Expression of an oncogenic mutant Gαi2 activates Ras in Rat-1 fibroblast cells

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Abstract It has been reported that expression of the active mutant of heterotrimeric GTP-binding protein α subunit Gαi2 transforms Rat-1 cells. However, the Gαi2-mediated mitogenic signaling pathways remain to be elucidated. Here, we demonstrate that inducible expression of the active mutant of Gαi2 (Gαi2(Q205L)) activates Ras and c-Jun N-terminal kinase (JNK) in addition to extracellular signal-regulated kinase (ERK) in Rat-1 cells. Our findings suggest that Ras may play a critical role in the Gαi2-induced transformation and Gαi2 can transduce signals from the Gi-coupled receptor to JNK and ERK in certain types of mammalian cells.

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Key words: G protein; *gip2*; Ras; c-Jun N-terminal kinase; Transformation; Inducible expression system

1. Introduction

Heterotrimeric GTP-binding proteins (G proteins) play key roles in the intracellular signal transductions from the cell surface receptors which possess seven transmembrane domains. The α subunit of G proteins (Gα) has a guanine nucleotide-binding site and an intrinsic GTPase activity. The GDP-bound Gα forms a complex with G protein βγ subunits (Gβγ). When the receptors are activated by stimuli, the activated receptors induce the exchange of the guanine nucleotide from GDP to GTP on Gα, and the Gαβγ heterotrimer dissociates into the GTP-bound Gα and the Gβγ heterodimer. The GTP-bound Gα can interact with the effectors, such as adenylyl cyclase, phospholipase (PLC) β, and cGMP phosphodiesterase. Gβγ can also interact with and regulate the effectors, such as certain types of adenylyl cyclases, PLC β and G protein-coupled inwardly rectifying K^+ channel (GIRK) [1,2]. In addition, it was reported that Gβγ can activate Ras-regulated mitogenic signaling cascades [3–6].

In certain human tumor cells, GTPase-deficient mutations of Gα which form a constitutively active form have been found. Mutation of the *Gαs* gene (designated *gsp* oncogene) was found in pituitary tumors and thyroid adenomas [7,8]. The *gsp* product constitutively activates adenylyl cyclase and elevates the intracellular cAMP level. Mutation of the *Gαi2* gene (designated *gip2* oncogene) was found in tumors of adrenal cortex and ovary [8]. The *gip2* product inhibits cAMP accumulation [9]. Expression of the mutant Gαi2 in Rat-1 cells induces transformation [10,11].

To clarify the mechanism of the Gαi2-mediated cell proliferation, several groups have investigated intracellular mitogenic signals and transcriptional regulation of immediate early genes. In Rat-1 cells expressing the constitutively active Gαi2, extracellular signal-regulated kinase (ERK) and its activator, MAPK/ERK kinases (MEK), are activated [12]. Microinjection of the antibody that interferes with the function of Gαi2 inhibits the lysophosphatidic acid (LPA)-induced activation of c-*fos* promoter in mouse fibroblasts [13]. In addition, transfection of the constitutively active Gαi2 activates c-*fos* promoter in CHO and Rat-1 cells [14], and enhances the activity of the collagenase promoter which is regulated by AP-1 in a c-Jun-dependent manner in Rat-1 cells [15]. However, the activation of molecules upstream of MEK and c-Jun, such as Ras and c-Jun N-terminal kinase (JNK), has not yet been investigated.

To study the mitogenic signals mediated by Gαi2, we constructed a Rat-1 transfectant expressing an oncogenic mutant of Gαi2 (Gαi2(Q205L)) in an isopropyl-1-β-thio-D-galactoside (IPTG)-dependent manner. In this transfectant, Ras was activated by expression of Gαi2(Q205L). Moreover, activation of JNK as well as ERK was found.

2. Materials and methods

2.1. Plasmids

A plasmid pEF-LAC-Gi2(Q205L) was constructed by insertion of cDNA of rat Gαi2 [16] whose glutamine-205 was substituted for leucine into the *Not*I site of pEF-LAC [17]. A plasmid pOPRSVI-Ras(V12) was constructed by insertion of cDNA of c-Ha-Ras whose glycine-12 was substituted for valine [18] into the *Not*I site of pOPRSVI (Stratagene). A lactose repressor expression vector p3′SS was obtained from Stratagene. An expression vector for wild type c-Ha-Ras, pCMV5-Ras(WT) [6], was obtained from T. Satoh.

2.2. Glutathione S-transferase (GST) fusion protein

GST-fused c-Jun (1–223) was produced as described elsewhere [19]. GST-fused c-Raf-1 Ras-binding domain (GST-RBD) produced in *Escherichia coli* was provided by K. Tago.

2.3. Cell culture and stable transfectants

Rat embryonic fibroblast Rat-1 cells were grown in Dulbecco's modified Eagle's (DME) medium supplemented with 10% fetal calf serum (FCS). Rat-1 cells were transfected with 4 μg of pEF-LAC-Gi2(Q205L) and 16 μg of p3′SS (for Rat-1·QL112), or 10 μg of pOPRSVI-Ras(V12) and 10 μg of p3′SS (for Rat-1·RasValA1) by the calcium phosphate coprecipitation technique. Stable transfectants were selected with 1 mg/ml G418 and 0.4 mg/ml hygromycin B.

2.4. Analysis of the protein expression

Membrane proteins were analyzed by immunoblotting using a rabbit anti-Gαi1/2 antibody (AS/7, NEN Life Science). To detect Gαi2(Q205L), a trypsin resistance assay was performed. Membrane proteins (20 μg) were solubilized in 8 μl of buffer (20 mM HEPES-

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Abbreviations: G protein, heterotrimeric GTP-binding protein; JNK, c-Jun N-terminal kinase; ERK, extracellular signal-regulated kinase; IPTG, isopropyl-1-β-thio-D-galactoside; GST, glutathione *S*-transferase

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NaOH (pH 8.0), 10 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT, 0.64% (w/v) Luburol PX). After the solubilized proteins were incubated with 111 μM GTP at 30°C for 30 min, TPCK-treated trypsin (Sigma) was added (200 μg/ml) and incubated at 30°C for 5 min. The reactions were terminated by adding trypsin-chymotrypsin inhibitor (2.2 mg/ml, Sigma) and Laemmli sample buffer. The samples were analyzed by immunoblotting.

2.5. Colony formation in soft agar

Cells (3×10^4 cells) were seeded into 60-mm dishes with DME medium supplemented with 0.27% agar and 10% FCS over the DME medium containing 0.53% agar and 10% FCS. After the cells were cultured for 12 days in the absence or presence of 5 mM IPTG, microphotographs were taken.

2.6. Analysis of Ras·GTP

The Ras-bound guanine nucleotide was analyzed as described previously [6,20]. An assay for Ras·GTP by determining the association between Ras·GTP and Ras-binding domain of c-Raf-1 in vitro was performed as described elsewhere [21] with minor modifications. In brief, cells were transfected with 5 μg of pCMV5-Ras(WT) using Lipofectamine (Gibco BRL). After transfection, the cells were incubated with or without 5 mM IPTG for 24 h in the absence of serum. After stimulation with agonists, the cells were harvested and lysed with Ras·GTP IP buffer (50 mM Tris-HCl (pH 7.5), 20 mM $MgCl_2$, 150 mM NaCl, 0.5% Nonidet P-40, 20 μg/ml aprotinin, 1 mM Na_3VO_4). Ras·GTP associated with GST-RBD was collected with glutathione-Sepharose 4B beads (Amersham-Pharmacia) and analyzed by immunoblotting with an anti-Ha-Ras antibody (sc-520, Santa Cruz Biotechnology).

2.7. ERK2 and JNK1 assays

The kinase activity of ERK2 or JNK1 was assessed by in vitro kinase assay as described previously [19,22,23]. Phosphorylation of ERK2 was assayed by electrophoretic mobility shift using a mouse anti-ERK2 antibody (05-157, Upstate Biotechnology) as described previously [6].

3. Results

3.1. IPTG-induced expression of Gαi2(Q205L) in Rat-1 transfectants

To explore the function of Gαi2 on the mitogenic signaling pathways, we constructed Rat-1 transfectants which express Gαi2(Q205L) inducibly with IPTG treatment. Immunoblot analysis using an antibody for Gαi suggested that the IPTG-dependent expression of the exogenous Gαi2(Q205L) is comparable to that of endogenous Gαi (Fig. 1a). It has been reported that the GTP-bound form of Gα is substantially protected from tryptic proteolysis [24]. To confirm the expression of Gαi2(Q205L), tryptic proteolysis of Gαi was performed. The exogenous Gαi2(Q205L) was cleaved into two fragments (of 2 and 38 kDa) by trypsin, whereas the endogenous wild type Gαi was completely digested under the same conditions. The 38-kDa tryptic fragment of Gαi was detected by the anti-Gαi antibody for the C-terminus region of Gαi protein. Fig. 1b shows that Gαi2(Q205L) was accumulated in one transfectant, designated Rat-1·QL112. Another type of Rat-1 transfectant, designated Rat-1·RasValA1, was also prepared. In this transfectant, constitutively active mutant Ras (Ras(G12V)) was accumulated after IPTG treatment (data not shown).

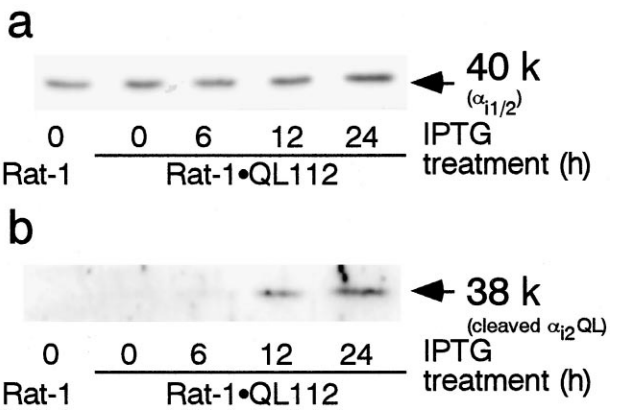

Fig. 1. IPTG-induced expression of Gαi2(Q205L) in the Rat-1 transfectant. a: Immunoblot analysis of the crude membrane of Rat-1·QL112 cells. The cells were treated with 5 mM IPTG for the indicated hours and the membrane protein was immunoblotted with an anti-Gαi antibody (AS/7). b: Tryptic proteolysis of Gαi. The membrane proteins were treated with trypsin as described in Section 2. The 38-kDa tryptic fragment was detected with AS/7 antibody.

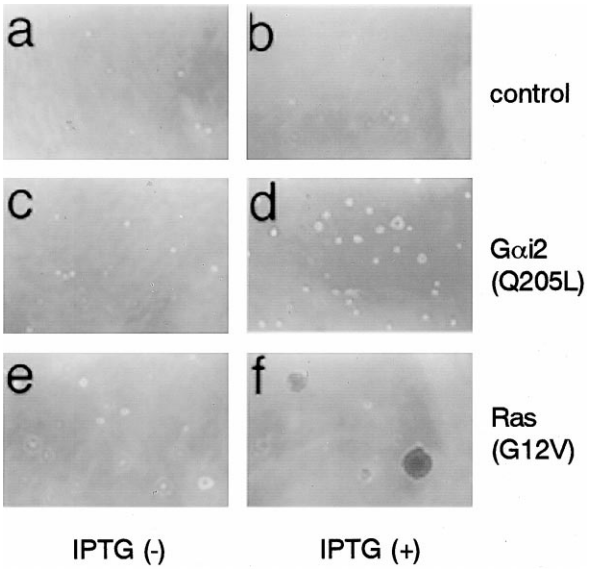

Fig. 2. Anchorage-independent growth of Rat-1 transfectants in soft agar. Parental Rat-1 (a and b), Rat-1·QL112 (c and d), and Rat-1·RasValA1 (e and f) cells were seeded in 0.27% soft agar over 0.53% bottom agar without (a, c, and e) or with 5 mM IPTG (b, d, and f). Microphotographs were taken after culture for 12 days.

3.2. Colony formation of Rat-1 transfectants in soft agar

It has been reported that transfection of the mutationally activated Gαi2 mutants (R179C, R179H, and Q205L) induces anchorage-independent growth of Rat-1 cells [10,11]. To test the transforming activity of the inducibly expressed Gαi2(Q205L), colony formation in soft agar was assayed. Parental Rat-1 cells and their transfectants, Rat-1·QL112 and Rat-1·RasValA1, were seeded into soft agar and treated with IPTG. The parental cells did not form any marked colonies in the soft agar (Fig. 2a,b). On the other hand, IPTG treatment induced the colony formation of Rat-1·QL112 cells (Fig. 2c,d). However, the size of colonies of Rat-1·QL112 was smaller than that of Rat-1·RasValA1 (Fig. 2d,f). This experiment shows that, in Rat-1·QL112 cells, inducibly expressed Gαi2(Q205L) has transforming activity and elicits certain mitogenic signals.

3.3. Ras activation in Gαi2(Q205L)-expressed Rat-1 cells

Ras is activated by various growth factors, cytokines,

and certain types of G protein-coupled receptor agonists, and plays a key role in mitogenic signaling cascades [1,25]. To evaluate the activity of Ras in Gαi2(Q205L)-expressed Rat-1 cells, Ras-bound guanine nucleotide was assayed. Rat-1·QL112 cells were labeled with ^{32}P, and treated with IPTG in serum-free medium. Ras was immunoprecipitated, and the bound guanine nucleotide was analyzed. As shown in Fig. 3a, the ratio of GTP versus GTP+GDP in the anti-Ras immunoprecipitate was increased after the cells were treated with IPTG for 24 h, or stimulated with epidermal growth factor (EGF) for 5 min. To confirm the Ras activation by expression of Gαi2(Q205L), the association of Ras·GTP with the Ras-binding domain (RBD) of c-Raf-1 was analyzed [21]. Since the expression level of endogenous Ras is very low, exogenous Ha-Ras was transfected into Rat-1·QL112 cells. A small amount of Ras·GTP from serum-deprived control cells was precipitated with GST-RBD in vitro (Fig. 3b). Treatment of the cells with EGF or LPA increased the complex formation of Ras and GST-RBD (Fig. 3b). In addition, IPTG treatment increased the amount of Ras precipitated from the lysates of Rat-1·QL112 cells (Fig. 3b), but not of parental Rat-1 cells (data not shown). These results indicate that Ras is activated in the Gαi2(Q205L)-expressed Rat-1 cells.

3.4. Activation of JNK1 in Gαi2(Q205L)-expressed Rat-1 cells

It has been reported that constitutive activation of Ras induces the activation of JNK [26]. In Rat-1·RasValA1 cells, IPTG treatment stimulated JNK1 activity (data not shown). To investigate whether the expression of Gαi2(Q205L) in Rat-1 cells induces JNK activation, an in vitro kinase assay was performed. Rat-1·QL112 cells were starved of serum and treated with or without IPTG. Then, JNK1 was immunoprecipitated from the cell lysate, and in vitro kinase activity was assayed. As shown in Fig. 4a, JNK1 activity was enhanced in IPTG-treated Rat-1·QL112 cells. ERK2 was also activated as reported previously [12] (Fig. 4b). Moreover, phosphorylation of ERK2 was detected 12 h after IPTG treatment (Fig. 4c). It was suggested that long-term activation of an ERK cascade might induce JNK activation through secretion of an extracellular mediator [26]. A recent study showed that activation of the ERK cascade induces JNK activation through the induction of heparin-binding epidermal growth factor (HB-EGF) [27]. To examine the possibility of ERK-dependent JNK activation, Rat-1·QL112 cells were treated with a MEK1 inhibitor, PD98059 [28–30]. Fig. 4 shows that Gαi2(Q205L)-induced JNK1 activation was not inhibited by PD98059, whereas ERK2 activation was completely inhibited. These results indicate that the active Gαi2 activates JNK1 in an ERK-independent manner.

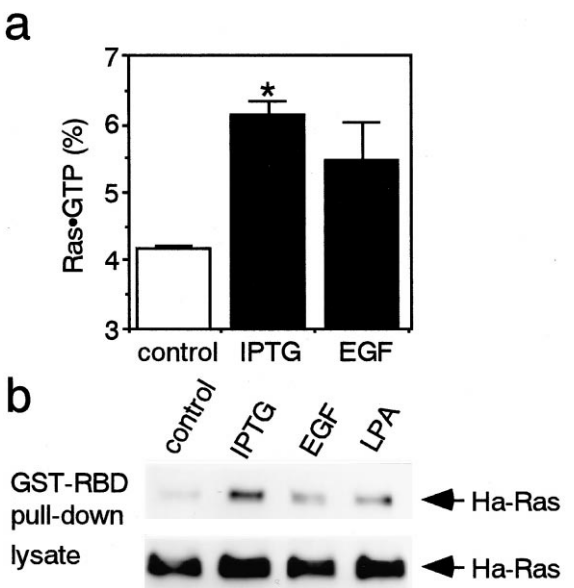

Fig. 3. IPTG-induced increase of GTP-bound Ras in Rat-1·QL112. a: Increase of Ras-bound GTP in Rat-1·QL112 cells. Confluent culture of Rat-1·QL112 cells was starved of serum and labeled with ^{32}P. The cells were treated with 5 mM IPTG for 24 h, or stimulated with 100 ng/ml EGF for 5 min. The Ras·GTP (%) was determined (GTP/(GDP+GTP) (%)). The bars show the mean ± S.E.M. of three to five independent experiments. An asterisk indicates $P < 0.01$ as compared with the control. b: In vitro association of Ras·GTP with c-Raf-RBD. Rat-1·QL112 cells were transfected with pCMV5-Ras(WT), and starved of serum. The cells were treated with 5 mM IPTG for 24 h, or stimulated with 100 ng/ml EGF or 100 μM LPA for 5 min. Ras·GTP associated with c-Raf-RBD (GST-RBD) was analyzed by immunoblotting with an anti-Ha-Ras antibody.

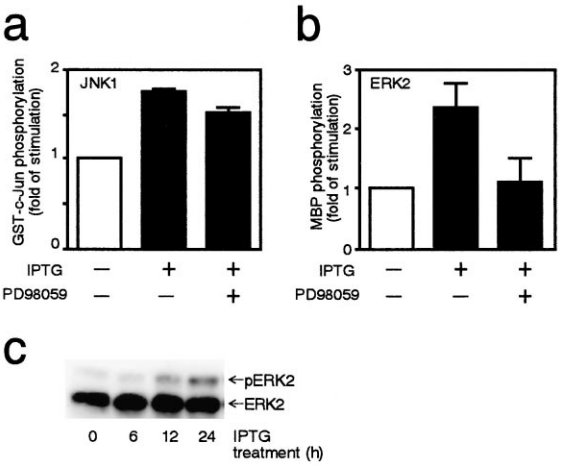

Fig. 4. Activation of JNK1 and ERK2 in the Gαi2(Q205L)-expressed cells. Rat-1·QL112 cells were starved of serum, and treated with 5 mM IPTG in the presence or absence of 50 μM PD98059 for 36 h. In vitro kinase activity of JNK1 (a) and ERK2 (b) was assayed. The results are shown as the mean ± S.E.M. from three independent experiments. c: ERK2 phosphorylation by expression of Gαi2(Q205L). Rat-1·QL112 cells were treated with 5 mM IPTG for the indicated hours. The phosphorylated ERK2 (pERK2) was detected by immunoblotting using an anti-ERK2 antibody.

4. Discussion

In this study, we show that expression of the constitutively active mutant of Gαi2 induces activation of Ras in Rat-1 cells. This result suggests that Ras may be a key molecule in the Gαi2-mediated mitogenic signaling pathways. However, the mechanism of Ras activation by Gαi2 remains to be elucidated. Ras activation is regulated by the activation of Ras-guanine nucleotide exchanging factors (GEF) and/or the inhibition of Ras-GTPase activating proteins (GAP). It has been reported that oncogenic Gαi2-induced transformation of Rat-1 cells was not inhibited by the expression of dominant-negative Ras (Ras(S17N)) which inhibits Ras activation by trapping GEFs in an inactive complex [11]. In neutrophils, f-Met-Leu-Phe (FMLP)-induced Ras activation is associated with inhibition of p120-GAP activity [31]. These results sug-

gest that Gαi2-induced Ras activation might be mediated by the inhibition of GAPs rather than by the activation of GEFs.

In Rat-1·QL112 cells, JNK1, as well as ERK2, was activated by induction of Gαi2(Q205L) (Fig. 4). It was previously reported that the long-term activation of Raf and ERK resulted in the activation of JNK through secretion of HB-EGF [27]. However, the JNK1 activation was not inhibited by the MEK1 inhibitor PD98059 (Fig. 4a), whereas ERK2 activation was completely inhibited (Fig. 4b). The culture supernatant from the IPTG-treated Rat-1·QL112 cells had no activity to induce JNK1 activation, whereas the culture supernatant from the IPTG-treated Rat-1·RasValA1 induced JNK1 activation (data not shown). These results indicate that Gαi2-induced JNK1 activation is independent of ERK2 activation. It was previously reported that, in Rat-1 cells, Gαi2-induced activation of collagenase promoter which contains AP-1-binding sites was c-Jun-dependent [15]. Our results suggest that this AP-1 activation may be mediated by the Gαi2-induced activation of JNK1. Recent studies have shown that Jun activity is essential for the transformation induced by oncoproteins, such as Ras and Bcr-Abl [32,33]. Hence, activation of JNK, as well as activation of ERK, may be critical for the induction of the transformation of Rat-1 cells by constitutively active Gαi2.

The present results obtained from the IPTG-induced expression of Gαi2 revealed that Ras and JNK1 are activated in Gαi2-transformed Rat-1 cells. It was suggested that Gαi2, in addition to Gβγ, may activate Ras-mediated signaling cascades upon receptor activation. The activation of Ras and JNK1 may be cell type-dependent. Indeed, *gip2* mutations have been detected only in tumors of adrenal cortex and ovary [8], whereas *ras* mutations have been detected in various types of human tumors. Clarification of the mechanism of Gαi2-mediated regulation of Ras activity should provide important findings that explain tumors induced by *gip2* mutations and cell proliferation regulated by Gαi2.

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