Critical Determinants of the G Protein γ Subunits in the Gβγ Stimulation of G Protein-activated Inwardly Rectifying Potassium (GIRK) Channel Activity*

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Luying Peng§§, Tooraj Mirshahi¶¶, Hailin Zhang§§, Jeanne P. Hirsch**, and Diomedes E. Logothetis‡ ‡‡

From the Departments of §§Physiology and Biophysics and **Molecular, Cell and Developmental Biology, Mount Sinai School of Medicine of the New York University, New York, New York 10029

The βγ subunits of G proteins modulate inwardly rectifying potassium (GIRK) channels through direct interactions. Although GIRK currents are stimulated by mammalian Gβγ subunits, we show that they were inhibited by the yeast Gβγ (Ste4/Ste18) subunits. A chimera between the yeast and the mammalian Gβ1 subunits (ymβ) stimulated or inhibited GIRK currents, depending on whether it was co-expressed with mammalian or yeast Gγ subunits, respectively. This result underscores the critical functional influence of the Gγ subunits on the effectiveness of the Gβγ complex. A series of chimeras between Gγ2 and the yeast Gγ revealed that the C-terminal half of the Gγ2 subunit is required for channel activation by the Gβγ complex. Point mutations of Gγ2 to the corresponding yeast Gγ residues identified several amino acids that reduced significantly the ability of Gβγ to stimulate channel activity, an effect that was not due to improper association with Gγ. Most of the identified critical Gγ residues clustered together, forming an intricate network of interactions with the Gβ subunit, defining an interaction surface of the Gβγ complex with GIRK channels. These results show for the first time a functional role for Gγ in the effector role of Gβγ.

Heterotrigeminer guanine nucleotide GTP-binding (G) proteins are composed of α, β, and γ subunits at a ratio of 1:1:1 (1). G proteins play important roles in a variety of transmembrane signaling pathways that are activated by extracellular hormones, neurotransmitters, chemokines, local mediators, and sensory stimuli (2–4). An agonist-bound G protein-coupled receptor activates the G proteins by generating GTP-bound α subunits and triggering the release of the βγ subunits from the heterotrimer. Both the α and βγ subunits are then able to regulate downstream effectors through direct interactions. Once the Ga-GTP subunit is inactivated through GTP hydrol-

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§ Present address: The Key Laboratory of Molecular Medicine, Ministry of Education, Shanghai Medical College of Fudan University, Shanghai, 200032, P. R. China.

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‡‡ To whom correspondence should be addressed. E-mail: diomedes.logothetis@mssm.edu.

The abbreviations used are: GIRK, G protein-activated inwardly rectifying potassium; ymβ, a chimera between the yeast and the mammalian Gβ1 subunits; mγ, mammalian Gγ2; yγ, yeast Gγ.

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**Yeas Gβγ inhibits GIRK4* channel activity.** In A, the upper panels show representative barium-sensitive current versus voltage relationships constructed by averaging data from oocytes expressing GIRK4* (left panel), GIRK4* + yeast βγ (middle panel), and GIRK4* + β1γ2 (right panel). The GIRK4* channel currents were inhibited by yeast Gβγ but were activated by mammalian β1γ2 subunits. The lower panels show IRK1 currents (left panel) IRK1 + yeast βγ and summary data for all experiments shown (right panel). In the summary plot, basal currents at −80 mV were averaged for a representative batch of oocytes tested (right, *, p < 0.01, unpaired Student’s t test, n = 7–9). In B, the yeast βγ inhibited, whereas the mammalian βγ stimulated, GIRK4* currents. Currents measured at −80 mV are shown. Yeast or mammalian Gβ and Gγ expressed alone or co-expressed with the complimentary subunit of the other species did not show any significant effect on GIRK4* currents (*, p < 0.01, n = 6–9).
GIRK1 (F137S) or GIRK4 (S143T, GIRK4*) channels results in highly active G protein-sensitive K⁺ currents (34). Use of the modified homomeric channel simplifies the experimental design for structure-function studies and provides an efficient approach to addressing the contribution of each of the subunits of the Gβγ dimer in signaling to the channel. Co-expression of Gα subunits with GIRK channels inhibits basal current activity, presumably through binding free Gβγ subunits that are responsible for the basal activity (25).

In the present study, we have found that the G protein subunits most distantly related to the mammalian ones, the yeast Gβγ (Ste4/Ste18) (5), inhibit GIRK4* currents. Combining biochemical and electrophysiological approaches, we have screened a series of chimeras between Gβγ1 or Gγ2 and their yeast counterparts. A minimal region localized in the middle of the primary amino acid sequence of γ2 was identified to be necessary for activation of GIRK4* channels. Ten residue differences between Gγ2 and its yeast counterpart, most of which are clustered together in the Gγ2 three-dimensional structure, proved critical for channel activation. This cluster of residues is located close to Gβ residues that have been previously identified to be functionally important (35). These studies define a molecular surface that is critical for Gβγ effects on GIRK channels, suggesting that multiple residues of γ2 play a role in regulating GIRK4* currents.

**EXPERIMENTAL PROCEDURES**

**Construction of Various Recombinant Plasmids—**Yeast genomic DNA was isolated from DY150 cells (Clontech) according to standard protocol. cDNAs encoding Ste4 and Ste18 (GenBank™ accession numbers: M23982 and M23983) were amplified using PCR with Pfu DNA polymerase (Stratagene) and subclone into the Xenopus expression vector pGEMHE (36). Similarly, cRNAs for GIRK4*, Gβ1, and Gγ4 were also subcloned into pGEMHE. Gene splicing by overlap extension was used to generate the chimeric cDNAs between Gβi, and STE4 or Gγ2 and STE18.

**Heterologous Expression in Xenopus Oocytes—**Oocytes were harvested from *Xenopus laevis* frogs and were placed in OR2 solution (82.4 mM NaCl, 2.5 mM KCl, 1 mM MgCl₂, 5 mM HEPES, pH 7.6). Following a 3–4 time wash with OR2 solution, oocytes were subjected to collagenase treatment (2 mg/ml). Once ~50% of the oocytes were defolliculated (1.5–2.5 h), they were rinsed and placed in an ND96 solution (96 mM NaCl, 2.5 mM KCl, 1 mM MgCl₂, 5 mM HEPES, 1.8 mM CaCl₂, pH 7.6) and were supplemented with penicillin (100 units/ml) and streptomycin (100 µg/ml). Oocytes were kept at 17–18 °C before and after injection of cRNA until they were used for electrophysiological recordings or biochemistry.

All constructs were linearized with NheI or SplI (New England Biolabs), and in vitro transcription of cRNA was performed using Ambion’s mMessage mMachine kit (Ambion) with T7 polymerase. cRNAs were electrophoresed on 1% formaldehyde gels, and concentrations were estimated from two dilutions compared with the RNA marker (Invitrogen). cRNAs were dissolved in nuclease-free water to a concentration of 1 mg/ml. One ng of each cRNA was generally injected into the following approximate quantities: GIRK channel subunits, 1.0 ng/species; IRK1 channel, 1.0 ng; m2 receptor, 1.5 ng; Gβγ, 1.0 ng; Gγ4 subunit, 1.0 ng; Gγ4 subunit, 1.0 ng; STE4, 1.0 ng; STE18, 1.0 ng; chimeras and mutants, 1.0 ng.

**Electrophysiology—**Whole-oocyte currents were measured by conventional two-microelectrode voltage clamp using a GeneClamp 500 amplifier (Axon Instruments). Agarose cushion microelectrodes were used with resistances between 0.1 and 1.0 megohms (37). Oocytes were constantly superfused with a high potassium solution containing 91 mM KCl, 1 mM NaCl, 1 mM MgCl₂, 5 mM KOH/HEPES (pH 7.4). To block GIRK currents, the oocyte chamber was perfused with solutions containing 3 mM BaCl₂. Typically, oocytes were held at 0 mV, the potas-
amplitudes were measured at the end of the 200-ms pulse at each oocytes, whereas Ba\(^{2+}\)–insensitive currents. Basal current represents the difference between the control and the 3 min after application of 3 m M Ba\(^{2+}\)–insensitive currents. Among the 71 amino acids, 11 (in the gray background) were lysed with a glass homogenizer in lysis buffer (150 m M NaCl, 50 mM Tris, 1 mM EDTA, pH 7.5, supplemented with a protease inhibitor mixture (Sigma). Next, they were incubated on ice for 1 h. Following electrophoresis, the samples were transferred to nitrocellulose membranes (Millipore) using a semidry electroblotter (120 mV for 45 min at room temperature). The membranes were blocked with blocking buffer (50 m M Tris-HCl (pH 7.5), 80 m M NaCl, 0.1% Tween 20, 5% non-fat dry milk, and 0.02% NaN\(_3\)), and the protein was immunoblotted with anti-β\(_2\) polyclonal antibody (T-20, Santa Cruz Biotechnology). The bound antibody was detected by horseradish peroxidase-conjugated goat anti-rabbit IgG (Santa Cruz Biotechnology), and chemiluminescence was used to visualize the bands (Amersham Biosciences).

RESULTS

**Yeast β2 Inhibits GIRK4** Basal Currents—We set out to test the effect of yeast β2 subunits on GIRK4 channels. Oocytes were injected with cRNA for GIRK4*. We measured basal K\(^+\) currents using a two-electrode voltage clamp (Fig. 1A). As expected, co-expression of the channel with Gβ1/γ2 subunits resulted in stimulated inwardly rectifying currents, as

![Image](https://via.placeholder.com/150)

**Preparation of Crude Membranes**—The injected or uninjected oocytes were lysed with a glass homogenizer in lysis buffer (150 m M NaCl, 50 m M Tris, 1 m M EDTA, pH 7.5, supplemented with a protease inhibitor mixture (Sigma). Next, they were incubated on ice for 1 h. Following two rounds of low speed centrifugation (900 \(\times\) g for 5 min at 4 °C), the insoluble debris was removed, and the clear supernatant was subjected to high speed centrifugation (100,000 \(\times\) g for 45 min at 4 °C). The pellet was dissolved in lysis buffer with 1% Nonidet P-40 in a volume of 2 \(\mu\)l/oocyte.

**Immunoprecipitation, Trypsin Assay, and Immunoblotting**—Crude membranes were mixed with the FLAG (M2) agarose affinity gel (Sigma) at 4 °C overnight. Immune complexes with beads were washed three times and mixed with the lysis buffer. A limited trypan digestion assay of the FLAG-β\(_2\)γ\(_2\) complexes was performed as described previously (38) with small modifications. Briefly, the protein bound to the beads was directly subjected to trypsin digestion (L-1-tosylamido-2-phenylethyl chloromethylketone-treated trypsin, Sigma T-8642) at 37 °C for 30 min. The reaction was terminated by the addition of equal volume of sample buffer (100 m M Tris- HCl (pH 6.8) 4% SDS, 5% 2-mercaptoethanol, 0.2% bromphenol blue, and 20% glycerol) and boiiled for 5 min. After removal of the beads by a transient spin, the same amount of protein was loaded on a 12% SDS-polyacrylamide gel. Following electrophoresis, the samples were transferred to nitrocellulose membranes (Millipore) using a semidry electroblotter (120 m V for 45 min at room temperature). The membranes were blocked with blocking buffer (50 m M Tris-HCl (pH 7.5), 80 m M NaCl, 0.1% Tween 20, 5% non-fat dry milk, and 0.02% NaN\(_3\)), and the protein was immunoblotted with anti-β\(_2\) polyclonal antibody (T-20, Santa Cruz Biotechnology). The bound antibody was detected by horseradish peroxidase-conjugated goat anti-rabbit IgG (Santa Cruz Biotechnology), and chemiluminescence was used to visualize the bands (Amersham Biosciences).

**RESULTS**

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comparing with control (34). Expression of yeast Gβγ subunits significantly inhibited basal (Fig. 1A) but not agonist-dependent GIRQ4* currents (data not shown). The effects of yeast Gβγ subunits on GIRQ currents appeared similar to those of the mammalian Gβγγγ subunits (35, 39). In contrast, the inwardly rectifying K+ channel IK1, which is insensitive to G proteins (25, 40), was not affected by the presence of yeast Gβγ subunits (Fig. 1A). We next mixed and matched mammalian and yeast Gβ and Gγ subunits and tested their effects on GIRQ4* currents. Only Gβ and Gγ dimers of the same species showed either stimulatory (mammalian) or inhibitory (yeast) effects on GIRQ4* currents (Fig. 1B).

**Yeast Gγ and Mammalian Gγγγ Exert Opposite Effects on the Function of GIRQ4* Channels by Associating with a Yeast/Mammalian Gβ Chimeric.** To determine the molecular determinants of the functional difference between the yeast Gγ and the mammalian Gγγγ on the Gβγγγ-mediated stimulation of GIRQ4* channels, we constructed chimeras between the yeast Gβ and the mammalian Gβ and screened for stimulatory or inhibitory effects on GIRQ4* currents. One chimera, where the first 35 amino acids of the yeast Gβ were added to the intact mammalian Gβ, subunit (ymβ), could yield the expected effect on GIRQ channel activity upon association with either the mammalian or the yeast Gγ subunit. cRNAs of the ymβ chimera, GIRQ4* channels, the mammalian Gγγγ (mγγγ), or the yeast Gγ (γγγ) were co-injected into oocytes (Fig. 2A). The controls, mammalian Gβγγ (mβγ) or yeast Gβγγ (γβγ), were tested each time on the same batch of oocytes. Fig. 2B shows the agonist-independent (basal) current measured by expression of GIRQ4* alone or together with mammalian and or yeast Gβγ subunits. The ymβ chimera stimulated GIRQ4* currents upon co-expression with the mammalian Gγ and inhibited currents upon co-expression with the yeast Gγ. This result strongly suggested that the N-terminal first 35 amino acids of the yeast Gβ is sufficient to confer the inhibitory phenotype of the yeast Gβγγ to GIRQ4* currents, without affecting the stimulatory effect of the mammalian Gγγγ. Moreover, this result underscores the importance of the Gγ subunit in the Gβγγ complex as the same Gβ subunit (ymβ) was stimulatory, when complexed with the mammalian Gγ subunit, or inhibitory, when complexed with the yeast Gγ subunit. When stoichiometric amounts of both the mammalian and yeast Gγ were co-expressed with the ymβ chimera, no significant effect on GIRQ4* currents was observed. However, when progressively greater amounts of

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**Fig. 4.** Ten residues on Gγγ reduced the ability of Gβγ to stimulate GIRQ4* activity. In A, 33 mutants of Gγγ (spanning the region from residue 35 to 71 of the subunit) in which residues were replaced with the corresponding yeast Gγ subunits were screened by measuring basal currents at ~80 mV from oocytes co-expressing GIRQ4* and wild-type Gβ. Ten mutants shown in boldface (A35L, E42S, D48N, P49H, P53G, V54L, P55K, P60S, P61S, R62N) significantly reduced the ability of Gβγ to stimulate GIRQ4* currents as compared with oocytes expressing GIRQ4* alone and the channel with wild-type Gγγγ, respectively. The remaining 23 mutants activated the channel by forming dimer with Gβγ in a manner similar to control. Data were expressed as normalized basal current of the control GIRQ4* (*, p < 0.01, n = 8–25). B, limited trypsin protection assay for the identified Gγγ mutants, which were co-expressed with FLAG-Gβγ (Gβγγ) in oocytes. The crude membrane proteins of oocytes were subjected to precipitation by anti-FLAG antibody and incubated either with or without trypsin for 30 min at 37°C, as indicated. Digested proteins were subsequently analyzed by SDS-polyacrylamide gel electrophoresis and immunoblotted with Gγγγ that was labeled with V54L, P55K, P60S, F61S, R62N) significantly reduced the ability of Gβγγ to stimulate GIRQ4* activity.

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**Experimental Procedures.**

To determine the molecular determinants of the functional difference between the yeast Gγ and the mammalian Gγγγ on the Gβγγγ-mediated stimulation of GIRQ4* channels, we constructed chimeras between the yeast Gβ and the mammalian Gβ and screened for stimulatory or inhibitory effects on GIRQ4* currents. One chimera, where the first 35 amino acids of the yeast Gβ were added to the intact mammalian Gβγγ subunit (ymβ), could yield the expected effect on GIRQ channel activity upon association with either the mammalian or the yeast Gγ subunit. cRNAs of the ymβ chimera, GIRQ4* channels, the mammalian Gγγγ (mγγγ), or the yeast Gγ (γγγ) were co-injected into oocytes (Fig. 2A). The controls, mammalian Gβγγ (mβγ) or yeast Gβγγ (γβγ), were tested each time on the same batch of oocytes. Fig. 2B shows the agonist-independent (basal) current measured by expression of GIRQ4* alone or together with mammalian and or yeast Gβγ subunits. The ymβ chimera stimulated GIRQ4* currents upon co-expression with the mammalian Gγ and inhibited currents upon co-expression with the yeast Gγ. This result strongly suggested that the N-terminal first 35 amino acids of the yeast Gβ is sufficient to confer the inhibitory phenotype of the yeast Gβγγ to GIRQ4* currents, without affecting the stimulatory effect of the mammalian Gγγγ. Moreover, this result underscores the importance of the Gγ subunit in the Gβγγ complex as the same Gβ subunit (ymβ) was stimulatory, when complexed with the mammalian Gγ subunit, or inhibitory, when complexed with the yeast Gγ subunit. When stoichiometric amounts of both the mammalian and yeast Gγ were co-expressed with the ymβ chimera, no significant effect on GIRQ4* currents was observed. However, when progressively greater amounts of...
mammalian Gγ were expressed relative to the yeast Gγ, then a correspondingly greater stimulatory effect was obtained. These data suggest that the competition of the appropriate Gβγ for the channel sites yields either a stimulatory effect (the mammalian phenotype) or an inhibitory effect (the yeast phenotype).

The C-terminal Half of the Gγ2 Subunit Is a Crucial Contributor to the GIRK4* Current Stimulation by Gβ1γ2—To identify a minimal region of Gγ2 responsible for activating GIRK4* channel currents, chimeric constructs between the mammalian Gγ2 and the yeast Gγ were made based on a sequence comparison between the two subunits (showing a 15% identity between the Gγ2 and the yeast Gγ proteins) (Fig. 3A). Each of these Gγ chimeras was co-injected with Gβ1, and their effects on GIRK4* currents were compared with control. Gγ chimeras GS-2, GS-6, and GS-11 showed significant stimulation of GIRK4* currents upon co-expression with Gβ1. All these chimeras had in common the Gγ2 amino acid region between residues 35 and 50, suggesting that this region contains important determinants for the stimulatory effects of Gγ2 on GIRK4* currents. Since the GS-11 chimera did not produce as much GIRK4* current as the GS-2 chimera or the wild-type Gγ2, it is likely that the residue 51–71 region contains determinants in addition to those found in the residue 35–50 region that are important for maximal Gβγ stimulation of GIRK4* currents. Another chimera, GS-10 incorporating the unique 13-residue insert Lys-68–Val-80 in the yeast Gγ sequence onto the mammalian Gγ (m1–44y68–80m45–71), did not interfere with the ability of the Gβγ/GS-10 complex to stimulate GIRK currents (data not shown). Thus, the 13-residue insert did not appear to be responsible for the inhibitory effect of the yeast Gγ on the Gβγ complex with the mammalian Gγ subunit.

Ten Amino Acids of Gγ2 Reduce the Ability of Gβγ to Stimulate GIRK4* Currents—We proceeded to identify the specific amino acids within the residue 35–71 region of Gγ2 responsible for the Gγ contribution to the stimulatory effects of the Gβγ complex on GIRK4* currents. Each residue of Gγ2 was mutated to its counterpart in the yeast Gγ and was co-expressed with GIRK4* channels in oocytes. Whole-cell currents were recorded, allowing us to screen for the ability of each mutant to increase channel currents relative to the wild-type Gβ1 (Fig. 4A). Of the 33 mutants of Gγ2 tested, 23 mutants showed the ability to enhance GIRK4* currents to levels that were not significantly different from those of the wild-type Gβ1. The remaining 10 single-point mutants (A35L, E42S, D48N, P49H, P53G, V54L, P55K, P60S, F61S, and R62N) of Gγ2, however, significantly decreased the ability to activate the channel current but did not completely abolish stimulation of channel activity, implying that negation of the contribution from multiple residues of Gγ2 might be required for disrupting full stimulation of the GIRK channel by Gβ1γ2 subunits. The inability of the mutants to cause full Gβγ stimulation raises the question of whether the impairment of the channel function could be due to a change in the native conformation of Gβ1γ2. To test for such a possibility, we linked a FLAG epitope at the N-terminus of Gβ1 to distinguish it from endogenous Gβ and identified the expressed Gβ1 in oocytes by immunoprecipitation with an anti-FLAG antibody conjugated to agarose beads (Sigma). The FLAG-tagged Gβ1 showed similar stimulation of K+ currents as compared with the non-tagged Gβ1 co-expressed with Gγ2 (data not shown). For the pulled-down proteins, limited trypsin digestion was performed (9, 38). Fig. 4B shows that a typical band of 26 kDa was protected from trypsin digestion. In contrast, expression of Gβ1 alone failed to confer trypsin resistance, which is accomplished by proper association of the Gβ with a Gγ subunit. (Fig. 4B, lanes 3 and 4). These results confirm that the Gγ2 mutants that show significant functional impairment still assemble properly with wild-type Gβ1 to produce Gβγ-stimulated currents. The weaker enhancement of the channel currents by the Gγ mutants is likely to be caused by a functional impairment rather than a global structural defect.

Gγ Residues Co-localize with Gβ Residues, All of Which Are Critical for Mediating the Gβγ Effects on GIRK Channel Activity—To better understand the nature of interactions between Gγ2 and GIRK channels, we mapped the identified critical residues onto the known crystal structure of the Gβ1γ2 complex. Fig. 5A (left panel) shows a ribbon diagram of the Gβ1γ2 complex adapted from the published coordinates (5). Gβ1 is shown in white, and Gγ2 is shown in yellow. Identified residues on Gγ2 are colored in cyan. Except for Ala-35 and Glu-42, the remaining 8 identified residues cluster in the same region of the complex. The Gγ2 structure is composed of two α-helical domains and two loops. Eight clustered residues are in a loop in close proximity to the lipid modification that is used to anchor the protein to the membrane. All 7 of the critical residues are located around a groove that is formed from the interaction of Gβ and Gγ. Ser-67 in Gβ, which was previously identified to play an important role in controlling GIRK channel activity (35), resides on the side of this groove defined by the majority of the identified mutations. Several residues in this region maintain interactions between Gβ and Gγ.
functionally important residues onto the Gβγ structure clearly indicates that these residues are surface-accessible (Fig. 5, right panel). Furthermore, these residues form a continuous surface with Ser-67 on Gβ. Overall, the positioning of these residues defines a surface on the Gβγ structure that is critical in the activation of GIRK channels.

The D36T and D48N Combination Mutant of Gγ2 Abolishes Gβγ-mediated K+ Currents—Based on the above evidence that no single-point mutant of Gγ2 could totally abolish activation of the GIRK channel, we next sought to address whether multiple residues were required for the Gβγ-mediated stimulation of channel currents. It has been shown that three consecutive amino acids in Gγ2, Ala-Asp-Leu (residues 35–37), conferred the ability to assemble with Gβγ (38). Does the triple-point mutant also impair the ability of the Gβγ to assemble into a dimer and thus in turn retard channel activation? Fig. 6A shows that both the double (A35L/D36T) and triple Gγ2 mutant (A35L/D36T/D48N) stimulated channel function, suggesting that mutation of these Gγ2 residues did not interfere with Gβγ assembly. Interestingly, in screening 23 multiple-point mu-

**Fig. 6.** A double-mutation of Gγ2 (D36T/D48N) completely abolished the Gβγ-mediated basal GIRK4* (G4*) currents. In A, whole-cell basal currents were measured at -80 mV to assess the effects of various combinations of mutations identified in Fig. 4 and normalized relative to the GIRK4* control. Every combination that included mutations D36T and D48N (underlined) abolished Gβγ activation of the GIRK channels. This suggests that the co-existence of Asp-36 and Asp-48 in Gγ2 was critical for Gβγ-stimulated activity. Other combination mutants containing either D36T or D48N significantly enhanced K+ currents relative to control (*, p < 0.01, n = 8–13). In B, the double-point mutant of Gγ2 (D36T/D48N) together with wild-type Gβ failed to stimulate K+ currents relative to control (*, p < 0.01, n = 6–10). C, trypsin protection assays of uninjected oocytes or oocytes injected with cRNA for FLAG-βγ, alone, FLAG-β1γ2, and FLAG-β1γ2(D36T/D48N). Crude membranes incubated with anti-FLAG beads and with or without trypsin. Lanes 1 and 2 from uninjected oocytes showed no protein bands. Oocytes injected with the cRNA of FLAG-βγ (lanes 3 and 4) showed a 38-kDa band that was completely digested by trypsin. The typical 26-kDa C-terminal Gβ fragment was protected in both the FLAG-tagged Gβ1γ2 and the FLAG-tagged Gβ1γ2 (D36T/D48N) (lanes 6 and 8). The data shown are representative of three similar experiments.
Gγ Determinants in Gβγ Activation of GIRK Currents

tants, we found that only the mutants which involved both the D36T and D48N residues abolished Gβγ stimulation of K+ currents (e.g. A35L/D36T/L37I/D48N, A35L/D36T/L37I/D48N/F61S, and A35L/D36T/D48N but not A35L/D48N, L37I/D48N, etc.) (Fig. 6A). Residue D36T alone did not impair stimulation of GIRK4* currents significantly, whereas residue Asp-48 did significantly reduce but did not abolish the ability of Gβγ to stimulate GIRK4 currents. Thus, we proceeded to test the double-point mutant (D36T/D48N) of Gγ2, which displayed current levels similar to those of the unstimulated control channel (Fig. 6B). These results suggest that the combination of the Gγ2 mutations D36T and D48N impair the ability of the Gβγ subunits to stimulate GIRK4* activity. The trypsin protection assay revealed a normal pattern by the Gβγ2γ2 complex, indicating that the defect of the double mutant did not result from a gross structural impairment.

**DISCUSSION**

In mammals, 20 Gα, 5 Gβ, and 13 Gγ subunits have been identified so far (4, 7). The possible diversity for G protein heterotrimeric complex formation in mammals is therefore great. Yet in simpler organisms such as yeast (e.g. *Saccharomyces cerevisiae*), only a single Gβ (STE4), a single Gγ (STE18), and two Gα (GPA1 and GPA2) gene copies are found (41–43), and thus, the possibility or need for diversity is limited. Sequence comparison of mammalian versus yeast G protein subunits reveals lower levels of sequence conservation than those encountered among mammalian subunits (44). It has been shown previously that K+ currents are directly stimulated by the mammalian Gβγ2γ2 complex (18, 45) by binding both the N and C termini of GIRK channels (24−33). In the present study, we have demonstrated that yeast Gβγ2, unlike the mammalian Gβγ2γ2 displayed inhibition on basal GIRK currents. The inhibitory effect of the yeast Gβγ2 is analogous to that seen with Gβγ2γ2, which when overexpressed binds the channel and prevents stimulatory Gβγ from activating GIRK currents (35, 39).

Recent work aiming to identify the interaction sites between the Gβγ subunits and the channel has focused primarily on the interacting surfaces of the Gβ subunits with the channels or of Gβ subunits with Gα (35, 46−48). Gβγ subunits alone were shown to bind to GIRK channels, but they failed to enhance channel currents without forming a dimer with the Gγ2 subunits (49), suggesting the importance of the Gγ2 subunits on channel activation. Here, we added the 35 N-terminal end amino acids of the yeast Gβ onto the mammalian Gβ 1 protein and produced a chimeric Gβ protein. This chimera stimulated the GIRK current upon association with the mammalian Gγ subunit, whereas it inhibited this current upon association with the yeast Gγ subunit. This result strongly suggested that the turn-on (activation) or turn-off (inhibition) of channel activity depended on the conformations of different Gβγ complexes influenced by different Gγ subunits. This finding prompted us to utilize a chimeric strategy to identify the region of the mammalian Gγ responsible for the stimulation of GIRK currents.

Our chimeric approach revealed that the C-terminal half of Gγ2 was critical for the stimulatory contributions of the mammalian Gγ subunit on the Gβγ complex. Through site-directed mutagenesis, we identified 10 mutants that significantly reduced stimulation of K+ currents without affecting proper interactions with the Gβ subunit, as assessed by a trypsin protection assay. In a recent study, we identified three functionally important Gβγ residues that interact with GIRK channels (35). One of those, Ser-67, which does not interact with Ga subunits, affected basal K+ currents when mutated. Mutation of the neighboring Gγ2 (Arg-62) to amino acids with distinct chemical features, such as Ala, Glu, Lys, or Phe, did not produce a phenotype significantly different from that of the wild-type Gγ2 (data not shown). Thus, the changes obtained with mutation of the 7 Gγ2 residues seem rather specific and may affect interactions of the Gβγ2(Ser-67) and other associated Gβ residues with the GIRK channel. These results suggest that more than one residue of Gγ2 may be involved in the activation of the channel by the Gβγ2γ2 complex.

Some attention has recently been paid to the role of different Gγ isoforms in the interactions of the Gβγ complex with effectors (50). Yet it remains unclear how the Gγ subunits influence effectors through their interactions with Gβ subunits. In the present study, we identified the Gγ2 double-point mutant (D36T/D48N), which totally abolished Gβγ stimulation of GIRK currents, without affecting proper interactions with the Gβ subunit. This finding suggests not only that Gγ is required for the Gβγ activation of GIRK channels (49) but also that it modulates effector function by fine-tuning the effectiveness of the Gβγ dimer. Structural evidence has shown that residues Asp-36 and Asp-48 of Gγ2 could form putative salt bridge and hydrogen bonds with Gβγ (5). This may mean that the strict stereoechemical requirements in the hydrogen bond-driven interactions between Gβ and Gγ had not been reached in the conformation of the Gβγ2γ2/D48N mutant, despite the fact that there were 7 other residues of Gγ2 forming hydrogen bonds with Gβγ (5). This change may explain why the mutation attenuated stimulation of the channel activity by the Gβγ complex.

In summary, yeast Gβγ specifically inhibited GIRK4* basal currents in *Xenopus* oocytes, but not the Gγ2-insensitive IRK1 currents. The region (residues 35−71) of Gγ is required for full stimulation of GIRK4* currents by the Gβγ2γ2 subunits. Ten amino acid residues within this region of the Gγ subunit form an intricate network of interactions with Gβ, specifying a critical region of the Gβγ heterodimer that determines the stimulatory effects on the GIRK4 channel. Two amino acids of Gγ2 (Asp-36 and Asp-48), which interact with the Gβγ subunit by putative salt bridge and hydrogen-bonding interactions, maintain the fine conformation of the Gβγ2γ2 subunits required for full stimulation of GIRK4* activity. The precise interactions of these functionally critical residues that have been identified in the Gγ and Gγ subunits will have to await structural determination of the GIRK channel with the Gγ complex.

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