G protein β and γ subunits (Gβ and Gγ) form a complex that is involved in various signaling pathways. We reported that the C-terminal 10 amino acids of Gβ are required for association with Gγ (Yamauchi, J., Kaziro, Y., and Itoh, H. (1995) Biochem. Biophys. Res. Commun., 214, 694–700). To evaluate further the significance of the C-terminal region of Gβ in the formation of a Gβγ complex and its signal transduction, we constructed several C-terminal mutants and expressed them in human embryonal kidney 293 cells. The mutant lacking the C-terminal 2 amino acids (ΔC2) failed to associate with Gγ, whereas deletion of the C-terminal amino acid (ΔC1), replacement of Trp at –2 position by Ala (W339A), and addition of six histidines ((His)6) at the C terminus did not affect the association with Gγ. We also studied the effect of these mutations on the activation of mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK) and c-Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK), which is composed of seven WD repeating units, each of which stretches along the β-propeller blades, forming the β-propeller structure, 4259 Nagatsuta-cho, Midori-ku, Yokohama 226, Japan.

C-terminal Mutation of G Protein β Subunit Affects Differentially Extracellular Signal-regulated Kinase and c-Jun N-terminal Kinase Pathways in Human Embryonal Kidney 293 Cells*

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Heterotrimeric guanine nucleotide-binding regulatory proteins (G proteins) mediate signals from a variety of cell surface receptors to effector molecules (1–5). G proteins are composed of α, β, and γ subunits. Binding of ligands to G protein-coupled receptors stimulates the dissociation of Go and Gβγ, which regulate, independently or cooperatively, a variety of effector molecules.

Normally, Gβ and Gγ associate tightly and function as a complex. Gβ contains seven WD repeating units, each of which consists of approximately 40 amino acids and ends mainly with Trp-Asp (WD) (6). Recently, Wall et al. (7) and Lambright et al. (8) reported that the x-ray crystallographic structure of heterotrimer of Gaαβγ in Gα1 and Gaαβγ was analyzed by Sondek et al. (9). These reports have revealed that the structure of Gβγ in the trimeric complex is not very different much from that in the dimeric complex. The WD repeat provides a rigid scaffold of β-propeller structure, which is composed of seven β-propeller blades containing four antiparallel β-sheets. The N terminus of Gγ forms an α-helical coiled structure with the N terminus of Gβ, and the remainder of Gγ stretches along the β-propeller blades, forming multiple interaction sites with Gβ. On the other hand, the N-terminal α-helix of Go binds with a β-propeller blade of Gβ, and a region designated switch II of Go fits into the top of the β-propeller.

In mammalian cells, Gβγ has been shown to modulate the activities of adenylyl cyclases (10), phospholipase Cβ isozymes (11, 12), phosphatidylinositol 3-kinase γ (13), inward rectifier potassium channels (14, 15), and N-type and P/Q-type calcium channels (16, 17). Furthermore, it has been reported that Gβγ binds directly with the C-terminal region of the pleckstrin homology domain to regulate β-adrenergic receptor kinases (18, 19) and Tec family protein-tyrosine kinases (20, 21). More recently, Gβγ was shown to stimulate the activities of the mitogen-activated protein kinase subfamilies MAPK/ERK (22–25) and JNK/SAPK (26). These studies have established a critical role of Gβγ in the intracellular signal transduction, although little is known about the region involved in the effector regulation.

In the present study, we demonstrated that a few amino acids at the C terminus of Gβ are involved in the complex formation with Gγ. Furthermore, we found that mutations in C-terminal amino acids influence differentially the stimulation of MAPK/ERK and JNK/SAPK activities.

MATERIALS AND METHODS

Antibodies—Rabbit polyclonal anti-Gαa antibody was produced against amino acids 94–108 of Gαa and purified by a peptide affinity column. Rabbit polyclonal antibody (06-238) that recognizes a peptide spanning amino acids 127–139 identical in Gβγ and Gβγ was purchased from Upstate Biotechnology, Inc. Mouse monoclonal antibodies (M2 and 12CA5) against FLAG epitope (8 amino acids, EYKKEEEEK) and HA epitope (9 amino acids, YPYDVPDYA) were from Eastman Kodak Co. and Boehringer Mannheim, respectively. Rabbit anti-mouse Ig antibody (55480) was from Cappel.

cDNA Construction—Rat Gαa 1 cDNA (27, 28) was inserted into mammalian expression vector pCMV5 (29), cDNAs of bovine Gβγ (30) and bovine Gγ (31, 32) were generously provided by M. L. Simon (California Institute of Technology) and T. Nukada (Tokyo Institute of Psychiatry), respectively. Wild type Gβγ and Gγ were subcloned into pCMV5 as described before (25). To construct mutants of Gβγ, the coding region of Gβγ was amplified by PCR using 5’ sense primer containing an...
EcoRI recognition site and 3′ antisense primer containing a HindIII site. PCR was carried out for 30 cycles, each at 94 °C for 1 min, 50 °C for 2 min, and 72 °C for 3 min, using Pfu polymerase (Stratagene). The following oligonucleotides were used as PCR primers: primer 1, 5′-CC-GGTTTACTGATAACCGACGTTT-3′; primer 2, 5′-TGGATCTGGCCGCTGCTCGATTCGCCCTC-3′; primer 3, 5′-CC-AAGCTTCACCTGTTGAGGACGCT-3′; primer 4, 5′-CACCAGGTTTGACGTTTGGAGGAC-3′; and primer 5, 5′-CC-AAGCTTCACCTGTTGAGGACGCT-3′.

Differential Activation of ERK and JNK by Gβ Mutants

The cDNAs encoding Gβ mutants were constructed by PCR and placed under the control of the pCMV5 promoter as described under “Materials and Methods.”

RESULTS

In a previous study, we found that the C-terminal region of Gβ is involved in the complex formation with Gγ (33). To identify which amino acid residues within the last six amino acids in the C-terminal region is required for the association with Gγ, we constructed several C-terminal deletion mutants that lack the last six (ΔC6), two (ΔC2), and one (ΔC1) amino acids (Fig. 1). Since Gγ tagged with FLAG epitope at the N terminus can be co-immunoprecipitated with Gβ, FLAG-Gγ was utilized to analyze the complex formation with the C-terminal mutants of Gβ (33). The Gβ mutants and FLAG-Gγ

Wild type • • • SWDSFLKIWN
ΔC1 • • • SWDSFLKIW
ΔC2 • • • SWDSFLKI
ΔC6 • • • SWDS
W339A • • • SWDSFLKIAN

(Hls)e • • • SWDSFLKIAN(Hs)e

FIG. 1. The C-terminal amino acid sequences of wild type and mutants of Gβ. The cDNAs encoding Gβ mutants were constructed by PCR and placed under the control of the pCMV5 promoter as described under “Materials and Methods.”
were expressed at a similar level in HEK 293 cells (Fig. 2B). Fig. 2A shows that the ΔC6 and ΔC2 mutants failed to associate with FLAG-Gγ, whereas the ΔC1 mutant could form a Gβγ complex. Next, we constructed a mutant (W339A) in which the second amino acid (Trp) from the C terminus was replaced by Ala (Fig. 1). The W339A mutant could form a complex with FLAG-Gγ (Fig. 2A), suggesting that the presence of an amino acid residue at the −2 position of Gβ appeared to be important for the complex formation with Gγ. The addition of six histidines (His)6 to the C terminus of Gβ was not inhibitory to the interaction of Gβ with Gγ (Fig. 2A).

The results of x-ray crystal structure analysis suggest that the C terminus of Gβ may be located near the N terminus of Gγ (7, 8). We analyzed the effect of the C-terminal mutations of Gβ on the interaction with Gα. Addition of the FLAG sequence to the N terminus of Gγ has no effect on either the association with Gβ or the formation of a Gαβγ complex (33). In the presence of FLAG-Gγ, the ΔC1, W339A, and (His)6 mutants retained their full ability to interact with Gα, (34) (Fig. 3). Furthermore, we examined the effect of the C-terminal mutations on the GTP-dependent dissociation of Gα from Gβγ. The cells were transfected with cDNAs of Gαg, C-terminal mutants of Gβ, and FLAG-Gγ. The cells were lysed, and the ternary complexes were immunoprecipitated with anti-FLAG antibody. The immune complexes were incubated with a buffer containing GDP (Fig. 4) and GTPγS (Fig. 4). The ternary complexes formed with Gβ mutants of ΔC1, W339A, and (His)6 showed the GTPγS-dependent dissociation similar to the one formed with wild type Gβ.

Gβγ has been shown to stimulate the MAPK/ERK signaling pathway (22–25). To explore the effect of the C-terminal mutations of Gβ on MAPK/ERK activation, cDNAs of Gβ mutants, wild type Gγ, and HA-ERK2 were co-transfected into HEK 293 cells. HA-ERK2 was immunoprecipitated with anti-HA antibody, and the kinase activity was assessed using myelin basic protein as a substrate. It has been shown that MAPK/ERK activation by Gβγ-dependent receptors is mediated by Gβγ, whereas the activation by Gq11 or Gq11 coupled receptors is mediated by Gγ (22–25). Carbachol stimulated the HA-ERK2 activity 2- and 5-fold in HEK 293 cells transfected with m2 muscarinic and m1 muscarinic acetylcholine receptors, respectively (data not shown). Crespo et al. (22) and Faure et al. (23) demonstrated that the ERK2 activity is stimulated 4- and 2-fold, respectively, by overexpression of Gβγ in COS cells. In HEK 293 cells, overexpression of Gβγ induces a weak phosphorylation of endogenous ERK2 (25). As shown in Fig. 5A, co-expression of wild type Gβ and Gγ activated HA-ERK2 about 1.5-fold, whereas the ΔC1 and W339A mutants had a relatively weak effect on ERK2 activation, and the ΔC2, ΔC6, and (His)6 mutants could not activate ERK2 at all.

Furthermore, we examined the effect of the C-terminal mutations of Gβ on MEK kinase activity of c-Raf. Cells were co-transfected with cDNAs of various Gβ mutants, wild type Gγ, and RafFH6, which is c-Raf-1 tagged with FLAG epitope and six histidines. The RafFH6 was immunoprecipitated with anti-FLAG antibody, and its MEK kinase activity was assayed using recombinant MEK and recombinant kinase-negative MAPK. The MEK kinase activity of RafFH6 was stimulated more than 1.5-fold by wild type Gβ and Gγ. On the other hand, the activation of RafFH6 by the ΔC1 and W339A mutants was less potent than that by the wild type Gβ and the ΔC2, ΔC6, and (His)6 mutants failed to activate RafFH6 (Fig. 5B). Effects

Fig. 2. Association of the C-terminal mutants of Gβ with Gγ. HEK 293 cells were transfected with vector alone (Mock) or plasmids carrying cDNA for wild type Gβ (wt), ΔC1, ΔC2, ΔC6, W339A, (His)6, and FLAG-Gγ (γ), as indicated. The cells were lysed as described under “Materials and Methods.” Aliquots of the cell lysates were immunoprecipitated with anti-FLAG antibody and immunoblotted with anti-common Gβ antibody (A). Other aliquots were immunoblotted with anti-common Gβ antibody (B, upper) or anti-FLAG antibody (B, lower). The results shown are representative of three or five independent experiments.

Fig. 3. Interaction of Gαg with the C-terminal mutants of Gβ and Gγ. Cells were transfected with vector alone (Mock) or plasmids carrying cDNA for Gαg (α), wild type Gβ (wt), ΔC1, ΔC2, ΔC6, W339A, (His)6, and FLAG-Gγ (γ), as indicated. The cells were lysed as described under “Materials and Methods.” Aliquots of the cell lysates were immunoprecipitated with anti-FLAG antibody, immunoblotted with anti-Gαg antibody (A, upper), and reimmunoblotted with anti-common Gβ antibody (A, lower). Other aliquots were immunoblotted with anti-Gαg antibody (B, upper), anti-common Gβ antibody (B, middle), or anti-FLAG antibody (B, lower). The results shown are representative of three independent experiments.

Fig. 4. Guanine nucleotide-dependent dissociation of Gαg from Gβγ. Cells were transfected with cDNAs for Gαg (α), wild type Gβ (wt), and FLAG-Gγ (γ); Gαg, ΔC1, and FLAG-Gγ (B); Gαg, W339A, and FLAG-Gγ (C); and Gαg, (His)6, and FLAG-Gγ (D). The cells were lysed as described under “Materials and Methods.” The lysates were divided into two halves and immunoprecipitated with anti-FLAG antibody. The immune complexes were incubated with a buffer containing GDP (left) or GTPγS (right). The washed immune complexes were immunoblotted with anti-Gαg antibody (upper) and reimmunoblotted with anti-common Gβ antibody (lower). The results shown are representative of three independent experiments.
It is noteworthy that the (His)6 mutant could stimulate neither bg overexpression of G activity was assayed using GST-c-Jun as a specific substrate. After lysis, HA-JNK1 was immunoprecipitated, and its kinase activity was assayed using recombinant MEK and recombinant kinase-negative MAPK in the presence of [γ-32P]ATP, and the radioactivities incorporated into the MAPK were measured as described under "Materials and Methods." Aliquots of the cell lysates were immunoprecipitated with anti-common Gγ antibody (A). Other aliquots were immunoprecipitated with anti-common Gβ antibody (B, upper), or anti-FLAG antibody (B, lower). The results shown are representative of three independent experiments.

of the C-terminal mutations on the stimulation of c-Raf-1 activity were comparable to those on the stimulation of ERK2 activity. Transfection of Gβ or Gγ alone fails to activate ERK2 (see Fig. 8A, and Refs. 22, 23, and 25) and c-Raf-1 (see Fig. 8B). It is noteworthy that the (His)6 mutant could stimulate neither ERK2 nor c-Raf-1.

Since the N-terminal region of c-Raf-1 associates physically with the C-terminal region of Gβ (37), we tested whether the C-terminal mutants of Gβ are able to interact with c-Raf-1. As shown in Fig. 6, the (His)6 mutant could not bind with c-Raf-1, but other mutants could bind. It is likely that six histidine residues added at the C terminus of Gβ may sterically inhibit the association of c-Raf-1 with Gβ. It is suggested that the association of Gβ with c-Raf-1 by itself is not sufficient for activation of c-Raf-1, although the association may be required for its activation.

It has recently been reported that the signaling from G protein-coupled receptors to JNK/SAPK involves Gβγ and that overexpression of Gβγ enhances JNK/SAPK activity (26). We examined the effect of the Gβ C-terminal mutants on JNK/SAPK stimulation. The cells were co-transfected with cDNAs of each Gβ mutant and wild type Gγ together with HA-JNK1. After lysis, HA-JNK1 was immunoprecipitated, and its kinase activity was assayed using GST-c-Jun as a specific substrate. Wild type Gβ and Gγ increased the activity of JNK1 approximately 5-fold (Fig. 7A). In contrast to the results of MAPK/ERK and c-Raf-1 activations (Fig. 5), the ΔC1 mutant had little ability to stimulate JNK1, whereas the ΔC2, ΔC6, and (His)6 mutants showed moderate stimulatory effect. The stimulatory effect of the W339A mutant on JNK1 was almost indistinguishable from that of the wild type Gβ (Fig. 7A). Although the ΔC2 and ΔC6 mutants had little ability to associate with Gγ, they activated JNK1 significantly. In order to examine whether Gβ may activate JNK1 in the absence of interaction with Gγ in the cells, we utilized a N-terminal deletion mutant of Gβ. Since the N-terminal region of Gβ is essential to form an α-helical coiled coil structure with the N terminus of Gγ, the deletion of this region of Gβ completely prevents the dimer formation with Gγ (9, 33, 38). As shown in Fig. 7B, the ΔN38 mutant, which lacks N-terminal 38 amino acids, stimulated the activity of JNK1 approximately 3-fold. Furthermore, we transfected with Gβ or Gγ alone and measured the JNK1 activity. Fig. 8C shows that the transfection of Gβ alone caused the activation of JNK1. These results suggested that the overexpression of Gβ alone can stimulate the JNK1 activity in the cells.

**FIG. 5. Effects of the C-terminal mutants of Gβ on c-Raf-1 and MAPK/ERK stimulations.** Cells were co-transfected with plasmid DNAs of HA-ERK2 (A) or RafFH6 (B) plus vector alone (Mock) or cDNA for wild type Gβ (wt), ΔC1, ΔC2, ΔC6, W339A, (His)6, Gγ (γ), and RafFH6 (Raf), as indicated. A, the immune complexes of the cell lysates were assayed by measuring the incorporation of radioactive phosphate into myelin basic protein as described under "Materials and Methods." B, the immunoprecipitates were incubated with recombinant MEK and recombinant kinase-negative MAPK in the presence of [γ-32P]ATP, and the radioactivities incorporated into the MAPK were measured as described under "Materials and Methods." Values shown represent the mean ± S.E. from three or four separate experiments.

**FIG. 6. Association of the C-terminal mutants of Gβ with c-Raf.** Cells were co-transfected with vector alone (Mock) or plasmid carrying cDNA for wild type β (wt), ΔC1, ΔC2, ΔC6, W339A, (His)6, Gγ (γ), and RafFH6 (Raf), as indicated. The cells were lysed as described under "Materials and Methods." Aliquots of the cell lysates were immunoprecipitated with anti-FLAG antibody and immunoblotted with anti-common Gβ antibody (A). Other aliquots were immunoprecipitated with anti-common Gβ antibody (B, upper) or anti-FLAG antibody (B, lower). The results shown are representative of three independent experiments.

**DISCUSSION**

The mutant (ΔC1) lacking the C-terminal amino acid of Gβ retained the full ability to associate with Gγ and to form a ternary complex with Go (Figs. 2–4). Although the C-terminal Asn-340 of Gβγ participates in the specific interaction with Asn-62 of Gγγ (corresponding to Asn-59 of Gγγ (9), the deletion of Asn-340 of Gβγ did not affect the association with Gγγ. The interaction does not seem to be necessary for the complex formation. Removal of the two amino acid residues from the Gβ C terminus dramatically abolished the binding of Gβγ with Gγ (Fig. 2). Since Gγ associates with Gγ at multiple sites (9), it was unexpected that the truncation of the last two amino acid residues resulted in the loss of the ability to form a Gγγ complex. We first thought that the Trp-339 may be important for the complex formation, but the replacement of Trp-339 by Ala (W339A) did not show any effect on the association of Gβγ with Gγ (Fig. 2). It appears that the presence of an amino acid residue at the −2 position of Gβγ is important for the Gγγ complex formation.

Genetic studies of the pheromone response pathway in Saccharomyces cerevisiae suggested that two regions of the Ste4 protein (S. cerevisiae Gβ) may be involved in the effector activation (39). The first region is localized in the α-helical structure of the N terminus and far from the binding sites with Go (7, 8, 39). If this region is involved in the binding with an effector, it is unclear how the activation of effector by Gγγ is inhibited by Go. The second region is found in the third WD repeat (39). We made a substitution mutant using Gγ at Val-135 that was well conserved between mammalian Gγ and yeast Ste4. However, the V135G mutant of Gβ retained the ability to
stimulate the MAPK/ERK and JNK/SAPK activities to an extent similar to that of the wild type Gβ in HEK 293 cells (data not shown). It is possible that the V135G mutation may affect other signaling pathways in mammalian cells or that other mutations in the second region of Gβ may affect ERK/MAPK and JNK/SAPK activities.

We found differential effects of the C-terminal mutations of Gβ on the MAPK/ERK and JNK/SAPK pathways in mammalian cells. The ΔC1 mutant of Gβ, together with Gγ, could stimulate the activity of c-Raf-1 and MAPK/ERK but showed only a slight activation of JNK/SAPK. On the other hand, the (His)6 mutant of Gβ failed to stimulate c-Raf-1 and MAPK/ERK activities in the presence of wild type Gγ, and the mutant retained the ability to activate JNK/SAPK (Figs. 5 and 7). The C-terminal amino acid residue of Gβ is localized on the outside of a Gβγ complex (9). It is possible that the C terminus of Gβ is involved in binding with effector molecule(s). We speculate that the differential effects of these mutations on the activation of MAPK/ERK and JNK/SAPK might be due to the difference of their direct effector(s). The putative effector molecule(s) of Gβγ in the MAPK/ERK and JNK/SAPK cascades are as yet unidentified, although phosphatidylinositol 3-kinase has been reported to be involved in the pathway from Gβγ to MAPK/ERK (40). Phosphatidylinositol 3-kinase γ (13) may be a candidate for its effector. It has been reported that calcium ion is important in Gγ-coupled receptor-mediated stimulation of JNK/SAPK (41). The direct regulation of phospholipase Cβ isozymes by Gβγ (11, 12) may be involved in the signaling pathway.

Recently, Coria et al. (42) reported that the C-terminal region of yeast Ste4 is essential in triggering the yeast phero-

Fig. 7. The C-terminal mutants of Gβ influence the stimulation of JNK/SAPK activity. Cells were co-transfected with plasmid carrying DNAs for HA-JNK1 plus vector alone (Mock) or plasmid carrying cDNAs for wild type Gβ (wt), ΔC1, ΔC2, ΔC6, W339A, (His)6, ΔN38, and Gγ (γ), as indicated (A and B). The immune complexes of the cell lysates were incubated with GST-c-Jun in the presence of [γ-32P]ATP and assayed by measuring the incorporation of radioactive phosphate into GST-c-Jun as described under “Materials and Methods.” Values shown represent the mean ± S.E. from three separate experiments (upper). The autoradiogram of GST-c-Jun is representative of three experiments (middle). Aliquots of the cell lysates were immunoblotted with anti-HA antibody to detect HA-JNK1 (lower).

Fig. 8. Effect of transfection with Gβ or Gγ alone on the MAPK/ERK, c-Raf-1, and JNK/SAPK stimulations. Cells were co-transfected with plasmid carrying DNAs for HA-ERK2 (A), RafFH6 (B), or HA-JNK1 (C) together with vector alone (Mock) or DNAs for wild type Gβ (β) and/or Gγ (γ), as indicated. The cells were lysed, and the tagged kinases were immunoprecipitated with anti-HA antibody (A and C) or anti-FLAG antibody (B). The immune complexes were used to measure the kinase activities as described under “Materials and Methods.” Values shown represent the mean ± S.E. from three separate experiments.
Differential Activation of ERK and JNK by Gβ Mutants

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