Differential Regulation of Mitogen-activated Protein Kinase Kinase 4 (MKK4) and 7 (MKK7) by Signaling from G Protein βγ Subunit in Human Embryonal Kidney 293 Cells

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The abbreviations used are: MAPK(s), mitogen-activated protein kinase(s); ERK, extracellular signal-regulated kinase; JNK/SAPK, p38 MAPK, and BMK1/ERK5. ERK is activated by many growth factors and cytokines and plays important functions as mediators of cellular responses to a variety of stimuli such as growth factors, cytokines, hormones, and environmental stresses (1–4). A GDP-bound Gα subunit (Gα) change of GDP to GTP on Ras and activates a sequential kinase

Mitogen-activated protein kinases (MAPKs) are proline-directed serine/threonine kinases and play important functions as mediators of cellular responses to a variety of stimuli such as growth factors, cytokines, hormones, and environmental stresses (1–4). In mammalian cells, MAPKs have been classified into at least four subfamilies: ERK/MAPK, JNK/SAPK, p38 MAPK, and BMK1/ERK5. ERK is activated by many growth factors and cytokines and phosphorylates transcription factors, cytoskeleton proteins, and other protein kinases (1, 2). ERK is implicated in cell growth, differentiation, and survival. Various stressors such as chemical agents and ultraviolet irradiation, tumor necrosis factor-α, interleukin-1, CD40 ligand, and Fas/CD95 ligand stimulate the activities of JNK and p38 MAPK, which appear to be involved in cell cycle arrest and apoptosis (2–4). JNK phosphorylates transcription factors including c-Jun, Elk-1, and ATF2, whereas p38 MAPK phosphorylates not only transcription factors such as CHOP/GADD153, MEF2C, Elk-1, and ATF2, but also MAPKAP kinases (2–4). BMK1 has been reported to be a redox-regulated kinase and phosphorylates a transcription factor MEF2C, although its physiological role has remained unclear (3).

MAPK cascades consist of three conserved components: a family of serine/threonine kinases called MAPKKK/MEKK, which activate MAPKK/MEK, which in turn activates MAPK by a simultaneous phosphorylation on threonine and tyrosine residues (1–4). RAF and Tpl-2 activate ERK through MEK1 and MEK2. On the other hand, it has been shown that overexpression of Tpl-2, MEK1, MEK2, MEK3, MKK4/MTK1, MAPKKK/ASK1, TAK1, and MLK family protein kinases induce the activation of JNK and/or p38 MAPK cascade(s). JNK and p38 MAPK are phosphorylated and activated by MKK4/SEK1/JNK1/SKK1 (2–4). More recently, MKK7/JNK2/SSK4 has been cloned and shown to phosphorylate and activate only JNK (4). In contrast, MKK3/SSK2 and MKK6/SSK3 have been known to phosphorylate and activate specifically p38 MAPK (2–4). MKK5/MEK5 has been reported to phosphorylate BMK1 (3).

Heterotrimeric G protein βγ subunit (Gβγ) mediates signals to two types of stress-activated protein kinases, c-Jun NH2-terminal kinase (JNK) and p38 mitogen-activated protein kinase, in mammalian cells. To investigate the signaling mechanism whereby Gβγ regulates the activity of JNK, we transfected kinase-deficient mutants MKK4 and 7 (MKK7) into human embryonal kidney 293 cells. Gβγ-induced JNK activation was blocked by kinase-deficient MKK4 and to a lesser extent by kinase-deficient MKK7. Moreover, Gβγ increased MKK4 activity by 6-fold and MKK7 activity by 2-fold. MKK4 activation by Gβγ was blocked by dominant-negative Rho and Cdc42, whereas MKK7 activation was blocked by dominant-negative Rac. In addition, Gβγ-mediated MKK4 activation, but not MKK7 activation, was inhibited completely by specific tyrosine kinase inhibitors PP2 and PP1. These results indicate that Gβγ induces JNK activation mainly through MKK4 activation dependent on Rho, Cdc42, and tyrosine kinase, and to a lesser extent through MKK7 activation dependent on Rac.

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On the other hand, it has been demonstrated that JNK is also activated by an agonist stimulation of m1 and m2 muscarinic acetylcholine receptors expressed in NIH3T3, Rat-1, and COS-7 cells (15–17). JNK activation by muscarinic acetylcholine receptors has been shown to be mediated primarily by Gβγ in COS-7 cells (17). However, the mechanism by which Gβγ induces JNK activation has not been fully understood, although it has been suggested that phosphatidylinositol 3-kinase γ, Ras and Rac, and STE20-like kinase Pak1 are involved in JNK activation by Gβγ in COS-7 cells (17, 18). Furthermore, overexpression of constitutively activated GoαγGα12, GoαγGα13, and GoαγGα13 has been reported to induce the activation of JNK in some cells (19–25).

During investigations on JNK activation by stimulation of the m1 muscarinic acetylcholine receptor, we found that its activation was mediated by both Gβγ and Goγγγ in human embryonal kidney (HEK) 293 cells. We have reported recently that JNK activation by the m1 muscarinic acetylcholine receptor and Goγγγ partially involves the activation of protein kinase C and Src family tyrosine kinases (26). To clarify the signaling mechanism of JNK activation by Gβγ, we investigated whether Gβγ stimulates the activities of two JNK kinases MKK4 and MKK7. In this paper, we describe that Gβγ regulates MKK4 and MKK7 differentially through different signaling pathways.

MATERIALS AND METHODS

Antibodies and Inhibitors—Mouse monoclonal antibody (B-14) against Schistosoma japonicum GST was purchased from Santa Cruz Biotechnology, Inc. Mouse monoclonal antibodies M2 and 12CA5 against FLAG epitope and HA epitope were obtained from Eastman Kodak Co. and Boehringer Mannheim, Inc., respectively. Rabbit polyclonal antibodies C-14 and GC-2 against Goαγγ were purchased from Santa Cruz Biotechnology, Inc. and New England Biolabs, respectively. Goat anti-mouse (NA931) and anti-rabbit (NA934) Ig antibodies conjugated with horseradish peroxidase were from Amersham Pharmacia Biotech. Tyrosine kinase inhibitors (PP2, AG1479 and PP1/AG1872) were kindly provided by A. Levitzki (Hebrew University, Jerusalem, Israel). Mouse monoclonal antibody (B-14) against human homolog of mouse MKK7, were amplified from a human fetal brain cDNA library (CLONTECH) by polymerase chain reaction using human homolog of mouse MKK7-specific primers. Five μg of SRα-HA-JNK, SRα-HA-ERK, pCMV-GST-MKK4, or pCMV-GST-MKK7 was transfected with 0.3 μg of pCMV-m1 muscarinic acetylcholine receptor, 5 μg of pCMV-Gβγ, 5 μg of pCMV-Gγ, 10 μg of pCMV-Gα, Q209L, 10 μg of pCMV-Gα, 10 μg of pCMV-FLAG-MKKK9δR, 10 μg of pCMV-FLAG-MKK7K63R, 15 μg of pCMV-RasS17N, 15 μg of pCMV-FLAG-RhoT19N, 15 μg of pCMV-FLAG-Rac1T17N, or 15 μg of pCMV-FLAG-Cdc42T17N. The cells werestarved with serum-free medium containing 1 mg/ml bovine serum albumin (Nacala) for 24 h before transfection.

Purification of Recombinant Proteins from Escherichia coli—E. coli strain BL21 (DE3) cells were transformed with pET15b-JNK, pET32a-c-Jun (1–223), or pGEX2T-c-Jun (1–223). An isolated colony was inoculated in 10 ml of LB medium containing 100 μg/ml carbenicillin and 50 μg/ml X-Gal. This culture was incubated at 37 °C until A600 reached 0.2 and then induced at 16 °C with isopropyl-1-thio-β-D-galactopyranoside and further incubated for 3 h. The cells were harvested by centrifugation, washed with phosphate-buffered saline, and stored at −80 °C until use. The frozen cells were suspended and sonicated briefly in 10 ml of extraction buffer A (20 mM HEPES-NaOH (pH 7.5), 1 mM phenylmethanesulfonyl fluoride, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 0.5% Nonidet P-40) for hexahistidine-JNK and Try-c-Jun (1–223), the supernatants were applied to NTA-agarose (Qiagen, Inc.) and washed twice with column buffer A (20 mM HEPES-NaOH, pH 7.5, 1 mM dithiothreitol, 1 mM phenylmethanesulfonyl fluoride, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 mM EDTA, 0.5% Nonidet P-40) for GST-c-Jun (1–223) on ice, and shaken for 20 min at 4 °C. Cell debris was removed by centrifugation at 150,000 × g for 30 min at 4 °C.

All purification steps were performed at 4 °C. For the purification of hexahistidine-JNK or Try-c-Jun (1–223), the supernatants were applied to NTA-agarose (Qiagen, Inc.) and washed with column buffer A (20 mM HEPES-NaOH (pH 8.0), 1 mM phenylmethanesulfonyl fluoride, 1 μg/ml aprotinin, 1 μg/ml leupeptin) containing 20 mM imidazole. Hexahistidine-JNK and Try-c-Jun (1–223) were eluted with column buffer A containing 200 mM imidazole. Hexahistidine-JNK was diluted using TBS (20 mM Tris-HCl (pH 7.5), 137 mM NaCl, 2 mM KCl) containing 0.5% Nonidet P-40, 1 mM EDTA, and 100 mM NaCl, and 10% glycerol. The eluate was dialyzed against column buffer B and stored at −80 °C until use.

MAPK Assays—After 24 h of serum starvation, the cells transfected together with SRα-HA-JNK or SRα-HA-ERK were treated with or without carbachol at 37 °C and 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 μg/ml leupeptin, 1 mM EDTA, 1 mM Na3VO4, 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. Aliquots (500 μg) of the supernatants were mixed with protein A-Sepharose CL-4B preabsorbed with a mouse anti-HA antibody for 1 h at 4 °C. The immune complexes were precipitated and washed twice with lysis buffer A and twice with reaction buffer A (20 mM HEPES-NaOH (pH 7.5), 1 mM dithiothreitol, 1 mM phenylmethanesulfonyl fluoride, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 mM EDTA) and eluted with column buffer B containing 10 mM glycerol. The eluate was dialyzed against column buffer B and stored at −80 °C until use.

MAPK Assays—After 24 h of serum starvation, the cells transfected together with pCMV-GST-MKK4 or pCMV-GST-MKK7 were

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cells were transfected with plasmids carrying cDNAs for HA-JNK (acetylcholine receptor is mediated by Gbg).

**FIG. 1.** JNK activation by stimulation of the m1 muscarinic acetylcholine receptor is mediated by Gbg and Gαq/11 in HEK 293 cells. To confirm that the m1 muscarinic acetylcholine receptor stimulated JNK activity, we transfected plasmids encoding the receptor and the HA-tagged JNK into HEK 293 cells. Using an anti-HA antibody, HA-JNK was immunoprecipitated from cell lysates and washed with lysis buffer A and reaction buffer A. The precipitates after centrifugation were mixed with glutathione-Sepharose 4B for 2 h at 4°C. GST-MKK4 or GST-MKK7 was precipitated by centrifugation and washed with lysis buffer A and reaction buffer A. The precipitates were incubated in 30 μl of reaction buffer A containing 2 μg of hexahistidine-JNK, 10 μg of Trx-c-Jun (1–223), 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 × Laemmli sample buffer and boiling. Samples were subjected to SDS-polyacrylamide gel electrophoresis, and the radioactivity incorporated into Trx-c-Jun (1–223) was measured by an imaging analyzer (Fuji BAS 2000) and detected by autoradiography. The bound antibodies were visualized by an enhanced chemiluminescence detection system, using anti-rabbit or mouse Ig antibody conjugated with horse-radish peroxidase as a secondary antibody.

**Protein Assay—**Protein concentrations were determined using Bradford reagent (Nacalai) with bovine serum albumin as the standard.

**RESULTS**

**JNK Activation by the m1 Muscarinic Acetylcholine Receptor Is Mediated by Both Gβγ and Gαq/11 in HEK 293 Cells.** To confirm that the m1 muscarinic acetylcholine receptor stimulated JNK activity, we transfected plasmids encoding the receptor and the HA-tagged JNK into HEK 293 cells. Using an anti-HA antibody, HA-JNK was immunoprecipitated from lysates of the transfected cells, and the kinase activity was assayed using recombinant GST-c-Jun (amino acids 1–223) as a specific substrate. Fig. 1A shows the time course of JNK activation after stimulation by carbachol, which is an agonist of the receptor. Mock-transfected cells did not respond to carbachol (data not shown). In each experiment, we examined the expression level of HA-JNK by immunoblotting to monitor the transfection efficiency (see Figs. 1–3). The persistent activation of JNK was observed from 10 min to at least 30 min after the receptor stimulation. JNK activation by the m1 muscarinic acetylcholine receptor was dependent on the concentration of carbachol to maximum response at 10 μM and decreased slightly at 100 μM (Fig. 1B).

Next, we examined whether JNK activation by the m1 muscarinic acetylcholine receptor is mediated by Gβγ. It has been demonstrated previously that ERK phosphorylation (30) and p38 MAPK activation (33) induced by G protein-coupled receptors and Gβγ are blocked by cotransfection of Goq and Gαo, indicating that both Gβγ and Gαq,11 may mediate the signal from the m1 muscarinic acetylcholine receptor to JNK. To verify that inhibition of the

Authors A–E), the m1 muscarinic acetylcholine receptor (panels A–C), Goq (α-, panels C–E), Gαq (β-, panels D), Gβγ (γ-, panels D), and Gαq,11Q209L (α1I2Q209L, panel E). JNK activity was measured as described under "Materials and Methods." JNK activity is shown at different time points after the addition of 10 μM carbachol (panel A), 15 min after the addition of increasing concentrations of carbachol (panel B), and 15 min after the addition of 10 μM carbachol (panel C). Values shown represent the mean ± S.E. from three separate experiments. The phosphorylation of GST-c-Jun and the expression of HA-JNK and G protein subunits in the cell lysates are shown.
MKK4 may play a major role in this cascade. In contrast, G bg (Fig. 3 C), GST-MKK7, which was tagged with GST at the NH2 terminus, functioned as MAPKK in the pathway from G bg to JNK, and Trx-tagged c-Jun (amino acids 1–223). In each experiment, we examined the expression level of GST-MKK4 or GST-MKK7 by immunoblotting to monitor the transfection efficiency (see Figs. 2–7). Activation of the m1 muscarinic acetylcholine receptor by carbachol stimulated MKK4 and MKK7 activities (Fig. 2, C and D). These results suggest that the m1 muscarinic acetylcholine receptor activates JNK through at least two JNK kinases, MKK4 and MKK7.

**MKK4 Is a Major Mediator for JNK Activation by Gbg**—Because JNK activation by the m1 muscarinic acetylcholine receptor appears to be mediated through MKK4 and MKK7, we investigated whether MKK4 and MKK7 may be involved in Gbg-mediated JNK activation. As shown in Fig. 3, A and B, JNK activation by Gbg was reduced by about 90 and 50% by cotransfection of kinase-deficient MKK4 and MKK7, respectively, raising the possibility that either MKK4 or MKK7 may function as MAPKK in the pathway from Gbg to JNK, and MKK4 may play a major role in this cascade.

Next, we investigated whether Gbg activates MKK4 and MKK7. Gbg stimulated MKK4 activity by more than 5-fold (Fig. 3C). In contrast, Gbg activated MKK7 by about 2-fold (Fig. 3D). On the other hand, Go11Q209L only weakly activated MKK4 and MKK7 (data not shown). Together with data using dominant-interfering mutants, it is suggested that Gbg stimulates JNK activity mainly through MKK4 and to a lesser extent through MKK7.

**Gbg Activates MKK4 and MKK7 in a Ras-independent Manner**—Ras is known to be essential for the ERK activation by Gbg (11, 30). It was reported that Gbg-mediated JNK activation was blocked by dominant-negative Ras (17). To examine whether Ras is involved in the pathway from Gbg to MKK4 and MKK7, we utilized dominant-negative mutant of Ras (RasS17N), which inhibits the activation of endogenous Ras by sequestering guanine nucleotide exchange factors. Cotransfection of RasS17N completely inhibited the ERK activation by Gbg (Fig. 4C), whereas RasS17N had no effect on the activations of MKK4 and MKK7 by Gbg (Fig. 4, A and B).

**MKK4 Activation by Gbg Is Dependent on Rho and Cdc42, whereas MKK7 Activation Is Dependent on Rac**—It has been reported that Rho family GTPases are also involved in JNK activation upon various stimuli (34). Therefore, we investigated the role of these proteins on the Gbg-mediated MKK4 and MKK7 activations by cotransfection of Rho119N, Rac117N, or Cdc42T17N, which act as dominant-negative mutants analogous to RasS17N (35, 36). The MKK4 activation by Gbg (Fig. 4C), whereas RasS17N had no effect on the activations of MKK4 and MKK7 by Gbg (Fig. 4, A and B).

**Fig. 2.** JNK activation by stimulation of the m1 muscarinic acetylcholine receptor is mediated by MKK4 and MKK7. The cells were transfected with plasmids carrying cDNAs for HA-JNK, GST-MKK4 (panel A), GST-MKK4K95R (panel B), GST-MKK7 (panel C), and GST-MKK7K63R (panel D). m1 muscarinic acetylcholine receptor (panels A–D), FLAG-MKK4K95R (panel A), and FLAG-MKK7K63R (panel B). The activities of JNK, MKK4, and MKK7 were measured at 15 min after the addition of 10 μM carbachol as described under “Materials and Methods.” 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, 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.
was blocked completely by RhoT19N and Cdc42T17N, but not RacT17N (Fig. 5). On the other hand, the MKK7 activation by Gbg was blocked by RacT17N, but not RhoT19N and Cdc42T17N (Fig. 5). These results suggest that Gbg regulates MKK4 and MKK7 differentially through different Rho family GTPases. We also examined the effect of dominant-negative mutants of Rho family GTPases on JNK1 activation by Gbg. RhoT19N, Cdc42T17N, and RacT17N reduced JNK activation by 80, 60, and 30%, respectively. Data using dominant-negative mutants of Rho family GTPases also support that Gbg stimulates JNK activity mainly through MKK4.

PP2 and PP1, Specific Tyrosine Kinase Inhibitors, Significantly Inhibit Gbg-induced MKK4 but Not MKK7 Activation—In a previous study (26), we demonstrated that JNK activation by the m1 muscarinic acetylcholine receptor was partially reduced by PP2 and PP1, which are known to be specific tyrosine kinase inhibitors (37). Therefore, we explored a possible involvement of tyrosine kinase in the signaling pathway from Gbg to MKK4 and MKK7. The transfected cells were incubated with various concentrations of PP2 or PP1. These inhibitors attenuated the MKK4 activation by Gbg in a dose-dependent manner (Fig. 6).
The IC₅₀ value of PP2 and PP1 for the inhibition of Gβγ-induced MKK4 activation is approximately 5 and 10 μM, respectively. On the other hand, the MKK7 activation by Gβγ was not inhibited by these inhibitors (Fig. 6). It is likely that Gβγ regulates MKK4 in a tyrosine kinase-dependent manner and MKK7 in an independent manner.

**Gβγ-induced MKK4 and MKK7 Activations Do Not Depend on Phosphatidylinositol 3-Kinase**—To test the possibility that phosphatidylinositol 3-kinase is involved in the MKK4 and MKK7 activations by Gβγ, we investigated the effect of wortmannin and LY294002, which are specific inhibitors of phosphatidylinositol 3-kinase. The transfected cells were treated with 100 nM wortmannin or 100 μM LY294002 as described previously (14, 18). These inhibitors had no effect on the MKK4
and MKK7 activations (Fig. 7). In addition, we also observed that JNK activation by the m1 muscarinic acetylcholine receptor was not inhibited by the treatment of these inhibitors (data not shown). Under the same experimental conditions, the ERK activation by $G_{bg}$ was effectively attenuated by these inhibitors, being consistent with reports that $G_{bg}$ activate ERK pathway via phosphatidylinositol 3-kinase (13, 14). Therefore, phosphatidylinositol 3-kinase appears not to be necessary for the JNK pathway mediated by $G_{bg}$ in HEK 293 cells.

**DISCUSSION**

MKK4 and MKK7 have been shown to respond to environmental stresses and inflammatory cytokines such as tumor...
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necrosis factor-α and interleukin-1 (27–29, 38). However, the regulation of MKK4 and MKK7 by signaling through G protein-coupled receptors remained to be elucidated. In this paper, we showed that in HEK 293 cells, JNK activation by the m1 muscarinic acetylcholine receptor was mediated by Gβγ as well as Gαq/11 (26). We next demonstrated that the receptor-induced JNK activation required at least two JNK kinases, MKK4 and MKK7. Thus, we attempted to determine which signal component, including MKKs, small GTPases, tyrosine kinases, and phosphatidylinositol-3-kinases, is involved in the signaling pathway from Gβγ to JNK. We found that JNK activation by Gβγ was mediated mainly by MKK4 and partially by MKK7. Neither MKK4 nor MKK7 activation by Gβγ was inhibited by dominant-negative Ras. However, Gβγ-induced MKK4 activation was blocked by dominant-negative Rho and Cdc42, whereas Gβγ-induced MKK7 activation was blocked by dominant-negative Rac. Furthermore, the MKK4 but not MKK7 activation by Gβγ was inhibited by tyrosine kinase inhibitors. Finally, Gβγ-induced activations of MKK4 and MKK7 were independent on phosphatidylinositol-3-kinase activity. These results indicate that Gβγ regulates MKK4 and MKK7 differentially through different signaling molecules.

Cotransfection of kinase-deficient MKK4 completely inhibited Gβγ-mediated JNK activation, whereas that of kinase-deficient MKK7 partially inhibited JNK activation (Fig. 3). However, we could not rule out the possibility that JNK kinase(s) other than MKK4 and MKK7 might be involved in the pathway. In fact, Moriguchi et al. (38) have reported that the activity of a JNK kinase rather than of MKK4 and MKK7 is detected in unabsorbed fraction of anion-exchange chromatography in the process of fractionating extracts from L5178Y and KB cells exposed to hyperosmolarity. Further studies are necessary for clarifying MAPKKK responsible to the signal from Gβγ in HEK 293 cells.

The number of MAPKKK involved in JNK pathway is currently growing, and the regulation of MAPKKK is very divergent and complicated (3, 4). Several groups reported recently that MEKK1, MEKK4, MLK2, and MLK3 specifically associate with Cdc42 and Rac and may mediate JNK activation through Cdc42 and Rac (39–41). Because Cdc42 and Rac were involved in the activations of MKK4 and MKK7 by Gβγ, respectively (Fig. 5), MEKK1, MEKK4, MLK2, and/or MLK3 might act upstream of MKK4 and MKK7 in these pathways.

We have shown previously that Gβγ increases the level of the GTP-bound form of Ras in HEK 293 cells (30). Furthermore, it has been reported that oncogenic Ras is a potent activator of the JNK pathway (35, 36). We thought that Gβγ might activate MKK4 and MKK7 through a Ras-dependent pathway. However, neither MKK4 nor MKK7 activation by Gβγ was blocked by cotransfection of dominant-negative Ras (Fig. 4). Collins et al. (22) reported that constitutively activated Gα12 stimulates JNK activity in a Ras-independent manner, although Gα12 is able to activate Ras in HEK 293 cells. It appears that Ras is not essential for the JNK pathway mediated by Gα12 and Gβγ in HEK 293 cells.

We found differential involvement of Rho family GTPases in Gβγ-induced MKK4 and MKK7 activation (Fig. 5). Because MKK4 activation by Gβγ is blocked by both dominant-negative Rho and Cdc42, it is possible that the MKK4 activation is mediated by a guanine nucleotide exchange factor specific for Rho and Cdc42, e.g. Dbl and Ost (34). On the other hand, MKK7 activation by Gβγ is blocked only by dominant-negative Rac. Thus, Gβγ may activate MKK7 through a guanine nucleotide exchange factor specific for Rac, e.g. Tiam1 (34).

It is likely that tyrosine kinase is required for the MKK4 but not MKK7 activation by Gβγ (Fig. 6). We observed that Gβγ and the m1 muscarinic acetylcholine receptor induced tyrosine phosphorylation of intracellular proteins, and tyrosine-phosphorylated proteins were reduced by the treatment with PP2. Many lines of evidence suggest that Src family tyrosine kinases act downstream of Gβγ in various cells (11, 12). In addition, PP2 and PP1 preferentially inhibit Src family tyrosine kinases, and the IC50 value of PP2 for the inhibition of Src is 15 μM in intact cells. Thus, we considered that Src family tyrosine kinases might contribute to Gβγ-mediated MKK4 activation, and we transfected plasmids encoding a negative regulator of Src family tyrosine kinases (Csk) and kinase-negative Fyn or Lyn into the cells. However, these plasmids did not affect the MKK4 pathway. It was also reported that Gβγ directly activated Tec family tyrosine kinases Tsk and Btk in the presence of plasma membrane fractions in vitro (10), and Btk regulated JNK activity in vivo (42). This is unlikely to be a general mechanism for JNK regulation by Gβγ because Tsk and Btk appear to be expressed in very limited tissue distribution. However, another Tec family tyrosine kinase may be involved in the MKK4 activation.

Crespo et al. (43) reported recently that tyrosine phosphorylation of Vav guanine nucleotide exchange factor promoted the exchange of GDP to GTP on Rac1 and to a lesser extent on Cdc42. It is conceivable that Gβγ may induce the tyrosine phosphorylation of guanine nucleotide exchange factors expressed on Rho family GTPases and may increase the intrinsic exchange activity, leading to the MKK4 activation.

Pretreatment of specific phosphatidylinositol-3-kinase inhibitors wortmannin and LY294002 failed to inhibit MKK4 and MKK7 activation by Gβγ in HEK 293 cells (Fig. 7). Very recently, Lopez-Ilasaca et al. (18) demonstrated that in COS-7 cells, JNK stimulation induced by Gβγ was effectively suppressed by wortmannin or LY294002 and partially blocked by coexpression of a kinase-deficient mutant of phosphatidylinositol-3-kinase γ. This discrepancy may be caused by the difference of cell types.

The present study presents some hints for elucidating the mechanism by which Gβγ induces MKK4 and MKK7 activations. In conclusion, Gβγ activates JNK through at least two distinct pathways: one pathway is dependent on Rho and Cdc42 and tyrosine kinase, and the other is dependent on Rac. Further studies are needed to prove how Gβγ differentially regulates the activities of MKK4 and MKK7.

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