G<sub>i</sub>-dependent Activation of c-Jun N-terminal Kinase in Human Embryonal Kidney 293 Cells

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Heterotrimeric G proteins stimulate the activities of two stress-activated protein kinases, c-Jun N-terminal kinase (JNK) and p38 mitogen-activated protein kinase in mammalian cells. In this study, we examined whether α subunits of G<sub>i</sub> family activate JNK using transient expression system in human embryonal kidney 293 cells. Constitutively activated mutants of Gα<sub>q</sub>, Gα<sub>o</sub>, and Gα<sub>z</sub> increased JNK activity. In contrast, constitutively activated Gα<sub>o</sub> and Gα<sub>z</sub> mutants did not stimulate JNK activity. To examine the mechanism of JNK activation by Gα<sub>i</sub>, kinase-deficient mutants of mitogen-activated protein kinase kinase (MKK) 4 (4MKK4) and 7 (MKK7), which are known to be JNK activators, were transfected into the cells. However, Gα<sub>i</sub>-induced JNK activation was not blocked effectively by kinase-deficient MKK4 and MKK7. In addition, activated Gα<sub>i</sub> mutant failed to stimulate MKK4 and MKK7 activities. Furthermore, JNK activation by Gα<sub>i</sub> was inhibited by dominant-negative Rho and Cdc42 and tyrosine kinase inhibitors, but not dominant-negative Rac and phosphatidylinositol 3-kinase 3-kinase inhibitors. These results indicate that Gα<sub>i</sub> regulates JNK activity dependent on small GTPases Rho and Cdc42 and on tyrosine kinase but not on MKK4 and MKK7.

Heterotrimeric guanine nucleotide-binding regulatory proteins (G proteins)<sup>1</sup> are composed of α, β, and γ subunits (Gα, Gβ, and Gγ), which are encoded by at least 15, 5, and 11 genes, respectively, in mammalian cells (1–4). In response to stimuli such as sensory signals, hormones, neurotransmitters, and chemokines, G protein-coupled receptors activate G proteins, which in turn modulate downstream effectors including adenyl cyclases, phospholipase Cβ<sub>2</sub>, phosphatidylinositol 3-kinases, ion channels, and β-adrenergic receptor kinases (1–4).

Mitogen-activated protein kinases (MAPKs) are serine/threonine kinases involved in cellular responses to various stimuli (5–8). MAPKs are grouped into four major classes: ERK/MAPK, BMK1/ERK5, JNK/stress-activated protein kinase, and p38 MAPK. ERK and BMK1 are activated mainly by growth factors and are involved in cell cycle progression and cell growth (5–8). Inflammatory cytokines and environmental stresses stimulate the activities of JNK and p38 MAPK, which appear to be implicated in cell cycle arrest and apoptosis (6–8). MAPKs are activated by dual phosphorylation on threonine and tyrosine residues catalyzed by MAPKK/MEK, which is phosphorylated and activated by serine/threonine kinases called MAPKK/MEKK (5–8). Raf activates ERK via MEK1/ MEK1 and MEK2/MKK2. MEK5/MKK5/SKK5 phosphorylates BMK1. JNK is phosphorylated and activated by MKK4/SEK1/JNK1/SKK1 and MKK7/JNK2/SKK4, while p38 MAPK is phosphorylated and activated by MKK3/SKK2, MKK6/SKK3, and MKK4. Since many MAPKK/MEKs including MEK1, MEK2, MEK3, MEK4, MAPKK5, and MAPKK6, induce the activation of JNK and/or p38 MAPK cascade(s), the linkage of MAPKK/MEKK to MAPK/MEK is more complicated than that of MAPK to MAPK.

Several lines of evidence suggest that G protein-coupled receptors stimulate the ERK pathway through some G protein subunits in various cells (9, 10). It is likely that G<sub>i</sub>-dependent ERK activation is mediated primarily by Gβγ (9, 10). Gβγ directly activates phosphatidylinositol 3-kinase γ and β-adrenergic receptor kinase 1, resulting in an increase of activities of Src family tyrosine kinases (9–14). Tyrosine-phosphorylated Shc permits the translocation of the Grb2-Sos complex to plasma membranes, leading to the promotion of the GDP-GTP exchange on Ras. Ras regulates the activity of Raf, which induces the activation of MEK1 and -2 and subsequently ERK.

Some G protein-coupled receptors are able to activate JNK in certain types of cells (9). JNK is activated by agonist stimulation of G<sub>i</sub>-coupled receptors in NIH3T3, Rat-1, and COS-7 cells (15–17). It has been reported that JNK activation by G<sub>i</sub>-coupled m2 muscarinic acetylcholine receptor is mediated mainly by Gβγ in COS-7 cells (17). Small GTPases Ras and Rac and phosphatidylinositol 3-kinase γ are involved in this Gβγ-induced JNK activation (17, 18). In the course of studying G<sub>i</sub>-dependent JNK activation in human embryonal kidney (HEK) 293 cells, we found that its activation was mediated by both Gα<sub>i</sub> and Gβγ. Here we show that constitutively activated Gα<sub>o</sub> mutant as well as Gβγ (19) stimulates JNK activity. Furthermore, we investigate whether JNK kinases, Rho family GTPases, tyrosine kinases, and phosphatidylinositol 3-kinases participate in this signal transduction pathway.

**EXPERIMENTAL PROCEDURES**

**Materials—**Mastoparan and pertussis toxin were purchased from Calbiochem-Novabiochem Co. and Kaken Pharmaceutical Co., respectively. Tyrosine kinase inhibitors PP1/AG1872 and PP2/AG1879 were kindly provided by A. Levitzki (Hebrew University). Phosphatidylinositol 3-kinase inhibitors wortmannin and LY294002 were purchased from Calbiochem-Novabiochem Co. and BIOMOL, respectively. Mouse monoclonal antibodies M2, 12CA5, and 9E10 against FLAG, HA, and Myc epitopes were obtained from Eastman Kodak Co., Roche Molecular Biochemicals, and Babco, respectively. Mouse monoclonal antibody

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1 The abbreviations used are: G protein, heterotrimeric guanine nucleotide-binding regulatory protein; Gα, G protein α subunit; Gβγ, G protein βγ subunit; ERK, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase; JNK, c-Jun N-terminal kinase; MEKK, MAPK/ERK kinase; MEKK; MAPK/ERK kinase; MEKK; MEK; GST, glutathione S-transferase; Trx, thioredoxin; HA, hemagglutinin; Hhek, human embryonal kidney; βARK1, β-adrenergic receptor kinase 1.
Regulation of JNK by Goi

B-14 against Schistosoma japonicum GST was purchased from Santa Cruz Biotechnology, Inc. Rabbit polyclonal antibodies T-20 and 06-238 against Gβδ were obtained from Santa Cruz Biotechnology and Upstate Biotechnology, Inc., respectively. Rabbit polyclonal antibodies AS7, EC22, and GO2 against Goαq, Goαo, and Goαv, respectively, were purchased from Life Science Products, Inc. Rabbit polyclonal antibodies against GoαqGαq and Goα (X264) were generously provided by T. Asano (Aichi Human Service Center) and P. C. Sternweis (University of Texas Southwestern Medical Center), respectively. Rabbit polyclonal anti-Cακ antibody C-20 was purchased from Santa Cruz Biotechnology. Goat anti-mouse and anti-rabbit IgG antibodies conjugated with horse-radish peroxidase were obtained from NEN Life Science Products.

Plasmids—Complementary DNAs of Goαq, GoαQ205L, Goαo, and GoαQ505L (20–22) were inserted into pCMV mammalian expression vector. pCMV-Goαq, pCMV-GoαQ205L, pCMV-Goαo, pCMV-GoαQ505L, pCMV-Goα, pCMV-G αq, pCMV-G αv, pCMV-carboxy terminus of β-adrenergic receptor kinase 1 (βARK1ct), pCMV-FLAG-Rho1T19N, pCMV-FLAG-Rac1T17N, pCMV-FLAG-Cdc42T17N, pCMV-GST-MK4K, pCMV-FLAG-MKK4K95R, pCMV-GST-MKK7, and pCMV-FLAG-MKK7K63R were constructed as described previously (23–25). pCMV-GoαQ205L was prepared by T. Yamaguchi and M. Tagaya (Tokyo University of Pharmacy and Life Science). cDNA of human orthologue (26) of mouse MKK7a (27) was inserted into a BamHI restriction site of pCMV-GST. SRa-HA-JNK1 and SRa-HA-ERK2 were kindly provided by M. Karin (University of California, San Diego). pEF-BOS-Clostridium botulinum C3 toxin (28) was generously provided by S. Narumiya (Kyoto University). Pak1 cDNA was a generous gift from L. Lim (National University of Singapore). A CRIB region, which interacts with active Cdc42 and Rac (29), was amplified by polymerase chain reaction using Pak1 cDNA as a template and was ligated into pCMV-Mye. Cακ cDNA (30) was kindly provided by M. Okada (Institute for Protein Research, Osaka University). pCMV-Cακ was constructed and generously provided by S. Mizutani. Hexahistidine tag expression plasmids, pET15b-JNK1 and pET32a-c-Jun (amino acids 1–223), were constructed as described before (19). pGEX2T-c-Jun (amino acids 1–223) was kindly provided by M. Karin (University of California, San Diego). All DNA sequences were confirmed by DNA sequencer L-4000L (LI-COR) according to the manufacturer’s protocol.

Cell Culture—HEK 293 cells (ATCC CRL 1573) were maintained in Dulbecco’s modified Eagle’s medium (Sigma) containing 100 μg/ml kanamycin (Nacalai) with 10% heat-inactivated fetal bovine serum (Life Technologies, Inc.). The cells were cultured at 37 °C in a humidified atmosphere containing 10% CO2.

Transfection—Plasmid DNA was transfected into HEK 293 cells by the calcium phosphate precipitation method. The final amount of the transfected DNA for a 60-mm dish was adjusted to 25 μg by empty vector. pCMV. Three μg of SRa-HA-JNK1, SRa-HA-ERK2, pCMV-GST-MKK4, pCMV-GST-MKK7, or pCMV-GST-MKK7β was cotransfected with 3 μg of pCMV-Mye-βARK1ct, 10 μg of each Gα wild type or QL mutant plasmid, 5 μg of pCMV-Gαq, and 5 μg of pCMV-Gαv, 10 μg of pCMV-FLAG-MKK4K95R, 10 μg of pCMV-FLAG-MKK7K63R, 10 μg of pCMV-Cακ, 10 μg of RAD51, and 1 μg of pCMV-GST-MKK7K63R. The medium was replaced 24 h after transfection, and the cells were starved in the serum-free medium containing 1 mg/ml mouse serum albumin (Nacalai) for 24 h.

Recombinant Proteins—Recombinant hexahistidine-JNK, Trx-c-Jun, and GST-c-Jun were purified from the transformed E. coli strain BL2 (DE3) cells as described before (19). Briefly, E. coli cells treated with isopropyl-thio-β-D-galactopyranoside were harvested by centrifugation and sonicated in extraction buffer A (20 mM HEPES-NaOH (pH 8.0), 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 0.5% Nonidet P-40) for hexahistidine-JNK and Trx-c-Jun or extraction buffer B (20 mM HEPES-NaOH (pH 7.5), 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 mM EDTA, 0.5% Nonidet P-40) for GST-c-Jun. The cell extracts were centrifuged at 150,000 × g for 30 min. All purification steps were performed at 4 °C.

Purification of GST-c-Jun, the supernatants were subjected to nickel-nitrilotriacetic acid-agarose (Qiagen, Inc.), and the resin was washed with column buffer A (20 mM HEPES-NaOH (pH 8.0), 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 200 mM NaCl containing 20 mM imidazole). Hexahistidine-JNK and Trx-c-Jun were eluted with column buffer A containing 200 mM imidazole. For the purification of GST-c-Jun, the supernatant was applied to glutathione-Sepharose 4B (Amersham Pharmacia Biotech), and the resin was washed with column buffer B (20 mM HEPES-NaOH (pH 7.5), 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 mM EDTA).

GST-c-Jun was eluted with column buffer B containing 10 mM glutathione. Hexahistidine-JNK was dialyzed against column buffer B containing 200 mM NaCl and stored at −80 °C until use. Under these storage conditions, hexahistidine-JNK retained the catalytic activity within at least 6 months. The eluate of GST-c-Jun and Trx-c-Jun was dialyzed against column buffer B and stored at −80 °C until use.

Kinase Assays—After 24 h of serum starvation, the cells transfected with SRa-HA-JNK1, SRa-HA-ERK2, pCMV-GST-MKK4, pCMV-GST-MKK7, or pCMV-GST-MKK7β were lysed in 600 μl of lysis buffer A (20 mM HEPES-NaOH (pH 7.5), 3 mM MgCl2, 100 mM NaCl, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 1 mM EDTA, 1 mM Na2VO3, 10 mM NaF, 20 mM β-glycerophosphate, and 0.5% Nonidet P-40) on ice. The lysates were centrifuged at 14,000 rpm for 10 min at 4 °C. For JNK and ERK assay, aliquots (500 μg) of the supernatants were mixed with protein A-Sepharose CL-4B (Amersham Pharmacia Biotech) preabsorbed with a mouse anti-HA antibody for 12 h at 4 °C. The immune complexes were washed twice with lysis buffer A and twice with reaction buffer A (20 mM HEPES-NaOH (pH 7.5), 10 mM MgCl2, 0.1 mM phenylmethylsulfonyl fluoride, 0.1 μg/ml leupeptin, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 mM EDTA, 10 mM Na2VO3, and 2 μM β-glycerophosphate) and incubated in 30 μl of reaction buffer A containing 3 μg of GST-c-Jun for JNK assay or 5 μg of myelin basic protein for ERK assay, 20 μM ATP, and 5 μCi of [γ-32P]ATP (Amersham Pharmacia Biotech) at 30 °C for 10 min. For MKK4, MKK7, or MKK7β assay, aliquots (500 μg) of the supernatants were mixed with glutathione-Sepharose 4B for 12 h at 4 °C and centrifuged. The precipitate was washed with lysis buffer A and with reaction buffer A and was incubated in 30 μl of reaction buffer A containing 2 μg of hexahistidine-JNK, 10 μg of Trx-c-Jun, 20 μM ATP, and 5 μCi of [γ-32P]ATP at 30 °C for 20 min. The reaction was stopped by adding 10 μl of 4 x Laemmli sample buffer and boiled, and the sample was subjected to SDS-polyacrylamide gel electrophoresis. The radioactivity incorporated into GST-c-Jun, Trx-c-Jun, and myelin basic protein was measured by an imaging analyzer (FUJI BAS 2000) and detected by autoradiography.

Immunoprecipitation and Immunoblotting—Aliquots (250 μg) of cell lysates were mixed with protein A-Sepharose CL-4B preabsorbed with a mouse anti-Myc antibody for 12 h at 4 °C. The immune complexes were precipitated by centrifugation and washed four times with lysis buffer B. Aliquots of cell lysates and immune complexes were boiled in Laemmli sample buffer. The boiled samples were separated by SDS-
polycrylamide gel electrophoresis, and the proteins were transferred
to nitrocellulose membranes (BA85; Schneider & Schnell). After the
membranes were blocked with phosphate-buffered saline containing
0.1% Tween 20 and 5 mg/ml bovine serum albumin, the separated
proteins were immunoblotted with various antibodies. The bound anti-
odies were detected using anti-rabbit or mouse IgG antibody conju-
gated with horseradish peroxidase.

RESULTS

Mastoparan-induced JNK Activation Is Mediated by both Ga
and Gβγ in HEK 293 Cells—We introduced plasmids en-
coding HA-tagged JNK with various cDNAs into HEK 293
cells. Using anti-HA antibody, the epitope-tagged JNK was
immunoprecipitated from lysates of the transfected cells. The
JNK activity was assessed as the radioactivity incorporated
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JNK activity was assessed as the radioactivity incorporated

Next, we investigated whether Gi is involved in mastoparan-
induced JNK activation. The cells were treated with pertussis
toxin, which ADP-ribosylates Gz and inhibits the coupling of
Gz with the receptors, for 24 h before the addition of mas-
toparan (Fig. 1A). The activation was almost completely inhib-
ited by the pretreatment of pertussis toxin. Since there is no Gz
in HEK 293 cells as described below, the inhibition by pertussis
toxin indicated that mastoparan increases JNK activity via Gz.

To determine whether mastoparan-induced JNK activation is
mediated by Gz and/or Gβγ, a plasmid encoding carboxy-
terminal peptide of β-adrenergic receptor kinase 1 (βARK1)
was cotransfected. It has been shown that βARK1 associates
with Gβγ and inhibits Gβγ-mediated ERK and JNK activations
by G protein-coupled receptors (11, 17). Mastoparan-induced
JNK activation was reduced approximately 50% by cotransfec-
tion of βARK1ct (Fig. 1B), suggesting that the JNK activation
is mediated by both Gz and Gβγ in HEK 293 cells.

GzQ205L Stimulates the Activity of JNK in HEK 293
Cells—We explored which α subunit of the G family increases
JNK activity. As shown in Fig. 2A, constitutively activated
mutants of Gα1, Gα2, and Gαz stimulated JNK activity by
approximately 3.5-, 5-, and 3.5-fold, respectively. On the other
hand, constitutively activated Gα1 and Gαz mutants did not
stimulate JNK activity. The activation of JNK by GαzQ205L
was comparable with that by Gβγ (Fig. 2A). The expression of
endogenous Gα1, Gα2, and Gαz in HEK 293 cells was not detected by
immunoblotting (Fig. 2A).

To confirm that the inhibitory effect of βARK1ct on masto-
paran-induced JNK activation results from the sequestration of
Gβγ, βARK1ct was cotransfected with GαzQ205L or Gβγ
(Fig. 2B). The activation of JNK by Gβγ, but not GαzQ205L,
was blocked completely by cotransfection of bARK1ct.

**Mastoparan-induced JNK Activation Is Dependent Partially on MKK4 and MKK7**—To clarify whether mastoparan activates JNK through two JNK kinases, MKK4 and MKK7, we cotransfected a plasmid of MKK4K95R or MKK7K63R. MKK4K95R and MKK7K63R are kinase-deficient mutants that sequester upstream components such as MEKK1. Mastoparan-induced JNK activation was suppressed partially by co-transfection of MKK4K95R or MKK7K63R (Fig. 3, A and B).

Next, we analyzed the activities of MKK4 and MKK7. The cells were cotransfected with a plasmid encoding GST-fused MKK4 or MKK7. GST-MKK4 or GST-MKK7 was precipitated from lysates of the transfected cells using glutathione-Sepharose 4B and immunoblotted with anti-GST antibody.

**Fig. 3.** Partial involvement of MKK4 and MKK7 in mastoparan-induced JNK activation. Cells were transfected with plasmids carrying cDNAs for HA-JNK (A and B), GST-MKK4 (C), GST-MKK7 (D), FLAG-MKK4K95R (MKK4K95R) (A), and FLAG-MKK7K63R (MKK7K63R) (B). The activities of JNK, MKK4, and MKK7 were measured at 15 min after the addition of 50 μM mastoparan as described under "Experimental Procedures." Values shown represent the mean ± S.E. from three or four separate experiments. The phosphorylation of GST-c-Jun and Trx-c-Jun and the expression of HA-JNK, GST-MKK4, GST-MKK7, FLAG-MKK4K95R, and FLAG-MKK7K63R in the cell lysates are shown. GST-MKK4 and GST-MKK7 were precipitated with glutathione-Sepharose 4B from the cell lysates and immunoblotted with anti-GST antibody.

**Fig. 4.** Gaα2Q205L activates JNK through an MKK4- and MKK7-independent pathway. Cells were transfected with plasmids carrying cDNAs for HA-JNK (A and B), GST-MKK4 (C), GST-MKK7 (D), Gaα2Q205L (α2Q205L) (A-E), Gβγ (β1γ2) (C-E), FLAG-MKK4K95R (MKK4K95R) (A), and FLAG-MKK7K63R (MKK7K63R) (B). The activities of JNK, MKK4, MKK7, and MKK7β were measured as described under "Experimental Procedures." Values shown represent the mean ± S.E. from at least three separate experiments. Statistical analysis was performed using Student’s t test. *, p < 0.01 (n = 6) compared with Gaα2Q205L without MKK7K63R. The phosphorylation of GST-c-Jun and Trx-c-Jun and the expression of HA-JNK, GST-MKK4, GST-MKK7, GST-MKK7β, G protein subunits, FLAG-MKK4K95R, and FLAG-MKK7K63R in the cell lysates are shown. GST-MKK4, GST-MKK7, and GST-MKK7β were precipitated with glutathione-Sepharose 4B from the cell lysates and immunoblotted with anti-GST antibody.
and incubated with recombinant JNK and c-Jun, and the radioactivity incorporated into c-Jun was measured. Mastoparan activated only weakly MKK4 and MKK7 (Fig. 3, C and D). This result, considered together with results of kinase-deficient mutants, indicates that JNK activation by mastoparan is dependent partially on MKK4 and MKK7.

Ga<sub>i</sub>Q205L Fails to Activate MKK4 and MKK7—We reported previously that Gβγ activates JNK mainly through MKK4 and to a lesser extent through MKK7 (19). Because JNK activation by mastoparan was dependent partially on MKK4 and MKK7, we considered that MKK4 and MKK7 might be involved in JNK activation mediated by Ga<sub>i</sub>. However, cotransfection of MKK4K95R failed to attenuate Ga<sub>i</sub>Q205L-induced JNK activation (Fig. 4A). Furthermore, Ga<sub>i</sub>Q205L failed to activate MKK4 (Fig. 4C). Although cotransfection of MKK7K63R inhibited partially Ga<sub>i</sub>Q205L-induced JNK activation, MKK7 activity was not stimulated by Ga<sub>i</sub>Q205L (Fig. 4, B and D). A human MKK7 gene appears to generate some alternative splicing forms. A MKK7β isoform has 86 extra amino acid residues at the N terminus of MKK7 (26) and is expressed mainly in HEK 293 cells (data not shown). We thought that Ga<sub>i</sub> might activate a MKK7β isoform. However, Ga<sub>i</sub>Q205L failed to stimulate MKK7β activity (Fig. 4E).

Ga<sub>i</sub>Q205L-mediated JNK Activation Is Dependent on Rho and Cdc42 but Not on Rac—Rho family GTPases have been shown to be implicated in the activation of JNK by various stimuli (32). In addition, JNK activation by Gβγ depends on Rho family GTPases (17–19). To test the possibility that Rho family GTPases are involved in the pathway from Ga<sub>i</sub> to JNK, we cotransfected each plasmid of dominant-negative Rho family GTPases. Mastoparan-induced JNK activation was inhibited...
by RhoT19N and Cdc42T17N but not RacT17N (Fig. 5, A–C). In addition, JNK activation by Ga\(_i2\)Q205L was also blocked by the dominant negative mutants of Rho and Cdc42 (Fig. 5, D–F), indicating that Ga\(_i2\) regulates JNK activity through Rho and Cdc42 in HEK 293 cells. To confirm that Rho and Cdc42 are involved in Ga\(_i2\)Q205L-induced JNK activation, we utilized C3 toxin and Pak1CRIB. C3 toxin ADP-ribosylates Rho and inhibits its Rho functions (28). Pak1CRIB is associated with active Rac or Cdc42 and inhibits the interaction of active Rac or Cdc42 with the effectors (29, 32). As shown in Fig. 5, G and H, JNK activation by Ga\(_i2\)Q205L was blocked by cotransfection of C3 toxin or Pak1CRIB. These results suggest that Rho and Cdc42 participate in the JNK pathway from Ga\(_i\).

Effect of Tyrosine Kinase Inhibitors on Ga\(_i2\)-mediated JNK Activation—To investigate whether tyrosine kinases are involved in Ga\(_i2\)-mediated activation of JNK, we used PP1 and PP2, which are tyrosine kinase inhibitors preferential for Src family tyrosine kinases (33), and Csk, which is a negative regulator of phosphatidylinositol 3-kinase (PI3K). Csk is a negative regulator of PI3K (30, 31, 34–36). As shown in Fig. 6, Ga\(_i2\)-mediated JNK activation depends on Src family tyrosine kinases. Cells were transfected with plasmids carrying cDNAs for HA-JNK (A–E), Ga\(_i2\)Q205L (a\(_i2\)Q205L) (B–D), Ga\(_i2\)Q205L (a\(_i2\)Q205L) (E and F). A and B, cells 48 h after transfection were treated with 50 \(\mu\)M mastoparan for the indicated time in the presence (open circle) or absence (closed circle) of 1 \(\mu\)M wortmannin. C and D, the transfected cells were pretreated with the indicated concentrations of wortmannin for 20 min and were treated with 50 \(\mu\)M mastoparan for 15 min. E and F, the transfected cells were pretreated with 100 nM wortmannin and 100 \(\mu\)M LY294002 for 20 min. Kinase activities of HA-JNK and HA-ERK were measured as described under “Experimental Procedures.” Values shown represent the mean ± S.E. from three or four separate experiments. The phosphorylation of GST-c-Jun and the expression of HA-JNK and Ga\(_i2\)Q205L in the cell lysates are shown.

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Regulation of JNK by G\textsubscript{\alpha}\text{i}

Two JNK kinase genes, MKK4 and MKK7, have been cloned to date. We showed previously that G\beta\gamma stimulates JNK via mainly MKK4 and to a lesser extent MKK7 (19). In contrast, JNK activation by G\alpha\text{bg}Q205L was not inhibited effectively by cotransfection of kinase-deficient MKK4 and MKK7 (Fig. 4). Moreover, cotransfection of G\alpha\text{bg}Q205L into the cells failed to stimulate the activities of MKK4, MKK7, and MKK7\beta, an alternative splicing form of MKK7 (Fig. 4). It has been reported that there is a JNK kinase other than MKK4 and MKK7 at the level of the fractionation by column chromatography with lysates of stress-stimulated cells (37). G\alpha\text{gip2} may regulate JNK activity through an unidentified JNK kinase.

A Rac and Cdc42-binding region is found in MAPKKKs including MEKK1, MEKK4, MLK2, and MLK3. MEKK1 activates primarily JNK pathway and appears to mediate JNK activation by Rac or Cdc42 (38). It has been shown that MEKK1 and ASK1 mediate JNK activation induced by G\alpha\text{bg} and G\alpha\text{i12} in COS-7 and HEK 293 cells (39). Additionally, Collins et al. (40) have reported that G\alpha\text{i2Q205L}-induced JNK activation partially involves Cdc42 in HEK 293 cells. It appears that a Cdc42-MEKK1 signaling unit functions upstream of JNK. Because G\alpha\text{bg}Q205L-induced JNK activation depended on Cdc42 (Fig. 5), MEKK1 might be a candidate of MAPKKK in the JNK pathway from G\alpha\text{i}, JNK activation by G\alpha\text{i} dependent on Rho (Fig. 5).

Although a MAPKKK regulated by Rho has not yet been identified, it is expected that there may exist a MAPK cascade linking Rho with a transcription factor SRF in c-fos promoter activation induced by lyso phosphatidic acid, a G\alpha-coupled receptor ligand (41).

G\alpha\text{bg}Q205L-induced JNK activation was inhibited by tyrosine kinase inhibitors PP1 and PP2 in a dose-dependent manner (Fig. 6). These inhibitors preferentially inhibit Src family tyrosine kinases, and the IC\textsubscript{50} value of PP2 for the inhibition of Src is 15 \muM in intact cells. Furthermore, cotransfection of Csk attenuated G\alpha\text{bg}Q205L-induced JNK activation (Fig. 6). Src family tyrosine kinases are likely to be involved in the signaling pathway from G\alpha\text{i} to JNK.

We reported previously that G\beta\gamma stimulates MKK4 activity in a Rho- and Cdc42-dependent manner (19). In contrast, G\alpha\text{bg}Q205L failed to activate MKK4, but activated JNK in a Rho- and Cdc42-dependent manner. This difference may be due to the difference of downstream signaling components of G\alpha\text{i} and G\beta\gamma. It must be noted that JNK activation by G\alpha\text{bg}Q205L, but not by G\beta\gamma, was reduced by Csk (Fig. 6) and dominant-negative Fyn. These results suggest that the relationships among tyrosine kinases, Rho, and Cdc42 may be more complex than a single sequential cascade.

Fig. 8 shows a proposed pathway from G\alpha to JNK. In the present study, we used mastoparan as an activator of G\alpha\text{i}. JNK activation by mastoparan, as well as G\alpha\text{bg}Q205L and G\beta\gamma (19), was almost completely inhibited by dominant negative mutants of Rho and Cdc42 (Fig. 5). Moreover, mastoparan-induced JNK activation was blocked partially by Src family inhibitor PP1 and the kinase-deficient mutant of MKK4 (Figs. 3 and 6). These results are consistent with the model in which G\alpha\text{gip2} and G\beta\gamma participate equivalently in the signaling pathway from G\alpha\text{i} to JNK.

Activating mutations of G\alpha\text{bg}

which were denoted as the gip2 oncogene, have been found in some types of tumor including ovarian sex cord stromal tumors, adrenal cortex tumors, and nonfunctional pituitary tumors (42). In addition, ectopic expression of the gip2 oncogene has been shown to cause oncogenic transformation of Rat-1 fibroblast cells. However, it appears that the gip2 oncogene fails to transform other fibroblast.

\textsuperscript{2}A. Levitski, personal communication.
cells such as NIH3T3 (42). Recently, we reported that conditional expression of active Goαq mutant in Rat-1 cells induces the colony formation on soft agar (36). We found that JNK is activated with the expression of active Goαq mutant in the cells (36). On the other hand, JNK activity was not stimulated in NIH3T3 cells expressing active Goαq mutant (43). Further elucidation of the Goαq-JNK pathway may clarify the relationship between the activation of JNK and the mechanism of oncogenic transformation by the hip2 oncogene.

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REFERENCES
1. Gilman, A. G. (1987) Annu. Rev. Biochem. 56, 615–649
2. Kaziro, Y., Itoh, H., Kozasa, T., Nakafuku, M., and Satoh, T. (1991) Annu. Rev. Biochem. 60, 349–400
3. Neer, E. J. (1995) Cell 80, 249–257
4. Hamn, H. E. (1998) J. Biol. Chem. 273, 669–672
5. Sato, T., and Nishida, E. (1993) Trends Biochem. Sci. 18, 128–131
6. Davis, R. J. (1994) Trends Biochem. Sci. 19, 470–473
7. Cobb, M. H., and Goldsmith, E. J. (1995) J. Biol. Chem. 270, 14843–14856
8. Kyriakis, J. M., and Avruch, J. (1996) J. Biol. Chem. 271, 24313–24316
9. Sugiyama, J. S. (1996) J. Biol. Chem. 271, 1839–1842
10. Luttrell, L. M., Daaka, Y., and Levkowitz, R. J. (1999) Curr. Opin. Cell Biol. 11, 177–183
11. van Biesen, T., Hawes, B. E., Luttrell, D. K., Krueger, K. M., Touhara, K., Perifir, E., Sakaue, M., Luttrell, L. M., and Levkowitz, R. J. (1995) Nature 376, 781–784
12. Luttrell, L. M., Hawes, B. E., van Biesen, T., Touhara, K., Wagenknecht, J. W., and Levkowitz, R. J. (1996) J. Biol. Chem. 271, 19443–19450
13. Lopez-Ilasaca, M., Crespo, M., Pellici, P., and Gutkind, J. S. (1998) Science 275, 394–397
14. Luttrell, L. M., Ferguson, S. S. G., Daaka, Y., Miller, W. E., Maudsley, S., Della Rocca, G. J., Lin, F.-H., Kawakatsu, H., Owada, K., Luttrell, D. K., Caron, M. G., and Levkowitz, R. J. (1999) Science 283, 655–661
15. Coas, O. A., Chiariello, M., Kalinac, G., Kyriakis, J. M., and Libertini, P., and Gutkind, J. S. (1996) J. Biol. Chem. 270, 5620–5624
16. Mitchell, P. M., Russell, M., and Johnson, G. L. (1995) Biochem. J. 309, 381–384
17. Coas, O. A., Teramoto, H., Simon, W. F., and Gutkind, J. S. (1996) J. Biol. Chem. 271, 3963–3966
18. Lopez-Ilasaca, M., Gutkind, J. S., and Wetzker, R. (1998) J. Biol. Chem. 273, 2505–2508
19. Yamauchi, J., Kaziro, Y., and Itoh, H. (1999) J. Biol. Chem. 274, 1957–1965
20. Itoh, H., Kazoz, T., Nakamura, S., Nakamura, S., Kado, T., Oh, M., Iwai, S., Ohtsuka, E., Kasahara, H., Suzuki, K., and Kaziro, Y. (1986) Proc. Natl. Acad. Sci. U. S. A. 83, 3776–3780
21. Itoh, H., Toyama, H., Kazoz, T., Tsukamoto, T., Matsuka, M., and Kaziro, Y. (1988) J. Biol. Chem. 263, 6656–6664
22. Matsuka, M., Itoh, H., Kazoz, T., and Kaziro, Y. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 5384–5388
23. Yamauchi, J., Kaziro, Y., and Itoh, H. (1997) J. Biol. Chem. 272, 7602–7607
24. Yamauchi, J., Nagao, M., Kaziro, Y., and Itoh, H. (1997) J. Biol. Chem. 272, 27771–27777
25. Sung, H., Yamauchi, J., Kaziro, Y., and Itoh, H. (1999) J. Biochem. (Tokyo) 125, 515–521
26. Wu, Z., Wu, J., and Jin, S. (1997) Mol. Cell. Biol. 17, 7407–7416
27. Tournier, M. H., and Goldsmith, E. J. (1997) Curr. Opin. Cell Biol. 9, 173–179
28. Sekine, A., Fujimura, M., and Narumiya, S. (1989) J. Biol. Chem. 264, 8602–8605
29. Burkel, P. D., Drechsel, D., and Hall, A. (1995) J. Biol. Chem. 270, 29071–29074
30. Nada, S., Okada, M., MacAuley, A., Cooper, J. A., and Nakagawa, H. (1991) Nature 351, 69–72
31. Higashijima, T., Burnier, J., and Ross, E. M. (1990) J. Biol. Chem. 265, 14176–14186
32. Zohn, I. M., Campbell, S. L., Khoros-Rafi, R., Rossman, K. L., and Der, C. J. (1998) Oncogene 17, 1415–1438
33. Hanke, J., Gardner, J. P., Dow, R. L., Changelian, P. S., Brissette, W. H., Winger, E. J., Pollok, B. A., and Connelly, P. A. (1996) J. Biol. Chem. 271, 685–701
34. Stoyanova, B., Volin, S., Hanek, T., Rubio, I., Loubtenenkov, M., Malek, D., Stoyanova, S., Zemsek, B., Dhand, R., Perneg, B., Gierschik, P., Seedor, K., Heuu, J., Watersfield, M. D., and Wetzker, R. (1995) Science 269, 690–693
35. Guo, J. H., Wang, H., and Malbon, C. C. (1998) J. Biol. Chem. 273, 16487–16493
36. Edamatsu, H., Kaziro, Y., and Itoh, H. (1999) FERS Lett. 440, 231–234
37. Morichi, T., Toyoshima, F., Masayama, N., Hanafusa, H., Gotoh, Y., and Nishida, E. (1997) EMBO J. 16, 7045–7053
38. Fang, G. R., Johnson, N. L., and Johnson, G. L. (1997) EMBO J. 16, 4961–4972
39. Berestetskaya, Y. V., Faure, M. P., Ichijo, H., and Yoyano-Yasentakaya, T. A. (1998) J. Biol. Chem. 273, 27816–27823
40. Collins, L. R., Minden, A., Karin, M., and Brown, J. H. (1996) J. Biol. Chem. 271, 1734–17355
41. Vojtek, A. B., and Cooper, J. A. (1995) Cell 82, 527–529
42. Dhanaleskar, N., Tsim, S., Dermott, J. M., and Onesime, D. (1998) Oncogene 17, 1383–1394
43. Prasad, M. V. V. S. V., Dermott, J. M., Heasley, L. E., Johnson, G. L., and Dhanaleskar, N. (1995) J. Biol. Chem. 270, 18655–18659
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