The bath solution contained 120 mM CsAsp, 1 mM $MgCl_2$, 50 mM Hepes (pH 7.5), and 0.1% glucose; the pipette solution contained 120 mM CsAsp, 1 mM $MgCl_2$, 50 mM MES (pH 5.7 to 6.3) or Hepes (pH 7.3), and 1 mM Mg–adenosine triphosphate (ATP). For the combined current-pH measurements, the pipette solution contained 140 mM CsAsp, 1 mM $MgCl_2$, 10 mM MES (pH 6.5), 1 mM MgATP, and 100 μM carboxy-SNARF-1.

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Stat3-Mediated Transformation of NIH-3T3 Cells by the Constitutively Active Q205L $G\alpha_o$ Protein

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Expression of Q205L $G\alpha_o$ ($G\alpha_o$*), an alpha subunit of heterotrimeric guanine nucleotide–binding proteins (G proteins) that lacks guanosine triphosphatase (GTPase) activity in NIH-3T3 cells, results in transformation. Expression of $G\alpha_o$* in NIH-3T3 cells activated signal transducer and activator of transcription 3 (Stat3) but not mitogen-activated protein (MAP) kinases 1 or 2. Coexpression of dominant negative Stat3 inhibited $G\alpha_o$*-induced transformation of NIH-3T3 cells and activation of endogenous Stat3. Furthermore, $G\alpha_o$* expression increased activity of the tyrosine kinase c-Src, and the $G\alpha_o$*-induced activation of Stat3 was blocked by expression of Csk (carboxyl-terminal Src kinase), which inactivates c-Src. The results indicate that Stat3 can function as a downstream effector for $G\alpha_o$* and mediate its biological effects.

Although downstream effectors that mediate the actions of G protein α subunits $G\alpha_s$, $G\alpha_i$, and $G\alpha_q$ have been elucidated, little is known about signaling pathways activated by $G\alpha_o$. Expression of the GTPase-deficient (and thus constitutively active) mutant of $G\alpha_o$ in which Gly^{205} is changed to Leu ($G\alpha_o$*) in NIH-3T3 cells results in transformation (*1*), but the molecular mechanisms underlying this phenomenon are not known. MAP kinases 1 and 2 participate in stimulation of proliferation and transformation of NIH-3T3 cells (*2*). The transcription factor Stat3 is activated and required for transformation of NIH-3T3 cells by the v-Src oncogene (*3*). Hence, the roles of MAP kinases and Stat3 in transformation of NIH-3T3 cells by $G\alpha_o$* were investigated.

The Stat family of proteins is implicated in the functions of a wide range of cells (*4*). When activated, Stat3 becomes phosphorylated, dimerizes, and translocates to the nucleus, where it binds DNA and modulates gene expression. To determine the effects of $G\alpha_o$* on the phosphorylation state of native Stat3, we transiently transfected NIH-3T3 cells with a $G\alpha_o$* expression vector, extracted the proteins, resolved them by SDS–polyacrylamide gel electrophoresis (SDS-PAGE), and blotted them with antibodies specific for Stat3 phosphorylated on Tyr^{705} (*5*). Expression of $G\alpha_o$* led to phosphorylation of Tyr^{705} on endogenous Stat3 proteins in NIH-3T3 cells (Fig. 1A). Expression of $G\alpha_o$* did not lead to phosphorylation of Stat1 (*6*). To further determine if the $G\alpha_o$*-induced phosphorylation of Stat3 reflected an increase in Stat3 transcriptional activity, we did a transcriptional activation assay in cells transfected with a Stat3-responsive luciferase reporter construct (*7*). Expression of $G\alpha_o$* resulted in activation of endogenous Stat3 (Fig. 1B), as evidenced by increased reporter gene expression. To determine that the reporter gene activity was in fact due to Stat3 activation, we coexpressed mutant Stat3 proteins that are not phosphorylated or fail to bind DNA and act in a dominant negative manner (*3*). Activation of Stat3 in cells expressing $G\alpha_o$* was inhibited by the coexpression of dominant negative Stat3 proteins (Fig. 1B). Additionally, expression of wild-type $G\alpha_o$ or G protein β and γ subunits had no effect on Stat3 activity (Fig. 1C). Expression of $G\alpha_{12}$* gave a small but consistent twofold increase in Stat3 activity (Fig. 1C). Activation of Stat3 by $G\alpha_q$* was varied (Fig. 1C), and cell density appears to play a role in this activation; further work is being done. Thus, $G\alpha_o$* appears to activate Stat3 in a specific manner in NIH-3T3 cells.

The role of MAP kinases 1 and 2 in cell proliferation and transformation has been extensively studied, and activation of these enzymes can transform NIH-3T3 cells (*2*). Receptor-mediated activation of $G\alpha_o$ leads to increased activity of MAP kinases 1 and 2 in Chinese hamster ovary cells (*8*). Therefore, we examined the effects of $G\alpha_o$* expression on MAP kinase activity in NIH-3T3 cells (*9*). Expression of $G\alpha_o$* did not activate MAP kinases 1 or 2 in NIH-3T3 cells (Fig. 2), as measured by immunoblot analysis with antibodies that specifically recognize the active forms of MAP kinases 1 and 2, which are phosphorylated on Thr^{202} and Tyr^{204}. Thus, the Stat3 signaling pathway and not the MAP kinase 1 and 2 pathway is activated by $G\alpha_o$* in NIH-3T3 cells.

Expression of $G\alpha_o$* transforms NIH-3T3 cells and leads to colony formation in soft agar (*1*). Expression of dominant negative Stat3 inhibits transformation of NIH-3T3 cells by v-Src but not transformation by H-Ras (*3*). To test the hypothesis that activation of Stat3 is necessary for transformation of NIH-3T3 cells by $G\alpha_o$*, we prepared transfected cells that expressed dominant negative Stat3 and $G\alpha_o$* and assayed colony formation (*10*). Expression of dominant negative Stat3 inhibited $G\alpha_o$* transformation of NIH-3T3 cells (Fig. 3). Thus, activation of Stat3 is necessary for transformation of NIH-3T3 cells by $G\alpha_o$*.

Stat3 is activated in response to cytokines and is tyrosine phosphorylated by Janus kinases (JAK) (*11*). We found that expression of $G\alpha_o$* did not activate JAK2 (*6*). However, Stat3 can also be directly phosphorylated and activated by c-Src or v-Src, which results in the proliferation and transformation of several cell types (*3*, *12*). Therefore, we tested whether $G\alpha_o$* activated endogenous c-Src. $G\alpha_o$* was expressed in NIH-3T3 cells. The cells were lysed, endogenous c-Src was immunoprecipitated, and an in vitro kinase assay was done with the immune complex (*13*). Expression of $G\alpha_o$* increased endogenous c-Src activity in NIH-3T3 cells (Fig. 4A). We

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then examined the role of endogenous Src in activation of Stat3 in response to $G\alpha_o$* and in the transformation of NIH-3T3 cells. Csk inhibited activation of Stat3 in cells expressing $G\alpha_o$* (Fig. 4B). Furthermore, expression of Csk also significantly inhibited transformation of NIH-3T3 cells expressing $G\alpha_o$* (Fig. 4C). These data show that c-Src is activated in cells expressing $G\alpha_o$* and that c-Src acts upstream of Stat3.

The activation of Stat3 by a G protein α-subunit, leading to expression of the transformed phenotype, demonstrates a connection between G protein and Stat pathways. What would be the biological relevance of such a connection? Most effects of Stat have been characterized in differentiated cells such as the effects of growth hormone, prolactin, and leptin. However, at least in NIH-3T3 cells, Stat-3 can trigger transformation (*3*). Proliferation and transformation of NIH-3T3 cells can be induced by many stimuli. Indeed, signaling components that trigger transformation in NIH-3T3 cells, when expressed in other cell types, can induce differentiation. One prominent example is the small GTPase Ras, which transforms NIH-3T3 cells but triggers neurite outgrowth in PC-12 cells (*14*), both effects being mediated by activation of MAP kinases 1 and 2. $G\alpha_o$ is abundantly expressed in growth cones of neurons (*15*), and expression of activated $G\alpha_o$ induces neurite outgrowth in PC-12 and NE-115 cells (*16*). Large amounts of c-Src are also found in nerve growth cones (*17*), and amounts of c-Src increase severalfold during neuronal differentiation (*18*). The molecular mechanisms involved in triggering of neurite outgrowth are currently not known. Several signaling pathways have been implicated, including the MAP kinase pathways in the effects of NGF and the Stat pathway in the effects of interleukin-6 (IL-6) (*19*, *20*). Thus, it is possible that $G\alpha_o$ might also stimulate Stat3 transcriptional activity in differentiated cells such as neurons to regulate neuronal plasticity.

It is unlikely that $G\alpha_o$ activation of c-Src and Stat3 represents the sole effector pathway of $G\alpha_o$ signaling. Studies from our laboratory show that wild-type $G\alpha_o$ directly interacts with

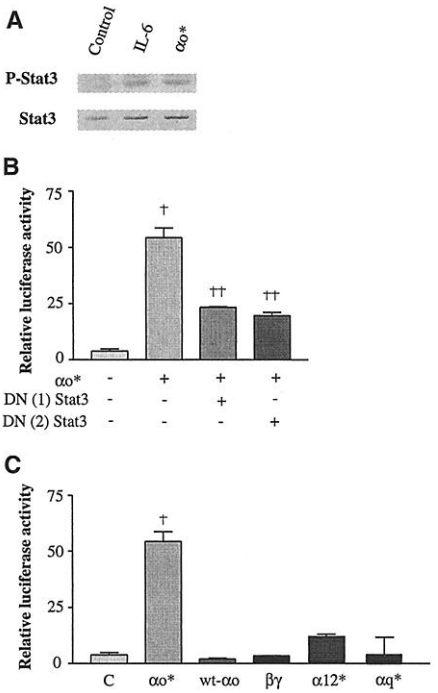

Fig. 1. Phosphorylation and activation of Stat3 induced by Q205L $G\alpha_o$. (**A**) Soluble proteins from cell lysates of NIH-3T3 cells treated with Il-6 or transfected with $G\alpha_o$* were probed with antibody specific for phosphorylated Stat3 at Tyr^{705} (top) or with antibody to Stat3 (bottom). (**B**) NIH-3T3 cells transfected with the Ly6E Stat3-responsive luciferase reporter construct were transfected with either $G\alpha_o$* alone or with $G\alpha_o$* and dominant negative Stat3 constructs VVV461-463AAA/EE434-435AA (DN 1) or Y705-F (DN 2). (Statistical differences between the treatments were assessed with the Newman-Keuls test: $P < 0.001$; †, compared with control; ††, compared with $G\alpha_o$*; $n = 4$ to 5 in duplicate transfections each time.) (**C**) Stat3 transcriptional assay performed as in (B). Cells were transfected with either $G\alpha_o$*, wild-type (wt) $G\alpha_o$, $G\beta_2\gamma_1$ $G\alpha_{12}$*, or $G\alpha_9$*. (Newman-Keuls test: $P < 0.001$; †, compared with control; $n = 2$ in duplicate transfections each time.)

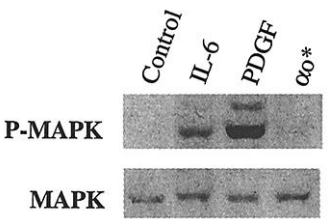

Fig. 2. Activation of MAP kinases 1 and 2 in cells expressing $G\alpha_o$*. Proteins from soluble cell lysates of NIH-3T3 cells treated with either IL-6 or PDGF or transfected with $G\alpha_o$* were probed with antibody specific for phosphorylated MAP kinases 1 and 2 on Thr^{202} and Tyr^{204} (top) or antibody to MAP kinases 1 and 2 (bottom).

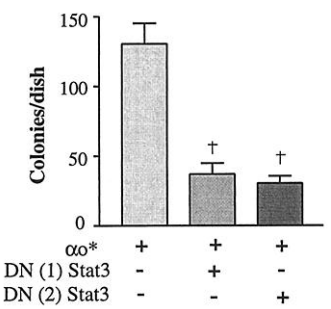

Fig. 3. Inhibition of $G\alpha_o$* transformation of NIH-3T3 cells by dominant negative Stat3. Colony formation in soft agar was assayed with $G\alpha_o$*-transformed cells that were co-transfected with either one of the two dominant negative Stat3 constructs. (Newman-Keuls test: $P < 0.001$; †, compared with $G\alpha_o$*-expressing cells.) The experiment was performed twice, with duplicate transfections each time.

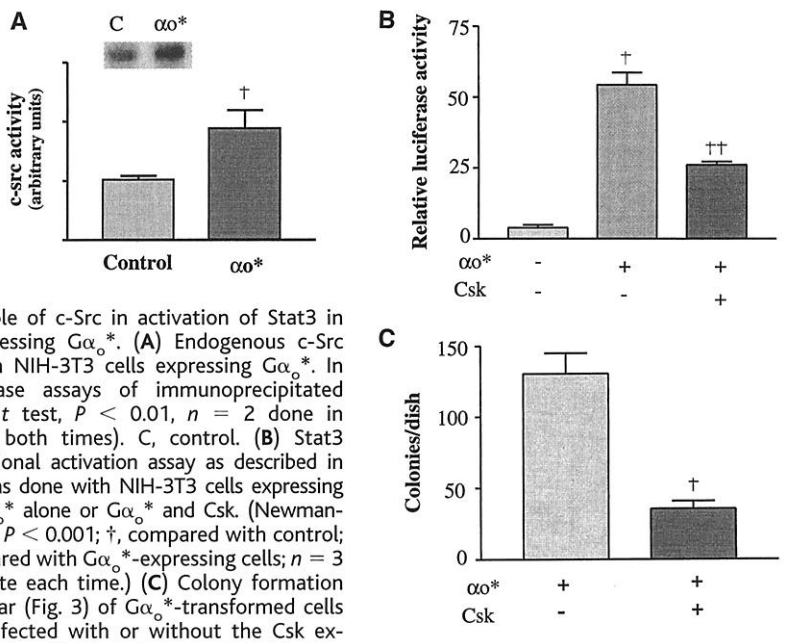

Fig. 4. Role of c-Src in activation of Stat3 in cells expressing $G\alpha_o$*. (**A**) Endogenous c-Src activity in NIH-3T3 cells expressing $G\alpha_o$*. In vitro kinase assays of immunoprecipitated c-Src (†, *t* test, $P < 0.01$, $n = 2$ done in duplicate both times). C, control. (**B**) Stat3 transcriptional activation assay as described in Fig. 1B was done with NIH-3T3 cells expressing either $G\alpha_o$* alone or $G\alpha_o$* and Csk. (Newman-Keuls test: $P < 0.001$; †, compared with control; ††, compared with $G\alpha_o$*-expressing cells; $n = 3$ in duplicate each time.) (**C**) Colony formation in soft agar (Fig. 3) of $G\alpha_o$*-transformed cells also transfected with or without the Csk expression plasmid (†, *t* test, $P < 0.01$). The experiment was performed twice, with duplicate transfections each time.

Rap1-GTPase–activating protein (Rap1-GAP) to modulate Rap1 activity (*21*). Thus, $G\alpha_o$ may be able to engage several distinct signaling pathways to elicit its biological effects.

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5. NIH-3T3 cells cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with bovine calf serum (5%) were transfected with either $G\alpha_o$* cloned into pcDNA3.1 ($G\alpha_o$*-pcDNA3.1) or pcDNA3.1 empty vector (Invitrogen) with the use of Lipofectamine (Gibco-BRL). The cells were cultured in DMEM containing bovine calf serum (1%) for 16 hours before stimulation with either IL-6 (10 ng/ml), platelet-derived growth factor (PDGF) (20 ng/ml), or vehicle alone for 5 min. Cells were lysed in lysis buffer [10 mM tris-HCl (pH 7.5), 1% sodium deoxycholate, 1% NP-40, 150 mM NaCl, 1 mM sodium orthovanadate, 0.5 mM phenylmethylsulfonyl sulfate (PMSF), leupeptin (10 μg/ml), and aprotenin (10 μg/ml)]. Proteins from the soluble lysate (50 μg) were resolved by SDS-PAGE (8% gel), transferred to Hybond C nitrocellulose membrane (Amersham), probed with antibody specific for phosphorylated Stat3 on Tyr^{705} (NEB) or antibody specific for Stat3 (NEB), and visualized with ECL reagents (Amersham).
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7. NIH-3T3 cells were transfected with $G\alpha_o$* or with $G\alpha_o$* and either dominant negative Stat3 or Csk (1:2 DNA ratio of $G\alpha_o$* to dominant negative Stat3 or Csk) in duplicate in six-well plates. All cells were also transfected with the Ly6E luciferase construct (1 μg) and β-galactosidase (β-Gal) construct (0.5 μg). Twenty-four to 28 hours after transfection, the cells were rinsed and cultured in fresh DMEM supplemented with bovine calf serum (1%) for 6 hours. The cells were then lysed in 1× luciferase assay buffer (Promega). Luciferase and β-Gal activity was measured in a luminometer, and the data were analyzed after normalization for the β-Gal activity.
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9. NIH-3T3 cells cultured in DMEM supplemented with bovine calf serum (5%) were transfected with either $G\alpha_o$*-pcDNA3.1 or pcDNA3.1 with Lipofectamine. The cells were cultured in DMEM containing bovine calf serum (1%) for 16 hours before stimulation with either IL-6 (10 ng/ml), PDGF (20 ng/ml), or vehicle alone for 5 min. Cells were lysed in lysis buffer (5), and proteins from the soluble lysate (50 μg) were resolved by SDS-PAGE (10% gel), transferred to Hybond C, and probed with antibody specific for phosphorylated MAP kinase 1 or 2 at Thr^{202} and Tyr^{204} (NEB) or antibody specific for MAP kinase 1 and 2 (NEB).
10. NIH-3T3 cells were transfected with $G\alpha_o$*-pcDNA3.1 and selected with G-418 (Gibco-BRL) for 10 to 14 days. The $G\alpha_o$*-expressing cells were then transfected with pcDNA3.1-Hygro (Invitrogen) and with either dominant negative Stat3 or Csk. Forty-eight hours later, 30,000 cells were plated in DMEM with bovine calf serum (5%) containing agar (0.3%), G-418, and Hygromycin, and the colonies were counted 2 to 3 weeks later. Cells were transfected in duplicate and were then grown in duplicate soft agar plates, resulting in four plates per condition.
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13. NIH-3T3 cells were transiently transfected with $G\alpha_o$*-pcDNA3.1, and the cells were cultured in DMEM supplemented with 1% bovine calf serum for 16 hours before the assay. Cells were lysed in lysis buffer (*5*), and endogenous c-Src was immunoprecipitated from soluble lysate (400 μg) with antibody to c-Src (3 μg) (Upstate Biotechnology) for 2 hours at 4°C. The immune complex was precipitated with protein G–agarose beads for 2 hours at 4°C. The complex was washed three times with lysis buffer and three times with kinase buffer [40 mM Hepes-NaOH (pH 7.5), 10 mM $MgCl_2$, 3 mM $MnCl_2$, 0.5 mM dithiothreitol, 1 mM sodium fluoride, 1 mM sodium orthovanadate, 0.5 mM PMSF, leupeptin (10 μg/ml), and aprotenin (10 μg/ml)]. The kinase assay was performed with [^{32}P-γ]adenosine triphosphate, and the resulting product was resolved by SDS-PAGE (10% gel). The autophosphorylated c-Src was visualized and quantitated on a phosphorimager. The experiment was done with duplicate transfections and immunoprecipitations.
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