Signal transducer and activator of transcription 3 (STAT3) can be stimulated by several Gs-coupled receptors, but the precise mechanism of action has not yet been elucidated. We therefore examined the ability of GaqQ226L (GaqlQL), a constitutively active mutant of Gaq, to stimulate STAT3 Tyr705 and Ser727 phosphorylations in human embryonic kidney 293 cells. Apart from GaqlQL, the stimulation of Gaq by chlordetrin toxin or b2-adrenergic receptor and the activation of adenyl cyclase by forskolin, (S)-cAMP, or dibutyryl-cAMP all promoted both STAT3 Tyr705 and Ser727 phosphorylations. Moreover, the removal of Gaq by RNA interference significantly reduced the b2-adrenergic receptor-mediated STAT3 phosphorylations, denoting its capacity to regulate STAT3 activation by a G protein-coupled receptor. The possible downstream signaling molecules involved were assessed by using specific inhibitors and dominant negative mutants. Induction of STAT3 Tyr705 and Ser727 phosphorylations by Janus kinase 2/3, and a similar profile was observed in response to b2-adrenergic receptor stimulation. In contrast to the GaqlQL-mediated regulation of STAT3 in HEK 293 cells (Lo, R. K., Cheung, H., and Wong, Y. H. (2003) J. Biol. Chem. 278, 52154–52165), the Gaq-mediated responses, including STAT3-driven luciferase activation, were resistant to inhibition of phospholipase Cβ. Surprisingly, Gaq-mediated phosphorylation at Tyr705, but not at Ser727, was resistant to inhibition of c-Src, Raf-1, and MEK1/2 as well as to the expression of dominant negative Ras. Therefore, as with other Ga-mediated activations of STAT3, the stimulatory signal arising from Gaq is transduced via multiple signaling pathways. However, unlike the mechanisms employed by Gaq and Gq14/16, Gaq distinctly requires protein kinase A, JNK, and phosphatidylinositol 3-kinase for STAT3 activation.

By virtue of their linkage to the superfamily of seven-transmembrane receptors, the heterotrimeric G proteins are critical players in the regulation of cellular functions ranging from cell proliferation to differentiation. There is increasing evidence to suggest that malfunctions in G protein signaling may be associated with various disease states such as bacterial infections (Vibrio cholera and Bordetella pertussis), pseudohypoparathyroidism, McCune-Albright syndrome, cancers, and night blindness (1). All four classes of G proteins (Gs, Gq, G12, and G13) possess the ability to stimulate mitogenesis and induce neoplastic growth (2–8), as illustrated by the expression of constitutively active mutants of the Ga subunits in different cell types. Attempts to elucidate the mechanisms by which G proteins transduce proliferative signals have revealed many new conduits for G protein signaling. These new pathways provide linkages to oncogenes, kinases, and transcription factors. It has now become apparent that many G protein-coupled receptors (GPCRs) can modulate the activities of mitogen-activated protein kinases (MAPKs), thereby allowing them to regulate cell proliferation and differentiation (9). Regulation of MAPKs by G proteins proceeds via complex signaling networks involving oncogenes such as Src tyrosine kinases (10) and the monomeric GTPases Ras and Rac1 (11, 12). Transcription factors such as signal transducers and activators of transcription (STATs) have also been shown to participate in the transduction of G protein-mediated proliferative signals (7).

STATs are key players in mitogenesis because all seven members of STATs are associated with major types of cancer (13). Although STATs are typically stimulated by cytokines or growth factor receptors via Janus kinases (JAKs) (14), at least 20 GPCRs have now been shown to possess the ability to activate STATs in a variety of cell types (15–20). STAT3 is one of the most widely studied STAT proteins, and its activation apparently involves multiple pathways. Both viral Src (v-Src) and cel-

### References

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3. The abbreviations used are: GPCRs, G protein-coupled receptors; AC, adenyl cyclase; β2AR, β2-adrenergic receptor; CaMKII, calmodulin-dependent protein kinase II; CCH, carbachol; CTX, cholera toxin; DN, dominant negative; Bt2cAMP, dibutyryl cAMP; EGF, epidermal growth factor; ERK, extracellular signal-regulated kinase; FBS, fetal bovine serum; Fsk, forskolin; GEF, guanine nucleotide exchange factor; HEL, human erythroleukemia; HEK, human embryonic kidney; HIP, human parathyroid; JAK, Janus kinase; JNK, c-Jun N-terminal kinase; JNKK-CA, c-Jun N-terminal kinase kinase constitutive active mutant; MAPK, mitogen-activated protein kinase; MEK, MAPK/ERK kinase; MKK, mitogen-activated protein kinase kinase; PI3K, phosphatidylinositol 3-kinase; PLCβ, phospholipase Cβ; PKA, protein kinase A; PTX, pertussis toxin; STAT3, signal transducer and activator of transcription 3; ANOVA, analysis of variance; RNAI, RNA interference; PKC, protein kinase C.

**Activation of STAT3 by Gaq Distinctively Requires Protein Kinase A, JNK, and Phosphatidylinositol 3-Kinase**

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lular Src (c-Src) tyrosine kinases can activate STAT3 in mammalian fibroblasts (21, 22). Moreover, MAPKs can phosphorylate Ser727 on STAT3 to modulate its transcriptional activity (23), whereas activation of p38 MAPK and c-Jun N-terminal kinase (JNK) is thought to be required for v-Src activation of STAT3 (24). These and other signaling molecules have been similarly implicated in the activation of STAT3 by G proteins. Neoplastic transformation of NIH-3T3 cells by constitutively active Gαs is mediated via STAT3 in an Src-dependent manner (7), although expression of a dominant negative mutant of Gα12 in the same cell type inhibits Src kinase activity and Tyr705 phosphorylation of STAT3 (25). The Gαq family members, Gα14 and Gα16, also employ c-Src for the activation of STAT3 (26, 27). Unlike Gα14/16-mediated stimulatory signals, Gα14/16 additionally requires the Ras/Raf/MEK/ERK and PLCβ/PKC/CaMKII pathways for STAT3 activation. Among the GPCRs that are known to be capable of activating STAT3, most are linked to either Gα11 or Gαq, with only a handful coupled to Gz. Although activation of the Gz-coupled human thyrotropin receptor and the prostacyclin receptor (hIP) can lead to STAT3 phosphorylation in FRTL-5 (28) and human thyrotropin receptor and the prostacyclin receptor (hIP) can also possess the ability to signal via Gq proteins (29, 30). This raises a concern as to whether the Gz protein can truly activate the STAT3 pathway because Gg proteins such as Gz14 and Gz16 are known activators of STAT3 (26, 27). In FRTL-5 cells, elevation of intracellular cAMP activates STAT3, but inhibition of protein kinase A (PKA) does not affect thyrotropin receptor-mediated STAT3 activation (28). In contrast, cytokine-triggered STAT3 Tyr705 phosphorylation and DNA binding are inhibited by cAMP in human mononuclear cells (31). Moreover, isoproterenol-induced phosphorylation of STAT3 in cultured cardiomyocytes can be markedly enhanced by the phosphodiesterase inhibitor amrinone, indicating that cAMP is critically involved in β-adrenergic receptor (β-AR)-mediated STAT3 activation (32). However, this Gz-induced CAMP signal may facilitate JAK/STAT3 signaling indirectly through the induction of cytokine expression such as interleukin-6 (33, 34). The issue of whether Gz can indeed directly stimulate STAT3 activity remains controversial.

We have recently demonstrated that the Gz/Gαq-coupled hIP is capable of activating STAT3 in HEL cells, and the signal propagation appears to require JNK (29). Because Gα16-mediated activation of STAT3 in HEL cells, as well as in HEK 293 cells, is independent of JNK signaling (26), we hypothesized that this difference is because of Gz-mediated signaling. In this study, we examined the ability of Gαz to activate STAT3 by expressing the constitutively active GαzQL or stimulating the Gz-coupled βzAR in HEK 293 cells and characterizing the molecular components involved in the signal transduction.

**EXPERIMENTAL PROCEDURES**

**Materials**—The cDNAs of human G proteins were obtained from Guthrie Research Institute (Sayre, PA). Cell culture reagents, including Lipofectamine PLUS and Lipofectamine 2000 reagents, were obtained from Invitrogen. All kinase inhibitors, their negative analogues, and other cAMP analogues were ordered from Calbiochem (Darmstadt, Germany). Isoproterenol, salbutamol, carbachol, ICI 118-551, epidermal growth factor (EGF), and cholera toxin (CTX) were purchased from Sigma. Pertussis toxin (PTX) was obtained from List Biological Laboratories (Campbell, CA). Gαz and Ras-GRF1 antisera were from Santa Cruz Biotechnology (Santa Cruz, CA), and other antisera were purchased from Cell Signaling (Beverly, MA). Nitrocellulose membrane and ECL kit were ordered from Bio-Rad and Amersham Biosciences, respectively. The Select Steal RNAi for Gαz and Select Steal RNAi negative control were products of Invitrogen, and the RNAi for Ras-GRF1 was purchased from Santa Cruz Biotechnology. The luciferase reporter gene, pSTAT3-TA-luc, was from Clontech (Palo Alto, CA). The luciferase substrate and its lysis buffer were purchased from Roche Diagnostics (Mannheim, Germany).

**Cell Culture and Transfection**—Human embryonic kidney (HEK) 293 cells were purchased from the American Type Culture Collection (CRL-1573, Manassas, VA) and were grown in Eagle's minimum essential medium at 5% CO2, 37 °C with 10% fetal bovine serum (FBS), 50 units/ml penicillin, and 50 μg/ml streptomycin. For Western blotting analysis, HEK 293 cells were seeded on 6-well plates at a density of 5 × 105 cells/well and were cultured in the growth medium for 18–24 h prior to transfection. They were co-transfected with various cDNAs using Lipofectamine PLUS reagents. The transfection mixtures in serum-free Opti-MEM (600 μl/well) contained 3 μl of both PLUS and Lipofectamine reagents and 500 ng of each G protein cDNA. For the experiments with other signaling molecules, 250 ng of each construct was added. 3 h after transfection, 300 μl of Opti-MEM containing 30% FBS was added into the wells. For Gαz knockdown experiments, HEK 293 cells were seeded into 6-well plates at 3 × 105 cells/well and again cultured in growth medium overnight. They were transfected and maintained in serum-free Opti-MEM containing 80 pmol of Stealth Select RNAi targeting Gαz RNAi targeting Ras-GRF1, or Stealth RNAi Negative Control Med GC and 5 μl Lipofectamine 2000 per well for 24 h. For luciferase assays, HEK 293 cells were seeded into 96-well white microplates designed for luminescent work at 1.5 × 104 cells/well and were cultured in minimum essential medium overnight. Cells were transiently transfected using Lipofectamine PLUS reagents (26). The transfection mixtures in serum-free Opti-MEM (100 μl/well) contained 0.2 μl of both PLUS and Lipofectamine reagents, 10 ng of cDNAs encoding G proteins or the control vector cDNAs, and 100 ng of pSTAT3-TA-luc. For the experiments with extra signaling molecules, 5 ng of each construct cDNA was added. After 3 h, 5 μl of Opti-MEM containing 30% FBS was added into the wells and cultured overnight.

**Western Blotting Analysis**—30 h after transfection, HEK 293 cells were serum-starved overnight. Prior to cell lysis, incubation with different kinase inhibitors for 30 min, if applicable, was performed. An extra 30 min of agonist exposure was performed for receptor-induced STAT3 activations. For Gαz knockdown experiments, transfectants were directly challenged with different ligands for 30 min. Cells were then lysed in 150 μl of lysis buffer and then gently shaken on ice for 30 min. Supernatants were collected by centrifugation at 16,000 × g for 5 min. Clarified lysates were resolved on 12% SDS-polyacrylamide gels and transferred to nitrocellulose membrane (35). STAT3, phospho-STAT3-Tyr705, phospho-STAT3-Ser727, and...
Data shown represent the mean luciferase activities, and the luminescence emitted was expressed as fold stimulation of the pcDNA1 control. Cells transfected with G\textsubscript{min}, Bt2cAMP (\textbullet), or vehicle (0.1% dimethyl sulfoxide) for 30 min. Cell lysates were immunoblotted with anti-phospho-STAT3-Tyr705 (upper panel), anti-phospho-STAT3-Ser\textsuperscript{727} (middle panel), or anti-STAT3 (lower panel) antisera. The result of the densitometric analysis is shown above the immunoblots; open bars represent the Tyr\textsuperscript{705} phosphorylation level of STAT3, and closed bars indicate the Ser\textsuperscript{727} phosphorylation level of STAT3. Numerical values shown above the immunoreactive bands represent relative intensities of G\textsubscript{\alpha}QL-induced STAT3 phosphorylations expressed as a ratio of the basal level (set as 1.0). B, for the luciferase assay, along with the G protein cDNAs, pSTAT3-TA-luc cDNA was transiently co-transfected in HEK 293 cells. Vehicle, H89, and (R\textsubscript{S})-cAMP treatment of cells was conducted overnight prior to cell lysis. Cell lysates were used to measure luciferase activities, and the luminescence emitted was expressed as fold stimulation of the pcDNA1 control. Data shown represent the mean \pm S.E. from four separate experiments performed in triplicate. C, serum-starved HEK 293 cells were challenged with CTX (100 ng/ml, 4 h), Fsk (50 \mu M, 30 min), (S\textsubscript{R})-cAMP (SP, 100 \mu M, 30 min), Bt2cAMP (DB, 100 \mu M, 30 min), (R\textsubscript{S})-cAMP (RP, 100 \mu M, 30 min), or vehicle (0.1% dimethyl sulfoxide) prior to cell lysis. D, HEK 293 cells were transiently transfected with pcDNA1, G\textsubscript{\alpha}s or G\textsubscript{\alpha}QL. Transfectants were treated with 10 \mu M U73122, 10 \mu M U73343, or vehicle (0.1% dimethyl sulfoxide) for 30 min. Cell lysates were subjected to Western blot analysis as in A. *, G\textsubscript{\alpha}s-QL induced (as well as drug-induced) STAT3 phosphorylations or transcriptional activation were significantly higher than the basal value (one-way ANOVA with Dunnett’s post-tests, \( p < 0.05 \)). Immunoblots shown represent one of three sets; two other sets yielded similar results.

G\textsubscript{\alpha}s were detected by specific primary antisera and horseradish peroxidase-conjugated secondary antisera. The immunoblots were visualized by chemiluminescence with the ECL kit, 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).

**Luciferase Assay**—Transfectants were grown in culture medium for 30 h and then maintained in serum-free medium. Where indicated, cells were treated with different kinase inhibitors overnight in serum-free medium. Cell lysates were analyzed as described previously (26).

**RESULTS**

**Activation of G\textsubscript{\alpha}s Induces STAT3 Activations in HEK 293 Cells**—We have previously demonstrated that hIP is coupled to both G\textsubscript{s} and G\textsubscript{q} for signal transduction (36, 37), and G\textsubscript{\alpha}s may also participate in mediating the hIP-induced STAT3 phosphorylations in HEL cells (29). Hence, we began our study by determining whether a constitutively active mutant of G\textsubscript{\alpha}s (G\textsubscript{\alpha}QL) possesses the ability to stimulate STAT3 phosphorylation and activation. HEK 293 cells were transiently transfected with pcDNA1, G\textsubscript{\alpha}s, or G\textsubscript{\alpha}QL. Total cell lysates prepared from the transfected cells were probed with anti-phospho-STAT3-Tyr\textsuperscript{705}, anti-phospho-STAT3-Ser\textsuperscript{727}, and anti-STAT3 antisera. Expression of either G\textsubscript{\alpha}s or G\textsubscript{\alpha}QL in HEK 293 cells did not affect the expression of total STAT3 as compared with the vector control (Fig. 1A). Anti-phospho-STAT3-Tyr\textsuperscript{705} and anti-phospho-STAT3-Ser\textsuperscript{727} antisera revealed very low levels of basal STAT3 phosphorylation at either Tyr\textsuperscript{705} or Ser\textsuperscript{727} sites in the vector control and G\textsubscript{\alpha}s-expressing cells. In contrast, expression of G\textsubscript{\alpha}QL led to a detectable increase in both Tyr\textsuperscript{705} and Ser\textsuperscript{727} phosphorylation of STAT3 (Fig. 1A). Pretreating the transfectants with P3K inhibitors, 10 \mu M H-89 or 100 \mu M (R\textsubscript{S})-cAMP, significantly inhibited G\textsubscript{\alpha}QL-induced STAT3 Tyr\textsuperscript{705} and Ser\textsuperscript{727} phosphorylations. Examination of the STAT3-driven transcriptional activity was also performed using a luciferase reporter gene assay. In HEK 293 cells transiently co-transfected with pSTAT3-TA-luc, G\textsubscript{\alpha}QL significantly induced STAT3 transcriptional activation as com-
Ser727 phosphorylations (Fig. 1C). Stimulation of AC by Fsk and the applications of cAMP analogues (Bt2cAMP and (S)p-cAMP) also increased the STAT3 Tyr705 and Ser727 phosphorylations, although using (R)p-cAMP did not induce any significant change in STAT3 phosphorylations. Thus, activation of the Gαs pathway could indeed result in STAT3 phosphorylation and transcriptional activation.

GαsQL Induces STAT3 Phosphorylations via MEK1/2, JNK, c-Src, and PI3K in HEK 293 Cells—A number of signaling molecules have been shown to be required for Gαc-induced activation of STAT3 (7, 26, 27). They include c-Src (for GαsQL, GαsQL, and Gα16QL), ERK, and PLCβ/PKC/CaMKII (for Gα14QL and Gα16QL). Hence, we asked if GαsQL-mediated STAT3 phosphorylations utilize the same signaling intermediates as other constitutively active Gα subunits. PLCβ/PKC/CaMKII signaling cascade is one of the critical pathways regulating Gα14QL- and Gα16QL-mediated STAT3 activations (26, 27). Because Gαc is unable to modulate the PLCβ cascade, inhibiting PLCβ should have no effect on GαsQL-mediated STAT3 phosphorylations. HEK 293 cells were transfected with pcDNA1, Gαs, or GαsQL and treated with different kinase inhibitors for 30 min (Figs. 1D, 2, 3, and 5). As expected, treatment of GαsQL-transfected cells with U73122 or U73343 (10 μM) did not affect STAT3 phosphorylations (Fig. 1D), suggesting the lack of involvement of PLCβ in GαsQL-mediated STAT3 activation. ERK has been shown to be a central component in GαsQL-induced activation of STAT3 (26). To study the requirement of ERK in the GαsQL-induced STAT3 phosphorylations, we used 10 μM U0126 to inhibit MEK1/2, the upstream regulators of ERK. Suppression of MEK1/2 activity by U0126 significantly blocked the Ser727, but not the Tyr705, phosphorylation of STAT3 (Fig. 2A). This is in agreement with the differential attenuation of hIP-induced STAT3 Ser727 phosphorylation by U0126 in HEL cells (29). Apart from ERK, the possible involvement of the other two MAPKs was also examined. Blockade of JNK and p38 MAPK by SP600125 (30 μM) and SB202190 (10 μM), respectively, produced very different results (Fig. 2A). Inhibition of

pared with the corresponding controls (Fig. 1B). Inhibition of PKA by H-89 or (R)p-cAMP completely abrogated the GαsQL-induced luciferase activity, indicating that Gαs/cAMP is able to mediate STAT3 phosphorylations and transcriptional activation.

To further confirm the specific involvement of Gαs/cAMP in the activation of STAT3, we utilized Gαs-activating CTX, adenylyl cyclase (AC)-stimulating forskolin (Fsk), and cAMP analogues (Bt2cAMP and (S)p-cAMP; Fig. 1C). Pretreating HEK 293 cells for 4 h with CTX to directly stimulate Gαs by ADP-ribosylation (38) significantly induced both STAT3 Tyr705 and

FIGURE 2. The requirement of MAPKs in GαsQL-induced STAT3 phosphorylations in HEK 293 cells. A and B, HEK 293 cells were transiently transfected with pcDNA1, Gαs, or GαsQL. Cells were treated with 10 μM U0126, 10 μM U0126, 30 μM SP600125 (SP600), 30 μM negative SP600125 (SP-ve), 10 μM SB202190 (SB202), 10 μM SB202474 (SB-ve), or vehicle (0.1% dimethyl sulfoxide) for 20 min. C and D, HEK 293 cells were transiently transfected with pcDNA1, Gαs, or GαsQL, along with cDNAs of wild type or dominant negative mutants (DN) of different signaling molecules, including Ras, Rac1, RhoA, or vector control (pcDNA1). Cell lysates were resolved and analyzed for STAT3, ERK, JNK, and c-Jun phosphorylations as described in Fig. 1A. Numerical values shown above the immunoreactive bands represent relative intensities of STAT3 phosphorylations expressed as a ratio of the basal level with dimethyl sulfoxide (set as 1.0). *, GαsQL-induced STAT3 phosphorylations were significantly higher than the basal value (one-way ANOVA with Dunnett’s post-tests, p < 0.05). Data shown represent the mean ± S.E. from three separate experiments, and the immunoblots shown represent one of three sets; two other sets yielded similar results.
JNK suppressed both Tyr^{705} and Ser^{727} STAT3 phosphorylations, whereas p38 MAPK inhibition was unable to alter any Gα_s-QL-induced STAT3 activations. To address the specificity of these kinase inhibitors, we analyzed the MEK/ERK and JNK/c-Jun activations using various antibodies following the treatment of cells with different kinase inhibitors. As shown in Fig. 2B, only the application of U0126 led to the attenuation of ERK phosphorylation, whereas SP600125 and the negative controls (U0124 and SP-ve) were ineffective. Inhibition of JNK by SP600125 suppressed the phosphorylation of c-Jun. The use of further studies on different monomeric GTPases associated with the MAPK cascades were performed by overexpressing their dominant negative mutants (Fig. 2C). The ability of Gα_s-QL to stimulate STAT3 phosphorylations was unaffected by the presence of wild type monomeric GTPases. Consistent with the results obtained with U0126 (Fig. 2A), inhibition of the Ras/Raf/MEK pathway by overexpressing the dominant negative Ras (RasDN) inhibited Ser^{727} STAT3 phosphorylation but failed to attenuate the Tyr^{705} STAT3 phosphorylation (Fig. 2C). Along with STAT3, Gα_s-QL-induced ERK phosphorylations were reduced as well, although the reductions were only partial (Fig. 2D), thus indicating that ERK activation can be attained via other pathways besides Ras.

Inhibition of the Rac1/JNK pathway by overexpression of Rac1 dominant negative mutant (Rac1DN) completely abolished Gα_s-QL-induced STAT3 phosphorylations at both Tyr^{705} and Ser^{727} (Fig. 2C) whereas wild type Rac1 had no effect STAT3 phosphorylation. Overexpression of Rac1DN markedly reduced the phosphorylations of JNK as well as c-Jun (Fig. 2D). Gα_s-QL-induced ERK phosphorylation was also partially inhibited by Rac1DN, denoting that Rac1 can regulate both JNK and ERK activities (Fig. 2D). As a control, we also examined monomeric RhoA GTPase on STAT3 regulations. Neither the expression of wild type RhoA nor dominant negative RhoA (RhoADN) elicited any inhibitory effect on Gα_s-QL-induced STAT3 phosphorylations.

Next we tested the involvement of c-Src because this tyrosine kinase is required for Gα_s-QL-induced STAT3 phosphorylations (29). As shown in Fig. 3A, inhibitors of c-Src (PP1 and PP2) were capable of reducing Gα_s-QL-induced STAT3 Ser^{727} phosphorylation. This reduction was also observed when the dominant negative mutant of c-Src (c-SrcDN) was overexpressed (Fig. 3B), whereas wild type c-Src neither affected the STAT3 expression nor the Gα_s-QL-induced STAT3 Tyr^{705} phosphorylation. However, unlike STAT3 Ser^{727} phosphorylation, STAT3 Tyr^{705} phosphorylation was insensitive to inhibi-
Data shown represent the mean ± S.E. from three separate experiments, and the immunoblots shown represent one of three sets; two other sets yielded similar results.

FIGURE 4. Modulating capacity of JNK, but not Ras/ERK, on JAK2 phosphorylation in HEK 293 cells. A, HEK 293 cells were transiently transfected with pcDNA1, GαQL, or GαQL along with cDNAs of wild type or dominant negative mutant (DN) of Ras or vector control (pcDNA1). Cell lysates were resolved and analyzed for c-Src phosphorylation. B, HEK 293 cells were transiently transfected with pcDNA1, GαQL, or GαQL along with cDNAs of wild type or dominant negative mutant (DN) of Ras or vector control (pcDNA1). Cell lysates were resolved and analyzed for c-Src and JAK2 phosphorylation. C, HEK 293 cells were transiently transfected with the constitutive active mutant of JNKK (JNKK-CA), MEK1, or vector control (pcDNA1). Cell lysates were resolved and analyzed for JAK2, c-Src, ERK, and JNK phosphorylations. Numerical values shown above the immunoreactive bands represent relative intensities of analyzed for JAK2, c-Src, ERK, and JNK phosphorylations. Numerical values shown above the immunoreactive bands represent relative intensities of STAT3 phosphorylations expressed as a ratio of the basal level (set as 1.0). *, GαQL-induced STAT3 phosphorylations as described in Fig. 1A. Numerical values shown above the immunoreactive bands represent relative intensities of STAT3 phosphorylations expressed as a ratio of the basal level with dimethyl sulfoxide (set as 1.0). **, GαQL-induced STAT3 phosphorylations as described in Fig. 1A. Numerical values shown above the immunoreactive bands represent relative intensities of STAT3 phosphorylations expressed as a ratio of the basal level with dimethyl sulfoxide (set as 1.0).

FIGURE 5. Involvement of PI3K in GαQL-induced STAT3 phosphorylations in HEK 293 cells. A, HEK 293 cells were transiently transfected with pcDNA1, GαQL, or GαQL along with cDNAs of wild type or dominant negative mutant (DN) of PI3K or vector control (pcDNA1). Overexpression of exogenous PI3K was confirmed by an anti-PI3K antiserum (lowest panel). Cell lysates were resolved and analyzed for STAT3 and ERK phosphorylations as described in Fig. 1A. Numerical values shown above the immunoreactive bands represent relative intensities of STAT3 phosphorylations expressed as a ratio of the basal level with dimethyl sulfoxide (set as 1.0). *, GαQL-induced STAT3 phosphorylations as described in Fig. 1A. Numerical values shown above the immunoreactive bands represent relative intensities of STAT3 phosphorylations expressed as a ratio of the basal level with dimethyl sulfoxide (set as 1.0). **, GαQL-induced STAT3 phosphorylations as described in Fig. 1A. Numerical values shown above the immunoreactive bands represent relative intensities of STAT3 phosphorylations expressed as a ratio of the basal level with dimethyl sulfoxide (set as 1.0).

PI3K has been shown to mediate wortmannin-sensitive activation of MAPKs by GPCRs (39). Thus, PI3K appears to be a prime candidate for signal integration in the activation of STAT3 by GαQL. GαQL-induced STAT3 Tyr705 and Ser727 phosphorylations were both attenuated upon suppression of PI3K by its inhibitors, wortmannin (100 nm) and LY294002 (10 μM; Fig. 5A). Similar suppressions were observed when the

PI3K has been shown to mediate wortmannin-sensitive activation of MAPKs by GPCRs (39). Thus, PI3K appears to be a prime candidate for signal integration in the activation of STAT3 by GαQL. GαQL-induced STAT3 Tyr705 and Ser727 phosphorylations were both attenuated upon suppression of PI3K by its inhibitors, wortmannin (100 nm) and LY294002 (10 μM; Fig. 5A). Similar suppressions were observed when the
dominant negative mutant of PI3K (PI3KDN) was transiently co-transfected into HEK 293 cells with Goalpha_QL (Fig. 5B). Overexpression of wild type PI3K had no effect on STAT3 activations, whereas the overexpression of PI3KDN completely abrogated the Goalpha_QL-induced STAT3 responses. In control experiments, application of PI3K inhibitors (wortmannin and LY294002) or overexpression of PI3KDN effectively removed Akt phosphorylation (data not shown). Suppression of PI3K activity weakly inhibited the ability of Goalpha_QL to induce ERK phosphorylation (Fig. 5, A and B), suggesting that the regulation of STAT3 by PI3K may in part be mediated via ERK.

Collectively, the Goalpha_QL-induced STAT3 Ser727 phosphorylation was independent of PLCβ and appeared to be mediated via multiple signaling intermediates, including Ras/MEK1/2, JAK2, and c-Src, whereas the Goalpha_QL-induced STAT3 Tyr705 phosphorylation did not require the participation of PI3K (26). To corroborate with their ability to block Goalpha_QL-induced STAT3 Tyr705 phosphorylation, we transiently co-transfected HEK 293 cells with Goalpha_QL and treated with various kinase inhibitors. As described earlier, Goalpha_QL significantly induced STAT3-driven luciferase expression (Fig. 1B). The Goalpha_QL-induced luciferase activity was, however, almost totally suppressed in the presence of 10 μM H-89, 30 μM SP600125, 100 μM AG490, 100 μg/ml WHI-P131, 100 nm wortmannin, or 10 μM LY294002 (Fig. 6A). Inhibition of STAT3-driven luciferase activity by these agents corroborated with their ability to block Goalpha_QL-induced STAT3 Tyr705 and Ser727 phosphorylations (Figs. 1–3 and 5). Given that STAT3 Tyr705 phosphorylation alone is sufficient to drive STAT3 transcriptional activity (14), inhibition of Ser727 phosphorylation by the inhibitors of c-Src and MEK should not completely suppress Goalpha_QL-induced STAT3-driven luciferase activity. As predicted, Goalpha_QL-induced STAT3 transcriptional activation was significantly but only partially (>65%) attenuated by 25 μM PP1, 25 μM PP2, 10 μM Raf-1 kinase inhibitor, or 10 μM U0124 (Fig. 6A). None of the corresponding inactive analogues, including inactive U0124 (10 μM), PP3 (25 μM), LY303511 (10 μM), and WHI-P258 (10 μM) showed any inhibitory effects.

The different dominant negative mutants of various signaling molecules, including the three small GTPases (Ras, Rac1, and RhoA), PI3K, and c-Src, were also examined for their effects on Goalpha_QL-induced STAT3-driven luciferase expression (Fig. 6, B and C). None of the wild type proteins elicited any effects on Goalpha_QL-induced STAT3-driven luciferase expression as compared with the vector control (pcDNA1). In line with the previous findings (Figs. 2 and 5), both Rac1DN (Fig. 6B) and PI3KDN (Fig. 6C) were capable of almost completely attenuating the Goalpha_QL-induced STAT3-driven luciferase expression. RasDN (Fig. 6B) and c-SrcDN (Fig. 6C) only partially reduced the Goalpha_QL-mediated luciferase responses. Finally, RhoADN was unable to suppress the Goalpha_QL-induced STAT3-driven luciferase expression (Fig. 6B).

**FIGURE 6.** Differential regulation by c-Src, MEK1/2, and PI3K in Goalpha_QL-induced STAT3-driven transcriptional activations in HEK 293 cells. A, HEK 293 cells were transiently co-transfected with pSTAT3-TA-luc, pcDNA1, Goalpha, or Goalpha_QL and treated with different kinase inhibitors. As described earlier, Goalpha_QL significantly induced STAT3-driven luciferase expression (Fig. 1B). The Goalpha_QL-induced luciferase activity was, however, almost totally suppressed in the presence of 10 μM H-89, 30 μM SP600125, 100 μM AG490, 100 μg/ml WHI-P131, 100 nm wortmannin, or 10 μM LY294002 (Fig. 6A). Inhibition of STAT3-driven luciferase activity by these agents corroborated with their ability to block Goalpha_QL-induced STAT3 Tyr705 and Ser727 phosphorylations (Figs. 1–3 and 5). Given that STAT3 Tyr705 phosphorylation alone is sufficient to drive STAT3 transcriptional activity (14), inhibition of Ser727 phosphorylation by the inhibitors of c-Src and MEK should not completely suppress Goalpha_QL-induced STAT3-driven luciferase activity. As predicted, Goalpha_QL-induced STAT3 transcriptional activation was significantly but only partially (>65%) attenuated by 25 μM PP1, 25 μM PP2, 10 μM Raf-1 kinase inhibitor, or 10 μM U0124 (Fig. 6A). None of the corresponding inactive analogues, including inactive U0124 (10 μM), PP3 (25 μM), LY303511 (10 μM), and WHI-P258 (10 μM) showed any inhibitory effects.

**Pivotal Role of PKA in Goalpha__-mediated STAT3 Activation**—Because the cAMP/PKA pathway is immediately downstream of Goalpha_, we further analyzed the effects of inhibiting PKA by H-89 on the various signaling molecules. Cell lysates from H-89-treated cells were resolved and analyzed for the activations of c-Src, JAK2, ERK, and JNK (Fig. 7A). In agreement with Fig. 1, overexpression of Goalpha_QL or the application of Bt,cAMP led to both STAT3 Tyr705 and Ser727 phosphorylations, and the STAT3 responses were abolished upon inhibition of PKA by H-89. H-89 also attenuated the Goalpha_QL- and Bt,cAMP-induced activations of c-Src, JAK2, ERK, and JNK (Fig. 7A). These results suggest that PKA is probably upstream of these signaling molecules.

Recently, an endogenously expressed GEF, named RasGRF1, has been shown to be activated by Goalpha-_coupled serotonin 5-HT2 receptor via PKA, and its activation leads to the stimu-
JNK cascades but not for the c-Src/JAK pathway. Ras-GRF1 plays a significant role in modulating ERK and STAT3 phosphorylations in HEK 293 cells. Taken together, these results indicate that transiently transfected with pcDNA1, GαQL, or GαQL. Transfectants were pre-treated with 10 μM H-89 or vehicle (0.1% dimethyl sulfoxide) for 30 min followed by a 30-min incubation with Bt2cAMP (DB) where appropriate. Cell lysates were resolved and analyzed for STAT3, ERK, JNK, JAK2, and c-Src phosphorylations as described in Fig. 1A. B, HEK 293 cells were transfected with pcDNA1, GαQL, or GαQL in the absence or presence of the siRNA of Ras-GRF1 (siRas-GRF1) or control siRNA (Ctrl siRNA). Effective knockdown of Ras-GRF1 was confirmed by an anti-Ras-GRF1 antiserum (lower panel). Cell lysates were resolved and analyzed for STAT3, ERK, JNK, JAK2, and c-Src phosphorylations as well as total expression of Ras-GRF1 as described in Fig. 1A. Numerical values shown above the immunoreactive bands represent relative intensities of STAT3 phosphorylations expressed as a ratio of the basal level with vector control (set as 1.0). Data shown represent the mean ± S.E. from three separate experiments, and the immunoblots shown represent one of three sets; two other sets yielded similar results.

FIGURE 7. The involvement of PKA and Ras-GEF in GαQL-mediated STAT3 phosphorylations in HEK 293 cells. A, HEK 293 cells were transiently transfected with pcDNA1, GαQL, or GαQL. Transfectants were pre-treated with 10 μM H-89 or vehicle (0.1% dimethyl sulfoxide) for 30 min followed by a 30-min incubation with Bt2cAMP (DB) where appropriate. Cell lysates were resolved and analyzed for STAT3, ERK, JNK, JAK2, and c-Src phosphorylations as described in Fig. 1A. B, HEK 293 cells were transfected with pcDNA1, GαQL, or GαQL in the absence or presence of the siRNA of Ras-GRF1 (siRas-GRF1) or control siRNA (Ctrl siRNA). Effective knockdown of Ras-GRF1 was confirmed by an anti-Ras-GRF1 antiserum (lower panel). Cell lysates were resolved and analyzed for STAT3, ERK, JNK, JAK2, and c-Src phosphorylations as well as total expression of Ras-GRF1 as described in Fig. 1A. Numerical values shown above the immunoreactive bands represent relative intensities of STAT3 phosphorylations expressed as a ratio of the basal level with vector control (set as 1.0). Data shown represent the mean ± S.E. from three separate experiments, and the immunoblots shown represent one of three sets; two other sets yielded similar results.

Gαs-Activates STAT3 Phosphorylation

Gαs-coupled β2AR-mediated STAT3 Activations Also Require MEK1/2, JNK, PI3K, and c-Src—Because GαsQL stimulated the phosphorylation and activation of STAT3, we next sought to investigate whether the Gαs-coupled β2AR is also capable of stimulating STAT3. HEK 293 cells are known to endogenously express β2AR, and application of isoproterenol, a selective β-AR agonist, leads to an increase in AC activity (42). As shown in Fig. 8A, weak levels of STAT3 Tyr705 and Ser727 phosphorylations were observed in HEK 293 cells incubated with 10 μM isoproterenol for 10 min or more. The magnitude of the isoproterenol-induced STAT3 phosphorylations (~2-fold) was similar to that obtained with GαsQL (Fig. 1A), and the phosphorylations were sustained up to 120 min; the level of phosphorylated STAT3 gradually decreased thereafter and returned to basal level at 4 h after isoproterenol challenge (data not shown). As isoproterenol is a nonselective agonist for both β1- and β2ARs, we employed the highly selective agonist for β2AR (salbutamol) to confirm the involvement of β2AR. Exposure of HEK 293 to salbutamol (10 μM) for 30 min significantly induced both Tyr705 and Ser727 STAT3 phosphorylations with the magnitude of stimulation comparable with that obtained with isoproterenol (Fig. 8B). Furthermore, isoproterenol-induced STAT3 phosphorylations were effectively blocked in the presence of a selective antagonist of β2AR. As shown in Fig. 8B, ICI 115-881 (1 mM) treatment did not have any effect on STAT3 stimulations, although it has been reported to elevate basal ERK activity (43). Co-treatment of ICI 115-881 with isoproterenol completely abrogated the isoproterenol-induced STAT3 responses.

In light of the capacity of β2AR to signal via Gs proteins (44) and the ability of Gαs to promote STAT3 activations (25), we next asked whether the Gαs signals contribute to isoproterenol-induced STAT3. To eliminate the possible coupling of β2AR to Gs proteins, HEK 293 cells were pretreated with PTX (100 ng/ml) overnight. The PTX pretreatment had no effect on basal levels nor did it suppress the isoproterenol-induced STAT3 phosphorylations (Fig. 8B). This suggests that the β2AR-mediated STAT3 phosphorylations are primarily dependent on Gs signaling.

To confirm this postulation, we introduced Gαs-targeting siRNA into HEK 293 cells (Fig. 9A). Introduction of siGαs into HEK 293 cells substantially reduced the Gαs expression level (>90%) as compared with parental HEK 293 cells (Fig. 9A). As reported in HeLa cells (45), compensatory increase of other Gs subunits was not detectable (data not shown). In contrast, the expression of Gαs was unaffected in cells transfected with the control siRNA. When HEK 293 cells were transfected with control siRNA, isoproterenol and salbutamol remained capable of stimulating STAT3. HEK 293 cells are known to endogenously express β2AR. Exposure of HEK 293 to salbutamol (10 μM) for 30 min significantly induced both Tyr705 and Ser727 STAT3 phosphorylations with the magnitude of stimulation comparable with that obtained with isoproterenol (Fig. 8B). Furthermore, isoproterenol-induced STAT3 phosphorylations were effectively blocked in the presence of a selective antagonist of β2AR. As shown in Fig. 8B, ICI 115-881 (1 mM) treatment did not have any effect on STAT3 stimulations, although it has been reported to elevate basal ERK activity (43). Co-treatment of ICI 115-881 with isoproterenol completely abrogated the isoproterenol-induced STAT3 responses.

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The siRNA studies suggest that the regulation of STAT3 by β2AR is mainly mediated via Goαs-dependent pathways. If so, β2AR-induced STAT3 phosphorylations should exhibit the same sensitivity to various kinase inhibitors as Goαs (si Goαs) in serum-free conditions for 24 h. Prior to cell lysis, transfectants were challenged with carbachol (CCh, 100 μM), isoproterenol (ISO, 10 μM), salbutamol (Sal, 10 μM), or EGF (50 ng/ml) for 30 min. Cell lysates were immunoblotted for STAT3 phosphorylations and analyzed as described in Fig. 1A. Numerical values shown above the immunoreactive bands represent relative intensities of agonist-induced STAT3 phosphorylations expressed as a ratio of the basal level (set as 1.0). *, agonist-induced STAT3 phosphorylations were significantly higher than the basal value (one-way ANOVA with Dunnett’s post-tests, p < 0.05). Data shown represent the mean ± S.E. from three separate experiments, and the immunoblots shown represent one of three sets; two other sets yielded similar results.

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**FIGURE 8.** Isoproterenol is capable of activating Tyr705 and Ser727 STAT3 phosphorylations in a time-dependent and PTX-insensitive manner mediated through β2AR. A, HEK 293 cells were challenged by isoproterenol (10 μM) for different time intervals. B, to test the specificity of β2AR to induce STAT3 activations by isoproterenol, HEK 293 cells were either stimulated with salbutamol (Sal, 10 μM) or co-treated with ICI 118551 (ICI, 1 μM) and isoproterenol (ISO, 10 μM) for 30 min. To eliminate the involvement of G proteins, HEK 293 cells were pretreated with PTX (100 ng/ml) overnight before the 30-min isoproterenol exposure. Cell lysates were immunoblotted for STAT3 phosphorylations and analyzed as described in Fig. 1A. Numerical values shown above the immunoreactive bands represent relative intensities of agonist-induced STAT3 phosphorylations expressed as a ratio of the basal level (set as 1.0). *, agonist-induced STAT3 phosphorylations were significantly higher than the basal value (one-way ANOVA with Dunnett’s post-tests, p < 0.05). Data shown represent the mean ± S.E. from three separate experiments, and the immunoblots shown represent one of three sets; two other sets yielded similar results.

**FIGURE 9.** Isoproterenol-stimulated β2AR-mediated STAT3 phosphorylations in HEK 293 cells are dependent upon Goαs. A and B, HEK 293 cells were transfected with either control siRNA (Ctrl siRNA) or siRNA targeting Goαs (si Goαs) in serum-free conditions for 24 h. Prior to cell lysis, transfectants were challenged with carbachol (CCh, 100 μM), isoproterenol (ISO, 10 μM), salbutamol (Sal, 10 μM), or EGF (50 ng/ml) for 30 min. Cell lysates were immunoblotted for STAT3 phosphorylations and analyzed as described in Fig. 1A. Numerical values shown above the immunoreactive bands represent relative intensities of agonist-induced STAT3 phosphorylations expressed as a ratio of the basal level (set as 1.0). *, agonist-induced STAT3 phosphorylations were significantly higher than the basal value (one-way ANOVA with Dunnett’s post-tests, p < 0.05). Data shown represent the mean ± S.E. from three separate experiments, and the immunoblots shown represent one of three sets; two other sets yielded similar results.

**DISCUSSION**

In recent years, it has become increasingly clear that G protein signaling can modulate gene transcription and thus affect c-Src by PP1 and PP2 (Fig. 10B) significantly attenuated the isoproterenol-induced Ser727 STAT3 phosphorylation, whereas the Tyr705 STAT3 phosphorylation was not affected. Inhibitions of JNK by SP600125 (Fig. 10A), JAKs by AG490 or WHI-P121 (Fig. 10B), and PI3K by wortmannin or LY294002 (Fig. 10C) completely abrogated both Ser727 and Tyr705 STAT3 phosphorylations induced by isoproterenol. Specific inhibitors against p38 MAPK (SB202190; Fig. 10A) and PLC (U73122; Fig. 10D) were unable to modify the patterns of β2AR-induced STAT3 phosphorylations. Additionally, none of the inactive analogues tested (U0124, SP-ve, SB-ve, PP3, WHI-ve, and U73343) had any suppressive effect on isoproterenol-induced STAT3 phosphorylations. In summary, the patterns of STAT3 phosphorylations generated by the isoproterenol-induced β2AR activation were identical to those elicited by the expression of GoαsQL in HEK 293 cells.

**FIGURE 10.** Isoproterenol-induced Ser727 and Tyr705 STAT3 phosphorylations in HEK 293 cells are dependent upon Goαs. A and B, HEK 293 cells were transfected with either control siRNA (Ctrl siRNA) or siRNA targeting Goαs (si Goαs) in serum-free conditions for 24 h. Prior to cell lysis, transfectants were challenged with carbachol (CCh, 100 μM), isoproterenol (ISO, 10 μM), salbutamol (Sal, 10 μM), or EGF (50 ng/ml) for 30 min. Cell lysates were immunoblotted for STAT3 phosphorylations and analyzed as described in Fig. 1A. Numerical values shown above the immunoreactive bands represent relative intensities of agonist-induced STAT3 phosphorylations expressed as a ratio of the basal level (set as 1.0). *, agonist-induced STAT3 phosphorylations were significantly higher than the basal value (one-way ANOVA with Dunnett’s post-tests, p < 0.05). Data shown represent the mean ± S.E. from three separate experiments, and the immunoblots shown represent one of three sets; two other sets yielded similar results.
**Gα\textsubscript{s} Activates STAT3 Phosphorylation**

**A**

![Graph A](image)

**B**

![Graph B](image)

**C**

![Graph C](image)

**D**

![Graph D](image)

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**FIGURE 10.** The involvement of JNK, PI3K, and JAK2 in isoproterenol-induced STAT3 phosphorylations in HEK 293 cells. HEK 293 cells were pretreated for 30 min at 37 °C with 10 μM Raf-1 kinase inhibitor (Raf-1), 10 μM U0126, 10 μM U0124, 30 μM SP600125 (SP600), 30 μM negative SP600125 (SP-ve), 10 μM SB202190 (SB202), or 10 μM SB202474 (SB-ve) (A); 25 μM PP1, 25 μM PP2, 25 μM PP3, 100 μM AG490, 100 μg/ml WHI-P258 (WHI-ve) (B); 100 nM wortmannin (Wort) or 10 μM LY294002 (LY294) (C); 10 μM U73122 or 10 μM U73343 (D). They were then stimulated by isoproterenol (10 μM) for another 30 min. Cell lysates were immunoblotted for STAT3 phosphorylations and analyzed as described in Fig. 1A. Numerical values shown above the immunoreactive bands represent relative intensities of isoproterenol-induced STAT3 phosphorylations expressed as a ratio of the basal level (set as 1.0). *, isoproterenol-induced STAT3 phosphorylations were significantly higher than the basal value (one-way ANOVA with Dunnett’s post-tests, p < 0.05). Data shown represent the mean ± S.E. from three separate experiments, and the immunoblots shown represent one of three sets; two other sets yielded similar results.

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Numerous biological processes. Among the many transcription factors identified to date, the STAT proteins are the most well established in terms of their regulation by G proteins. Members of the G\textsubscript{α}\textsubscript{q}, G\textsubscript{α}\textsubscript{s}, and G\textsubscript{α}\textsubscript{QL} families have been reported to stimulate STAT3 Tyr\textsuperscript{705} and Ser\textsuperscript{727} phosphorylations through several common signaling intermediates such as Src and MAPKs (26, 27, 46, 47). Despite the fact that a number of G\textsubscript{s}-coupled receptors have been reported to activate STAT3 (28, 29, 48), very little is known with regard to their mechanism of action. Moreover, because many GPCRs can activate multiple G proteins simultaneously, there remains a possibility of G\textsubscript{s}-independent activation of STAT3 (e.g., via G\textsubscript{βγ} or other G\textsubscript{α} subunits) by these receptors. In this study, we have unequivocally demonstrated that activated G\textsubscript{s} is indeed fully capable of stimulating STAT3 phosphorylations as well as STAT3-driven gene expression. This conclusion is based on several lines of evidence. 1) Expression of G\textsubscript{sQL} in HEK 293 cells led to STAT3 phosphorylations and transcriptional activation. 2) Manipulation of G\textsubscript{s}-dependent signals can stimulate or inhibit STAT3 activity accordingly. 3) Stimulation of endogenous G\textsubscript{s}-coupled β\textsubscript{2}-AR resulted in STAT3 activation, and the sensitivities of STAT3 responses to specific inhibitors were identical to those obtained with G\textsubscript{sQL}. 4) Knockdown of G\textsubscript{s} expression by RNAi effectively abolished the receptor-induced STAT3 responses. More interestingly, our study revealed that the G\textsubscript{s}-mediated STAT3 regulations involve a complex mechanism uniquely requiring PKA, PI3K, and JNK.

Previous studies on the regulation of STAT3 by members of the G\textsubscript{s} proteins (26, 27) have indicated that the PLC\textsubscript{β}/PKC/CA MKII cascade plays a crucial role in stimulating STAT3. Because the primary signal generated upon activation of G\textsubscript{s} is cAMP instead of inositol 1,4,5-trisphosphate/Ca\textsuperscript{2+}, it is hardly surprising that G\textsubscript{s}-mediated STAT3 phosphorylations employ components of the cAMP pathway (Fig. 1A) and are refractory to the inhibition of PLC\textsubscript{β} (Figs. 1D, 6A, and 10D). We have used a number of approaches to verify that activation of the AC/PKA pathway can lead to STAT3 Tyr\textsuperscript{705} and Ser\textsuperscript{727} phosphorylations as well as its transcriptional activation in HEK 293 cells. These include stimulation of endogenous G\textsubscript{s} by CTX (Fig. 1C) or β\textsubscript{2}-AR (Fig. 8A), direct activation of AC by Fsk, mimicking CAMP actions with Bt\textsubscript{2}cAMP and (S\textsubscript{35})-cAMP (Fig. 1C), and inhibiting PKA by H-89 and (R\textsubscript{s})-cAMP (Fig. 1A). These results illustrate that the G\textsubscript{s}/AC/PKA pathway is indeed capable of activating STAT3 and may provide the molecular basis for the regulation of STAT3 by G\textsubscript{s}-coupled receptors such as luteinizing hormone receptor (49) and β-AR (32).

Extensive studies have been performed on G protein-mediated STAT3 regulations where the involvement of ERK and
c-Src/JAK signaling pathways are evident (26, 27). In Goαs-mediated STAT3 regulation, PKA is likely to be involved in the activation of STAT3 because inhibition of PKA suppressed the phosphorylation of STAT3 at both Tyr705 and Ser727 (Fig. 1). Indeed, PKA appears to play a pivotal role in STAT3 regulation because of its ability to modulate various signaling cascades. Blockade of PKA resulted in the attenuation of ERK, JNK, c-Src, and JAK2 phosphorylations (Fig. 7A), signifying that PKA is a key control for several downstream cascades important for Goαs-induced STAT3 regulations.

Despite their primary linkage to different effector pathways, Goαs and Go16 share some common features in activating STAT3. As in Goαs-induced STAT3 activation, the participation of c-Src/JAK pathway also appears to be important in Goαs-mediated STAT3 activations. Goαs has been reported to stimulate the activity of c-Src (50), whereas in terms of signaling intermediates, c-Src and JakS are well known regulators of STAT3 (51). It is therefore conceivable that c-Src/JAK acts as a site for the integration of signals from GPCRs to STAT3. Our findings support the participation of c-Src and Jak in Goαs-induced STAT3 phosphorylations because the application of selective inhibitors of c-Src (PP1 and PP2) and JAK2/3 (AS490 and WHI-131) (Fig. 3A) as well as expression of c-SrcDN (Fig. 3B and 6C) suppress these responses. We further delineated that c-Src appears to be upstream of JAK2, as illustrated by the ability of c-Src inhibitors (Fig. 3C) or overexpression of c-SrcDN (Fig. 3D) to inhibit Goαs-induced JAK2 phosphorylation. Yet, unlike C5a/Goαs-induced responses (26), the Goαs/QL- or isoproterenol-induced STAT3 Tyr705 phosphorylation was not affected upon inhibition of c-Src (Fig. 3, A and B, and 10B). The insensitivity of Goαs/QL-linked STAT3 Tyr705 phosphorylation to c-Src inhibitors suggests that activation of Goαs may trigger an alternative route for the phosphorylation of STAT3 at Tyr705 by a tyrosine kinase other than c-Src. Indeed, Etk, a Bruton tyrosine kinase, was reported to directly regulate STAT3 activation through Goαs (52), whereas the cAMP/PKA cascade is able to modulate spleen tyrosine kinase (53). Nevertheless, much remain to be elucidated, especially the identity and specificity of JAK isoforms. Apart from JAK2/3 (as illustrated in the present study), other JAKs may also be involved in the regulation of STAT3.

A large body of evidence has illustrated that PKA can activate MAPks (54) such as ERKs which are crucial in STAT3 regulations. The importance of MEK/ERK in the regulation of the Ser727 STAT3 phosphorylation has long been suggested (23), and this regulation appears to be equally critical in G protein-mediated STAT3 activation (26, 27, 46). At least two different routes are available for the activation of MEK/ERK by PKA (Fig. 11). First, MEK/ERK activations by PKA can be mediated through a small G protein named Rap1 (55). Rap1 is stimulated by PKA and consequently leads to the excitation of Raf-1 kinase that is directly upstream of the MEK/ERK pathway. Another route to ERK activation involves Ras. Its involvement was demonstrated by the overexpression of RasDN that attenuated Goαs/QL-mediated ERK phosphorylations (Fig. 2D). As observed for the Goαs-coupled serotonin 5-HT7 receptor (40), the activation of PKA promotes Ras activation and subsequent MEK/ERK stimulation via the recruitment of a GEF named Ras-GRF1. Our findings confirm the involvement of the MEK/ERK pathway in Goαs-mediated STAT3 responses. However, unlike the G16-mediated STAT3 stimulations (26), the Goαs/QL- or isoproterenol-induced STAT3 Tyr705 phosphorylation is insensitive to inhibition of MEK1/2 by U0126 (Figs. 2A and 10A) or to blockade by RasDN (Fig. 2C). The Ras/Raf/MEK/ERK pathway thus appears to selectively modulate the Ser727 site on STAT3. Because the precise mechanism by which MEK/ERK mediates STAT3 Tyr705 phosphorylation has not been elucidated, it is difficult to envisage how Goαs and Goαs signals can produce different degrees of dependence on MEK/ERK.

Rac1 represents another monomeric GTPase that can regulate STAT3 signaling. This monomeric GTPase is known to activate JNK through mitogen-activated kinase kinase 4/7 (MKK4/7; like MEK in ERK pathway) (56); indeed, overexpression of Rac1DN significantly attenuated the activity of JNK as illustrated by the suppression of c-Jun phosphorylation (Fig. 2D). As compared with Rac1-dependent but JNK-independent Goαs/16-induced STAT3 activations, remarkably, our results show that both Rac1 and its downstream JNK signaling cascade play significant regulatory roles in Goαs-mediated STAT3 phosphorylations (Fig. 2, A and C, and 10A) and transcriptional acti-
ivation (Fig. 6, A and B); expression of the Rac1DN as well as inhibition of JNK by SP600125 abolished the GαQL- or isoproterenol-induced STAT3 phosphorylations. Rac1 is able to directly bind STAT3. The expression of the activated Rac1 stimulated STAT3 phosphorylation at both Tyr705 and Ser727 residues (57); nevertheless, it remains to be determined whether this activation is mediated through direct phosphorylation because indirect activation of STAT3 by Rac1 has also been suggested (58). The participation of JNKs in the modulation of Ser727 STAT3 phosphorylation upon cytokine induction has been demonstrated previously (59), whereas STAT3 Tyr705 phosphorylation can be regulated through JAK2 as the expression of either activated Rac1 (60) or constitutively active JNK (JNKK-CA) is sufficient to drive JAK2 stimulation (Fig. 3C). This specific requirement of JNKs in Gs-mediated STAT3 activations, but not in G16-mediated responses, might result from the distinctive activation of the AC/PKA pathway (Fig. 11). The ability of the Gαi-coupled parathyroid hormone receptor to stimulate Rac1 in opossum kidney cells was documented more than a decade ago (61), whereas Rac1 regulation by PKA required for the growth factor-stimulated migration of carcinoma cells was also demonstrated (62). Yet the mechanism has only recently been elucidated to also require the Ras-GRF1. Interestingly, this Ras-activating GEF contains two separate structural domains for stimulating Ras and Rac1 (41). Indeed, the ability of Rac1DN to inhibit STAT3 phosphorylations at both Tyr705 and Ser727 (Fig. 2C) points to the involvement of Rac1 in GαQL-induced STAT3 activation. However, it should be noted that Gα16QL-induced STAT3 activations require Rac1 but not JNK (26). It is not clear as to why JNK is distinctively required for GαQL-induced STAT3 activation, when Rac1 is apparently activated in both GαQL- and Gα16QL-induced responses.

Recently, the role of Ras-GRF1 has been suggested to be crucial in regulating monomeric GTPases through different structural domains (41). In line with the established studies that both Ras (40) and Rac1 (63) are modulated by this GEF, ERK and JNK (downstream of Ras and Rac1, respectively) were inhibited upon the knockdown of endogenous Ras-GRF1 in HEK 293 cells by the RNAi technique (Fig. 7B). Furthermore, c-Src also participates in the Ras-GRF1 regulation in that its phosphorylation by c-Src is required for Rac1 induction (63). Hence, as Ras-GRF1 is located downstream of c-Src, it is not surprising that its reduction had negligible effect on c-Src and JAK2 phosphorylations (Fig. 7B). It is also important to note that only a partial inhibition of GαQL-induced STAT3 phosphorylations was observed upon the introduction of siRas-GRF1. This may be due to either the incomplete removal of Ras-GRF1 by RNAi or the involvement of other parallel signaling cascades such as the Ras-GRF1-independent c-Src/JAK pathway (Fig. 11).

Another noteworthy observation relates to the apparent involvement of PI3K in Gαs-mediated STAT3 phosphorylation in HEK 293 cells. The Gαs-induced Tyr705 and Ser727 STAT3 phosphorylations and transcriptional activity are abrogated upon inhibition of PI3K by wortmannin or LY294002 (Figs. 5A, 6A, and 10C), or by overexpression of PI3KDN (Fig. 5B and 6C). This is distinctively different from those of Gα16QL- (26), Gα14QL- (27), or Gα1-mediated (46) STAT3 phosphorylations, wherein the same approaches failed to implicate PI3K. PI3K has been shown to play a critical role in mediating STAT3 Ser727 phosphorylation (64), possibly via the cross-talk between the ERK signaling cascade as blockade of PI3K by wortmannin, LY294002, or PI3KDN produced partial but significant attenuation of the phosphorylated ERKs (Fig. 5). Other possible involvement, for instance the downstream effectors Akt/mTOR of PI3K, has also been suggested (65). However, little is known as to how PI3K regulates STAT3 phosphorylation at Tyr705 site. Although it is generally accepted that PI3K can be activated by Gβγ subunits (39), our results illustrate that the expression of GαQL alone is fully capable of eliciting PI3K-sensitive STAT3 phosphorylations. In fact, there is considerable literature available to support a linkage between AC/PKA and PI3K. First, PI3K can be stimulated by c-Src or JNK (Fig. 3, D and E) as well as by overexpression of PI3KDN (Fig. 5C). Second, stimulation of PKA by Gαi-coupled thyrotropin receptor has been shown to promote the formation of the PI3K-Ras complex and the subsequent activation of PI3K in thyroid FRTL-5 cells (67). In neutrophils, the cytosolic regulator of AC that binds PI3K products via a pleckstrin homology domain is essential for chemoattractant-mediated activation of AC (68). Finally, PI3K can serve as a scaffolding protein and contribute to the control of cAMP levels in the cardiac system (69). Collectively, these studies illustrate that elaborate cross-talk interactions exist between the AC/PKA and PI3K pathways, some of which may form part of the circuitry in Gαs-mediated activation of STAT3. Additional studies are required to discern their functional relationship.

A notable feature of Gαs-induced changes in STAT3 phosphorylations is the relative resistance of Tyr705 to suppression by the kinase inhibitors. It is generally believed that Tyr705 phosphorylation is required for STAT3 transcriptional activity, whereas Ser727 phosphorylation enhances transcriptional activity (14). Hence, blockade of Ras, c-Src, and MEK signals would be expected to partially inhibit GαQL-induced STAT3 transcriptional activity (Fig. 6), as opposed to complete suppressions seen with Gα16QL- or Gα14QL-induced activity (26, 27). Our results do conform to such a postulation. In depth experimentation and analysis are required to fully appreciate the significance of differential phosphorylation of STAT3 on its transcriptional activity.

Based on the known signaling properties and abilities of various signaling components to regulate their respective targets, we have generated a mechanistic model for Gαs-mediated STAT3 phosphorylation (Fig. 11). Given that the production of cAMP and stimulation of PKA are direct consequences of Gαs activation, they may serve as a point of divergence for the regulation of other signaling cascades that are pertinent for the stimulation of STAT3 by Gαs. The involvement of Ras-GRF1 can lead to the stimulation of MEK/ERK and JNK cascades via Ras and Rac1. A number of signaling molecules are also capable of stimulating ERK and can thus induce STAT3 Ser727 phosphorylation; they include Rac1 (Fig. 2D) (12), c-Src (Fig. 3), and PI3K (Fig. 4) (70). JAK2-mediated STAT3 Tyr705 phosphorylation, on the other hand, can be elicited via c-Src or JNK (Fig. 3, B and C). It should also be noted that the proposed models are simplistic views of the actual signaling networks because paral-
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lel and alternative pathways may well be involved, for instance the regulation of Ras by Rac1 (71) and the release of Gβγ to modulate Src activity (72) and PI3K (39). In summary, this study has highlighted the role of Goαs in regulating STAT3 activity and provided a rudimentary network for the possible participating signaling. However, a number of details remain to be determined. The most outstanding question lies in what other tyrosine kinase participates in the phosphorylation of STAT3 Tyr705 and provides alternative routes for stimulation when the MEK and c-Src pathways are blocked. Because such an alternative pathway does not appear to be engaged in Goαs- and Gβγ-mediated STAT3 activation (both STAT3 Tyr705 and Ser727 phosphorylations are abolished upon inhibition of MEK and c-Src (26, 27, 46)), presumably it is evoked by the AC/PKA signals. A search for novel signaling molecules along the AC/PKA pathway may provide some useful clues. Nevertheless, this study has provided conclusive evidence that Goαs can indeed transduce GPCR signals to regulate STAT3 activity.

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