The pertussis toxin (PTX) insensitive heterotrimeric G protein \(\text{G}_{\alpha_{12}}\) has been implicated in mitogenesis and transformation, but its direct effectors remain unknown. To define potential signaling pathways utilized by \(\text{G}_{\alpha_{12}}\), we expressed an activated mutant of its \(\alpha\) subunit, \(\text{G}_{\alpha_{12}}(Q229L)\), in HEK293 cells and examined its effects on Ras and mitogen-activated protein kinases (MAPKs). Transient expression of activated \(\text{G}_{\alpha_{12}}\) increased the percentage of Ras in the active, GTP-bound state, stimulated c-jun NH$_2$-terminal kinase (JNK) activity, and enhanced the transcriptional activity of c-jun. Dominant negative Ras (N17Ras) inhibited \(\text{G}_{\alpha_{12}}\)-mediated JNK activation in NIH3T3 cells but failed to do so in HEK293 cells. In contrast, dominant negative Rac (N17Rac1) inhibited JNK activation by \(\text{G}_{\alpha_{12}}\) in HEK293 cells as well as three other cell lines. In 1321N1 cells, where thrombin stimulates \(\text{G}_{\alpha_{12}}\)-dependent mitogenesis, coexpression of N17Rac1 or a dominant negative mutant of MEKK1 (MEKK1(ΔK432M)) inhibits c-jun/AP-1 sensitive reporter gene expression stimulated by thrombin or \(\text{G}_{\alpha_{12}}\). These data demonstrate that the \(\alpha\) subunit of the low molecular weight G protein \(\text{G}_{\alpha_{12}}\) like tyrosine kinase growth factor receptors, activates Ras and recruits a signal transduction pathway involving the small GTP-binding protein Rac that leads to JNK activation.

The c-jun NH$_2$-terminal kinases (JNKs), which belong to the mitogen activated family of protein kinases (MAPKs), stimulate the transactivation potential of the c-jun component of the transcription factor AP-1 by phosphorylating it at residues Ser-63 and Ser-73 (1, 2). The JNKs are strongly activated by exposure to UV irradiation and osmotic stress (2–4). The JNKs are also activated by mitogens including epidermal growth factor and the oncogenic v-Src tyrosine kinase and v-Ras, and by proinflammatory cytokines such as tumor necrosis factor \(\alpha\) (2, 5, 6). The activation of JNK by growth factors and cytoplasmic oncogenes suggests a role for JNK in cellular proliferation or differentiation (7). Consistent with this hypothesis, blockade of the JNK pathway inhibits transformation of NIH3T3 fibroblasts by v-Src (5).

JNK regulation by cytoplasmic oncogenes and growth factors has recently been shown to require activation of the low molecular weight G protein Ras (8) and two Rho subfamily low molecular weight G proteins, Cdc42 and Rac (5, 9). While Ras is also an efficient activator of the extracellular signal regulated kinases (ERKs), Rac and Cdc42 are involved only in activation of JNK and the related p38MAPK (5, 10–12). It has also been demonstrated that G protein-coupled muscarinic receptors expressed in NIH3T3 and Rat 1a cells can activate JNKs (13, 14). A recent report demonstrates that M$_1$ and M$_3$ muscarinic acetylcholine receptors (mAChRs) activate JNKs through the G$_\alpha_q$ subunit, suggesting this as the mechanism of JNK regulation for other G protein-coupled receptors (15).

Although its effectors are unknown, the pertussis toxin (PTX) insensitive heterotrimeric G protein \(\text{G}_{\alpha_{12}}\) has been implicated in cellular transformation and proliferation. Expression of an activated form of \(\text{G}_{\alpha_{12}}\), \(\text{G}_{\alpha_{12}}(Q229L)\) (referred to here as activated \(\text{G}_{\alpha_{12}}\)) induces transformation in NIH3T3 and Rat1 cells (16, 17). We recently demonstrated by microinjection of antibodies to \(\text{G}_{\alpha_{12}}\) that the PTX insensitive DNA synthesis induced by thrombin in 1321N1 astrocytoma cells requires \(\text{G}_{\alpha_{12}}\) (18). In addition, transient expression of activated \(\text{G}_{\alpha_{12}}\) can dramatically stimulate AP-1-dependent gene expression in a Ras-dependent manner (18). These results suggest that \(\text{G}_{\alpha_{12}}\) stimulates a Ras-dependent signaling pathway leading to mitogenesis and AP-1 activation. The studies reported here examine the possibility that the \(\alpha\) subunit of \(\text{G}_{\alpha_{12}}\) activates Ras and Rac-dependent pathways and thereby stimulates JNK and AP-1 activity.

**MATERIALS AND METHODS**

**Transfection**

HEK293 cells were plated at 2 × 10$^5$ cells per 60-mm dish 3 days before transfection and grown at 5% CO$_2$ in a minimum essential medium supplemented with 10% fetal calf serum and penicillin/streptomycin. Cells were transfected by calcium phosphate coprecipitation (19) using a total of 12 μg of DNA. After 48 h, cells were harvested either for immunoblotting, Ras-GTP, kinase, or reporter gene assays. NIH3T3, HeLa, and COS1 cells were grown on 35-mm dishes in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, 1 mM glutamate, and penicillin/streptomycin. Cells were transfected by the LipofectAMINE method (5) using a total of 2 μg of DNA. After 48 h, cells were harvested for immunoblotting or kinase assays. 1321N1 cells were plated at 6 × 10$^5$ cells/60-mm dish and grown at 10% CO$_2$ in Dulbecco’s modified Eagle’s medium supplemented with 5% fetal calf serum and penicillin/streptomycin. Cells were transfected by calcium phosphate coprecipitation (19) using the AP-1-dependent reporter gene 2× TRE-luciferase and other constructs as described. After 48 h, cells were harvested and results were obtained by analytical luminescence.

**Immunoblotting**

Verification of \(\text{G}_{\alpha_q}\) Subunit Expression—48 h after transfection, membranes were isolated from HEK293 cells as described previously (20). Membrane proteins were denatured by boiling in Laemmli buffer and...
resolved by 12% SDS-PAGE. Proteins were transferred to Immobilon membranes which were then blocked and probed with specific Go subunit antibodies (Santa Cruz). G proteins were visualized by chemiluminescence (ECL, Amersham).

Determination of JNK and ERK Expression—48 h after transfection, cells were harvested in JNK lysis buffer described below. HA-JNK or HA-ERK was immunoprecipitated from 200 g of HEK293 lysate using an anti-HA antibody (Boehringer Mannheim). Immunoprecipitates were washed twice in lysis buffer and twice in kinase buffer (see below). After the final wash, beads were boiled in 1 × Laemmli buffer and resolved on 12% SDS-PAGE and transferred to Immobilon as above. Blots were blocked and then probed with the appropriate specific antibodies (either anti-JNK1 or anti-ERK2, Santa Cruz) and visualized by chemiluminescence.

Determination of Ras-GTP Loading—

Cells were transfected by calcium phosphate coprecipitation with wild type Ha-Ras and either vector or activated Go subunits. As a positive control for the assay, one plate of cells was transfected with activated Ha-Ras (V12) and processed simultaneously with the experimental conditions. The transfection of wild type Ha-Ras was necessary because of the low levels of endogenous Ha-Ras in HEK293 cells (21). Forty-eight hours after transfection, cells were labeled for 3 h with 1 mCi/ml of [32P]orthophosphate in phosphate-free culture media, and the content of Ras-associated nucleotides was assayed as described previously (22). Briefly, Ras was immunoprecipitated using Y13–259 antibody (Santa Cruz), immunoprecipitates were washed extensively, and guanine nucleotides bound to Ras were eluted and separated by thin layer chromatography (Bakerflex PEI cellulose). After autoradiography, GDP and GTP spots were isolated from the plates and quantitated by scintillation counting. The percentage of Ras that was bound to GTP was determined by the following formula: Ras-GTP = (GTP cpm/ (GTP cpm + GDP cpm)).

Protein Kinase Assays—HA-JNK1 and HA-ERK1 were assayed by collecting transiently transfected cells in lysis buffer containing 20 mM Tris, pH 7.6, 250 mM NaCl, 3 mM EDTA, 3 mM EGTA, 20 mM β-glycerophosphate, 0.5% (v/v) Nonidet P-40. The following inhibitors were added to the lysis buffer immediately before use: 100 µM Na3VO4, 1 µM p-nitrophenylphosphate, 2 mM di thiothreitol, 1 mM phenylmethylsulfonyl fluoride, 10 µg/ml leupeptin, and 10 µg/ml apro tin. Kinases were immunoprecipitated in lysis buffer using anti HA antibodies (Boehringer Mannheim). Immunoprecipitates were washed twice in lysis buffer and twice in a kinase buffer containing 20 mM Hepes, pH 7.6, 20 mM β-glycerophosphate, 10 mM MgCl2, 100 µM Na3VO4, 2 mM dithiothreitol, and 1 mM p-nitro phenylphosphate, were added to kinase buffer before use. HA-JNK1 activity was determined in a 25-μl reaction mixture of kinase buffer with 1 μM ATP, 5 μCi of [γ-32P]ATP, and 2 μg GST-c-jun (1-19) as the substrate for 20 min at 30 °C. HA-JNK1 activity was determined similarly, but 2 μg of myelin basic protein per sample were used as the substrate, and the reactions were performed for 15 min at 30 °C. Phosphorylated substrate was resolved by SDS-PAGE, gels were then dried, and results were quantitated either by AMBIS or PhosphorImager.

Results—The plasmids pCisGoR183C, and pCisGo12-Q229L plasmids were the gifts of Dr. Gary Bokoch. The plasmids encoding MEKK1 were the gift of Dr. Gary Johnson (8).

Plasmids—The plasmids pCisGoR183C, and pCisGo12-Q229L plasmids were the gifts of Dr. M. E. Simon. The plasmids encoding N17Ras and N17Rac1 were described previously (23, 24). HA-JNK, MEKK1Δ (K423M), Gal4c-Jun, 5XGalLuc, and SR α12 were also described previously (2, 8, 25). N17Rac1 were described previously (23, 24). HA-JNK, MEKK1 and pCis were transiently transfected cells. Each bar on the graph represents the mean ± S.E. of four separate experiments. Results are normalized to the percent Ras-GTP observed in the presence of control vector (pCis) which had an average value of 5.8%. Expression of GoR183C did not increase percent Ras-GTP in any experiment. Expression of Go12-Q229L increased the percent Ras-GTP from 150 to 300% of control in three of the four experiments. The stimulation by Go12-Q229L was found to have a p value less than 0.25 (by nonparametric analysis of variance) when all experiments (including one experiment in which there was no increase) were included, or p < 0.05 when the three experiments in which there was a response were statistically analyzed.

Stimulation of JNK through Small G Proteins Ras and Rac

We previously demonstrated that both the thrombin receptor and a constitutively activated mutant of Go12-Q229L stimulate AP-1-mediated gene expression in 1321N1 astroglial cells (18, 19). We have also demonstrated that these responses are inhibited by coexpression of dominant negative Ras (18, 22). In this system, thrombin activates Ras and requires both Go12 and Ras to induce proliferation (18, 22). These observations suggest that Go12 may be involved in thrombin-stimulated Ras activation.

Since 1321N1 astrocytoma cells have a very low transfection efficiency, we utilized HEK293 cells to determine directly whether Go12 could activate Ras. In these experiments, we expressed constitutively activated Go12 and examined its effects upon Ras-GTP loading. For comparison, the effects of a similarly activated mutant of GoR183C (α12*), or control vector (pCis) and wild type Ras. Cells were processed 48 h later as described under “Materials and Methods.” A, Go subunit expression in transfected cells was examined by immunoblot analysis. The arrow shows the position of the 46-kDa marker indicating that the Go subunit bands migrate at the appropriate molecular mass. B, Ras-GTP levels in pCis, GoR183C, or Go12-Q229L transfected cells. Each bar on the graph represents the mean ± S.E. of four separate experiments. Results are normalized to the percent Ras-GTP observed in the presence of control vector (pCis) which had an average value of 5.8%. Expression of GoR183C did not increase percent Ras-GTP in any experiment. Expression of Go12-Q229L increased the percent Ras-GTP from 150 to 300% of control in three of the four experiments. The stimulation by Go12-Q229L was found to have a p value less than 0.25 (by nonparametric analysis of variance) when all experiments (including one experiment in which there was no increase) were included, or p < 0.05 when the three experiments in which there was a response were statistically analyzed.

Possible downstream effectors of Ras include ERK and JNK (1, 10, 26). We therefore asked whether Go12 can activate these MAPKs. The activated mutants of Go12 and GoR183C were expressed in HEK293 cells along with hemagglutinin-tagged ERK2 (HA-ERK2). We next determined kinase activity and verified by immunoblotting that HA-ERK2 was expressed to similar extents in Go12- and GoR183C-transfected cells (2A). While transfected v-Raf stimulates HA-ERK2 activity 7-fold (data not shown) demonstrating that the HA-tagged kinase is functional, neither Go12 nor GoR183C significantly stimulate ERK2 activity. In contrast, expression of Go12 increases HA-JNK1 activity by 10-fold as assessed by phosphorylation of a specific protein kinase. Further studies are needed to determine whether Go12 increases JNK activity in vivo. The stimulation by Go12-Q229L was found to have a p value less than 0.25 (by nonparametric analysis of variance) when all experiments (including one experiment in which there was no increase) were included, or p < 0.05 when the three experiments in which there was a response were statistically analyzed.
values from four experiments performed in duplicate. The immunoblots shown in A and B were prepared from anti-HA immunoprecipitates of transfected cells and demonstrate the effect of activated Gα12 on HA-JNK1 and control vector (pcIS) transfected cells. After 48 h, the activity of transfected kinase was determined as described under “Materials and Methods.” Results are normalized to the kinase or luciferase activity observed in vector (pcIS) transfected cells.

A reporter system used to assess JNK phosphorylation-dependent transcription has been developed (1). This system utilizes a chimeric transcription factor in which the DNA binding domain of Gal4 is fused to the transactivation domain of c-Jun and a reporter gene containing five Gal4 binding sites upstream of the luciferase gene. When the transactivation domain of c-Jun is phosphorylated, luciferase production increases (1). Reporter activity is increased 15-fold by Gα12 demonstrating that Gα12 can regulate transcription through c-Jun. More modest stimulation was observed with Gα12 (Fig. 2C). The activation of JNK-dependent transcription by Gα12 is consistent with our earlier finding that this G protein induces AP-1-dependent gene expression (18).

Since Gα12 stimulates both Ras and JNK activity, and oncogenic Ras is able to stimulate JNK activity in HEK293 cells (data not shown), we hypothesized that Gα12 might stimulate JNK through a Ras-dependent pathway. Surprisingly, Gα12-induced JNK activity in HEK293 cells is not inhibited by coexpression of either of two dominant negative Ha-Ras mutants, N17Ras or A15Ras (Fig. 3A). Expression of dominant negative Ras in Gα12-transfected HEK293 cells was confirmed by Western blot (data not shown). Moreover, coexpression of N17Ras inhibited Gα12-induced JNK activation in NIH3T3 cells, demonstrating that the dominant negative Ras is functional (Fig. 3B).

Recent work has shown that the JNKs can be activated by two members of the Rho family, Cdc42Hs and Rac, but not by a third member RhoA (5, 9). Consistent with this observation, a dominant negative form of RhoA (N19rhoA) failed to inhibit JNK activation by Gα12 in HEK293 cells (data not shown). However, coexpression of a dominant interfering Rac mutant, N17Rac1, reduced JNK activation by Gα12 by approximately 90% compared to vector control in HEK293 cells (Fig. 4A). Inhibition of Gα12-mediated JNK activation by N17Rac1 was also observed in three other cell lines. As shown in Fig. 4, B–D, N17Rac1 reduced Gα12-mediated JNK activity by 60% in NIH3T3s, 75% in COS1 cells, and 93% in HeLa cells. These data demonstrate that activated Gα12 signals to Rac1 and through this signal communicate to the JNK cascade. Expression of a dominant negative mutant of Cdc42, N17Cdc42, was somewhat less effective in HEK293 and COS1 cells (35% and 30% reduction, respectively). Therefore, Gα12 can regulate both Ras and JNK activity in HEK293 cells, and oncoproteins can be activated by Gα12, but not by Rac.
24% inhibition, respectively), but nevertheless reduced Gα12 stimulation of HA-JNK to similar extents as N17Rac1 in 3T3 and HeLa cells (71% and 94%, respectively, data not shown).

In 1321N1 astrocytoma cells, thrombin and Gα12 stimulate AP-1-mediated reporter gene (23TRE luciferase) expression in a Ras-dependent manner. In addition, thrombin-stimulated mitogenesis in these cells requires Gα12. Since Gα12 stimulates JNK activity, we hypothesized that thrombin might stimulate AP-1 gene expression and mitogenesis through JNK. We first determined whether thrombin stimulates JNK activity in 1321N1 cells. In three experiments, thrombin stimulation of 1321N1 cells for 20 min produced a 4.4 ± 0.5 fold stimulation of JNK activity (data not shown). AP-1-dependent gene expression is regulated by JNK (6, 27). As shown in Fig. 5A, AP-1 activity induced by thrombin was markedly attenuated by expression of a dominant inhibitory form of Rac, N17Rac1. Expression of a dominant negative form of MEKK1 (MEKK1D(K432M)) almost completely inhibits both thrombin and Gα12-stimulated AP-1 gene expression (Fig. 5, A and B). Since both MEKK1 and Rac1 have been reported to preferentially mediate activation of the JNK cascade (5, 8, 9), these data suggest that the thrombin receptor stimulates JNK through Gα12, Rac, and MEKK1.

**DISCUSSION**

The purpose of this work was to examine the signal transduction pathways by which Gα12 effects AP-1-dependent gene expression. Since our previous work demonstrated that Ras mediates thrombin and Gα12-induced AP-1 activation, we examined the possibility that Gα12 increases the level of Ras in the active state (18, 22). We demonstrate here that expression of constitutively activated Gα12 stimulates Ras-GTP loading in HEK293 cells, while Gαq, another PTX-insensitive Gα subunit that can interact with the thrombin receptor (22, 28), does not. To our knowledge, this is the first demonstration that expression of a specific Gα subunit can increase formation of Ras-GTP.

The mechanism of Ras activation by Gα12 is currently unknown. It is possible that expression of activated Gα12 activates Ras by stimulating Ras exchange factors or inhibiting Ras-GAPs; alternatively, prolonged expression of Gα12 may activate Ras through an indirect mechanism such as inducing the expression of other proteins that regulate Ras. Regardless of the mechanism, these data demonstrate the existence of a pathway by which a heterotrimeric G protein α subunit can communicate with small G proteins of the Ras family. The communication between Ras and Gα12 is consistent with previous observations that Gα12-mediated responses are Ras-dependent (18, 29) and may explain the ability of Gα12 to transform fibroblasts (16, 17) and its requirement for thrombin-stimulated mitogenesis (18).

A recent report demonstrated that expression of Gβ1γ in...
increases JNK activity (15), presumably due to the ability of free Gbg subunits to activate Ras (21). While this appears to be the mechanism of JNK activation by M1 and M2 muscarinic acetylcholine receptors (M1 and M2 mAChRs) (15), our data and that of Prasad et al. (29) demonstrate that expression of a Gα subunit, Gα12, stimulates JNK providing another pathway by which G protein-coupled receptors may activate JNK. The thrombin receptor, which induces proliferation in several cell types including fibroblasts, vascular smooth muscle cells, and astrocytes (22, 30–32), can couple to Gα12 (20, 28), while the M3 mAChR appears to do so only poorly relative to the thrombin receptor. A combined signal input from both Gα12 and Gbg subunits could explain our observation that thrombin activates Ras and induces mitogenesis in 1321N1 cells while the M3 mAChR, which does not appear to couple to Gα12, cannot (20).

The finding that Ras is required for JNK activation by Gα12 in 3T3 cells is in agreement with the observations reported by Prasad et al. (29) in COS1 cells. However, since dominant negative Ras does not affect Gα12-induced JNK activation in HEK293 cells, the requirement for Ras to activate JNK is cell type-specific. In contrast, the low molecular weight G protein Rac is required for JNK activation by Gα12 in all the cell lines examined. Furthermore, the observation that both thrombin- and Gα12-induced AP-1-dependent gene expression in 1321N1 cells require Rac and MEKK1 activity suggests that the thrombin receptor signals through Gα12, Rac, and MEKK to stimulate JNK and AP-1. It has recently been reported that Gα12 stresses stress fiber and focal adhesion formation through the low molecular weight G protein Rho (33). Since Rho may be activated subsequent to Ras and Rac activation (24, 34), our data are consistent with a pathway in which Gα12 activates Rac and Rho.

In addition to regulating the activity of the JNKs and the cytoskeleton, the low molecular weight G proteins may be involved in proliferation. Olson et al. (35) have demonstrated that Rac and Cdc42 are required for Swiss 3T3 cells to progress through the G1 phase of the cell cycle, and that activated forms of Rac and Cdc42 promote DNA synthesis; the effectors responsible for this phenomenon are unknown. The ERK family of MAP kinases are required for differentiation and proliferation in response to several growth factors (36, 37), but Rac and Cdc42 stimulate DNA synthesis without activating the ERKs (35). Since both Rac and Cdc42 stimulate JNK activity, it was suggested that while not the ERKs mediate the effects of Rac and Cdc42 on cell proliferation (5, 35). In support of this hypothesis, JNKs are required for v-src-induced transformation of NIH3T3 cells (5). Our observations that Gα12 stimulates JNK in a Rac-dependent manner without appreciably stimulating ERK also support a model in which Gα12 promotes its proliferative and transforming effects through Rac and JNK. We are currently testing such a model.

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