Parallel Regulation of Mitogen-activated Protein Kinase Kinase 3 (MKK3) and MKK6 in \( G_q \)-signaling Cascade*

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Heterotrimeric G protein \( G_q \) stimulates the activity of p38 mitogen-activated protein kinase (MAPK) in mammalian cells. To investigate the signaling mechanism whereby \( \alpha \) and \( \beta \gamma \) subunits of \( G_q \) activate p38 MAPK, we introduced kinase-deficient mutants of mitogen-activated protein kinase kinase 3 (MKK3), MKK4, and MKK6 into human embryonal kidney 293 cells. The activation of p38 MAPK by \( G_q \) and \( G\beta\gamma \) was blocked by kinase-deficient MKK3 and MKK6, but not by kinase-deficient MKK4. In addition, \( G_q \) and \( G\beta\gamma \) stimulated MKK3 and MKK6 activities. The MKK3 and MKK6 activations by \( G_q \), but not by \( G\beta\gamma \), were dependent on phospholipase C and c-Src. \( G_q \) stimulated MKK3 in a Rac- and Cdc42-dependent manner and MKK6 in a Rho-dependent manner. On the other hand, \( G\beta\gamma \) activated MKK3 in a Rac- and Cdc42-dependent manner and MKK6 in a Rho-, Rac-, and Cdc42-dependent manner. \( G\beta\gamma \)-induced MKK3 and MKK6 activations were dependent on a tyrosine kinase other than c-Src. These results suggest that \( G_q \) and \( G\beta\gamma \) stimulate the activity of p38 MAPK by regulating MKK3 and MKK6 through parallel signaling pathways.

Mitogen-activated protein kinase (MAPK) plays a major role in the mechanism whereby intracellular signals are transduced to nucleus with MAPK kinase (MAPKK, also called MEK) and MAPKK kinase (MAPKKK, also called MEKK) (1–4). Three MAPK subgroups, ERK, JNK, and p38 MAPK, have been well characterized in mammalian cells. ERK phosphorylates many transcription factors, translation factors, and other kinases (1, 2) and participates in gene expression, protein synthesis, and cell cycle progression (1, 2). ERK is activated by MEK1 and MEK2, which are activated by Raf (1, 2). On the other hand, JNK and p38 MAPK were originally identified as cellular stress-activated protein kinases. Recently, these kinases were shown to be cooperatively and independently involved in various biological functions such as cytoskeletal regulation, cell movement, and development (1–4). JNK phosphorylates transcription factors c-Jun and ATF2, whereas p38 MAPK phosphorylates many transcription factors including ATF2 and MEF2C and other kinases (3, 4). MKK4/SEK1/JNKK1/SKK1 phosphorylates and activates both JNK and p38 MAPK, whereas MKK7/JNKK2/SKK4 specifically phosphorylates and activates JNK (3). MKK3/SKK2 and MKK6/SKK3 phosphorylate and activate p38 MAPK but not JNK (4). To date, more than 15 MAPKKKs including MEKK1, MEKK2, MEKK3, MEKK4/MTK1, and MLK family kinases have been reported to mediate the activation of JNK and/or p38 MAPK cascades (3, 4).

Heterotrimeric G proteins are commonly used to transduce the signals across seven-transmembrane receptors. G proteins are composed of \( \alpha \), \( \beta \) and \( \gamma \) subunits (\( G\alpha \), \( G\beta \), and \( G\gamma \)) and activated by the G protein-coupled receptors, which respond to sensory signals, hormones, neurotransmitters, and chemokines in mammalian cells (5–8). Many types of G protein-coupled receptors activate ERK in mammalian cells (8). \( G\alpha_q \)-coupled m1 muscarinic acetylcholine and \( \alpha_1 \)-adrenergic receptors have been reported to stimulate ERK activity mainly through \( G\alpha_q \) (8). \( G\alpha_q \) directly activates phospholipase C, leading to induction of PKC activation. The PKC activation is involved in the activation of some receptor and nonreceptor types of tyrosine kinases (9–11). A tyrosine phosphorylation of Shc induces the translocation of Grb2-mSos complex to the plasma membranes, where mSos promotes the exchange of GTP for GDP bound on small GTPase Ras (10). Ras mediates the activation of c-Raf, which in turn activates MEK1 and MEK2. It is likely that MEK1 and MEK2 equally activate ERK.

Some types of G protein-coupled receptors have been reported to stimulate the activity of JNK (12, 13). An agonist stimulation of m1 muscarinic acetylcholine receptor increases JNK activity through \( G\beta\gamma \) (14). Furthermore, it has been shown that \( G\beta\gamma \) stimulates JNK activity mainly through MKK4 dependent on a tyrosine kinase not in the Src family and Rho family small GTPases Rac and Cdc42 and to a lesser extent through MKK7 dependent on Rho family small GTPase Rac (15). Additionally, it has been reported that constitutively activated \( G_{\alpha_q} \) activates JNK (16), and its activation requires PKC and Src family tyrosine kinases (17).

In previous study, we found that m1 muscarinic acetylcholine receptor stimulates the activity of p38 MAPK, and its activation is mediated by both \( G\alpha_q \) and \( G\beta\gamma \) (18). However, it remains unclear how G protein-coupled receptors and G protein subunits activate p38 MAPK. Here we demonstrate that m1 muscarinic acetylcholine receptor induces p38 MAPK activation mediated by MKK3 and MKK6, but not by MKK4.
Furthermore, we show that \( \text{Go}_q \) and \( \text{G}\beta \) differentially regulate MKK3 and MKK6 through parallel signaling pathways.

**MATERIALS AND METHODS**

**Antibodies**—Mouse monoclonal antibodies M2, 12CA5, and 9E10 against FLAG, HA, and Myc epitopes were obtained from Sigma, Roche Molecular Biochemicals, and Babco, respectively. A mouse monoclonal antibody B-14 against *Schistosoma japonicum* GST was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). A rabbit polyclonal antibody C-19 against \( \text{Go}_{q/11} \) was obtained from Santa Cruz Biotechnology. Rabbit polyclonal antibodies T-20 and 06–238 against \( \text{G}\beta \) were purchased from Santa Cruz Biotechnology and Upstate Biotechnology, Inc. (Lake Placid, NY), respectively. Rabbit polyclonal anti-Ras (C-20) and anti-Csk (C-20) antibodies were obtained from Santa Cruz Biotechnology. A mouse monoclonal anti-c-Src antibody (05-184) and a rabbit polyclonal anti-phosphorylated c-Src (Tyr(P)418) antibody (44-660) were purchased from Upstate Biotechnology, Inc. and Biogenesis International, respectively. Goat anti-mouse and anti-rabbit IgG antibodies conjugated with horseradish peroxidase were obtained from PerkinElmer Life Sciences.

**Inhibitors**—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 and Biomol, respectively. Edelfosine/ET-18-OCH\(_3\) was obtained from Calbiochem.

**Plasmids**—A complementary DNA of \( \text{Go}_q \) (GenBank\textsuperscript{TM} accession number U40038) was isolated by polymerase chain reaction using human technology, Inc. (Lake Placid, NY), respectively. Rabbit polyclonal anti-Ras (C-20) and anti-Csk (C-20) antibodies were obtained from Santa Cruz Biotechnology. A mouse monoclonal anti-c-Src antibody (05-184) and a rabbit polyclonal anti-phosphorylated c-Src (Tyr(P)418) antibody (44-660) were purchased from Upstate Biotechnology, Inc. and BIORC Source, respectively. Goat anti-mouse and anti-rabbit IgG antibodies conjugated with horseradish peroxidase were obtained from PerkinElmer Life Sciences.

**Fig. 1.** p38 MAPK activation by m1 muscarinic acetylcholine receptor is mediated through MKK3 and MKK6, but not MKK4. HEK 293 cells were transfected with the plasmids encoding m1 muscarinic acetylcholine receptor (A–C), HA-p38 MAPK (A–C), FLAG-MKK3K64R (A), FLAG-MKK4K95R (B), and FLAG-MKK6K82R (C). The p38 MAPK activity was measured at 10 min after the addition of 10 \( \mu \text{M} \) carbachol as described under "Materials and Methods." Cells were transfected with the plasmids encoding m1 muscarinic acetylcholine receptor (D and E), GST-MKK3 (D), and GST-MKK6 (E). The MKK3 and MKK6 activities were measured at 10 min after the addition of 10 \( \mu \text{M} \) carbachol as described under "Materials and Methods." Values shown represent the mean \( \pm \) S.E. from three or four separate experiments. The phosphorylation of GST-ATF2 and KD-p38 MAPK and the expression of GST-MKK3 and GST-MKK6 in the cell lysates are shown. GST-MKK3 and GST-MKK6 were precipitated with glutathione-Sepharose 4B and immunoblotted with anti-GST antibody.

**Fig. 2.** Go\(_q\)-induced p38 MAPK activation is mediated through MKK3 and MKK6, but not MKK4. Cells were transfected with the plasmids encoding Go\(_q/Q209L\) (A–C), HA-p38 MAPK (A–C), FLAG-MKK3K64R (A), FLAG-MKK4K95R (B), and FLAG-MKK6K82R (C). The p38 MAPK activity was measured as described under "Materials and Methods." Cells were transfected with the plasmids encoding Go\(_q/Q209L\) (D and E), GST-MKK3 (D), and GST-MKK6 (E). The MKK3 and MKK6 activities were measured as described under "Materials and Methods." Values shown represent the mean \( \pm \) S.E. from three or four separate experiments. The phosphorylation of GST-ATF2 and KD-p38 MAPK and the expression of Go\(_q/Q209L\), HA-p38 MAPK, FLAG-MKK3K64R, FLAG-MKK4K95R, and FLAG-MKK6K82R in the cell lysates are shown. GST-MKK3 and GST-MKK6 were precipitated with glutathione-Sepharose 4B and immunoblotted with anti-GST antibody.
**Parallel Regulation of MKK3 and MKK6 by Gαq and Gβγ**

**Materials and Methods.** Values shown represent the mean ± S.E. from three or four separate experiments. The phosphorylation of GST-ATF2 and KD-p38 MAPK and the expression of Gαq, HA-p38 MAPK, FLAG-MKK3K64R, FLAG-MKK4K95R, and FLAG-MKK6K82R in the cell lysates are shown. GST-MKK3 and GST-MKK6 were precipitated with glutathione-Sepharose 4B and immunoblotted with anti-GST antibody.

**Fig. 3. Gβγ-induced p38 MAPK activation is mediated through MKK3 and MKK6 but not MKK4.** Cells were transfected with the plasmids encoding Gαq (A–C), Gβ1 (D), and Gγ2 (E) expression plasmids encoding the kinase-deficient form of Mpk2, a Xenopus orthologue of mouse p38α, was kindly provided by E. Nishida (Kyoto University). pGEX-ATF2 (amino acids 1–96) was constructed as described previously (18). All DNA sequences were confirmed by DNA sequencer L-4000L (LI-COR).

Recombinant Proteins—A recombinant hexahistidine-tagged KD-p38 MAPK was purified from the transformed Escherichia coli strain BL21 (DE3) plyS cells. E. coli cells treated with isopropyl-thiogalactoside sonicated in extraction buffer A (20 mM HEPES-NaOH (pH 8.0), 1 mM phenylmethylsulfonyl fluoride, 1 μM aprotinin, 1 μM leupeptin, and 0.5% Nonidet P-40) on ice. All purification steps were performed at 4 °C. The cell lysate was centrifuged at 150,000 × g for 30 min. The supernatant was applied to nitrotetraacetic acid-agarose (Qiagen, Inc.), and the resin was washed with column buffer A (20 mM HEPES-NaOH (pH 8.0), 1 mM phenylmethanesulfonyl fluoride, 1 μM aprotinin, 1 μM leupeptin, and 200 mM NaCl) with 20 mM imidazole. KD-p38 MAPK was eluted with column buffer A with 200 mM imidazole. The elute was dialyzed against column buffer B (20 mM HEPES-NaOH (pH 7.5), 1 mM dithiothreitol, 1 mM phenylmethanesulfonyl fluoride, 1 μM aprotinin, 1 μM leupeptin, and 1 mM EGTA) and stored at −80 °C until use. GST-ATF2 (1–96) was purified as described previously (18) and stored at −80 °C until use.

Cell Culture and Transfection—Human embryonic kidney (HEK) 293 cells (ATCC CRL 1573) were maintained in Dulbecco’s modified Eagle’s medium containing 100 μg/ml kanamycin and 10% heat-inactivated fetal bovine serum. The cells were cultured at 37 °C in humidified atmosphere containing 10% CO2. Plasmid DNAs were 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 pCMV-HA-p38 MAPK, pCMV-GST-MKK3, or pCMV-GST-MKK6 was cotransfected with 0.3 μg of pCMV-m1 muscarinic acetylcholine receptor, 10 μg of plasmids encoding Gαq, wild type or mutants, 5 μg of pCMV-Gαq or pCMV-Gα1T143A and 5 μg of pCMV-Gγ2, 10 μg of pCMV-FLAG-MKK3K64R, 10 μg of pCMV-FLAG-MKK4K95R, 10 μg of pCMV-MKK6K82R, 10 μg of pCMV-MKK3 or pCMV-MKK6, 10 μg of pCMV-FLAG-GαqQ209L(R256A, T257A) mutants were constructed by S. Mizutani (Gunma University). pEFBOS-Csk was generously provided by S. Narumiya (Kyoto University). Csk cDNA was ampliﬁed from human fetal brain cDNA library and inserted into the BamHI restriction site of pCMV. pEFBOS-v-Src was generously provided by M. Okada (Osaka University), and pCMV-Csk was generously provided by Y. Fukami (Kobe University). A hexahistidine tag expression plasmid encoding the kinase-deficient form of Mpk2, a Xenopus orthologue of mouse p38α, was kindly provided by E. Nishida (Kyoto University). pGEX-ATF2 (amino acids 1–96) was constructed as described previously (18). All DNA sequences were confirmed by DNA sequencer L-4000L (LI-COR).

Kinase Assays—The transfected cells 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 phenylmethanesulfonyl fluoride, 1 μM leupeptin, 1 μM EDTA, 1 mM NaN3, 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 the p38 MAPK assay, aliquots (50 μg of protein) of the supernatants were mixed with anti-GST antibody or GST-Gαq-4B (Amersham Pharmacia Biotech) preabsorbed with a mouse anti-HA antibody for 2 h at 4 °C. The immunocomplexes 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 phenylmethanesulfonyl fluoride, 0.1 μM leupeptin, 0.1 mM EDTA, 10 μM NaN3, 2 mM β-glycerophosphate, and 0.5% Nonidet P-40) on ice. The precipitates were washed with 50 μl of reaction buffer A containing 1 μg of GST-ATF2 (1–96), 20 μM ATP, and 5 μCi of [γ-32P]ATP at 30 °C for 20 min. For MKK3 and MKK6 assays, aliquots (500 μg of protein) of the supernatants were mixed with glutathione-Sepharose 4B (Amersham Pharmacia Biotech) for 2 h at 4 °C and centrifuged. The precipitates were washed with lysis buffer A and with reaction buffer A and incubated in 30 μl of reaction buffer A

![Diagram of parallel regulation of MKK3 and MKK6 by Gαq and Gβγ](image-url)
containing 4 µg of KD-p38 MAPK, 20 µM ATP, and 5 µCi of [γ-32P]ATP at 30 °C for 20 min. The reactions were stopped by adding 10 µl of 4 × Laemmli sample buffer. After they were boiled, the samples were subjected to 15% SDS-polyacrylamide gel electrophoresis. The radioactivities incorporated into GST-ATF2 (1–96) or KD-p38 MAPK were measured by an imaging analyzer (Fuji BAS 2000) and detected by autoradiography using X-Omat film (Eastman Kodak Co.).

**Immunoprecipitation**—The cells transfected with the plasmids encoding Myc-Pak1CRIB were lysed in lysis buffer A. Aliquots (500 µg of protein) of the cell lysates were mixed with protein A-Sepharose CL-4B preabsorbed with a mouse anti-Myc antibody for 2 h at 4 °C. The immune complexes were precipitated by centrifugation and washed three times with lysis buffer A.

**Immunoblotting**—Aliquots of the cell lysates and immune complexes were boiled in Laemmli sample buffer. The boiled samples were separated by 8 or 15% SDS-polyacrylamide gel electrophoresis. The separated proteins were transferred to nitrocellulose membranes. After the membranes were blocked with phosphate-buffered saline containing 0.1% Tween 20 and 5 mg/ml bovine serum albumin, the membranes were subjected to immunoblotting with various antibodies. The bound antibodies were detected using Renaissance (PerkinElmer Life Sciences) with anti-mouse or -rabbit IgG antibody conjugated with horseradish peroxidase.

**RESULTS**

The Pathway from m1 Muscarinic Acetylcholine Receptor, Goq and Gβγ to p38 MAPK Is Mediated by MKK3 and MKK6, but Not by MKK4—To examine a signal transduction pathway linking m1 muscarinic acetylcholine receptor to p38 MAPK, we transfected plasmids encoding HA-tagged p38 MAPK with various cDNAs into human embryonal kidney 293 cells. Using an anti-HA antibody, the epitope-tagged p38 MAPK was immunoprecipitated from the cell lysate, and the in vitro kinase activity was assessed as the radioactivity incorporated into recombinant ATP2. The expression level of HA-p38 MAPK was confirmed by immunoblotting in each experiment to compare each transfection efficiency (see Figs. 1–3). The p38 MAPK activation by stimulating m1 muscarinic acetylcholine receptor with an agonist, carbachol, was specifically blocked by co-transfection of MKK3K64R or MKK6K82R but not by that of MKK4K95R (Fig. 1, A–C). MKK3K64R, MKK4K95R, and MKK6K82R are kinase-deficient constructs and act as dominant negative mutants by sequestering upstream components (25).

Next, we attempted to measure the kinase activities of MKK3 and MKK6. The cells were co-transfected with plasmids encoding GST-MKK3 or GST-MKK6, which are tagged with GST at the N terminus. GST-MKK3 and GST-MKK6 were precipitated from the cell lysate using glutathione-resin, and the kinase activities were assessed as the radioactivity incorporated into recombinant kinase-deficient p38 MAPK (KD-p38 MAPK). In each experiment, we examined the expression level of GST-MKK3 or GST-MKK6 to monitor each transfection efficiency (see Figs. 1–11). As shown in Fig. 1, D and E, m1 muscarinic acetylcholine receptor stimulated the activities of MKK3 and MKK6. These results indicate that m1 muscarinic acetylcholine receptor stimulates the activity of p38 MAPK through two p38 MAPK kinases, MKK3 and MKK6.

The stimulation of m1 muscarinic acetylcholine receptor induces p38 MAPK activation through Goq and Gβγ (18). In addition, p38 MAPK activation by m1 muscarinic acetylcholine receptor appeared to be mediated by MKK3 and MKK6 (Fig. 1). Thus, we examined whether Goq and Gβγ induce p38 MAPK activation through MKK3 and MKK6. Co-transfection of MKK3K64R or MKK6K82R, but not of MKK4K95R, inhibited p38 MAPK activation induced by constitutively activated Goq (Goq, Q209L) (Fig. 2, A–C). Moreover, Goq, Q209L stimulated the activities of MKK3 and MKK6 (Fig. 2, D and E). Co-transfection of MKK3K64R or MKK6K82R, but not of MKK4K95R, blocked Gβγ-induced p38 MAPK activation (Fig. 3, A–C). In addition, Gβγ induced MKK3 and MKK6 activations (Fig. 3, D and E). These results suggest that Goq and Gβγ activate p38 MAPK through MKK3 and MKK6.

Goq and Gβγ Induce the Activations of MKK3 and MKK6 in a Ras-independent Manner—Oncogenic Ras (RasG12V) activates p38 MAPK (26–28). Additionally, RasG12V transfection resulted in increase of the MKK3 and MKK6 activities (data not shown). Furthermore, signal-dependent p38 MAPK activation has been effectively blocked by co-transfection of dominant negative Ras (RasS17N) (29). In order to examine whether m1 muscarinic acetylcholine receptor stimulates the activities of MKK3 and MKK6 via Ras, we co-transfected the plasmid encoding RasS17N into the cells. However, co-transfection of RasS17N did not block MKK3 and MKK6 activations by m1 muscarinic acetylcholine receptor (Fig. 4, A and B). Similarly, RasS17N failed to suppress Goq, Q209L- and Gβγ-induced activations of MKK3 and MKK6 (data not shown). These results suggest that Ras is not required for the pathway from Goq and Gβγ to MKK3 and MKK6.

**Differential Regulation of the Signaling Pathways from Goq and Gβγ to MKK3 and MKK6 by Rho Family Small GTPases—**Rho family small GTPases Rac and Cdc42 have been reported to function upstream of p38 MAPK signaling cascade (1–4, 26–28). We investigated the involvement of Rho family small GTPases in m1 muscarinic acetylcholine receptor-induced MKK3 and MKK6 activations by using the dominant negative mutants, RhoT19N, RacT17N, and Cdc42T17N. Co-transfection of RacT17N or Cdc42T17N, but not that of RhoT19N, reduced the receptor-induced MKK3 activation (Fig. 5, A, C, and E), while co-transfection of RhoT19N, RacT17N, or Cdc42T17N reduced the receptor-induced MKK6 activation (Fig. 5, B, D, and F). Next, we tested whether MKK3 and MKK6 activations mediated by Goq and Gβγ involve Rho family small GTPases. Co-transfection of RacT17N or Cdc42T17N, but not of RhoT19N, inhibited Goq, Q209L-induced MKK3 activation (Fig. 5, G, I, and K). Similarly, Gβγ-induced MKK3
FIG. 5. Differential regulation of the pathways from Goq and Goβγ to MKK3 and MKK6 by Rho family small GTPases. Cells were transfected with the plasmids encoding m1 muscarinic acetylcholine receptor (A–F), GST-MKK3 (A, C, and E), GST-MKK6 (B, D, and F),
Parallel Regulation of MKK3 and MKK6 by Goα and Gβγ

**Fig. 6. Effects of C. botulinum C3 exoenzyme and Pak1CRIB on the pathways from Goα and Gβγ to MKK3 and MKK6.** Cells were transfected with the plasmids encoding GoQ209L (A–D), GST-MKK3 (A and C), GST-MKK6 (B and D), C3 exoenzyme (A and B), and Myc-Pak1CRIB (C and D). Cells were transfected with plasmids encoding Gβ1 (E–H), Gγ2 (E–H), GST-MKK3 (E and F), GST-MKK6 (F and H), C3 exoenzyme (E and F), and Myc-Pak1CRIB (G and H). The MKK3 and MKK6 activities were measured as described under "Materials and Methods." Values shown represent the mean ± S.E. from at least three separate experiments. Statistical analysis was performed using Student's t test. *p < 0.001 (n = 10) compared with GoQ209L without Pak1CRIB. The phosphorylation of KD-p38 MAPK and the expression of GoQ209L, Gβ1, and Myc-Pak1CRIB in the cell lysates are shown. Myc-Pak1CRIB was immunoprecipitated and immunoblotted with anti-Myc antibody. GST-MKK3 and GST-MKK6 were precipitated with glutathione-Sepharose 4B and immunoblotted with anti-GST antibody.

**Activation** was also blocked by RacT17N or Cdc42T17N, but not by RhoT17N (Fig. 5, M, O, and Q). In contrast, GoQ209L-induced MKK6 activation was blocked only by co-transfection of RhoT17N (Fig. 5, H, J, and L), while Gβγ-induced MKK6 activation was blocked by three dominant negative mutants (Fig. 5, N, P, and R). These results suggest that Goα and Gβγ activate MKK3 in a Rac- and Cdc42-dependent manner, whereas GoQ209L activates MKK6 in a Rac-dependent manner and Gβγ activates MKK6 in a Rho-, Rac-, and Cdc42-dependent manner.

To confirm that Rho family small GTPases are involved in MKK3 and MKK6 activations mediated by Goα and Gβγ, we further utilized C. botulinum C3 exoenzyme and Pak1CRIB. The C3 exoenzyme ADP-ribosylates Rho and inhibits its cellular function (30). The Pak1CRIB binds to an active form of Rac or Cdc42 (31) and inhibits the interaction of Rac or Cdc42 with the effectors. GoQ209L-induced MKK3 activation was reduced by co-transfection of Pak1CRIB (Fig. 6C) but not by C3 exoenzyme (Fig. 6A). Similarly, Gβγ-induced MKK3 activation was inhibited by Pak1CRIB (Fig. 6G) but not by C3 exoenzyme (Fig. 6E). On the other hand, GoQ209L-induced MKK6 activation was blocked by C3 exoenzyme (Fig. 6B) but not by Pak1CRIB (Fig. 6D). Gβγ-induced MKK6 activation was blocked by C3 exoenzyme (Fig. 6F) and Pak1CRIB (Fig. 6H). Again, these results suggest that Goα and Gβγ activate MKK3 in a Rac- and Cdc42-dependent manner, whereas GoQ209L activates MKK6 in a Rho-dependent manner and Gβγ activates MKK6 in a Rho-, Rac-, and Cdc42-dependent manner.

**Differential Involvement of Tyrosine Kinases in MKK3 and MKK6**

**Activations by Goα and Gβγ—p38 MAPK activation by m1 muscarinic acetylcholine receptor was suppressed by PP1 and PP2 (17), which are known to be tyrosine kinase inhibitors preferential for Src family tyrosine kinases. MKK3 and MKK6 activations by m1 muscarinic acetylcholine receptor were inhibited by treatment with PP1 (Fig. 7, A and B). Next, we investigated the involvement of tyrosine kinase in MKK3 and MKK6 activations induced by Goα and Gβγ. Treatment of the cells with PP1 inhibited MKK3 and MKK6 activations induced by Goα and Gβγ.**
by Gq, Q209L and Gβγ in a dose-dependent manner (Fig. 7, C–F). PP2 also inhibited the activations in the same manner (data not shown). It is likely that Gαq and Gβγ activate MKK3 and MKK6 via tyrosine kinase.

We previously showed that GαqQ209L-mediated p38 MAPK activation is attenuated by co-transfection of Csk (17), a negative regulator of Src family tyrosine kinases. In order to test whether Src family tyrosine kinases mediate the pathway from Gαq, and Gβγ to MKK3 and MKK6, we co-transfected the plasmid encoding Csk into the cells. Csk resulted in attenuation of MKK3 and MKK6 activations induced by GαqQ209L (Fig. 8, A and B), but not by Gβγ (Fig. 8, C and D).

Next, we attempted to evaluate Gq-mediated c-Src activation using an antibody that recognizes a phosphorylated state of c-Src. Involvement of c-Src in MKK3 and MKK6 activations mediated through Gαq but not Gβγ. Cells were transfected with the plasmids encoding GαqQ209L (A and B), Gβ1 (C and D), Gγ2 (E and F), GST-MKK3 (C and E), and GST-MKK6 (D and F) and treated with the indicated concentrations of PP1 (C–F) for 24 h. The MKK3 and MKK6 activities were measured as described under "Materials and Methods." Values shown represent the mean ± S.E. from at least three separate experiments. Statistical analysis was performed using Student's test. *, p < 0.001 (n = 10) compared with GαqQ209L without Csk. The phosphorylation of c-Src (Tyr(P)418) and the expression of GαqQ209L, Gβ1, and endogenous c-Src in the cell lysates are shown.

FIG. 7. Involvement of tyrosine kinases in MKK3 and MKK6 activations mediated through Gαq and Gβγ. Cells were transfected with the plasmids encoding m1 muscarinic acetylcholine receptor (A and B), GST-MKK3 (A), and GST-MKK6 (B) and pretreated with 10 μM PP1 for 1 h. The MKK3 and MKK6 activities at 10 min after the addition of 10 μM carbachol are shown. Cells were transfected with the plasmids encoding GαqQ209L (C and D), Gβ1 (E and F), Gγ2 (E and F), GST-MKK3 (C and E), and GST-MKK6 (D and F) and treated with the indicated concentrations of PP1 (C–F) for 24 h. The MKK3 and MKK6 activities were measured as described under "Materials and Methods." Values shown represent the mean ± S.E. from three or four separate experiments. The phosphorylation of KD-p38 MAPK and the expression of GαqQ209L and Gβ1 in the cell lysates are shown. GST-MKK3 and GST-MKK6 were precipitated with glutathione-Sepharose 4B and immunoblotted with anti-GST antibody.
tyrosine 418 on c-Src. The tyrosine 418 is known to be an autophosphorylation site essential for its activation (32). Fig. 8E shows that GaqQ209L transfection induced c-Src phosphorylation. Treatment with PP1 reduced GaqQ209L-induced c-Src phosphorylation (Fig. 8E). We observed the weak but inconsistent tyrosine phosphorylation of c-Src by overexpression of Gbg (Fig. 8F). These results suggested that Gaq may activate MKK3 and MKK6 through Src family tyrosine kinases, and Gbg may activate MKK3 and MKK6 through tyrosine kinases not in the Src family.

c-Src Functions Upstream of Rho Family Small GTPases in MKK3 and MKK6 Signaling Pathways—To determine whether c-Src acts as an upstream or downstream component of Rho family small GTPases in the pathway from Gaq to MKK3 and MKK6, we co-transfected the plasmids encoding v-Src and dominant negative mutants of Rho family small GTPases into the cells. Co-transfection of RacT17N or Cdc42T17N suppressed the v-Src-induced MKK3 activation (Fig. 9, A and B), and RhoT19N co-transfection blocked MKK6 activation by v-Src (Fig. 9C), indicating that c-Src acts upstream of Rac and Cdc42 in the MKK3 signaling pathway and upstream of Rho in the MKK6 signaling pathway.

RhoG14V, RacG12V, and Cdc42G12V are constitutively activated mutants of Rho family small GTPases (26–28). As shown in Fig. 9, D–H, RacG12V and Cdc42G12V activated MKK3, whereas RhoG14V, RacG12V, and Cdc42G12V activated MKK6. In contrast, RhoG14V failed to activate MKK3 (data not shown). Since Rho family small GTPases might function downstream of c-Src (Fig. 9, A–C), we investigated whether MKK3 and MKK6 activations by the constitutively activated mutants of Rho family small GTPase are inhibited by PP1. Pretreatment of this inhibitor failed to attenuate MKK3 and MKK6 activations induced by RhoG14V, RacG12V or Cdc42G12V (Fig. 9, D–H), indicating that Rho family small GTPases function downstream of tyrosine kinases in MKK3 and MKK6 signaling cascades.

MKK3 and MKK6 Activations by Gaq, but Not by Gbg, Involve Phospholipase C—Phospholipase C is known to be an effector of Gq (7). To explore whether phospholipase C is involved in MKK3 and MKK6 activations mediated by Gaq and
Gβγ, we utilized Goq and Gβ mutants incapable of activating phospholipase C. Mutations of amino acid residues 256 and 257 in Goq retain the ability to bind GTP but impair the capability to activate phospholipase C in HEK 293 cells (33). These mutations dramatically reduced the ability of constitutively activated Goq to stimulate the activities of MKK3 and MKK6 (Fig. 10, A and B). A mutation of amino acid residue 143 in Gβ results in impairment of its ability to stimulate phospholipase C (34). This mutation in Gβ had no effect on Gβγ-induced MKK3 and MKK6 activations (Fig. 10, C and D). Furthermore, a phospholipase C inhibitor edelfosine, 1-O-octadecyl-2-O-methyl-rac-glycerol-phosphocholine (ET-18-OCH3), inhibited the activation of MKK3 and MKK6 by Goq, Q209L but not by Gβγ (data not shown). These results suggested that phospholipase C may be involved in MKK3 and MKK6 activations mediated by Goq, but not by Gβγ.

Gβγ-mediated MKK3 and MKK6 Activations Do Not Depend on Phosphatidylinositol 3-Kinase Activity—It has been shown that Gβγ participates in activating phosphatidylinositol 3-kinase (35, 36). To examine the involvement of phosphatidylinositol 3-kinase in MKK3 and MKK6 activations by Gβγ, the transfected cells were treated with phosphatidylinositol 3-kinase inhibitors wortmannin and LY294002 as described previously (37, 38). Wortmannin had no effect on carbachol- and Goq-induced MKK3 and MKK6 activations (Fig. 11). Similarly, LY294002 did not inhibit the MKK3 and MKK6 activations (data not shown). These results suggested that phosphatidylinositol 3-kinase is not implicated in MKK3 and MKK6 activations by Gβγ.

DISCUSSION

MKK3 and MKK6 have been shown to be activated by physical and chemical stresses and inflammatory cytokines such as tumor necrosis factor-α and interleukin-1 (21, 24), although only limited information is known about how signals from G protein-coupled receptors are linked to the activation of these kinases. In the present study, we showed that m1 muscarinic acetylcholine receptor and G protein subunits, Goq and Gβγ, stimulated p38 MAPK activity dependent on MKK3 and MKK6, but not on MKK4. Goq induced MKK3 and MKK6 activations in a phospholipase C-dependent manner. In addi-
Many MAPKKKs induce the activation of the p38 MAPK cascade, although it is likely that MAPKKK selects specific MAPKK as its partner. For example, MEKK1 preferentially activates MKK3, MKK4, and MKK6 rather than MKK7 (39), whereas MEKK4 activates MKK3 and MKK6 rather than MKK4 (40). In addition, MEKK3 couples to MKK6 and MKK7 but not to MKK3 and MKK4 (41). Since MKK3 and MKK6 activations by Gαq signaling are mediated through distinct pathways, different MAPKKKs might be implicated in the regulation of MKK3 and/or MKK6.

MEKK1 and MEKK4 contain a CRIB domain that interacts with Rac and Cdc42 (42). Additionally, constitutively activated mutants of Rac and Cdc42 stimulate p38 MAPK activity (26–28). Rac and Cdc42 were involved in Gαq-mediated MKK3 activation and Gβγ-mediated MKK3/6 activations (Figs. 5 and 6). Therefore, MEKK1 and MEKK4 might be candidates of MAPKKKs in these signaling pathways. On the other hand, MKK6 activation mediated by Gαq and Gβγ was dependent on Rho (Figs. 5 and 6). A recent report has indicated that a transcription factor, SRF, is phosphorylated through a p38 MAPK-dependent mechanism (43), and Rho has been suggested to be involved in SRF phosphorylation (44). Although MAPKKK regulated by Rho has not yet been clarified, a putative Rho-regulated MAPKKK may participate in the MKK6 signaling pathway mediated by Gαq and Gβγ.

Mammalian guanine-nucleotide exchange factors (GEFs) for Rho family small GTPases have been grouped (45). Some members of Rho family GEFs exhibit the exchange activity for a broad range of Rho family small GTPases, whereas others are more specific. We found differential involvement of Rho family small GTPases in the activations of MKK3 and MKK6 mediated by Gαq protein subunits (Figs. 5 and 6). The differential involvement of Rho family small GTPases in the pathways might be due to the different Rho family GEFs. Since the MKK3 activation mediated by Gαq and Gβγ is dependent on Rac and Cdc42 (Figs. 5 and 6), it is possible that its MKK3 activation is mediated by Rho family GEF specific for Rac and Cdc42, e.g. Vav and αPix/Cool-2. However, it must be noted that MKK3 activation by Gαq involves c-Src, whereas MKK3 activation by Gβγ involves a tyrosine kinase not in the Src family (Figs. 7 and 8). It is conceivable that diverse Rho family GEFs may function downstream of Gαq and Gβγ in the MKK3 signaling pathway. On the other hand, MKK6 activation by Gαq only depended on Rho (Figs. 5 and 6). Thus, Rho-specific GEF (e.g. Lbc and Lfc) may play a role in Gαq-mediated MKK6 activation. In contrast, Gβγ-mediated MKK6 activation required Rho, Rac, and Cdc42 (Figs. 5 and 6). It is possible that Gβγ activates MKK6 through Rho family GEFs (e.g. Abr and Bcr) that have a broad specificity for Rho family small GTPases.

Gαq-mediated MKK3 and MKK6 activations involved c-Src (Figs. 7 and 8). In addition, Gαq activated MKK3 and MKK6 pathways via phospholipase C (Fig. 10) and PKC (17). Since Pyk2/CAKβ/RAFTK/CADTK/FAK2 is associated with c-Src and activated upon the stimulation of Gαq-coupled bradykinin and α1-adrenergic receptors (9, 10), Pyk2 might connect Gαq with c-Src to lead to MKK3 and MKK6 activations. A recent report that Pyk2 increases p38 MAPK activity (46) is consistent with this possibility. On the other hand, it is likely that Gβγ activates MKK3 and MKK6 through a tyrosine kinase not in the Src family (Figs. 7 and 8). It has been reported that Gβγ directly activates Btk in the presence of plasma membrane fraction (47). We tried to introduce a Btk construct with Gβγ into the cells and measure Btk activity. Although Gβγ failed to activate Btk (data not shown), we could not rule out the involvement of another Tec family tyrosine kinase in the pathway from Gβγ to MKK3 and MKK6.

Tyrosine kinases appear to act as the upstream regulator not only of Ras but also of Rho family small GTPases (48). For example, the Vav group of Rho family GEF has been shown to be activated by its tyrosine phosphorylation (49–51). Rho subfamily-specific GEFs Vav-2 and Vav-3 have been reported to be activated directly by Src family tyrosine kinases (50, 51). This is in agreement with our result that tyrosine kinases including c-Src function upstream of Rho family small GTPases in the signaling pathway linking G protein subunits to MKK3 and MKK6 (Fig. 9).

The signaling components of MAPK cascade may be organized into a module in vivo (52). It has been reported that JSAp1 provides a scaffold of the JNK signaling module containing only MKK4 as JNK kinase and facilitates JNK activation in mammalian cells (53). In contrast, JIP family scaffold proteins such as JIP1, JIP2, and JIP3 (an alternative splicing form of JSAp1) are specifically associated with another JNK

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J. Yamauchi, G. Tsujimoto, Y. Kaziro, and H. Itoh, unpublished data.
kinase MKK7 and modulate JNK activation (54–56). In the present study, we demonstrated that Goq and Gbg differentially regulate MKK3 and MKK6. It is conceivable that such a scaffold protein may contribute to the communication between each G protein subunit and MAPKks.

p38 MAPK pathway has been implicated in various cellular functions such as gene expressions, cytoskeletal regulations, and morphological changes by stimuli through some Gq-coupled receptors (57–59). In this study, we presented some clues for solving the mechanism whereby Gq activates MKK3 and MKK6 and in turn p38 MAPK. Further studies are needed to elucidate how Gq stimulates tyrosine kinases and regulates Rho family small GTPases.

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REFERENCES

1. Kyriakis, J. M., and Avruch, J. (1996) Bioessays 18, 567–577
2. Robinson, M. J., and Cobb, M. H. (1997) Curr. Opin. Cell Biol. 9, 180–186
3. Ip, Y. T., and Davis, R. J. (1998) Curr. Opin. Cell Biol. 10, 205–219
4. New, L., and Han, J. (1998) Trends. Cardiovasc. Med. 8, 220–229
5. Gilman, A. G. (1987) Annu. Rev. Biochem. 56, 615–649
6. Kaziro, Y., Itoh, H., Kozasa, T., Nakafuku, M., and Satoh, T. (1991) Annu. Rev. Biochem. 60, 349–400
7. Ham, H. E. (1996) J. Biol. Chem. 271, 669–672
8. Ulevitch, R. J. (1996) J. Biol. Chem. 271, 383–386
9. Ulevitch, R. J. (1996) J. Biol. Chem. 271, 383–386
10. Ulevitch, R. J. (1996) J. Biol. Chem. 271, 383–386
11. Ulevitch, R. J. (1996) J. Biol. Chem. 271, 383–386
12. Ulevitch, R. J. (1996) J. Biol. Chem. 271, 383–386
13. Ulevitch, R. J. (1996) J. Biol. Chem. 271, 383–386
14. Ulevitch, R. J. (1996) J. Biol. Chem. 271, 383–386
15. Ulevitch, R. J. (1996) J. Biol. Chem. 271, 383–386
16. Ulevitch, R. J. (1996) J. Biol. Chem. 271, 383–386
17. Ulevitch, R. J. (1996) J. Biol. Chem. 271, 383–386
18. Ulevitch, R. J. (1996) J. Biol. Chem. 271, 383–386
19. Ulevitch, R. J. (1996) J. Biol. Chem. 271, 383–386
20. Ulevitch, R. J. (1996) J. Biol. Chem. 271, 383–386
21. Ulevitch, R. J. (1996) J. Biol. Chem. 271, 383–386
22. Ulevitch, R. J. (1996) J. Biol. Chem. 271, 383–386
23. Ulevitch, R. J. (1996) J. Biol. Chem. 271, 383–386
24. Ulevitch, R. J. (1996) J. Biol. Chem. 271, 383–386
25. Ulevitch, R. J. (1996) J. Biol. Chem. 271, 383–386
26. Ulevitch, R. J. (1996) J. Biol. Chem. 271, 383–386
27. Ulevitch, R. J. (1996) J. Biol. Chem. 271, 383–386
28. Ulevitch, R. J. (1996) J. Biol. Chem. 271, 383–386
29. Ulevitch, R. J. (1996) J. Biol. Chem. 271, 383–386
30. Ulevitch, R. J. (1996) J. Biol. Chem. 271, 383–386
31. Ulevitch, R. J. (1996) J. Biol. Chem. 271, 383–386
32. Ulevitch, R. J. (1996) J. Biol. Chem. 271, 383–386
33. Ulevitch, R. J. (1996) J. Biol. Chem. 271, 383–386
34. Ulevitch, R. J. (1996) J. Biol. Chem. 271, 383–386
35. Ulevitch, R. J. (1996) J. Biol. Chem. 271, 383–386
36. Ulevitch, R. J. (1996) J. Biol. Chem. 271, 383–386
37. Ulevitch, R. J. (1996) J. Biol. Chem. 271, 383–386
38. Ulevitch, R. J. (1996) J. Biol. Chem. 271, 383–386
39. Ulevitch, R. J. (1996) J. Biol. Chem. 271, 383–386
40. Ulevitch, R. J. (1996) J. Biol. Chem. 271, 383–386
41. Ulevitch, R. J. (1996) J. Biol. Chem. 271, 383–386
42. Ulevitch, R. J. (1996) J. Biol. Chem. 271, 383–386
43. Ulevitch, R. J. (1996) J. Biol. Chem. 271, 383–386
44. Ulevitch, R. J. (1996) J. Biol. Chem. 271, 383–386
45. Ulevitch, R. J. (1996) J. Biol. Chem. 271, 383–386
46. Ulevitch, R. J. (1996) J. Biol. Chem. 271, 383–386
47. Ulevitch, R. J. (1996) J. Biol. Chem. 271, 383–386
48. Ulevitch, R. J. (1996) J. Biol. Chem. 271, 383–386
49. Ulevitch, R. J. (1996) J. Biol. Chem. 271, 383–386
50. Ulevitch, R. J. (1996) J. Biol. Chem. 271, 383–386
51. Ulevitch, R. J. (1996) J. Biol. Chem. 271, 383–386
52. Ulevitch, R. J. (1996) J. Biol. Chem. 271, 383–386
53. Ulevitch, R. J. (1996) J. Biol. Chem. 271, 383–386
54. Ulevitch, R. J. (1996) J. Biol. Chem. 271, 383–386
55. Ulevitch, R. J. (1996) J. Biol. Chem. 271, 383–386
56. Ulevitch, R. J. (1996) J. Biol. Chem. 271, 383–386
57. Ulevitch, R. J. (1996) J. Biol. Chem. 271, 383–386
58. Ulevitch, R. J. (1996) J. Biol. Chem. 271, 383–386
59. Ulevitch, R. J. (1996) J. Biol. Chem. 271, 383–386