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Activation of c-*fos* Promoter by Gβγ-Mediated Signaling: Involvement of Rho and c-Jun N-Terminal Kinase[1]

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Several extracellular stimuli mediated by G protein-coupled receptors activate c-*fos* promoter. Recently, we and other groups have demonstrated that signals from G protein-coupled receptors stimulate mitogen-activated protein kinases (MAPKs), extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and p38 MAPK. The activation of these three MAPKs is mediated in part by the G protein βγ subunit (Gβγ). In this study, we characterized the signals from Gβγ to c-*fos* promoter using transient transfection of c-*fos* luciferase into human embryonal kidney 293 cells. Activation of m2 muscarinic acetylcholine receptor and overexpression of Gβγ, but not constitutively active $G\alpha_{12}$, stimulated c-*fos* promoter activity. The c-*fos* promoter activation by m2 receptor and Gβγ was inhibited by β-adrenergic receptor kinase C-terminal peptide (βARKct), which functions as a Gβγ antagonist. MEK1 inhibitor PD98059 and kinase-deficient mutant of JNK kinase, but not p38 MAPK inhibitor SB203580, attenuated the m2 receptor- and Gβγ-induced c-*fos* promoter activation. Activated mutants of Ras and Rho stimulated the c-*fos* promoter activity, and the dominant negative mutants of Ras and Rho inhibited the c-*fos* promoter activation by m2 receptor and Gβγ. Moreover, c-*fos* promoter activation by m2 receptor, Gβγ, and active Rho, but not active Ras, was inhibited by botulinum C3 toxin. These data indicated that both Ras- and Rho-dependent signaling pathways are essential for c-*fos* promoter activation mediated by Gβγ.

Key words: c-*fos* promoter, G protein, HEK-293 cells, MAP kinase, signal transduction, small GTPase.

G protein-coupled receptors are a large family of cell surface receptors. Signals induced by the receptors communicate directly with heterotrimeric G proteins (*1, 2*). The G protein α (Gα) and βγ subunits (Gβγ)-stimulate the mitogen-activated protein kinase (MAPK) cascade (*3*), which plays an important role in the signal transduction from membrane to nucleus. MAPK in mammals has been grouped into four subfamilies: ERK/MAPK, JNK/SAPK, p38 MAPK, and BMK1/ERK5 (*4*). The targets of MAPKs are known to include several transcription factors. Transcription of c-*fos* is rapidly activated by stimuli through growth factor receptors as well as G protein-coupled receptors. Serum response element (SRE) is an important regulatory sequence present in c-*fos* promoter and well characterized. The c-*fos* SRE binds a ternary complex composed of serum response factor (SRF) (*5*) and a ternary complex factor (TCF) (*6*). TCF proteins are a family including Elk-1, SAP-1, and SAP-2. Phosphorylation of TCF by MAPKs potentiates DNA binding activity and transcriptional activation (*7, 8*).

The signaling pathway linking G protein-coupled receptors to c-*fos* promoter is still poorly understood. Although constitutively activated mutants of $G\alpha_s$ (*9*), $G\alpha_{12}$ (*10*), and $G\alpha_{12}$ (*11*), have been shown to activate c-*fos* promoter in certain cells, the involvement of Gβγ in c-*fos* promoter activation has not yet been investigated. Recent studies have shown that members of Rho family GTPases, RhoA, Rac, and Cdc42, are involved in signaling pathway leading to c-*fos* SRE activation independent on TCF (*12*). RhoA is important for c-*fos* SRE activation by serum, lysophosphatidic acid, and aluminium fluoride (*13*). Constitutively activated mutants of the Rho family GTPases are capable of inducing c-*fos* transcriptional activation in the absence of TCF. On the other hand, we and other groups have found that the Rho family GTPases are involved in Gβγ-mediated JNK activation pathway (*14, 15*).

In this study, we introduced the c-*fos* luciferase reporter gene into human embryonal kidney 293 cells expressing Gi-coupled m2 muscarinic acetylcholine receptor or Gβγ. We show that m2 receptor-induced activation of c-*fos*

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Abbreviations: G protein, heterotrimeric guanine nucleotide-binding regulatory protein; Gα, G protein α subunit; Gβγ, G protein βγ subunit; MAPK/ERK, mitogen-activated protein kinase/extracellular signal-regulated kinase; JNK/SAPK, c-Jun N-terminal kinase/stress-activated protein kinase; βARKct, β-adrenergic receptor kinase C-terminal peptide; PMSF, phenylmethane sulfonylfluoride; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

promoter is mediated by $G\beta\gamma$ but not $G\alpha_i$. The $G\beta\gamma$-mediated c-*fos* promoter activation is dependent on Ras and Rho. Moreover, ERK and JNK, but not p38 MAPK, appear to be necessary for the $G\beta\gamma$-mediated promoter activation. Taken together, these findings suggest that $G\beta\gamma$ transduces signals to c-*fos* promoter through both Rho/JNK and Ras/ERK pathways in HEK 293 cells.

MATERIALS AND METHODS

Cell Culture and Transfection—Human embryonal kidney (HEK) 293 cells (ATCC CRL 1573) were maintained in Dulbecco's modified Eagle's medium (Sigma) containing 100 μg/ml streptomycin, 50 units/ml penicillin with 10% heat-inactivated fetal bovine serum (GIBCO BRL). Plasmid DNAs were transfected into HEK 293 cells by calcium phosphate precipitation. The final amount of transfected DNA per 35-mm dish was adjusted to 3.5 μg by use of the empty vector pCMV5 (*16*). The medium was replaced 18 h after transfection, and the cells were starved in the serum-free medium containing 1 mg/ml bovine serum albumin for 24 h.

Plasmids—c-*fos* luciferase reporter plasmid that contains human c-*fos* promoter and firefly luciferase (*17*) was kindly provided by K. Kaibuchi (Nara Institute of Science and Technology). To construct the control plasmid for normalization of each luciferase transfection, a 1.3-kb DNA fragment of the human elongation factor 1α promoter was isolated with *Hin*dIII and *Eco*RI from pEF-BOS (*18*) and ligated upstream of *Renilla* luciferase by using the *Hin*dIII/*Eco*RI site of pRL-null (Promega). This control plasmid is designated as pEF-RL. Plasmids of human m1 and m2 muscarinic acetylcholine receptors, bovine $G\beta_1$ and $G\gamma_2$, mouse $G\alpha_{11}$, constitutively activated mutants of $G\alpha_{i2}$ and $G\alpha_{11}$ (α_{i2}Q205L and α_{11}Q209L), and Ha-RasS17N were prepared as described previously (*19-22*). cDNAs of RhoA and Rac1, and Cdc42Hs were kindly provided by K. Kaibuchi (Nara Institute of Science and Technology), and R.A. Cerione (Cornell University), respectively. Plasmids of FLAG-RhoAT19N, FLAG-Rac1T17N, FLAG-Cdc42-HsT17N, MKK4K95R, and MKK7K63R were prepared as described elsewhere (*15*). cDNA encoding β-adrenergic receptor kinase c-terminal peptide (βARKct) was amplified by polymerase chain reaction using Myc-tagged primer from rat brain cDNA library, and subcloned into pCMV5. The plasmid of C3 exoenzyme was kindly provided by S. Narumiya (Kyoto University). The isolated DNAs and the mutations were confirmed by dideoxynucleotide sequencing using LI-COR 4000L.

Antibodies and Inhibitors—Rabbit polyclonal antibodies against $G\beta$ (T-20) and $G\alpha_{i2}$ (AS/7) were purchased from Upstate Biotechnology Inc. and NEN Life Science Product, respectively. Rabbit polyclonal antibodies against $G\alpha_{11}$ (C-14) and Ha-Ras (C-20) were from Santa Cruz Biotechnology. Mouse monoclonal antibodies against FLAG epitope (M2) and c-Myc epitope (9E10) were purchased from Eastman Kodak and BAbCO, respectively. Anti-mouse (NA931) and anti-rabbit (NA934) Ig antibodies conjugated with horseradish peroxidase were from Amersham Pharmacia Biotech. MEK inhibitor PD98059 was purchased from Biomol Research Laboratories. p38 MAPK inhibitor SB203580 was from Calbiochem-Novabiochem.

c-fos Luciferase Assay—The cells were cotransfected with c-*fos* luciferase plasmid (100 ng) and pEF-RL control plasmid (3.3 ng) together with the indicated expression plasmid. After 42 h, the transfected cells were washed once with phosphate-buffered saline and lysed in 400 μl of lysis buffer [25 mM Tris-HCl (pH 7.8), 2 mM dithiothreitol, 2 mM EDTA, 10% glycerol, 1% Triton X-100] at room temperature for 15 min. The cell lysates were centrifuged at 12,000 rpm for 5 min at 4°C. The activity of the firefly and *Renilla* luciferases was analyzed sequentially using Dual-Luciferase Reporter Assay System (Promega). In brief, 10 μl of the supernatant was mixed with 50 μl of beetle luciferin reagent. The luminescence was recorded for 20 s using a Berthold Lumat LB9501 luminometer. Then 50 μl of Stop and GloR reagent was added, and the specific luminescence of the *Renilla* luciferase was recorded for 20 s. The firefly luciferase activity under control of c-*fos* promoter was normalized to the internal control activity of the *Renilla* luciferase. Data are the mean ± SD from three to six independent experiments.

Immunoblotting—Cell lysates were boiled in Laemmli sample buffer [50 mM Tris-HCl (pH 6.8), 2% SDS, 30 mM dithiothreitol, and 10% glycerol]. The boiled samples were separated by SDS-PAGE, and the proteins were transferred to nitrocellulose membranes. After the membranes were blocked, the separated proteins were immunoblotted with each antibody. The bound antibodies were visualized by an enhanced chemiluminescence detection system with anti-Ig antibody conjugated with horseradish peroxidase as a secondary antibody.

RESULTS

$G\beta\gamma$ Mediates c-fos Promoter Activation by m2 Muscarinic Acetylcholine Receptor—To examine the activation of c-*fos* promoter through Gi-coupled receptors, HEK-293 cells were transfected with a plasmid encoding m2 muscarinic acetylcholine receptor together with c-*fos* luciferase and *Renilla* luciferase plasmids. The transfected cells were treated with 100 μM carbachol for 8 h, and cell lysates were prepared. We measured the luminescence of firefly and *Renilla* luciferases, which are controlled under c-*fos* promoter and elongation factor 1α promoter, respectively. *Renilla* luciferase activity was used in each experiment to normalize the transfection efficiency.

Figure 1A shows that the c-*fos* luciferase activity was increased by stimulation of m2 receptor. Mock transfected cells did not respond to carbachol (data not shown). Next, we examined whether $G\alpha_i$ or $G\beta\gamma$ induces the activation of c-*fos* promoter. Overexpression of $G\beta\gamma$ but not constitutively active $G\alpha_i$ stimulated the activity of c-*fos* luciferase (Fig. 1, B and C). To confirm the involvement of $G\beta\gamma$ in the c-*fos* promoter activation by Gi-coupled receptor, we examine the effect of β-adrenergic receptor kinase c-terminal peptide (βARKct), which is known to bind and sequester free $G\beta\gamma$ upon receptor stimulation. As shown in Fig. 2, coexpression of βARKct completely inhibited the $G\beta\gamma$- and m2 receptor-induced activation of c-*fos* luciferase. In contrast, the activation of c-*fos* luciferase by constitutively active $G\alpha_{11}$ was not inhibited by βARKct. The m1 muscarinic receptor-induced activation was only slightly attenuated. These results indicate that c-*fos* promoter activation by Gi-coupled receptor is mediated by $G\beta\gamma$ but not $G\alpha_i$ in HEK-293 cells.

Involvement of Mitogen-Activated Protein Kinase Cascades in Gβγ-Mediated Activation of c-fos Promoter—Recently, we and other groups found that Gβγ can activate ERK (*19, 23, 24*), JNK (*14, 15*), and p38 MAPK (*21*), which have been shown to phosphorylate TCF (*7, 8*). Therefore, we examined the involvement of three MAPK cascades in Gβγ-mediated c-*fos* promoter activation using specific inhibitors and dominant negative mutants. First, we used MEK1 inhibitor PD98059, which specifically blocks the MEK/ERK pathway (*25*). Activation of c-*fos* luciferase by Gβγ and m2 receptor was inhibited by treatment with PD98058 (Fig. 3A). MKK7 has recently been cloned and shown to phosphorylate and activate JNK specifically (*26, 27*). We constructed the kinase-deficient mutant of MKK7, MKK7K63R, and coexpressed it with Gβγ and m2 receptor. As shown in Fig. 3B, coexpression of MKK7K63R partially attenuated the Gβγ- and m2 receptor-induced activation. We used also the kinase-deficient mutant of MKK4, which is known to be another JNK activator. The Gβγ-induced activation of c-*fos* promoter was inhibited by the MKK4 kinase-deficient mutant (data not shown). Next, we examined the involvement of the p38 MAPK pathway in c-*fos* promoter activation by Gβγ and m2 receptor using a specific inhibitor of p38 MAPK, SB203580 (*28*). Treatment with SB203580 had no effect on the activation of c-*fos* luciferase by m2 receptor and Gβγ (Fig. 3C). In contrast, c-*fos* luciferase activation by overexpression of p38 MAPK was inhibited by treatment with SB203580 under the same conditions (data not shown). These results suggest that ERK and JNK, but not p38 MAPK, participate in the c-*fos* promoter activation by m2 receptor and Gβγ.

Involvement of Small GTPases in c-fos Promoter Activation by the Signaling from Gβγ—We have previously shown that Gβγ induces the Ras activation in HEK-293 cells (*19*). Coexpression of a dominant negative mutant of Ras, RasS17N, reduced the c-*fos* promoter activation by Gβγ and m2 receptor (Fig. 4). The expression level of Gβ was not affected by coexpression of RasS17N. Recently, the

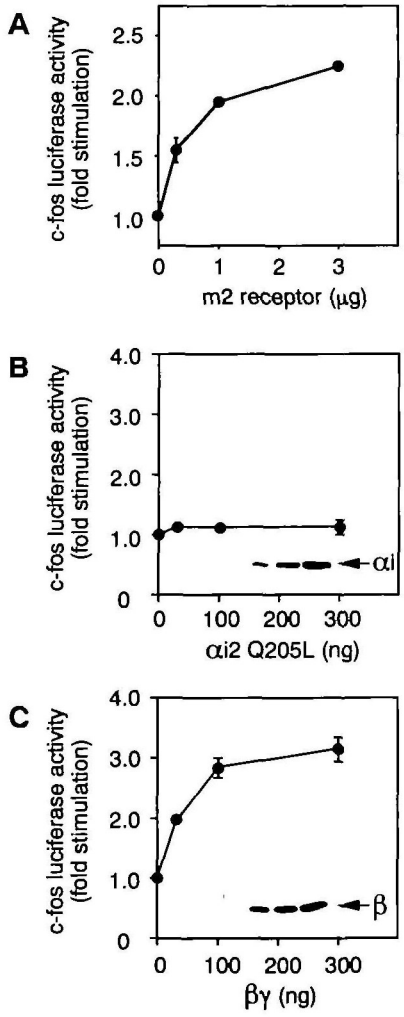

Fig. 1. **m2 muscarinic acetylcholine receptor and Gβγ induce c-*fos* promoter activation in HEK-293 cells.** The indicated amounts of plasmids of m2 receptor (A), constitutively activated $G\alpha_{i2}$ (αi2Q205L) (B), and Gβγ (C) were transfected into HEK-293 cells together with plasmid of c-*fos* luciferase and pEF-RL. c-*fos* luciferase activity was measured by Dual-Luciferase Reporter Assay System, and normalized to *Renilla* luciferase activity. Data are expressed as fold stimulation over empty vector-transfected controls. Values represent the mean±SD from four to six separate experiments. The expression of αi2Q205L and Gβ in the cell lysate is shown in the inset of (B) and (C), respectively.

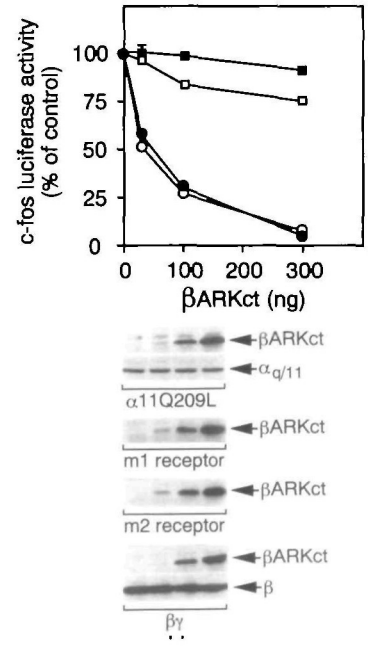

Fig. 2. **Effect of βARKct on c-*fos* promoter activation.** c-*fos* luciferase plasmid and pEF-RL were cotransfected with expression vectors for constitutively activated $G\alpha_{11}$, α11Q209L (100 ng, ■), Gβγ (300 ng ●), m1 receptor (1 μg, ▽), and m2 receptor (3 μg, ○) together with Myc-βARKct. c-*fos* luciferase activity was normalized to *Renilla* luciferase activity. Fold stimulations of c-*fos* luciferase activity by α11Q209L, Gβγ, m1 receptor, and m2 receptor were 10.8±0.9, 4.1±0.3, 29.3±1.1, and 2.5±0.1, respectively. Data represent the mean±SD from three independent experiments, and values are expressed as percent of c-*fos* luciferase in the absence of βARKct (upper). Zero percent represents the basal c-*fos* promoter activity without stimulation of m1 and m2 receptors, or in the absence of α11Q209L and Gβγ. Cell lysates were resolved by 10% SDS-PAGE, and expression of α11Q209L, Gβ, and Myc-βARK was detected by immunoblotting with each antibody (lower).

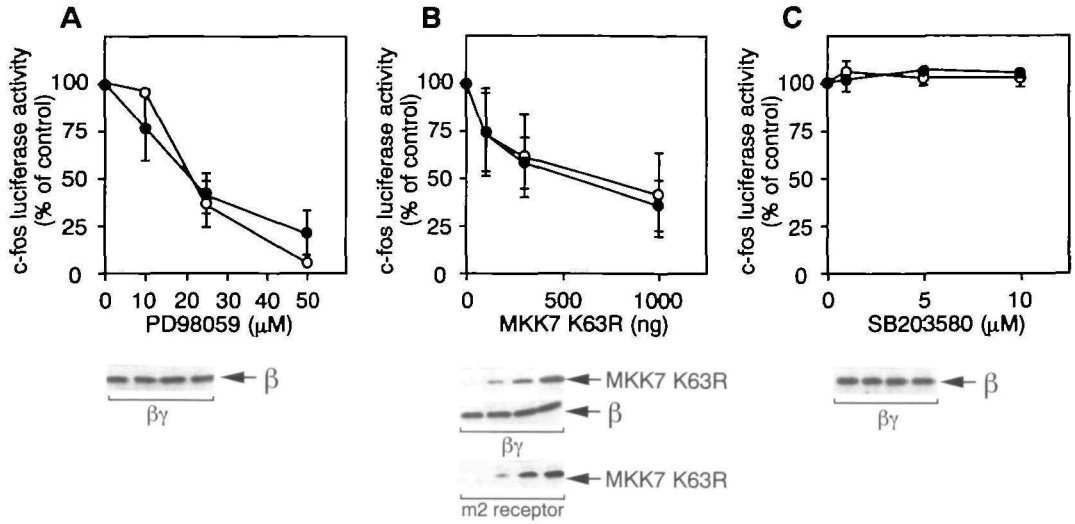

Fig. 3. **Involvement of MAP kinase pathways in Gβγ- and m2 receptor-mediated c-*fos* promoter activation.** (A) HEK-293 cells were cotransfected with c-*fos* luciferase plasmid and pEF-RL control vector plus expression vectors for Gβγ (300 ng, ●) or m2 receptor (3 μg, ○). The transfected cells were treated with the indicated concentrations of PD98059 for 24 h and harvested. c-*fos* luciferase activity was measured and normalized to *Renilla* luciferase activity. Values shown represent the mean±SD of four independent experiments. Data are expressed as the percent of c-*fos* luciferase activity in the absence of PD98059 (upper). Cell lysates were resolved by SDS-PAGE, and Gβ was detected by immunoblotting with anti-Gβ-antibody (lower). (B) c-*fos* luciferase plasmid and pEF-RL were cotransfected with vectors for Gβγ or m2 receptor together with the dominant negative mutant of MKK7, MKK7K63R. Values shown represent the mean±SD of four independent experiments. Data are expressed as the percent of c-*fos* luciferase activation by Gβγ or m2 receptor in the absence of MKK7K63R (upper). Cell lysates were resolved by SDS-PAGE, and Gβ and FLAG-MKK7K63R were detected by immunoblotting with anti-Gβ-antibody or anti-FLAG-antibody, respectively (lower). (C) Transfected cells were treated with the indicated concentrations of SB203580 for 24 h. Values shown represent the mean±SD of four independent experiments. Data are expressed as the percent of c-*fos* luciferase activation by Gβγ or m2 receptor in the absence of SB203580 (upper). Expression of Gβ was detected by immunoblotting (lower). Fold stimulations by Gβγ and m2 receptor were 4.0±0.3 and 2.4±0.2, respectively.

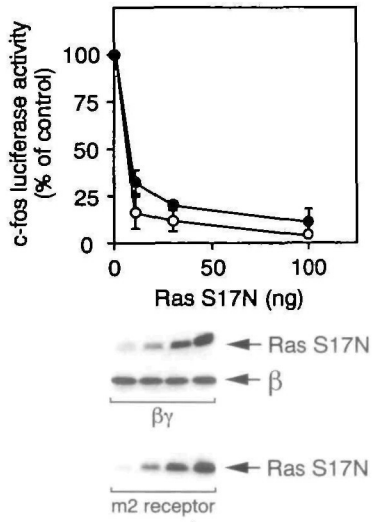

Fig. 4. **Dominant negative Ras inhibits the c-*fos* promoter activation by Gβγ-induced signals.** c-*fos* luciferase plasmid and pEF-RL were cotransfected with vectors for Gβγ (300 ng, ●) or m2 receptor (3 μg, ○) together with dominant negative mutant of Ras, RasS17N. c-*fos* luciferase activity was measured and normalized. Data are expressed as the percent of Gβγ- and m2 receptor-stimulated activity in the absence of RasS17N. Values shown represent the mean±SD from three independent experiments (upper). Fold stimulations by Gβγ and m2 receptor were 3.9±0.5 and 1.9±0.1, respectively. Cell lysates were resolved by 10% SDS-PAGE, and Gβ and Ras were detected by immunoblotting with anti-Gβ- and anti-Ras-antibodies, respectively (lower).

Rho family GTPases have been shown to be involved in the JNK activation (*12, 29, 30*). To assess the effect of Rho family GTPases on the c-*fos* promoter activation, we used FLAG-tagged mutants that had the same epitope attached at the N-terminal. At a comparable expression level of dominant negative mutants of Rho family GTPases, RhoT19N inhibited completely the c-*fos* promoter activation by Gβγ and m2 receptor (Fig. 5). In contrast, Cdc42T-17N had no inhibitory effect on the activation. RacT17N mutant showed a significant inhibitory effect. To confirm the involvement of Rho in the c-*fos* promoter activation by the signaling from Gβγ, we examined the effect of botulinum C3 toxin. HEK-293 cells were transfected with a plasmid of the C3 toxin. Figure 5C shows that the transfection with C3 toxin inhibited the c-*fos* promoter activation by m2 receptor, Gβγ, and active Rho (RhoG14V), but not by active Ras (RasG12V). These results strongly suggested the involvement of Rho in the Gβγ-induced activation of c-*fos* promoter.

Next, we examined whether JNK mediates the signal from Ras and Rho to c-*fos* promoter. The activation of c-*fos* luciferase by active mutants of Ras and Rho was reduced by coexpression of kinase-deficient MKK7 in a dose-dependent manner (Fig. 6B). It was suggested that the activated Ras and Rho stimulate c-*fos* promoter activity partly through JNK.

DISCUSSION

The c-*fos* gene is one of major nuclear targets for signal transduction. It has been shown that the c-*fos* gene is

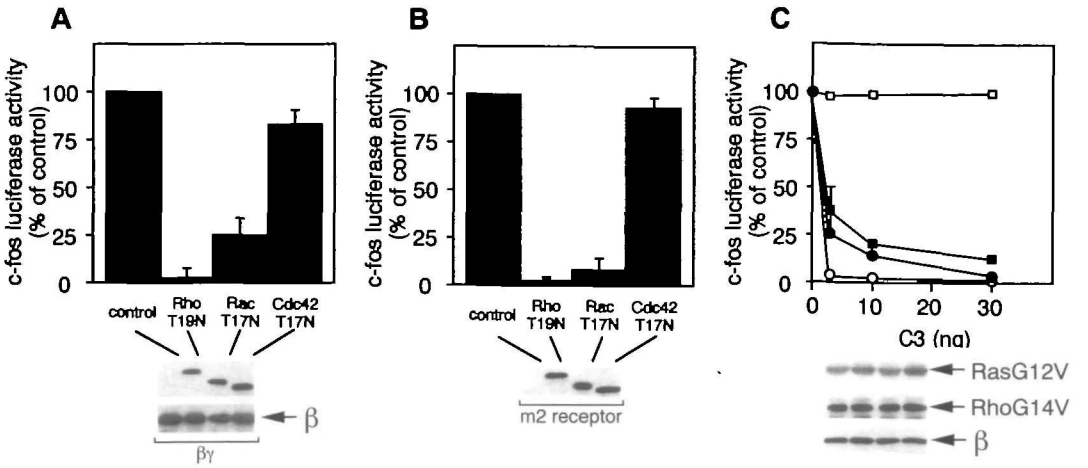

Fig. 5. **Effects of dominant negative mutants of Rho family GTPases and C3 toxin on Gβγ- and m2 receptor-induced c-*fos* promoter activation.** c-*fos* luciferase plasmid and pEF-RL were cotransfected with vectors for Gβγ (300 ng, A) or m2 receptor (3 μg, B) together with 1 μg of plasmid of FLAG-RhoT19N, FLAG-RacT17N, or FLAG-Cdc42T17N. c-*fos* luciferase activity was measured and normalized. The data are expressed as the percent of Gβγ- and m2 receptor-induced activation of c-*fos* luciferase activity in the absence of dominant negative mutants. Values shown represent the mean ± SD from three independent experiments. Cell lysates were resolved by 10% SDS-PAGE, and the FLAG-tagged mutants were detected by immunoblotting with anti-FLAG-antibody. (C) The indicated amounts of C3 toxin plasmid were cotransfected with RasG12V (30 ng, □), FLAG-RhoG14V (100 ng, ■), Gβγ (300 ng, ●), and m2 receptor (3 μg, ○). Fold stimulations of RasG12V, RhoG14V, Gβγ, and m2 receptor were 14.4±1.1, 9.5±1.5, 3.0±0.3, and 2.5±0.6, respectively.

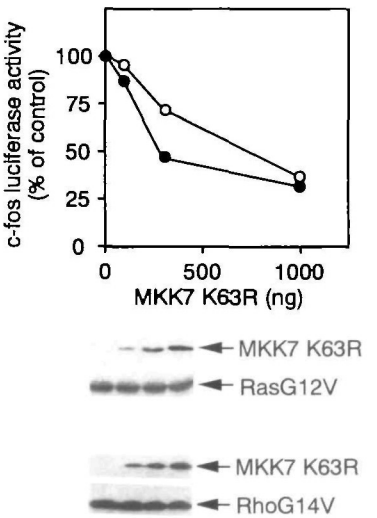

Fig. 6. **Involvement of JNK in Rho- and Ras-induced activation of c-*fos* promoter.** c-*fos* luciferase plasmid and pEF-RL were cotransfected with vectors for active forms of Ras (30 ng, ●) and Rho (100 ng, ○) together with kinase-deficient mutant of MKK7 (FLAG-MKK7K63R). c-*fos* luciferase activity was measured and normalized. Values shown represent the mean ± SD of five independent experiments. Data are expressed as the percent of c-*fos* luciferase activity by RasG12V or RhoG14V in the absence of MKK7K63R (upper). Cell lysates were resolved by SDS-PAGE, and FLAG-MKK7K63R and FLAG-RhoG14V were detected using anti-FLAG-antibody, and Ras was observed by anti-Ras-antibody (lower). Fold stimulations of RasG12V and RhoG14V were 24.5±0.6 and 9.5±1.5, respectively.

activated by diverse stimuli including the stimulation of G protein-coupled receptors (*13, 31*). Although it was known that some of the constitutively activated mutants of Gα stimulate c-*fos* promoter activity (*9-11*), the involvement of Gβγ in c-*fos* gene activation by signaling from G protein-coupled receptors remained to be elucidated. In this paper, we first showed that in HEK-293 cells, the signaling from m2 receptor to c-*fos* promoter is mediated by Gβγ. As shown in Fig. 1A, c-*fos* promoter activity was enhanced by the activation of m2 receptor, which is coupled with Gi. We expressed an activated mutant for $G\alpha_{12}$ and Gβγ. The $G\alpha_{12}$ mutant did not stimulate c-*fos* promoter activity at all (Fig. 1B), whereas expression of Gβγ induced the activation of c-*fos* promoter (Fig. 1C). Moreover, coexpression of βARKct almost completely abolished Gβγ- and m2 receptor-induced activation of the promoter (Fig. 2). The m1 receptor induced-activation was inhibited moderately by βARKct, and the activation by the activated mutant of $G\alpha_{11}$ was not reduced. These findings indicate that the signal from the m1 receptor to c-*fos* promoter is mediated mainly by $G\alpha_{q/11}$, whereas the signal from the m2 receptor is mediated mostly by Gβγ.

We have previously shown that p38 MAPK is activated by the signaling from Gβγ in HEK-293 cells (*21*). p38 MAPK has been shown to phosphorylate and activate Elk and SAP-1 (*32*). We expected that the c-*fos* promoter activation by Gβγ would be blocked by a specific inhibitor of p38 MAPK, SB203580. However, SB203580 did not affect the Gβγ-mediated activation (Fig. 3). Raingeaud *et al.* (*33*) have reported that stimulation by UV irradiation causes the activation of p38 MAPK, but not marked redistribution of p38 MAPK from the cytoplasm to the nucleus. It is likely that p38 MAPK activation by Gβγ does not contribute to the c-*fos* promoter activation.

In this study, we provide the first evidence that Rho- and JNK-dependent pathways are required for the Gβγ-mediated activation of c-*fos* promoter. We have demonstrated that a Gβγ-mediated signal induces the MKK4 and JNK1 activation dependent on Rho in HEK-293 cells (*15*). In contrast, Coso *et al.* (*14*) have reported that signaling from the G protein-coupled receptors to JNK involves Gβγ,

acting on a Ras- and Rac-dependent pathway in COS-7 cells. This discrepancy may reflect either cell-type differences and/or expression levels of dominant negative mutants in the cells.

In addition to Rho- and JNK-dependent pathways, Ras- and ERK-dependent pathways are also required for the c-*fos* promoter activation by G$\beta\gamma$-mediated signaling. But the relationship between Ras-ERK and Rho-JNK pathways in the c-*fos* promoter activation is unclear. In Swiss 3T3 fibroblasts, there is significant cross-talk between the Ras and Rho subfamilies (*34*). We have demonstrated that the JNK activation by G$\beta\gamma$ is independent of Ras (*15*). Hill *et al.* (*12*) have reported that function of Rho is required for the lysophosphatidic acid- and aluminum fluoride-induced activation of c-*fos* promoter with SRF independent on TCF. Taken together, these findings indicate that the pathways leading to c-*fos* promoter from Ras and Rho are probably independent of each other, and both pathways are essential for the activation of c-*fos* promoter by G$\beta\gamma$-mediated signaling.

Rho plays crucial roles in diverse cellular events including cytoskeletal organization and gene expression (*34-37*). It has been reported that Rho participates in the Gα_{12} signaling to c-*fos* promoter (*10*), and p115RhoGEF links G$\alpha_{12/13}$ to Rho (*38, 39*). Similarly, it is possible that G$\beta\gamma$ regulates guanine-nucleotide exchange factors specific for Rho family GTPases. Some RhoGEFs contain both DH and PH domains (*40*). Although the direct interaction of Rho and G$\beta\gamma$ using the recombinant proteins has been reported (*41, 42*), the molecular mechanism of Rho activation by G$\beta\gamma$ remains to be clarified. Further studies should provide a new insight into the implication of Rho family GTPases in the signaling from G protein-coupled receptors.

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