Constitutively Active Go\textsubscript{16} Stimulates STAT3 via a c-Src/JAK- and ERK-dependent Mechanism*  

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The hematopoietic-specific Go\textsubscript{16} protein has recently been shown to mediate receptor-induced activation of the signal transducer and activator of transcription 3 (STAT3). In the present study, we have delineated the mechanism by which Go\textsubscript{16} stimulates STAT3 in human embryonic kidney 293 cells. A constitutively active Go\textsubscript{16} mutant, Go\textsubscript{16QL}, stimulated STAT3-dependent luciferase activity as well as the phosphorylation of STAT3 at both Tyr\textsuperscript{705} and Ser\textsuperscript{727}. Go\textsubscript{16QL}-induced STAT3 activation was enhanced by overexpression of extracellular signal-regulated kinase 1 (ERK1), but was inhibited by U0126, a Raf-1 inhibitor, and coexpression of the dominant negative mutants of Ras and Rac1. Inhibition of phospholipase C\textgreek{g}, protein kinase C, and calmodulin-dependent kinase II by their respective inhibitors also suppressed Go\textsubscript{16QL}-induced STAT3 activation. The involvement of tyrosine kinases such as c-Src and Janus kinase 2 and 3 (JAK2 and JAK3) in Go\textsubscript{16QL}-induced activation of STAT3 was illustrated by the combined use of selective inhibitors and dominant negative mutants. In contrast, c-Jun N-terminal kinase, p38 MAPK, RhoA, Cdc42, phosphatidylinositol 3-kinase, and the epidermal growth factor receptor did not appear to be required. Similar observations were obtained with human erythroleukemia cells, where STAT3 phosphorylation was stimulated by CsA in a PTX-insensitive manner. Collectively, these results highlight the important regulatory roles of the Ras/Raf/MEK/ERK and c-Src/JAK pathways on the stimulation of STAT3 by Go\textsubscript{16} activation. Demonstration of the involvement of different kinases in Go\textsubscript{16QL}-induced STAT3 activation supports the involvement of multiple signaling pathways in the regulation of transcription by G proteins.

In addition to their classical roles as second messenger regulators, heterotrimeric G proteins have been implicated as mitogenic signal transmitters. The discovery of activating G protein mutations in various disease states highlights their roles in normal and aberrant growth (1). To date, a number of Go subunits have been shown to stimulate mitogenesis and induce neoplastic growth via initiation of intracellular signal cascades that lead to the activation of mitogen-activated protein kinases (MAPKs, 1 Refs. 2 and 3). In addition to MAPKs, other critical molecules such as signal transducers and activators of transcription (STATs) have also been shown to participate in the transduction of proliferative signals (4). STATs are latent cytoplasmic transcription factors that transduce signals from the cell membrane to the nucleus upon tyrosine phosphorylation (5). They were first identified as mediators of cellular responses to cytokines (6), but later it became apparent that they are also involved in mitogenic growth factor signaling (7). Binding of cytokines or growth factors to their cognate receptors leads to receptor dimerization and activation of receptor-associated Janus kinases (JAKs), resulting in the recruitment and homo- or heterodimerization of STAT proteins. Activated STAT proteins are then translocated to the nucleus to regulate gene expression. STAT activation by other non-receptor tyrosine kinases has also been demonstrated. Transformation of mammalian fibroblasts by viral Src (v-Src) specifically induces constitutive activation of STAT3 (8). Cellular Src (c-Src) tyrosine kinase is involved in the activation of both STAT1 and STAT3 in platelet-derived growth factor (PDGF)-stimulated NIH-3T3 cells (9). Additionally, it has recently been demonstrated that MAPKs can phosphorylate Ser\textsuperscript{727} on STAT3 to modulate its transcriptional activity (10), while activation of p38 MAPK and c-Jun N-terminal kinase (JNK) is thought to be required for v-Src activation of STAT3 (11).

Although activation of STAT proteins has generally been associated with cytokine and mitogenic growth factor signaling, ligands acting on G protein-coupled receptors (GPCRs) can also activate STAT proteins. Angiotensin II has been shown to induce c-Src-dependent tyrosine (Tyr\textsuperscript{705}) phosphorylation of STAT3 via activation of the G protein-coupled AT\textsubscript{1} receptor in vascular smooth muscle cells (12). \textalpha-Melanocyte-stimulating hormone, which enhances cellular proliferation, has been found to activate JAK2 and STAT1 in B-lymphocytes via stimulation of the melanocortin 5 receptor (13). Likewise, activation of \textalpha\textsubscript{1} adrenoceptors and protease-activated receptor 1 has been shown to induce tyrosine phosphorylation of JAK2, Tyk2, and STAT1 in vascular smooth muscle cells (14).

While activation of STATs in response to GPCR stimulation has been reported, the involvement of STATs in Go-activated transformation of cells is beginning to emerge (15). Expression of constitutively active Go\textsubscript{16} in NIH-3T3 cells results in Src-de-
pendent activation of STAT3, which leads to cellular transformation. Similarly, expression of constitutively active Gaα12 in NIH-3T3 cells increases STAT3 activity (4). Conversely, expression of a dominant negative mutant of Gaα12 inhibits Src kinase activity and Tyr705 phosphorylation of STAT3, leading to a reduction of v-fms-induced proliferation in NIH-3T3 cells (16). These data suggest that the STAT3 pathway may play a vital role in the Go subunit regulation of cell proliferation and transformation.

With the recent demonstration of STAT3 involvement in Gaα12 and Gaα13-induced cell transformation, it is reasonable to deduce that other Go subunits may also regulate mitogenesis via STAT3 activation. Gaα12, being unique in its restricted expression in hematopoietic cells (17), is also expressed in poorly differentiated leukemia cells, suggesting an association with hematopoietic cell growth and differentiation. Expression of a constitutively active Gaα16 mutant has been shown to induce cell differentiation in rat pheochromocytoma PC12 (3) and aortic vascular smooth muscle cells, although the same mutant was found to inhibit cell growth in Swiss 3T3 cells (18). Such observations suggest that Gaα16 may regulate cell growth and differentiation via activation of cell type-specific signal transduction pathways.

As a promiscuous G protein (19), Gaα16 possesses the ability to link a variety of GPCRs to the regulation of MAPKs. Recently, Gaα16 has been shown to activate JNK (3, 20). Interestingly, in addition to its ability to phosphorylate c-Jun, JNK can also phosphorylate STAT3 at Ser727 (21). It is therefore plausible that activated Gaα16 can influence cell differentiation via MAPK-induced STAT3 signaling. Based on the exclusivity of Gaα16 expression in hematopoietic cells and the involvement of STAT pathways in both normal and perturbed hematopoiesis (22), phosphorylation of STAT3 via Gaα16 activation may represent an important pathway for cell differentiation and development in the immune system. Indeed, we have recently shown that Gaα16 can support receptor-mediated activation of STAT3 in human embryonic kidney 293 (HEK 293) cells (23). In the present study, we examined the mechanism by which Gaα16,QL, a constitutively active Gaα16 mutant, stimulates STAT3 in HEK 293 cells and provide evidence for the involvement of various intermediate signaling molecules including c-Src, JAKs, and extracellular signal-regulated kinase (ERK).

**EXPERIMENTAL PROCEDURES**

**Reagents—**HEK 293 cells were obtained from the American Type Culture Collection (CRL-1573, Rockville, MD). Human erythroblastic (HEL) cells were from German Collection of Microorganisms and Cell Cultures (ACC11, Braunschweig, Germany). Cell culture reagents, including LipofectAMINE PLUS, were purchased from Invitrogen. The cDNAs of Ras, Rac1, and Cdc42 were obtained from Guthrie Research Institute (Sayre, PA). Constitutively activated mutants of Rac1 (Rac1G12V) and Cdc42 (Cdc42G12V) were kindly provided by Dr. Christopher L. Carpenter (Harvard Medical School, Boston). Constitutively activated RasG12V was a gift from Dr. Jeffery Field (University of Pennsylvania School of Medicine, Philadelphia, PA). RhoA and the various mutants (RhoA14V and RhoAT19N) as well as the dominant negative mutant of Cdc42 (Cdc42T17N) were generous gifts from Dr. Marc Symons (Pioueeer Institute for Medical Research, New York). The origins of plasmids encoding the dominant negative mutant of c-Src (c-Src-DN), phosphatidylinositol-3-kinase gamma (p110γ) and its dominant negative mutant (p110γ-DN) were as described previously (24). The p38 MAPK cDNA was obtained from Dr. Zhenguo Wu (Hong Kong University of Science and Technology, Hong Kong, China). The cDNAs of STAT3 and its dominant negative mutants STAT3Y705F and STAT3Y727A were kindly donated by Dr. Nancy C. Reich (State University of New York). Protein kinase C (PKC) α and ε dominant negative mutant cDNAs, PKCαKR and PKCεKR, were kindly obtained from Dr. Bernard Weinstein (Columbia University, New York). The construction or sources of other cDNAs encoding wild-type and mutant forms of Gaα12, Ras, Rac1, and JNK1 cDNAs were as described previously (20, 25). The luciferase reporter genes, pSTAT3-TA-luc, pGaS-7A-TA-luc and pJNKRE-TA-luc, were obtained from Chonect laboratories, Inc. (Palo Alto, CA). The luciferase substrate and its lysis buffer were purchased from Roche Diagnostics (Mannheim, Germany). All antibodies were obtained from Cell Signaling and kinase inhibitors were from Calbiochem (Darmstadt, Germany). C5a was purchased from Sigma Aldrich.

**Cell Culture and Transfection—**HEK cells were maintained at 5% CO2, 37°C in RPMI 1640 with 10% fetal bovine serum, 50 units/ml penicillin and 50 μg/ml streptomycin. HEK 293 cells were maintained at 5% CO2, 37°C in Eagle’s minimum essential medium (growth medium) with 10% fetal bovine serum, 50 units/ml penicillin and 50 μg/ml streptomycin. HEK 293 cells were seeded on 96-well microtiter plates at a density of 15,000 cells/well and were cultured in the growth medium at 18 to 24 h prior to transfection. They were co-transfected with various cDNAs using LipofectAMINE PLUS reagents. The transfection mixture was prepared by mixing 100 μg/ml of each cDNA, 0.1 μg of pSTAT3-TA-luc and 0.2 μg of both PLUS and LipofectAMINE reagents. After 3 h of transfection, 50 μl of OPTI-MEM medium containing 10 ng of G proteins, small GTPases or the control vector cDNAs, was added to the wells and incubated for another 30 h.

**Western Blot Analysis—**HEK 293 cells were transfected on 6-well plates at a density of 5 × 105 cells/well and were kept in the growth medium the day before transfection. They were co-transfected with various cDNAs using LipofectAMINE PLUS reagents following the supplier’s instructions. After 48 h of transfection, the transfected cells were serum-starved or treated with different kinase inhibitors overnight. The cells were lysed in 150 μl of lysis buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 5 mM EDTA, 40 mM NaP2O7, 1 mM dithiothreitol, 200 μg/ml Na3Vo4, 100 μg/ml phosphomethylsulfonyl fluoride, 2 μg/ml leupeptin, 4 μg/ml aprotinin, and 0.1% (v/v) phosphatase and then gently shaken on ice for 30 min. For detection, cell lysates in 25 μl of lysis buffer and 25 μl of luciferase substrate were measured by a microtiter plate luminometer MicroLumatPlus LB96V from EG&G Berthold.

**RESULTS**

**Activation of STAT3 by Constitutively Active Gaα16 in HEK 293 Cells**—It has previously been shown that expression of constitutively active Gaα12 and Gaα16 in NIH-3T3 cells induced STAT3 activation (4, 16). In the present study, we sought to investigate the ability of constitutively active Gaα16 to stimulate STAT3 phosphorylation and activation. Mutation of glutamine 212 to leucine (Q212L) in the conserved GTP/GDP binding domain of Gaα16 inhibits its intrinsic GTPase activity and results in constitutive activation of Gaα16 (3, 25). To investigate the effect of Gaα16QL mutant on the phosphorylation state of STAT3, HEK 293 cells were transiently transfected with cDNAs encoding pcDNA1 (vector control), Gaα16 or Gaα16QL. Total cell lysates prepared from the transfected cells were probed with anti-STAT3, anti-phospho-STAT3-Tyr705 and anti-phospho-STAT3-Ser727 antisera. Expression of either Gaα16 or Gaα16QL in HEK 293 cells did not affect the expression of total STAT3 as compared with the vector control (Fig. 1A). Further studies using anti-phospho-STAT3-Tyr705 and anti-phospho-STAT3-Ser727 antisera revealed basal levels of Ser727 phosphorylation of STAT3 protein in both the vector control and Gaα16QL expressing cells, while there was little or no Tyr705 phosphorylation of STAT3 in these cells. In contrast, expres-
sion of Gα₁₆QL led to a striking increase in both Tyr 705 and Ser727 phosphorylation of STAT3 protein (Fig. 1A). The observed Gα₁₆QL-induced phosphorylation of STAT3 is likely to induce STAT3 transcriptional activity, as demonstrated with Gα₁₆QL-induced phosphorylation and activation of STAT3 (4). In order to verify a similar correlation between Gα₁₆QL-induced STAT3 phosphorylation and modulation of STAT3 transcriptional activity, we performed reporter gene assays using a pSTAT3-TA-luc construct in combination with various cDNAs (pcDNA1, Gα₁₆, or Gα₁₆QL). As indicated in Fig. 1B, the magnitude of STAT3-mediated gene expression was unaffected by control vector or Gα₁₆ expression. On the contrary, expression of Gα₁₆QL induced an increased level of STAT3 activation as evidenced by a significant elevation of reporter gene expression. These results suggest that activation of Gα₁₆ can indeed lead to the phosphorylation of STAT3 at both Tyr 705 and Ser727 as well as the induction of STAT3 transcriptional activity. Using two other reporter gene constructs (pGAS-TA-luc and pISRE-TA-luc), we examined the ability of Gα₁₆QL to similarly activate STAT1 and STAT1/2 transcriptional activities.
STAT1-mediated gene expression was significantly elevated in Gα16QL-expressing cells, but not in Gα16 or vector-transfected cells (Fig. 1B). In contrast, STAT1-mediated gene expression was not enhanced in Gα16QL-expressing cells (Fig. 1B).

The specificity of Gα16QL-induced STAT3 activation was further characterized by the dose-dependent relationship between the amount of Gα16QL expression and STAT3 stimulation. As shown in Fig. 1C, the magnitude of STAT3-dependent luciferase activity was positively correlated to the concentration of Gα16QL cDNA transfected into the HEK 293 cells, whereas no correlation with activity could be observed for the cDNA of Gα16. The progressive increase in STAT3-dependent luciferase activity was eminent at the concentration range of 3–30 ng/ml of Gα16QL cDNA. Increasing the Gα16QL cDNA concentration beyond 30 ng/ml failed to induce further increase in luciferase activity, presumably because Gα16QL was no longer a limiting factor. The observed saturation of STAT3-dependent luciferase activity was likely to be caused by the limited amount of pSTAT3-TA-luc available in the transfected cells.

As both Tyr705 and Ser727 of STAT3 appeared to be phosphorylated in Gα16QL-expressing cells, we examined whether these modifications can occur independently. Using the reporter gene assay, the ability of Gα16QL to induce the phosphorylation of wild-type and phosphorylation-resistant mutants of STAT3 (STAT3Y705F and STAT3S727A) was examined in HEK 293 cells. Coexpression of STAT3 and Gα16QL resulted in an elevation of luciferase activity that was significantly higher than those obtained with Gα16 and vector control, and the magnitude of the response was identical to that of cells lacking recombinant STAT3 (Fig. 1D). In cells coexpressing the Y705F mutant of STAT3, although Gα16QL was still capable of inducing STAT3-dependent luciferase activity beyond the controls, such stimulation was significantly attenuated as compared with cells coexpressing wild-type STAT3 (Fig. 1D). Similar results were obtained with the S727A mutant of STAT3. Overexpression of STAT3 and its mutants was detected with anti-STAT3 antisera (Fig. 1D). Lack of phosphorylation of the Y705F and S727A mutants of STAT3 at Tyr705 and Ser727, respectively, was confirmed with anti-phospho-STAT3 antisera. Both wild-type and Gα16QL-dependent Tyr705 phosphorylation were prominently observed in STAT3- and S727A-mutant expressing cells, whereas a reduction in Tyr705 phosphorylation was seen with the Y705F mutant. Conversely, elevations in the levels of Ser727 phosphorylation were detected in cells expressing the wild-type and Y705F mutant of STAT3, while little or no phosphorylation at this site was detectable in S727A-expressing cells. This indicated that Gα16QL-induced phosphorylation of STAT3 at Tyr705 and Ser727 could occur independently.

Effects of MAPKs on STAT3 Phosphorylation—The MAPK pathway has been shown to play an important role in the regulation of STAT3 signaling. Both ERKs and JNK1 have been shown to phosphorylate STAT3 at Ser727 (10, 21). Likewise, p38 MAPK has been shown to play a key role in the Ser727 phosphorylation of STAT3 (11). Since Gα16 has been shown to activate JNK (4, 24), we asked if it can similarly stimulate ERK and p38 MAPK. Anti-phospho-MAPK antisera were used to probe the activities of MAPKs in Gα16QL-transfectants. The activities of all three branches of MAPKs were stimulated by Gα16QL, but not Gα16 (Fig. 2A). Next, we investigated whether the effects of MAPKs were required for Gα16QL-induced STAT3 activation. Firstly, we examined the effect of MAPKs expression on STAT3 activity. As illustrated in Fig. 2B, overexpression of ERK1 significantly stimulated STAT3 activation. In contrast, overexpression of JNK1 or p38 MAPK had no effect on STAT3 stimulation, suggesting that these two pathways did not play a significant role in STAT3 regulation in HEK 293 cells.

We used a panel of kinase inhibitors to further confirm the functional role of various MAPKs on Gα16QL-induced STAT3 phosphorylation and activation. The MAPK/ERK kinase 1/2 (MEK1/2) inhibitor, U0126 (10 μM), inhibited Gα16QL-induced STAT3 activation whereas the p38 MAPK/JNK inhibitors, SB202190 (10 μM) and SB203580 (10 μM), did not affect STAT3-dependent luciferase activity (Fig. 2C). The ineffectiveness of both SB202190 and SB203580 further substantiated the lack of involvement of p38 MAPK and JNK in STAT3 activation. The inhibitory effect of U0126 reflects a possible involvement of MEK1/2 in STAT3 regulation. Inhibition of the upstream activator of MEK1/2, Raf-1, by its specific inhibitor (10 μM) also suppressed the Gα16QL-induced STAT3 activation (Fig. 2C). These observations provided additional evidence to support the regulatory role of ERK on STAT3 activation. The effects of the various inhibitors on the state of phosphorylation of STAT3 were examined with anti-phospho-STAT3 antisera. Both U0126 and the Raf-1 inhibitor, but not SB202190 and SB203580, attenuated the Gα16QL-induced STAT3 phosphorylation at Ser727 (Fig. 2C). Interestingly, Gα16QL-induced STAT3 phosphorylation at Tyr705 was also suppressed by U0126 and the Raf-1 inhibitor, but not by SB202190 and SB203580 (Fig. 2C). Since ERK is a Ser/Thr-specific kinase, these results implied that the ERK pathway might indirectly activate STAT3 by phosphorylation at Tyr705.

Roles of Small GTPases on Gα16QL-induced STAT3 Activation—Next, we attempted to identify other intracellular signaling molecules involved in Gα16QL-induced STAT3 activation. Small GTPases have been shown to play critical roles in growth regulation and there is considerable evidence to suggest that GPCR activation can regulate cell growth through the engagement of these monomeric GTPases. In order to determine the role of small GTPases on STAT3 phosphorylation, constitutively activated mutants of Ras (RasG12V), Cdc42 (Cdc42G12V), Rac1 (Rac1G12V), and RhoA (RhoAG14V) were utilized to examine their effects on STAT3 activity. STAT3-dependent luciferase activity was significantly stimulated in cells expressing constitutively active RasG12V or Rac1G12V (Fig. 3A). In contrast, neither Cdc42G12V nor RhoAG14V was able to stimulate STAT3 activity (Fig. 3A).

The functional role of Rac and Ras in the regulation of Gα16QL-induced STAT3 activation was further examined in HEK 293 cells. If these small GTPases serve as intermediate signaling molecules for Gα16-mediated STAT3 activation, their dominant negative mutants should inhibit Gα16QL-induced STAT3 activity. Using STAT3 driven luciferase reporter gene assays, we coexpressed the various mutants of Ras and Rac1 with either Gα16 or Gα16QL in HEK 293 cells and determined the luciferase activity of the transfectants. In the Gα16-transfectants, the presence of RasG12V significantly elevated STAT3 activity as compared with either the vector control or wild-type Ras (Fig. 3B). The dominant negative mutant of Ras (RasS17N) slightly suppressed the STAT3 activity in Gα16-transfectants, the presence of Rac1G12V or Rac1S17N) significantly elevated STAT3 activity as compared with either the vector control or wild-type Ras (Fig. 3B). The dominant negative mutant of Ras (RasS17N) slightly suppressed the STAT3 activity in Gα16-transfectants, the presence of Rac1G12V or Rac1S17N) significantly elevated STAT3 activity as compared with either the vector control or wild-type Ras (Fig. 3B). The inhibitory effect of RasS17N on Gα16QL-induced STAT3 phosphorylation was also demonstrated by immunoblotting with
anti-phospho-STAT3 antisera. As compared with the vector control or Gα16-transfectants, much higher levels of Tyr705 and Ser727 phosphorylation of STAT3 were observed with HEK 293 cells expressing Gα16QL alone, RasG12V alone, RasG12V with Gα16, or Gα16QL together with either wild-type Ras or RasG12V (Fig. 3C). In the presence of RasSi17N, the Gα16QL-induced Tyr705 and Ser727 phosphorylation of STAT3 were completely suppressed (Fig. 3C). These studies confirmed that activation of Ras leads to STAT3 phosphorylation and that Ras is downstream of Gα16 in the STAT3 activation pathway. Similar results were obtained with the use of Rac1G12V and Rac1T17N (a dominant negative mutant of Rac1), although some differences were observed (Fig. 3, B and C). Unlike RasG12V, constitutively active Rac1G12V was unable to induce STAT3 phosphorylation at Ser727 (Fig. 3C). Nevertheless, Rac1T17N was able to attenuate Gα16QL-induced phosphorylation as well as the activation of STAT3 (Fig. 3C).

Roles of Protein Kinases on Gα16QL-induced STAT3 Activation—The participation of Ras, Rac1, and ERK in Gα16QL-induced STAT3 activation raises the possibility that this signaling pathway may require other molecules directly or indirectly associated with these molecules. To gain further insight into the mechanism of Gα16QL-induced STAT3 activation, the effects of a panel of inhibitors for various kinases and enzymes on STAT3 phosphorylation and activation were investigated. As a member of the Gα family, Gα16 stimulates phospholipase Cβ (PLCβ) and triggers the mobilization of intracellular Ca2+ and the subsequent activation of protein kinase C (PKC) and calmodulin-dependent protein kinase II (CaMKII). Given that this is a primary pathway for Gα16-mediated re-
FIG. 3. Regulation of STAT3 activity by Ras and Rac1. A, pSTAT3-TA-luc cDNA was transiently transfected into HEK 293 cells along with pcDNA1, wild-type or constitutively activated mutants of Ras, Cdc42, Rac1, or RhoA. B, HEK 293 cells were transiently co-transfected with cDNAs of pSTAT3-TA-luc and pcDNA1, and either Gα16 or Gα16QL in combination with Ras or Rac1 mutants. For luciferase reporter gene assays, STAT3-dependent luciferase activity was determined as in the legend to Fig. 1. Data shown represent the mean ± S.E. from four separate experiments performed in triplicates. *, STAT3 activity was significantly higher than pcDNA1 control (Dunnett t test, p < 0.05). C, HEK 293 cells were transiently co-transfected as in B. Cell lysates from the transfectants were immunoblotted with anti-STAT3 (upper panel), anti-phospho-STAT3-Tyr705 (middle panel), or anti-phospho-STAT3-Ser727 (lower panel) antiserum. The immunoblots shown represent one of three sets of immunoblots; two other sets yielded similar results. *, the level of STAT3 phosphorylation was significantly higher than that obtained with pcDNA1 control (Dunnett t test, p < 0.05). #, STAT3 phosphorylation at Tyr705 was significantly lower than that obtained with pcDNA1 control (Dunnett t test, p < 0.05).
sponses, we examined the possible requirement of PLCβ, PKC, and CaMKII in Goα16QL-induced STAT3 activation. Inhibition of PLCβ by 10 μM U73122 abolished the ability of Goα16QL to induce STAT3-dependent luciferase activity as well as the induction of STAT3 phosphorylation at both Tyr^705^ and Ser^727^ (Fig. 4A). Similar results were obtained with the CaMKII-specific inhibitor, KN62 (10 μM; Fig. 4A), and two inhibitors of PKC, 200 nM staurosporin and 100 nM calphostin C (Fig. 4B). Given that some of these inhibitors can exhibit non-selective actions, we further employed kinase-deficient mutants to determine the role of PKC in Goα16QL-induced STAT3 activation. The α and ε isoforms of PKC were selected as representatives of Ca^2+^-dependent (26) and -independent (27) members. In HEK 293 cells expressing the kinase-deficient mutant of either PKCa (PKCα-KR) or ε (PKCε-KR), Goα16QL-induced STAT3 activity was suppressed to control levels, and phosphorylation levels of Tyr^705^ and Ser^727^ were not significantly different than those of the Goα16 and vector controls (Fig. 4C). Collectively, these results suggest that activation of STAT3 by Goα16QL probably requires signaling via the PLCβ pathway.

In cytokine receptor signaling, stimulation of STATs is mediated by the phosphorylation and activation of JAKs (7). Interestingly, a number of GPCRs have now been shown to activate JAK2 (14–16) and JAK3 (28) that subsequently associate with STAT proteins. Hence, we examined the roles of JAK2 and JAK3 in Goα16QL-mediated STAT3 activation using selective inhibitors against these tyrosine kinases. As shown in Fig. 4A, Goα16QL-induced STAT3 activity and its phosphorylation at Tyr^705^ and Ser^727^ were significantly inhibited by 100 μM AG490, a specific inhibitor of JAK2, and by 100 μg/ml of a JAK3 inhibitor (WHI-P131). These results suggest that JAK2/3 activity was suppressed to control levels, and phosphorylation levels of STAT3-Tyr^705^ and STAT3-Ser^727^ were significantly inhibited by 100 μM AG1478 (data not shown), indicating that transactivation of the EGF receptor. We over, it has recently been demonstrated that GPCRs can induce Ras-dependent mitogenesis and activate STAT3 (30). More-including Src have been shown to activate PI3K. In addition, small GTPases such as Ras, Rac1, and Cdc42 are also known to interact with PI3K, and PI3K has recently been shown to mediate wortmannin-sensitive activation of MAPKs by GPCRs (34). Thus, PI3K appears to be a prime candidate for signal integration in the activation of STAT3 by Goα16QL. In the present study, the role of PI3K on Goα16QL-induced STAT3 activation was examined using the PI3K inhibitors wortmannin and LY294002. Goα16QL-induced STAT3 phosphorylation and activation were unaffected by 100 nM wortmannin or 10 μM LY294002 treatment (Fig. 5D), indicating that PI3K was not an essential regulator for STAT3 activation by Goα16QL. The lack of involvement of PI3K in Goα16QL-induced STAT3 activation was further confirmed with the use of a dominant negative mutant of PI3K (PI3KΔ). Indeed, coexpression of PI3KΔ with Goα16QL had no effect on the ability of Goα16QL to induce STAT3-dependent luciferase activity (Fig. 5E).

C5α Induces STAT3 Phosphorylation in a PTX-insensitive Manner in HEL Cells—Lastly, we examined whether STAT3 phosphorylation can be stimulated by Goα16-coupled receptors in human erythroleukemia (HEL) cells which are known to express Goα16 (35). The complement C5α receptor (C5αR) is capable of activating Goα16 (36) and is expressed in hematopoietic cells (37). We therefore treated HEL cells with 100 nM C5α and determined the phosphorylation status of STAT3 by using anti-phosphospecific antibodies. As illustrated in Fig. 6A, C5α significantly stimulated the phosphorylation of STAT3 at both Tyr^705^ and Ser^727^ residues as compared with the untreated cells. Since the C5αR can utilize both PTX-sensitive and -insensitive G proteins for signal transduction, the possible involvement of G proteins in mediating the C5α-induced STAT3 phosphorylation was eliminated by pretreating the HEL cells with PTX (100 ng/ml, overnight). C5α remained fully capable of inducing STAT3 Tyr^705^ and Ser^727^ phosphorylations in PTX-treated cells (Fig. 6A), suggesting that these C5α-induced responses were mediated by PTX-insensitive G proteins such as Goα16. If Goα16 was indeed responsible for mediating the C5α-induced STAT3 activation in HEL cells, then it may require the same signaling intermediates as in HEK 293 cells. Indeed, C5α-induced STAT3 Tyr^705^ and Ser^727^ phosphorylations were...
significantly inhibited by AG490, Raf-1 inhibitor, U0126, and KN62 in PTX-treated HEL cells (Fig. 6B). These findings illustrated that C5a-induced STAT3 phosphorylations in HEL cells required JAK2, Raf-1, ERK, and CaMKII.

**DISCUSSION**

G proteins are major players in numerous biological processes. In addition to their classical role in second messenger activation, they have recently been implicated in the regulation...
of gene expression. The hematopoietic-specific G\textsubscript{16} can regulate cell differentiation and apoptosis through modulation of MAPKs (3) and transcription factors such as NF-\kappaB (38). Since expression of G\textsubscript{16} is regulated during human myeloid differentiation as well as following T-cell activation (39), it represents a prime candidate for the regulation of STAT pathways that are often associated with cytokine signaling. We have recently demonstrated that activation of G\textsubscript{16} is indeed capable of directly phosphorylating and activating STAT3 (23). The present study confirms that G\textsubscript{16}, like G\textsubscript{6} and G\textsubscript{12} (4), is indeed capable of activating STAT3 through a complex mechanism involving multiple intermediates.

Expression of the constitutively active G\textsubscript{16} in HEK 293 cells reproducibly resulted in increased phosphorylations of STAT3 at Tyr\textsuperscript{705} and Ser\textsuperscript{727} as well as an induction of STAT3-dependent luciferase activity. G\textsubscript{16}Q1L-induced Tyr\textsuperscript{705} phosphorylation appeared to be more pronounced than that of Ser\textsuperscript{727} because the latter site exhibited higher basal phosphorylation. Phosphorylation of Tyr\textsuperscript{705} is required for cytokine-induced STAT3 dimerization, nuclear translocation, and DNA binding, while full transcriptional activity of the homodimer is manifested only when Ser\textsuperscript{727} in the transactivation domain is also phosphorylated (40). Since G\textsubscript{16}Q1L induced STAT3 phosphorylation at both sites, STAT3-dependent transcriptional activity was easily detected by luciferase reporter gene assays. As a signal transducing GTPase, G\textsubscript{16}Q1L must rely on other tyrosine and serine kinases to activate STAT3. By using a combination of selective inhibitors and constitutively active and dominant negative mutants, a number of signaling molecules were found to participate in G\textsubscript{16}Q1L-induced activation of STAT3 (Fig. 7). They included an effector (PLC\textgamma), small GTPases (Ras and Ral1), non-receptor tyrosine kinases (c-Src and JAK2/3), serine kinases (Raf-1, PKC\textgammah, PKCe, and CaMKII), and MAPK (ERK). Most of these molecules can be aligned into the Ras/RAF/MEK/ERK and c-Src/JAK pathways that directly or indirectly phosphorylate STAT3 at Ser\textsuperscript{727} and Tyr\textsuperscript{705}, respectively.

Although there is increasing evidence to support the participation of STAT3 in G protein-regulated pathways, detailed mapping of the molecular mechanisms involved in such pathways has yet to be completed. Nevertheless, ample evidence is available to support the modular role of MAPKs in STAT3 regulation (41). In the present study, we have investigated the regulatory roles of only three members of MAPKs on G\textsubscript{16}Q1L-induced STAT3 transcription. Our data suggested that only the ERK cascade participated in G\textsubscript{16}Q1L-induced activation of STAT3, despite the fact that G\textsubscript{16}Q1L also stimulated JNK and p38 MAPK. Participation of the ERK cascade was supported by several observations. Firstly, ERK was activated by G\textsubscript{16}Q1L and overexpression of ERK1, but not JNK1 or p38 MAPK, in HEK 293 cells was associated with increased STAT3-dependent luciferase activity. Modulation by ERK of the transcriptional activity of STAT3 via Ser\textsuperscript{727} phosphorylation has indeed been reported (10). Secondly, inhibition of MEK1/2 by U0126
led to the suppression of Go16QL-induced phosphorylation and activation of STAT3. Moreover, the inhibitory effect of a Raf-1 inhibitor on Go16QL-induced STAT3 transcription highlighted the requirement of Raf-1 (acting as an MEKK in the ERK cascade) in STAT3 activation. Lastly, the involvement of Ras was demonstrated with the use of wild-type and mutant Ras; RasG12V alone stimulated STAT3 activities while RasS17N suppressed the Go16QL-induced phosphorylation and activation of STAT3. Indeed, it has recently been reported that Go16QL can activate Ras via direct interaction with a novel adaptor protein known as tetratricopeptide repeat 1 (35). Constitutively active Ras protein has long been associated with pathogenesis of human cancer. The role of this small GTPase in signal transduction has been extensively studied and its crucial role in the initiation of the ERK cascade is well established. The stimulatory effect of RasG12V and inhibitory action of RasS17N on STAT3 transcriptional activity further revealed and strengthened the important connection between MAPK pathway and STAT3 activation. Unlike Go16-induced STAT3 activation in NIH-3T3 fibroblasts (4), the classical Ras/Raf/MEK/ERK pathway commonly employed by mitogens thus appeared to play an essential role in Go16QL-induced STAT3 activation in HEL 293 cells. Interestingly, although neither Ras nor Raf-1 acts as a tyrosine kinase, inhibition of the activity of either Ras or Raf-1 significantly attenuated Go16QL-induced phosphorylation of STAT3 at Tyr^705. These observations reiterated the possible existence of complex regulatory mechanisms involved in MAPK-mediated regulation of STAT proteins.

Apart from Ras, the Rho family of GTPases has also been shown to regulate STAT3 activity. Activation of STAT3 by Rac1 has been reported (11) and this pathway is actually employed by GPCRs (42). Recently, Rac1 has been shown to induce STAT3 phosphorylation directly (43). Complementary to the existing literature, we have also demonstrated an increase in STAT3 phosphorylation and activation induced by constitutively active Rac1. The dominant negative mutant of Rac1, on the other hand, prevented Go16QL from activating STAT3. However, not all small GTPases are involved in STAT3 regulation. RhoA and Cdc42 did not affect STAT3 activation in the present study. In view of the effects of various GTPases on STAT3 activity, it is likely that small GTPases may play a key role in network signaling. By acting as signal activators, small GTPases may provide a significant linkage between different signaling pathways. Ras and Rac1 might eventually converge at the level of ERK since Rac1G12V has been shown to activate ERK in Rat-2 fibroblasts (44). It is believed that intimate interactions between different signaling pathways contribute to the appropriate control of intracellular signaling (45).

One of the mechanisms by which heterotrimeric G proteins regulate the activities of monomeric GTPases is via second messengers. As a member of the G_i family, Go16QL constitutively stimulates the activity of PLCβ (18), subsequently leading to the activation of downstream effectors such as PKC and CaMKII. Given that Go16QL-induced STAT3 activation was effectively inhibited by U73122, KN62, staurosporin and calphostin C, PLCβ and its downstream effectors might constitute an important signaling pathway in mediating the actions of Go16QL. Linkages to STAT3 from the PLCβ pathway can be traced back to ERK. Group I metabotropic glutamate receptors have been shown to activate ERK via CaMKII in striatal neurons (46), and CaMKII-dependent activation of ERK in vascular smooth muscle requires the participation of tyrosine kinases such as Pyk2 and Src (47). As for PKC-dependent activation of ERK, gonadotrophin-releasing hormone-induced phosphorylation of ERK in hypothalamic neurons also requires Pyk2 and Src (48). Gonadotrophin-releasing hormone stimulation caused the translocation of PKCa and PKCe to the cell membrane and enhanced the association of Src with PKCs and PKCe. The same two isoforms of PKC appeared to play a role in Go16QL-induced STAT3 activation because their dominant negative mutants were effective in suppressing the phosphorylation and activation of STAT3. It should also be noted that PKC and CaMKII can also activate ERK via Raf-1 (49) and Ras (50), respectively. Indeed, both PKCa (51) and PKCe (52) have been shown to activate Raf-1, leading to the stimulation of ERK. Irrespective of the loci for signal integration, activation of STAT3 by Go16QL apparently involved a number of intermediates along the PLCβ pathway.

There is considerable evidence to support the involvement of cross talk between multiple signaling pathways and certain protein tyrosine kinases are thought to play important cooperative roles in mitogenic STAT3 signaling (53). Non-receptor tyrosine kinases such as JAKs have been shown to participate in signaling from a range of cell-surface receptors such as those for cytokines and growth factors. Activation of these receptors results in the recruitment and activation of JAKs, leading to downstream binding, phosphorylation and activation of STAT3 (7). In addition to JAKs, the Src family of non-receptor kinases has been implicated in the phosphorylation and activation of STAT proteins (8). The inhibitory effects of PP2, AG490 and JAK3 inhibitor on STAT3 activation observed in the present study verified the important roles of c-Src and JAK2/3 in Go16QL-induced STAT3 activation. Involvement of JAKs in c-Src-dependent STAT3 activation has previously been shown in PDGF-induced signaling (53). It is possible that Go16QL-induced STAT3 activation may involve cooperative association between c-Src and JAKs. The ability of Go16QL to activate

FIG. 6. Complement C5a-induced STAT3 Tyr^705 and Ser^727 phosphorylation in HEL cells was resistant to PTX treatment. A. HEL cells were seeded at a density of 1 × 10^4 cells using serum-free medium with or without PTX (100 ng/ml) treatment overnight. Cells were incubated with 100 mM C5a at 37 °C for 20 min. B. HEL cells were pre-treated with PTX in serum-free medium overnight. Prior to C5a treatment, cells were incubated with 0.1% Me2SO (v/v), 10 μM Raf-1 inhibitor, 10 μM U0126, and 10 μM KN62 for 30 min. Cell lysates were immunoblotted with anti-STAT3 (upper panel), anti-phospho-STAT3-Tyr^705 (middle panel) or anti-phospho-STAT3-Ser^727 (lower panel) antisera. The immunoblots shown represent one of three sets of immunoblots; two other sets yielded similar results.
JAKs provide a means to phosphorylate STAT3 at Tyr^{705}, while Ser^{727} can be phosphorylated via the Ras/Raf/MEK/ERK axis. Moreover, the involvement of JAK2/3 in mediating the G_{16QL} signals to STAT3 is in good agreement with their ability to activate Ras and ERK (54).

A requirement for c-Src in G_{16QL}-induced STAT3 activation can be predicted from the Src-dependences of G_{16QL} and G_{siQL} activation of STAT3 (4). As a ubiquitously expressed non-receptor tyrosine kinase, c-Src is well positioned to serve as a key signaling molecule in a variety of pathways. The inhibitory effects of the selective inhibitor and the dominant negative c-Src mutant demonstrate the involvement of c-Src in G_{16QL}-induced STAT3 activation. One of the most obvious consequences of the regulation of c-Src by G_{16QL} is the provision of a direct linkage to the JAK/STAT pathway. In addition, direct activation of STAT3 by v-Src has been demonstrated (8, 31). Yet, v-Src can also activate Ras and PI3K and thus regulate STAT3 via these intermediates. In G_{16QL}-induced STAT3 activation, c-Src did not appear to require PI3K because STAT3 activity was unaffected by wortmannin, a potent PI3K inhibitor. c-Src did not appear to require PI3K for the activation of STAT3 by v-Src, whereas P in the open circle depicts Ser^{727} phosphorylation. Solid-lined arrows illustrate findings based on previous studies. Dash-lined arrows indicate the interactions are either putative or involve multiple steps.

As a member of a family of latent cytoplasmic transcription factors, STAT3 has long been implicated in the normal processes of cell growth and development. More recently, evidence for a role of STAT3 in oncogenesis has become increasingly apparent. Constitutive activation of STAT3 in v-Src-transformed fibroblasts (8), the imperative requirement of STAT3 in v-Src-induced transformation (31), and cellular transformation induced by constitutively activated STAT3 (56), have provided strong evidence to support the tumorigenic potential of STAT3. In NIH-3T3 cells, G_{16QL} and G_{siQL} induce neoplastic transformation mediated via Src and STAT3 (4, 16). In contrast to G_{siQL} and G_{16QL}, persistent activation of G_{16QL} leads to inhibition of cell growth in Swiss 3T3 fibroblasts (18) and to cell differentiation in PC12 cells (3), probably due to prolonged activation of JNK. It remains to be determined if activation of STAT3 by G_{16} can indeed lead to neoplastic transformation in specific cell types. It is noteworthy that the mechanisms by which G_{16} and G_{16QL} activate STAT3 differ significantly in their dependences on ERK, although both mechanisms require c-Src. Importantly, the ability of G_{16QL} to activate STAT3 (and perhaps STAT1) offers GPCRs the opportunity to modulate cytokine signaling in hematopoietic cells. In this regard, the chemokine CXCR4 receptor has been shown to stimulate the JAK/STAT pathway independently of G proteins in human T cells (28). Since G_{16} is expressed in T cells (39), this response may well be mediated by G_{16}. In HEL cells that express both G_{16} and the C5aR (35, 37), we demonstrated that the C5a-induced STAT3 phosphorylation was PTX-insensitive (Fig. 6). Since the C5aR is capable of stimulating both G_{16} and G_{16QL}, the observed PTX-insensitivity verified that C5a-induced STAT3 phosphorylation in HEL cells are G_{16}-independent and may well be regulated by G_{16QL}. Although we did not unequivocally demonstrate the involvement of G_{16QL} in mediating the C5a response,
The sensitivity of the pathway to various inhibitors suggested that it is similar, if not identical, to the one employed by Gα16QL in HEK 293 cells. Both pathways require the participation of JAK2, Raf-1, MEK1/2, and CamKII. Nevertheless, additional studies are needed to confirm the involvement of Gα16 in GPCR-induced STAT3 activation in hematopoietic cells. Such studies would necessitate the use of Gα16-specific siRNA or antibodies. The demonstrated ability of Gα16QL to stimulate STAT3 activity in HEK 293 cells provides a mechanism by which C5α may elicit its potent proinflammatory actions.

Based on our observations, it is unlikely that a single signaling pathway is entirely responsible for Gα16QL-induced STAT3 activation.

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