK/STAT3 pathway through the PLCβ/PKC cascade (Fig. 10). If the PLCβ/PKC/CaMKII cascade constitutes a primary signal for $G\alpha_{14}$QL-induced STAT3 activation, other members of G_{q} subfamily may also activate STAT3. Indeed, we have demonstrated that several GPCRs can activate STAT3 in the presence of $G\alpha_{16}$ (Lo et al., 2003; Wu et al., 2003). It should be noted that the PLCβ/PKC/CaMKII cascade is not required for $G\alpha_{i2/o}$-induced STAT3 activation (Corre et al., 1999; Ram et al., 2000).

Small GTPases seem to occupy a critical juncture in signaling network that leads to STAT3 activation. Ras and Rac1

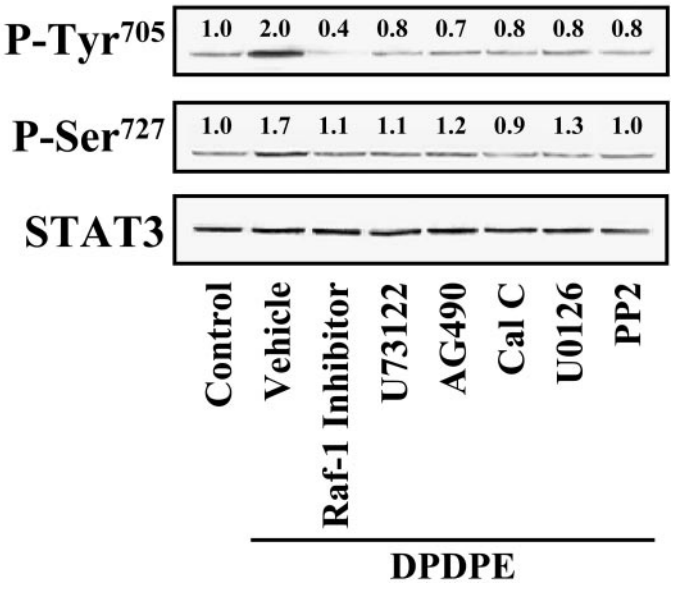

Fig. 9. The involvement of various signaling intermediates in DPDPE-induced STAT3 phosphorylation in HEL cells. HEL cells were seeded at a density of 1×10^6 cells using serum-free medium with 100 ng/ml PTX treatment overnight. Before100 nM DPDPE treatment for 30 min, cells were preincubated with 0.1% DMSO (v/v), 10 μM Raf-1 inhibitor, 10 μM U73122, 100 μM AG490, 100 nM calphostin C, 10 μM U0126, or 25 μM PP2 for 30 min. Cell lysates were immunoblotted with anti-phospho-STAT3-Tyr^{705} (top), anti-phospho-STAT3-Ser^{727} (middle), or anti-STAT3 (bottom) antiserum. Numerical values shown above the immunoreactive bands represent the relative intensities of STAT3 phosphorylation compared with the corresponding basal. The immunoblots shown represent one of three sets of immunoblots; two other sets yielded similar results.

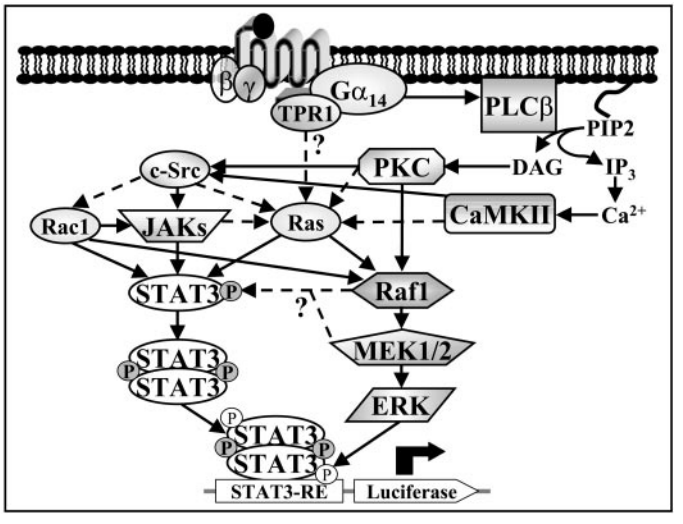

Fig. 10. Proposed signaling pathways leading to STAT3 activation by DPDPE-stimulated DOR via $G\alpha_{14}$. The PLCβ and c-Src/JAK pathways stimulated by $G\alpha_{14}$ converge at Ras and activate STAT3 via the Ras/Raf/MEK/ERK cascade. $G\alpha_{14}$ may directly interact with TPR1 and in turn activate the ERK cascade. Full activation of STAT3 by the activated $G\alpha_{14}$ is achieved by costimulation of c-Src/JAK- and ERK-dependent pathways. P in solid circle represents Tyr^{705} phosphorylation, whereas P in open circle represents Ser^{727} phosphorylation. Broken lines demonstrate that the protein regulations involve multiple steps. The experimental evidence identifying individual pathways and the interactions between their intermediates is described in the text.

act as molecular switches for numerous extracellular stimuli. In the present study, the inhibition of $G\alpha_{14}$QL-induced STAT3 activation by Ras and Rac1 dominant-negative mutants verified the importance of these GTPases. Because Ras is downstream of Src, JAK, and CaMKII (Abe and Berk, 1999; Odajima et al., 2000), it raises the possibility that $G\alpha_{14}$QL-stimulated c-Src/JAK and PLCβ pathways converge at Ras to activate STAT3 via the Ras/Raf/MEK/ERK cascade. Apart from Ras, Rac1 activation by c-Src has been demonstrated (Timokhina et al., 1998). Activated Rac1 induces JAK2 and STAT3 activation in COS-1 cells (Simon et al., 2000). ERK activation by Rac1 is not without precedence. It has been shown that ERK is situated downstream of Rac1 (Woo and Kim, 2002) and Rac1-induced ERK activation occurs in a PAK- and Raf-dependent manner (Tang et al., 1999). Because PAK is activated mainly by Rac1, it may represent a link between Rac1 and ERK cascade. Recent studies demonstrated that both Ras and Rac1 are involved in lysophosphatidic acid-induced ERK activation (Stahle et al., 2003). It is reasonable to suggest that $G\alpha_{14}$QL-induced stimulatory signals of Ras and Rac1 may converge at the level of ERK and ultimately activate STAT3.

There is a large body of evidence to illustrate the regulatory role of MAPKs in STAT3 activation. STAT3 activation can be induced by various components along the ERK, JNK, and p38 MAPK pathways (Haq et al., 2002). Although $G\alpha_{14}$QL can activate ERK, JNK, and p38 MAPK, only the ERK pathway is required for $G\alpha_{14}$QL-induced STAT3 activation (Fig. 6). Linkages to ERK from PLCβ cascades can be traced back to PKC and CaMKII, both of which can activate the Ras/Raf/MEK/ERK cascades (Yang et al., 2003). PKC is known to phosphorylate Raf-1 (Kolch et al., 1993), whereas CaMKII is required for ERK activation of vascular smooth muscle (Ginnan and Singer, 2002). The possibility of $G\alpha_{14}$QL activating Ras via TPR1 should also be considered. It is therefore plausible that ERK serves as a convergent point for multiple signals (e.g., Src, PLCβ, PKC, and CaMKII) that originate from $G\alpha_{14}$QL. In contrast, $G\alpha_o$QL-mediated STAT3 activation is ERK-independent (Ram et al., 2000). G_i and G_q subfamily members may used overlapping yet distinct mechanisms to stimulate STAT3, because they both require c-Src but differ in their dependencies on ERK. Another observation in the present study pertains to the lack of PI3K involvement. Recent studies have revealed that PI3K can phosphorylate STAT3 at Ser^{727} (Fung et al., 2003), and it acts downstream of Ras and c-Src (Kumar et al., 2003). It is surprising that PI3K inhibition in the present study could not abolish the ability of $G\alpha_{14}$QL to induce STAT3 activation, implying that PI3K does not participate in $G\alpha_{14}$QL-induced STAT3 activation.

The present study provides the first evidence that $G\alpha_{14}$ can link GPCR stimulation to STAT3 activation via complicated signaling pathways. Our data indicate that $G\alpha_{14}$QL-induced STAT3 activation involves multiple pathways ranging from PLCβ to c-Src/JAK to ERK. A rudimentary map for the signaling network can be constructed from the experiments described herein (Fig. 10). It is unclear why JNK and p38 MAPK do not participate in $G\alpha_{14}$QL-induced STAT3 activation even though they have been demonstrated to stimulate STAT3 (Turkson et al., 1999). Because the use of $G\alpha_{14}$QL eliminates any potential contributions by G$\beta\gamma$, STAT3 activation by $G\alpha_{14}$-coupled receptors may exhibit different characteristics, especially when G$\beta\gamma$ can modulate a host of effectors. For GPCRs that activate both $G_{i/o}$ and $G\alpha_{14}$ (e.g., DOR and ORL_1 receptors), their mechanisms for activating STAT3 will be more complicated because the two G protein subfamilies may use different pathways.

The biological implications of $G\alpha_{14}$-mediated STAT3 activation are currently unclear, but the present study might provide a framework for such explorations. Because STAT3 regulates gene transcription in cell proliferation and differentiation, it plays a prominent role in hematopoiesis (Coffer et al., 2000). Little is known regarding the capability of GPCRs to regulate hematopoiesis. Among GPCRs recognized by $G\alpha_{14}$, DOR is expressed in immune cells in which it modulates T-lymphocytes activation via PTX-sensitive G proteins (Sharp et al., 1998). However, the possibility that PTX-insensitive G proteins may also participate in the regulation of DOR-stimulated immune responses cannot be eliminated. In HEL cells, we illustrated that DPDPE-induced STAT3 Tyr^{705} and Ser^{727} phosphorylations are resistant to PTX, implying that these phosphorylations are $G_{i/o}$-independent. Because HEL cells express both $G\alpha_{14}$ and $G\alpha_{16}$ and that the latter can also interact productively with DOR, it remains to be demonstrated whether either or both Gα subunits are required for DPDPE-induced STAT3 phosphorylation. Nevertheless, DPDPE-mediated STAT3 phosphorylation required Raf-1, PLCβ, JAK2, c-Src, PKC, and MEK1/2 in much the same manner observed in HEK 293 cells. With new small-interfering RNA technology, it should be possible to test whether $G\alpha_{14}$-mediated STAT3 activation plays a role in hematopoiesis. Another direction for further exploration of the $G\alpha_{14}$/STAT3 pathway is in chemokine signaling. Chemokine receptors can couple to $G\alpha_{14}$ and $G\alpha_{16}$ in a cell type-specific manner (Arai and Charo, 1996), and they have already been shown to regulate transcriptional activity via nuclear factor-κB in a $G\alpha_{16}$-dependent manner. It is likely that these chemokine receptors can regulate STAT3 activity via either $G\alpha_{16}$ or $G\alpha_{14}$. In addition,because STAT3 constitutive activation can lead to cellular transformation, $G\alpha_{14}$QL may be oncogenic by virtue of its stimulatory effect on STAT3. Indeed, we have obtained preliminary results indicating that NIH-3T3 cells overexpressing $G\alpha_{14}$QL exhibit higher growth rate and saturation density than those harboring $G\alpha_{14}$. It remains to be investigated whether $G\alpha_{14}$QL-induced STAT3 activation can lead to neoplastic transformation of cells.

Acknowledgments

We are extremely grateful to the following persons for kindly providing the various cDNAs: Christopher Carpenter, Bernard Weinstein, Jeffery Field, Marc Symons, and Nancy Reich. We thank Maurice Ho and Joy Chan for helpful discussions.

References

Abe J and Berk BC (1999) Fyn and JAK2 mediate Ras activation by reactive oxygen species. *J Biol Chem* **274:**21003–21010.

Arai H and Charo IF (1996) Differential regulation of G-protein-mediated signaling by chemokine receptors. *J Biol Chem* **271:**21814–21819.

Chan ASL and Wong YH (2000) Regulation of c-Jun N-terminal kinase by the ORL_1 receptor through multiple G proteins. *J Pharmacol Exp Ther* **295:**1094–1100.

Coffer PJ, Koenderman L, and de Groot RP (2000) The role of STATs in myeloid differentiation and leukemia. *Oncogene* **19:**2511–2522.

Corre I, Baumann H, and Hermouet S (1999) Regulation by G_{i2} proteins of v-fms-induced proliferation and transformation via Src-kinase and STAT3. *Oncogene* **18:**6335–6342.

Fung MM, Rohwer F, and McGuire KL (2003) IL-2 activation of a PI3K-dependent

STAT3 serine phosphorylation pathway in primary human T cells. *Cell Signal* **15:**625–636.
Ginnan R and Singer HA (2002) CaMKII-dependent activation of tyrosine kinases and ERK1/2 in vascular smooth muscle. *Am J Physiol* **282:**C754–C761.
Haq R, Halupa A, Beattie BK, Mason JM, Zanke BW, and Barber DL (2002) Regulation of erythropoietin-induced STAT serine phosphorylation by distinct mitogen-activated protein kinases. *J Biol Chem* **277:**17359–17366.
Hirano T, Ishihara K, and Hibi M (2000) Roles of STAT3 in mediating the cell growth, differentiation and survival signals relayed through the IL-6 family of cytokine receptors. *Oncogene* **19:**2548–2556.
Ho MKC, Yung LY, Chan JSC, Chan JHP, Wong CSS, and Wong YH (2001) $G\alpha_{14}$ links a variety of G_i- and G_s-coupled receptors to the stimulation of phospholipase C. *Br J Pharmacol* **132:**1431–1440.
Kam AYF, Chan ASL, and Wong YH (2003) Rac and Cdc42-dependent regulation of c-Jun N-terminal kinases by the δ-opioid receptor. *J Neurochem* **84:**503–513.
Kolch W, Heidecker G, Kochs G, Hummel R, Vahidi H, Mischak H, Finkenzeller G, Marme D, and Rapp UR (1993) Protein kinase Cα activates Raf-1 by direct phosphorylation. *Nature (Lond)* **364:**249–252.
Kumar P, Amin MA, Harlow LA, Polverini PJ, and Koch AE (2003) Src and phosphatidylinositol 3-kinase mediate soluble E-selectin-induced angiogenesis. *Blood* **101:**3960–3968.
Liu AM, Ho MK, Wong CS, Chan JH, Pau AH, and Wong YH (2003) $G\alpha_{16/z}$ chimeras efficiently link a wide range of G protein-coupled receptors to calcium mobilization. *J Biomol Screen* **8:**39–49.
Lo RKH, Cheung H, and Wong YH (2003) Constitutively active $G\alpha_{16}$ stimulates STAT3 via a c-Src/JAK-and ERK-dependent mechanism. *J Biol Chem* **278:**52154–52165.
Lowes VL, Ip NY, and Wong YH (2002) Integration of signals from receptor tyrosine kinases and G protein-coupled receptors. *Neurosignals* **11:**5–19.
Ma YC, Huang J, Ali S, Lowry W, and Huang XY (2000) Src tyrosine kinase is a novel direct effector of G proteins. *Cell* **102:**635–646.
Maeda S, Wu S, Juppner H, Green J, Aragay AM, Fagin JA, and Clemens TL (1996) Cell-specific signal transduction of parathyroid hormone (PTH)-related protein through stably expressed recombinant PTH/PTHrP receptors in vascular smooth muscle cells. *Endocrinology* **137:**3154–3162.
Marty C, Browning DD, and Ye RD (2003) Identification of tetratricopeptide repeat 1 as an adaptor protein that interacts with heterotrimeric G proteins and the small GTPase Ras. *Mol Cell Biol* **23:**3847–3858.
McWhinney CD, Hunt RA, Conrad KM, Dostal DE, and Baker KM (1997) The type I angiotensin II receptor couples to STAT1 and STAT3 activation through JAK2 kinase in neonatal rat cardiac myocytes. *J Mol Cell Cardiol* **29:**2513–2524.
Odajima J, Matsumura I, Sonoyama J, Daino H, Kawasaki A, Tanaka H, Inohara N, Kitamura T, Downward J, Nakajima K, et al. (2000) Full oncogenic activities of v-Src are mediated by multiple signaling pathways. Ras as an essential mediator for cell survival. *J Biol Chem* **275:**24096–24105.
Pelletier S, Duhamel F, Coulombe P, Popoff MR, and Meloche S (2003) Rho family GTPases are required for activation of JAK/STAT signaling by G protein-coupled receptors. *Mol Cell Biol* **23:**1316–1333.
Ram PT, Horvath CM, and Iyengar R (2000) Stat3-mediated transformation of NIH-3T3 cells by the constitutively active Q205L $G\alpha_o$ protein. *Science (Wash DC)* **287:**142–144.
Saito Y, Hojo Y, Tanimoto T, Abe J, and Berk BC (2002) PKCα and PKCε are required for Grb2-associated binder-1 tyrosine phosphorylation in response to platelet-derived growth factor. *J Biol Chem* **277:**23216–23222.
Shah BH, Soh JW, and Catt KJ (2003) Dependence of gonadotropin-releasing hormone-induced neuronal MAPK Signaling on epidermal growth factor receptor transactivation. *J Biol Chem* **278:**2866–2875.
Sharp BM, McKean DJ, McAllen K, and Shahabi NA (1998) Signaling through δ-opioid receptors on murine splenic T cells and stably transfected Jurkat cells. *Ann NY Acad Sci* **840:**420–424.
Simon AR, Vikis HG, Stewart S, Fanburg BL, Cochran BH, and Guan KL (2000) Regulation of STAT3 by direct binding to the Rac1 GTPase. *Science (Wash DC)* **290:**144–147.
Soriano SF, Serrano A, Hernanz-Falcon P, De Ana AM, Monterrubio M, Martinez-A C, Rodriguez-Frade JM and Mellado M (2003) Chemokines integrate JAK/STAT and G-protein pathways during chemotaxis and calcium flux responses. *Eur J Immunol* **33:**1328–1333.
Stahle M, Veit C, Bachfischer U, Schierling K, Skripczynski B, Hall A, Gierschik P, and Giehl K (2003) Mechanisms in LPA-induced tumor cell migration: critical role of phosphorylated ERK. *J Cell Sci* **116:**3835–3846.
Tang Y, Yu J, and Field J (1999) Signals from the Ras, Rac and Rho GTPases converge on the Pak protein kinase in Rat-1 fibroblasts. *Mol Cell Biol* **19:**1881–1891.
Timokhina I, Kissel H, Stella G, and Besmer P (1998) Kit signaling through PI 3-kinase and Src kinase pathways: an essential role for Rac1 and JNK activation in mast cell proliferation. *EMBO (Eur Mol Biol Organ)J* **17:**6250–6262.
Tsai YT, Su YH, Fang SS, Huang TN, Qiu Y, Jou YS, Shih HM, Kung HJ, and Chen RH (2000) Etk, a Btk family tyrosine kinase, mediates cellular transformation by linking Src to STAT3 activation. *Mol Cell Biol* **20:**2043–2054.
Turkson J, Bowman T, Adnane J, Zhang Y, Djeu JY, Sekharam M, Frank DA, Holzman LB, Wu J, Sebti S, and Jove R (1999) Requirement for Ras/Rac1-mediated p38 and c-Jun N-terminal kinase signaling in STAT3 transcriptional activity induced by the Src oncoprotein. *Mol Cell Biol* **19:**7519–7528.
Turkson J, Bowman T, Garcia R, Caldenhoven E, De Groot RP, and Jove R (1998) STAT3 activation by Src induces specific gene regulation and is required for cell transformation. *Mol Cell Biol* **18:**2545–2552.
Wang Y, Mishra R, and Simonson MS (2003) Ca^{2+}/Calmodulin-dependent protein kinase II stimulates c-fos transcription and DNA synthesis by a Src-based mechanism in glomerular mesangial cells. *J Am Soc Nephrol* **14:**28–36.
Wilkie TM, Scherle PA, Strathmann MP, Slepak VZ, and Simon MI (1991) Characterization of G-protein α subunits in the G_q class: expression in murine tissues and in stromal and hematopoietic cell lines. *Proc Natl Acad Sci USA* **88:**10049–10053.
Woo CH and Kim JH (2002) Rac GTPase activity is essential for lipopolysaccharide signaling to extracellular signal-regulated kinase and p38 MAP kinase activation in rat-2 fibroblasts. *Mol Cells* **13:**470–475.
Wu EHT, Lo RKH, and Wong YH (2003) Regulation of STAT3 activity by G_{16}-coupled receptors. *Biochem Biophys Res Commun* **303:**920–925.
Yang CM, Chien CS, Ma YH, Hsiao LD, Lin CH, and Wu C (2003) Bradykinin B_2 receptor-mediated proliferation via activation of the Ras/Raf/MEK/MAPK pathway in rat vascular smooth muscle cells. *J Biomed Sci* **10:**208–218.
Yang M, Sang H, Rahman A, Wu D, Malik AB, and Ye RD (2001) $G\alpha_{16}$ couples chemoattractant receptors to NF-κB activation. *J Immunol* **166:**6885–6892.

Address correspondence to: Dr. Yung H. Wong, Department of Biochemistry, Hong Kong University of Science and Technology, Clear Water Bay, Kowloon, Hong Kong, China. E-mail: boyung@ust.hk