G protein $\beta\gamma$ subunit activates Ras, Raf, and MAP kinase in HEK 293 cells

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Abstract Using transient transfection of HEK 293 cells, we have studied the activation of Ras, c-Raf, and MAP kinase by G protein-coupled receptors, activated G protein α subunit (Gα), and $\beta\gamma$ subunits (G$\beta\gamma$). The expression of constitutively activated Gsα, Giα, and G11α did not have any effect on MAP kinase phosphorylation. In contrast, overexpression of G$\beta\gamma$ could stimulate the phosphorylation of MAP kinase and enhance the MEK kinase activity of c-Raf. Coexpression of dominant negative Ras inhibited G$\beta\gamma$-induced phosphorylation of MAP kinase. Furthermore, the GTP-bound form of Ras was increased by overexpression of G$\beta\gamma$. These results strongly suggest that the G$\beta\gamma$ may play an important role in signaling from G protein-coupled receptors to the MAP kinase pathway, and the activation of Ras and c-Raf may be involved in this signaling cascade in HEK 293 cells.

Key words: G protein; $\beta\gamma$ Subunit; Ras; Raf; MAP kinase

1. Introduction

Mitogen-activated protein kinase (MAP kinase) is involved in a variety of cellular signal transduction systems, leading to proliferation and differentiation of eukaryotic cells [1,2]. MAP kinase, which is also named Erk (extracellular signal-regulated kinase), is activated by phosphorylation of both threonine and tyrosine residues [3]. This phosphorylation is catalyzed by MEK (MAP kinase/Erk kinase). MEK is phosphorylated and activated by another serine/threonine kinase, Raf [4] or MEK kinase (MEKK) [5]. Signaling pathways from growth factor receptors to MAP kinases through Ras and Raf have been clarified [6]. Growth factor signals are transmitted from receptors to Ras through the signaling components including intrinsic or associated tyrosine kinases, SH2/SH3 adaptors proteins and Ras-guanine nucleotide exchange factors. The GTP-bound Ras can associate with Raf to recruit it to the membrane fractions [7,8]. Raf is subsequently activated by a still unknown mechanism, and the activated Raf transmits signals through a protein kinase cascade as described above.

Heterotrimeric guanine nucleotide-binding proteins (G proteins), consisting of α, β, and γ subunits, transduce signals from seven transmembrane receptors to various intracellular effectors in eukaryotic cells (reviewed in refs. [9,10,11,12]). Agonist binding to the receptors induces the dissociation of GDP from the α subunit, and thereby the binding of GTP to the α subunit. The GTP-bound α subunit acts upon downstream effector molecules, including subtypes of adenylyl cyclase, phospholipase C, and ion channels. The $\beta\gamma$ subunits dissociated from the α subunit also control some effector molecules, including type I and type II adenylyl cyclase, phospholipase Cβ, and the inward rectifier K^+ channel.

Recently, there is accumulating evidence which directs to the presence of cross-talks between two major signaling pathways, i.e. those involving heterotrimeric G proteins and receptors with tyrosine kinases. In Rat-1 cells, lysophosphatidic acid (LPA) activates the Ras-MAP kinase pathway through the pertussis toxin-sensitive G proteins [13]. Constitutively activated Gi2α, *gip2*, is oncogenic [14] and elevates the MAP kinase activity [15] in the fibroblast cells. On the other hand, cyclic AMP inhibits MAP kinase activation through Ras and Raf in the same cells [16]. More recently, it has been reported that transient expression of G protein $\beta\gamma$ subunits (G$\beta\gamma$) stimulates the MAP kinase activity in COS cells [17], and coexpression of dominant negative Ras attenuates its activation [18]. Nevertheless, there is no direct evidence which shows the activation of Ras and Raf by G$\beta\gamma$.

We investigated the mechanism of regulation of the MAP kinase pathway by G proteins using transient transfection of HEK 293 cells. In this report we show that the overexpression of G$\beta\gamma$ stimulates MAP kinase through activation of Ras and c-Raf.

2. Materials and methods

2.1. Cells and transfection

Human embryonic kidney (HEK) 293 cells were grown at 37°C in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum. Transfections were performed using calcium-phosphate precipitation. The final amount of transfected DNA for 10 cm^2 dish was adjusted to 40 μg by adding pCMV5 DNA [19].

2.2. Plasmid DNAs

Complementary DNA coding for the full length Gi1α was isolated from the rat brain cDNA library using human Gi1α genomic DNA [20] as a probe and sequenced. Mouse G11α cDNA was cloned by RT-PCR as described elsewhere. To make a constitutively activated G protein α subunit, we substituted a conserved Gln to Leu by site-directed mutagenesis. The mutations were confirmed by dideoxynucleotide sequencing. The mutated cDNAs of rat Gi1α, mouse G11α, and bovine Gsα short form [21] were subcloned into the pCMV5 vector. DNAs of human wild type, activated mutant Ha-Ras (G12V) [22], and dominant negative Ha-Ras (S17N) [23] were excised from respective plasmids and inserted into pCMV5. Complementary DNAs coding for bovine G protein β_1 [24] and γ_2 subunits were provided by M.I. Simon (California Institute of Technology) and T. Nukada (Tokyo Institute of Psychiatry), respectively, and subcloned into the above expression vector. Plasmids containing human m1 muscarinic acetylcholine receptor and m2 muscarinic acetylcholine receptor DNA [25] subcloned into the pCMV vector were given by E.M. Ross (University of Texas Southwestern Medical Center). Rat secretin receptor cDNA inserted into the CDM8 vector [26] was a gift from S. Nagata (Osaka Bioscience Institute). Mouse β type PDGF receptor cDNA [27] was also subcloned into pCMV5.

2.3. MAP kinase mobility shift

Cells were transfected with the indicated expression plasmids.

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Twenty four hours after transfection, the cells were serum starved in medium containing 1 mg/ml bovine serum albumin in DMEM. After 24 h starvation, the cells were stimulated with agonists for 20 min, and lysed on ice with buffer containing 50 mM Hepes, pH 7.0, 150 mM NaCl, 10% glycerol, 1% Nonidet P-40, 2 mM $MgCl_2$, 1 mM EDTA, 100 mM NaF, 10 mM sodium phosphate, 1 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride, 20 mM β-glycerophosphate, 10 μg/ml aprotinin, and 1 μg/ml leupeptin. The lysate was added to 5× Laemmli buffer (0.5 M Tris-HCl, pH 6.8, 10% SDS, 16.6% glycerol, 8.4% 2-mercaptoethanol, and 0.5% bromophenol blue) and sonicated. The mixture was electrophoresed on 10% SDS-polyacrylamide gel and transferred to a nitrocellulose filter. The filter was incubated with Anti-MAPK (Erk-2) antibody (UBI), and bands were visualized after treatment with a peroxidase-labeled second antibody by an ECL Detection System (Amersham).

2.4. Raf kinase (MEKK) assay

Cells were transfected with the indicated expression plasmids together with pLNC-Raf-1 : FH6, which was constructed to express wild-type human c-Raf with flag epitope tag and six histidine residues. One day after transfection, cells were starved for 16 h and then stimulated with agonists. The medium was removed, and a buffer (20 mM Hepes, pH 7.4, 150 mM KCl, 10% glycerol, 0.5% Triton X-100, 5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 10 mM NaF, 1 mM Na_3VO_4, 25 mM β-glycerophosphate, 20 μg/ml aprotinin, 10 μg/ml leupeptin, and 10 μg/ml pepstatin A) was added. The cells were scraped off with a rubber policeman and harvested by centrifugation. Anti-flag M2 antibody (IBI) was mixed with rabbit anti-mouse IgG antiserum (Cappel Laboratories) and protein A-Sepharose CL-4B (Pharmacia). The cell lysate was added to the antibody protein A-Sepharose complex and mixed gently at 4°C for 2 h. The precipitate was washed twice with washing buffer (50 mM Tris-HCl, pH 7.5, 2 mM $MgCl_2$, and 150 mM NaCl) and once with kinase buffer (20 mM Hepes, pH 7.3, 5 mM $MgCl_2$ and 0.5 mM $MnCl_2$). The precipitate was incubated at 30°C for 30 min with 3.2 μg of the wild-type *Xenopus* MEK [28], 6.4 μg of the kinase negative *Xenopus* MAP kinase [29], 0.28 mM ATP and 4 μCi [γ-^{32}P]ATP. Recombinant histidine-tagged MEK and GST-kinase deficient MAP kinase were produced in *E. coli* and purified as described [28,29]. The reaction was stopped by adding 5× Laemmli buffer and heating at 95°C for 5 min. The samples were resolved by 10% SDS-polyacrylamide gel. The radioactivity incorporated into MAP kinase was detected by autoradiography and measured by an image analyzer (FUJI BAS 2000).

2.5. Analysis of GDP and GTP bound to Ras

Cells were transfected with the indicated expression plasmid together with 1 μg of pCMVc-Ha-Ras containing wild type c-Ha-Ras. After serum starvation, HEK 293 cells (6×10^6) were labeled for 7 h with 0.5 mCi/ml [^{32}P]Pi (NEN) in phosphate-free DMEM supplemented with 1 mg/ml bovine serum albumin and 50 μM Na_3VO_4. After 5 min stimulation with or without agonists, the cells were harvested by centrifugation. Analysis of guanine nucleotides bound to Ras was performed as described [30].

3. Results

3.1. Stimulation of MAP kinase phosphorylation

We transfected HEK 293 cells with several receptors including PDGF, m1 muscarinic acetylcholine, m2 muscarinic acetylcholine, and secretin receptors. m1 muscarinic, m2 muscarinic, and secretin receptors activate Gq, Gi, and Gs, respectively. Activation of MAP kinase was assessed by the electrophoretic mobility shift [31]. The relevant agonists caused the mobility shifts of the MAP kinase (data not shown). Then we tested the possibility whether the transfection of activated mutants of G protein α subunit or βγ subunit can stimulate the phosphorylation of MAP kinase. As shown in Fig. 1, β1γ2 but neither the activated mutant of Gsα nor Gi1α could stimulate the MAP kinase phosphorylation. Phosphorylation of MAP kinase elicited by transfecting an activated Ras (RasG12V) was greater than that by βγ subunits. Under the same condition, the expression of activated G11α did not affect the phosphorylation (data not shown). Transfection with both β and γ subunit cDNA was required to show the phosphorylation of MAP kinase; neither β nor γ subunit cDNA alone being effective (Fig. 2). Cotransfection with 1 μg γ DNA and β DNA showed MAP kinase phosphorylation. This suggested that small amount expression of free βγ complex may be sufficient to activate MAP kinase. Coexpression of wild type Goα abolished the βγ-induced phosphorylation of MAP kinase. These results indicate that free Gβγ is able to activate the MAP kinase pathway.

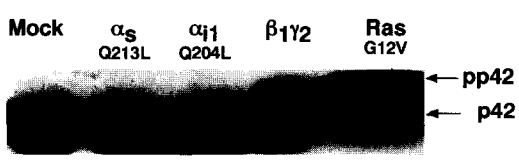

Fig. 1. Effect of G protein α and βγ subunits on MAP kinase phosphorylation. Cells were transfected with 10 μg of Gsα-sQ213L DNA, Gi1αQ204L DNA, 10 μg of β1 DNA and 10 μg of γ2 DNAs, and 10 μg of RasG12V DNA. Two days after transfection, the cell lysates were processed as described in section 2.

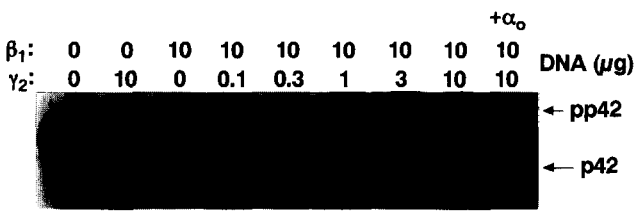

Fig. 2. MAP kinase phosphorylation by cotransfection of G protein β and γ subunits. Cells were transfected with the indicated amount of β1, γ2 and Goα1 DNAs. Two days later, the cell lysates were processed as described in section 2.

3.2. Inhibition of MAP kinase phosphorylation by dominant negative Ras

To investigate whether G protein-coupled receptors and Gβγ stimulate the MAP kinase pathway either through Ras or independent of Ras, we transfected the dominant negative Ras in which Ser 17 was replaced by Asn [32]. Immunoblot analysis with anti-Ras antibody (Has 6) indicated the dose-dependent expression of the dominant negative Ras (Fig. 3B). The MAP kinase phosphorylation induced by PDGF, secretin, and Gβγ was blocked by overexpression of dominant negative Ras. On the other hand, the dominant negative mutant did not show any inhibitory effect on m1 muscarinic receptor-induced phosphorylation. This suggests that the signals from m1 receptor do not require Ras function for MAP kinase activation in HEK 293 cells.

3.3. Activation of c-Raf kinase activity by βγ subunits

HEK 293 cells were transfected with a cDNA coding for a human c-Raf tagged with the flag epitope at its C terminus. The c-Raf activity was measured by an immune complex kinase assay. The immune complex was incubated with [γ-^{32}P]ATP in the presence of MEK and kinase-deficient MAP kinase, and the incorporation of ^{32}P into the MAP kinase was quantitated. As shown in Fig. 4, EGF and carbachol stimulated the MEK

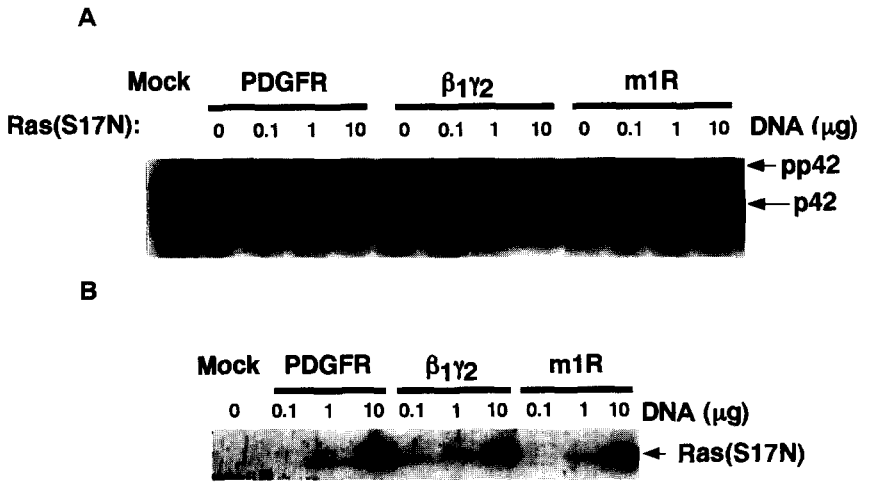

Fig. 3. Dominant negative Ras inhibits MAP kinase phosphorylation. Indicated amount of dominant negative Ras (RasS17N) DNA was cotransfected with receptors and Gβγ DNAs. Two days later, transfected cells were stimulated with 100 ng/ml PDGF, and 100 μM carbachol for 20 min. (A) Inhibition of MAP kinase phosphorylation. Cell lysates were subjected to immunoblot analysis with anti-Erk2 antibody. (B) Expression of dominant negative Ras proteins. Immunoblot analysis was performed with anti-Ras antibody (Has 6) [36].

kinase activity of c-Raf. Expression of Gβγ increased the kinase activity, although to a lesser extent.

3.4. Increase of GTP-bound Ras by overexpression of Gβγ

We examined the activation of Ras by Gβγ in HEK 293 cells. Since we could not obtain the sufficient amount of immune precipitates with the endogenous Ras proteins, we transfected the cells with the wild type c-Ha-Ras cDNA, the expressed Ras proteins were immunoprecipitated by anti-Ras monoclonal antibody (Y13–259), and the activation of Ras was measured. As shown in Fig. 5, overexpression of Gβγ caused a modest but definite increase in the level of Ras·GTP. Treatment with EGF increased the ratio of Ras·GTP to Ras·GDP + Ras·GTP by 2-fold. Carbachol also increased the level of Ras·GTP in the cells expressing the m2 muscarinic acetylcholine receptor.

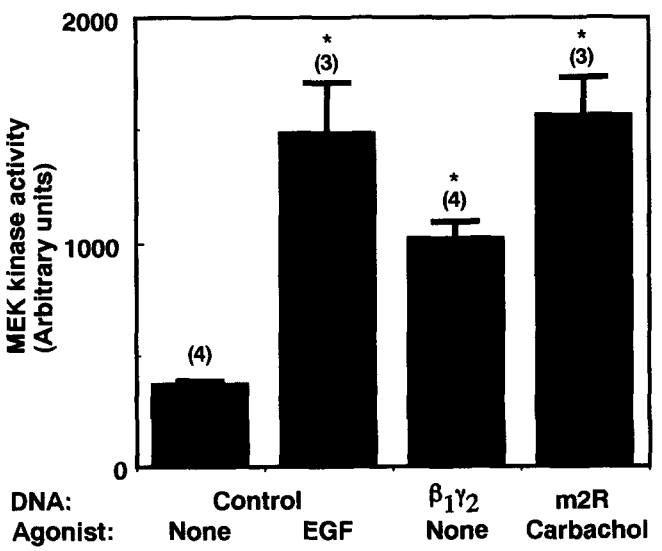

Fig. 4. Stimulation of Raf kinase activity in 293 cells. Cells were cotransfected with indicated plasmids plus wild type c-Raf cDNA. The cells were stimulated with 100 ng/ml EGF, or 100 μM carbachol for 10 min. Cell lysates were prepared and c-Raf proteins were immunoprecipitated as described in section 2. The immunoprecipitate was incubated with purified recombinant MEK and kinase deficient MAP kinase in the presence of [γ-^{32}P]ATP at 30°C for 30 min. Radioactivity incorporated into MAP kinase was measured by image analyzer. Data are shown as the mean ± S.E. from three to four independent experiments (n = 3–4, indicated in parentheses). Asterisks indicate $P < 0.01$ as compared with the control value.

4. Discussion

In the present study, we examined the activation of Ras, c-Raf, and MAP kinase. Overexpression of Gβγ induced the activation of all three molecules, although the activation by Gβγ was not so striking. Transient expression of the activated mutants of G protein α-subunits (Gsα, Gi1α, and G11α) did not show any stimulatory effect on the MAP kinase activation in HEK 293 cells. This is consistent with the results from an other laboratory using transient transfection of COS cells with an epitope-tagged MAP kinase [18]. Crespo et al. [18] indicated that the MAP kinase activation by G protein coupled-receptors and by overexpression of Gβγ was blocked by the dominant negative Ras. However, the difference between their results and ours is that the m1 muscarinic receptor-mediated activation of the MAP kinase was inhibited by transfecting dominant negative Ras in their case. In contrast, our results suggest that the Ras function is not required for the MAP kinase activation elicited by the m1 muscarinic receptor. In our experiments, m1 receptor-mediated MAP kinase activation was attenuated by prolonged treatment with phorbol ester, which downregulated PKC (data not shown). Furthermore, a PKC inhibitor, calphostin C, had an inhibitory effect on the m1 receptor-mediated MAP kinase activation in the cells. In HEK 293 cells, it is likely that the Gq/G11-coupled receptor may induce MAP kinase activation through the PKC-dependent pathway. In contrast, Gβγ-induced MAP kinase activation appears to be PKC-independent, since the activation by Gβγ was insensitive to the pretreatment with phorbol ester (data not shown).

The membrane localization of Gβγ is necessary for transducing the signals to effector molecule(s) because coexpression of

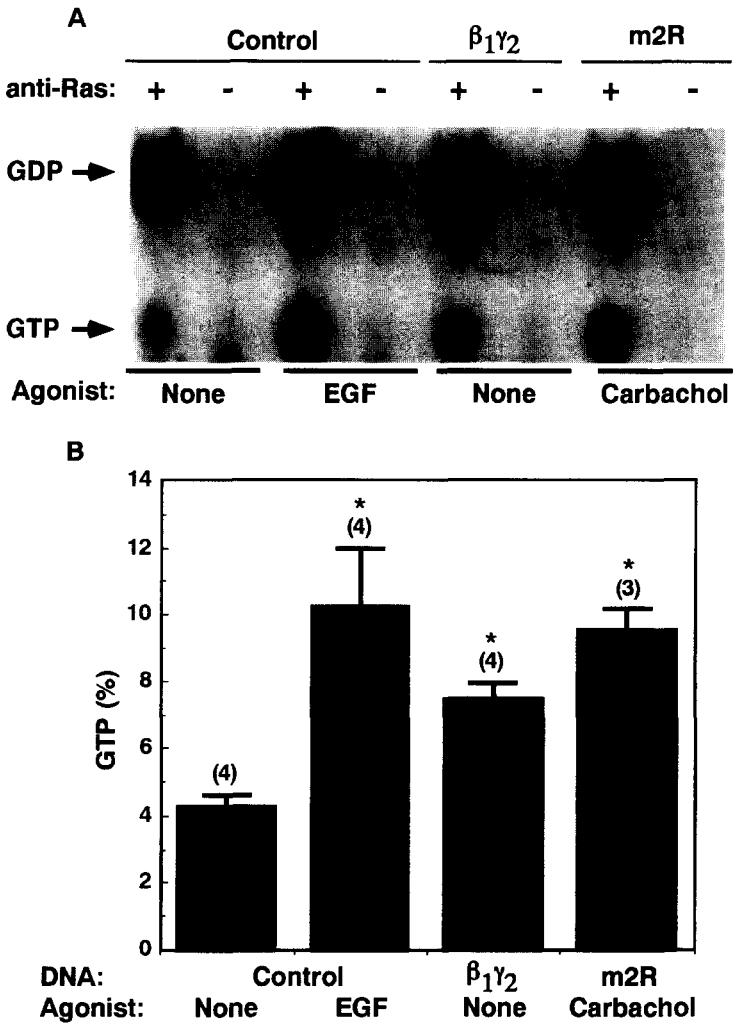

Fig. 5. Activation of Ras by transfecting G protein $\beta\gamma$ subunits and agonist stimulation. 293 cells were transfected with the indicated expression plasmids plus wild type c-Ha-Ras cDNA. Forty eight hours later, quiescent cells were labeled with [^{32}P]Pi for 7 h and stimulated with 100 μM carbachol for 7 min, and 100 ng/ml EGF for 5 min. After preparation of cell lysates, Ras proteins were immunoprecipitated with anti-Ras monoclonal antibody (Y13–259). The guanine nucleotides bound to the Ras proteins were resolved using thin layer chromatography. (A) Autoradiograph of GTP and GDP bound to Ras on TLC plate. (B) Increase of percent ratio of GTP/(GTP + GDP) by transfecting G$\beta\gamma$ and agonist stimulation. Bars show the mean ± S.E. from three to four separate experiments (n = 3–4, indicated in parentheses). Asterisks indicate $P < 0.01$ as compared with the control value.

the isoprenylation-deficient mutant of Gγ with wild type Gβ failed to activate the MAP kinase pathway [18, and data not shown]. Recently, it has been reported that G$\beta\gamma$ interacts with several polypeptides containing pleckstrin homology (PH) domains, i.e. β-adrenergic receptor kinase (βARK), Ras-GAP, PLCγ [33], and Btk [34]. Expression of G$\beta\gamma$-binding polypeptide of βARK showed an inhibitory effect on the activation of Ras and MAP kinase induced by G protein-coupled receptor [35]. This finding also supports our conclusion that free G$\beta\gamma$ released from Gα upon receptor activation transduces the signal to Ras proteins in the membranes. Demonstration and identification of the putative effector molecule(s) which mediate the signals from the G protein $\beta\gamma$ subunits to Ras activation is under investigation.

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