Binding of the G protein βγ subunit to multiple regions of G protein-gated inward-rectifying K^+ channels

Chou-Long Huang*, Yuh Nung Jan, Lily Y. Jan

Howard Hughes Medical Institute, Departments of Physiology and Biochemistry and Biophysics, the University of California at San Francisco, CA 94143-0724, USA

Received 31 January 1997

Abstract We have previously shown that direct binding of the βγ subunit of G protein (Gβγ) to both the N-terminal domain and the C-terminal domain of a cloned G protein-gated inward-rectifying K^+ channel subunit, GIRK1, is important for channel activation. We have now further localized the Gβγ binding region in the N-terminal domain of GIRK1 to amino acids 34–86 and the Gβγ binding region in the C-terminal domain of GIRK1 to two separate fragments of amino acids 318–374 and amino acids 390–462. Of the four cloned mammalian GIRK subunits, GIRK1–4, GIRK1 and 4 form heteromeric K^+ channels in the heart and similar channels in the brain include heteromultimers of GIRK1 and 2, and possibly other GIRK homomultimers and heteromultimers. We found that the N-terminal and the C-terminal domains of all four GIRKs bound Gβγ. The Gβγ binding activities for the C-terminal domains of GIRK2–4 were lower than that for the C-terminal domain of GIRK1. The higher Gβγ binding activity for the C-terminal domain of GIRK1 is due to amino acids 390–462 which are unique to GIRK1. We also found that the N-terminal and C-terminal domains of GIRKs interacted with each other, and the N-terminal domain of either GIRK1 or GIRK4 together with the C-terminal domain of GIRK1 exhibited much enhanced binding of Gβγ. These results are consistent with the idea that the N- and C-terminal domains of the cardiac G protein-gated K^+ channel subunits may interact with each other to form higher affinity binding site(s) for Gβγ.

Key words: Direct protein-protein interaction; *Weaver* mutation; G-protein-gated inwardly rectifying K^+ channel; Fusion protein; Gβγ binding; Glutathione-*S*-transferase

1. Introduction

Activation of G protein-gated inwardly rectifying K^+ channel by G protein-coupled receptors represents one mechanism of inhibitory synaptic transmission [1]. In the heart, activation of the muscarinic K^+ channel by acetylcholine results in slowing of the heart rate [2–4]. In the nervous system, activation of similar G protein-activated K^+ channels may mediate the inhibitory action of neurotransmitters such as somatostatin, adenosine, serotonin, opioid peptides and γ-aminobutyric acid ($GABA_B$) [1,5,6]. The βγ subunit of G protein (Gβγ) activates these K^+ channels via a membrane-delimited pathway that does not involve water-soluble cytoplasmic factors [7–9].

The cDNAs for G protein-gated inward-rectifying K^+ channel subunits, GIRK1–5, which share ~60% identity in amino acid sequence, have recently been isolated [10–14]. GIRK5 is cloned from *Xenopus* and no mammalian homologs of GIRK5 have been reported. While GIRK2 appears to form homomeric K^+ channels in oocyte and probably in dopamine neurons in the midbrain [15–19], G protein-activated K^+ channel in many tissues are likely heteromultimers of different subunits of the isoforms. The atrial muscarinic K^+ channel is a heteromultimer of GIRK1 and GIRK4 [13,20–22]. Neuronal G protein-activated K^+ channels include heteromultimers of GIRK1 and GIRK2 [15,19,20,23]. Indeed, a point mutation of the GIRK2 gene is associated with *weaver* mutation which causes loss of midbrain dopamine neurons as well as cerebellar neurons [24]. Heteromultimers of GIRK2 and 4 may also be functional K^+ channels [15,20]. The role of GIRK3 in the formation of the G protein-gated K^+ channels, however, is less clear. While several studies reveal no functional activity of homomultimers of GIRK3 or heteromultimers of GIRK3 and other GIRK subunits [12,15,20], other investigations reported functional expression of heteromultimers of GIRK1 and GIRK3 [16] and heteromultimers of GIRK3 and GIRK4 [21] and inhibition of GIRK2 channel activity by GIRK3 [16]. One possibility raised as a plausible explanation for this variability is that GIRK3 does not contain Gβγ binding sites and the activity of the heteromultimer depends upon the stoichiometry of GIRK3 in the heteromultimers [16].

To understand the mechanism for channel activation by Gβγ, it would be important to first identify regions of GIRK channel subunits that interact with Gβγ. Previously, we and others reported direct binding of Gβγ to both the N- and C-terminal domains of GIRK1 [25–27] and that binding of Gβγ to both domains of GIRK1 is physiologically important for channel activation [25,27–31]. Direct binding of Gβγ to GIRK4 has also been reported [32]. Whether GIRK2 and 3 also bind Gβγ, however, is not known. In the present study, we show that Gβγ binds to both the N- and C-terminal domains of GIRK2, GIRK3 and GIRK4. The Gβγ binding regions of GIRK1 have been further localized to amino acids (A.A.) 34–86 in the N-terminal domain, and A.A. 318–374 and A.A. 390–462 in the C-terminal domain of GIRK1. The amino acid sequences of the first two Gβγ binding regions of GIRK1 (34–86 and 318–374) are highly conserved among GIRK1–GIRK4. Furthermore, we found that the N-terminal domains of GIRK1 and GIRK4 interact with the C-terminal domain of GIRK1, and these interactions potentiate the binding of Gβγ to the channel fragments.

*Corresponding author (present address): University of Texas Southwestern Medical Center at Dallas, H5-112, MC 8856, 5323 Harry Hines Blvd, Dallas, TX 75235-8856, USA. Fax: (1) (214) 648-2071. E-mail: chuan1@mednet.swmed.edu

0014-5793/97/$17.00
PII S0014-5793(97)00197-X

2. Materials and methods

2.1. Materials

IPTG and molecular biological reagents were from Boehringer Mannheim. Glutathione 4B Sepharose beads, reduced glutathione and pGEX-2T vector were from Pharmacia. Iminodiacetic acid coupled-Sepharose beads charged with nickel ion were from Invitrogen. Donkey anti-rabbit IgG coupled with horseradish peroxidase and enhanced chemiluminescence (ECL) system for western blotting were from Amersham. Other chemicals including imidazole were from Sigma. *E. coli* DE 3 strain was used for fusion protein expression.

2.2. Construction of plasmid and expression of GST fusion protein

PGEX-2T vector was used for expression of recombinant bacterial fusion proteins. Nucleotide sequences corresponding to the peptide fragment of GIRK K^+ channel subunits of interest were amplified by polymerase chain reaction (PCR) using forward primer containing an in-frame *Bam*HI site immediately preceding the coding sequence of interest and reverse primer containing an *Eco*RI site following the coding sequence of interest. PCR products were subcloned into *Bam*HI and *Eco*RI sites of the pGEX-2T vector and confirmed by nucleotide sequencing. The in-frame start and stop codons within the pGEX-2T vector were used for protein translation. Expression of fusion proteins was induced by 0.4 mM IPTG at 37°C for 1–4 h as previously described [25]. Purification of GST fusion proteins containing GIRK fragments involve binding of the fusion proteins to glutathione 4B Sepharose beads and eluting with 10 mM reduced glutathione according to the Pharmacia product instruction manual as previously described [25,33]. Fusion proteins were dialyzed in phosphate-buffered saline (PBS) at 4°C and stored at −70°C. The purity and integrity of fusion proteins were checked by SDS-PAGE and Coomassie Blue staining. The concentration of fusion protein is determined using a Bio-Rad protein assay kit (Bradford).

2.3. Coprecipitation assay and western blotting

For binding of Gβγ to GST fusion proteins, purified bovine brain Gβγ (40 nM) [34] was incubated with GST fusion proteins at the indicated concentrations and 20 μl of glutathione Sepharose beads (suspended in an equal volume of PBS) in 50–100 μl of PBS containing 0.1% of the detergent Lubrol PX at 4°C for 30 min. Sepharose beads were spun down using a table-top micro-centrifuge and washed by resuspending beads in PBS followed by centrifugation. The wash with PBS was repeated 3 times. After the final wash, the Sepharose beads and precipitated proteins were resuspended in 20 μl of SDS-gel sample buffer and heated to 70°C for 10 min. Samples (10 μl) were separated by 10% SDS-PAGE and transferred to nitrocellulose membranes for western blotting and probing with specific antibodies and detection by ECL. Nitrocellulose filter containing precipitated proteins was stained with Ponceau S to verify that the appropriate amount of each fusion protein was precipitated by affinity beads in each experiment. The production and characterization of the polyclonal antibodies against GIRK1, GIRK4 and Gβ have been described previously [19,25,35].

For binding of Gβγ to H6-tagged fusion protein of GIRKs, Gβγ (40 nM) was incubated with GST-GKC-H6 at the indicated concentrations and 20 μl of iminodiacetyl-coupled Sepharose beads charged with nickel (nickel affinity beads). Imidazole, a competitive antagonist for binding to the nickel beads, was included in the reaction to prevent non-specific binding of proteins to the beads.

For binding of the N-terminal fusion protein to the C-terminal fusion protein, GST-GKN or GST-G4N (1 μM) was incubated with GST-GKC-H6 (200 nM) and 20 μl of nickel affinity beads in the presence of imidazole (50 mM). The apparent half-binding constant ($K_{1/2}$) for binding of GKN or G4N to GST-GKC-H6 was estimated to be $\sim 10^{-6}$ M according the following equation, $K_{1/2}$=[GST-GKN]$_{free}$/[GST-GKN–GST-GKC-H6]$_{bound}$ × [GST-GKC-H6]$_{free}$. The ratio of [GST-GKN]$_{free}$ to [GST-GKN–GST-GKC-H6]$_{bound}$ was determined from the immunoreactivity of GST-GKN in the supernatant vs. that of GST-GKN in the pellet containing the nickel affinity beads and precipitated proteins. When 1 μM GST−GKN was incubated with 200 nM GST-GKC-H6 and co-precipitated with nickel affinity beads, we found that the ratio of the immunoreactivity of GST-GKN in the supernatant to that in the pellet was ∼10:1. $K_{1/2}$ is, therefore, equal to 1.1 μM (910 nM [10/11 × 1000] of GST-GKN$_{free}$ divided by 90 nM [1/11 × 1000] of GST-GKN–GST-GKC-H6$_{bound}$ and multiplied by 110 nM [200−90] of GST-GKC-H6$_{free}$).

3. Results

3.1. Gβγ binding site in the C-terminal domain of GIRK1

Using the coprecipitation assay to detect interaction between purified Gβγ and hexahistidine (H6) tagged fusion proteins containing the fragment of GIRK1, we have previously shown that Gβγ binds to the C-terminal domain of GIRK1 [25]. Using glutathione-*S*-transferase (GST) fusion protein of GIRK1, Inanobe et al. [26] and Kunkel and Peralta [27] have also demonstrated binding of Gβγ to the C-terminal domain of GIRK1. Previously, we localized the Gβγ binding site on the C-terminal domain of GIRK1 to the region of A.A. 273–462. To define further the Gβγ binding region in the C-terminal domain of GIRK1, we made additional fusion proteins containing smaller fragments of GIRK1 and examined coprecipitation of these proteins with the purified Gβγ.

In analyzing a series of deletion constructs, we found that deletion of A.A. 273–317 (as in GST-GKC$_{318-462}$) did not affect binding of the fusion protein to Gβγ (Fig. 1). Further deletion of A.A. 318–339 (as in GST-GKC$_{340-462}$), however, reduced the Gβγ binding activity of the fusion protein, indicating that A.A. 318–339 either was part of the binding site or was important for the stability of the fusion protein. A fusion protein containing A.A. 180–374 (GST-GKC$_{180-374}$) showed reduced Gβγ binding activity. Amino acids from 374–384 of GIRK1 have been suggested to be important for binding with Gβγ based on its limited sequence homology with adenylate cyclase 2 [36]. We therefore made a GST fusion protein of the C-terminal domain of GIRK1 with an internal deletion of A.A. 375–389 (GST-GKC$_{180-374;\ 390-462}$ with the fragment of amino acids 180–374 fused to the fragment of amino acids 390–462) and found that the binding of Gβγ to this fusion protein was comparable to that of Gβγ to GST-GKC$_{180-462}$, indicating that A.A. 375–389 is not critical for the Gβγ interaction. Taken together, these results indicate that the regions of A.A. 318–374 and A.A. 390–462 of the C-terminal domain of GIRK1 are important for Gβγ binding. A smaller fusion protein containing A.A. 390–462 (GST-GKC$_{390-462}$), however, did not show detectable Gβγ binding activity (not shown). We do not know whether this fusion protein is folded properly. Previously, we have shown that a peptide derived from amino acids 434–462 of GIRK1 (peptide GC) [25] partially inhibited binding of Gβγ to the entire C-terminal domain of GIRK1. We found that peptide GC partially inhibited the binding of Gβγ to GST-GKC$_{340-462}$, but not that of Gβγ to GST-GKC$_{180-374}$ (Fig. 1C). This partial inhibition could be an indication that other amino acids besides 434–462 in the region of amino acids 390–462 are also important for Gβγ binding.

3.2. Gβγ binding activity of GIRK2–4

The Hill coefficient for Gβγ activation of atrial muscarinic K^+ channel is >1, indicating that more than one Gβγ is required for full activation of the G protein-gated K^+ channels [37]. Since the atrial and neuronal G protein-gated inward-rectifying K^+ channels may include heteromultimers of GIRK1 and 4 and heteromultimers of GIRK1 and 2, respectively, it would be important to determine whether GIRK2 and 4 also bind Gβγ. The findings that Gβγ can activate the

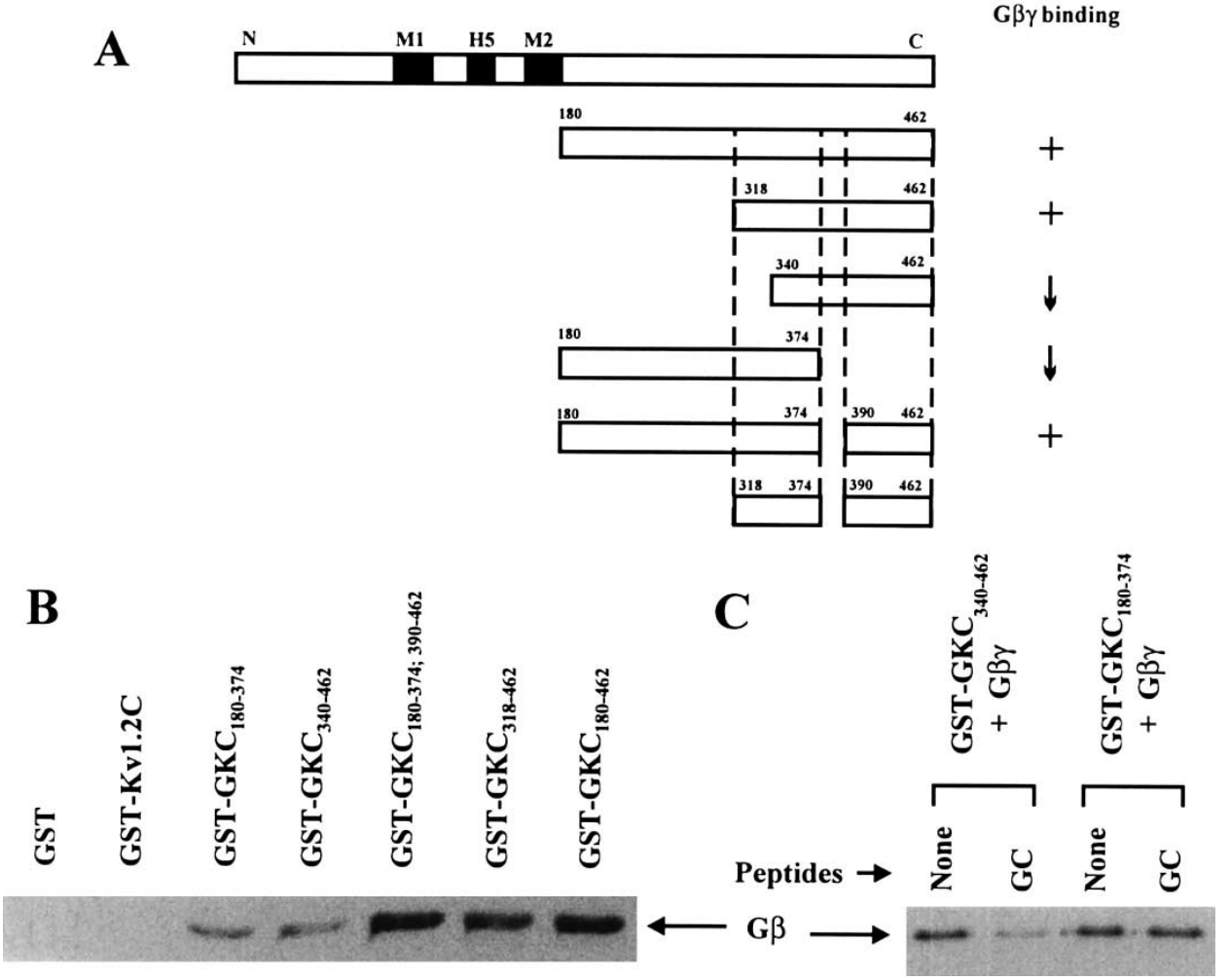

Fig. 1. Gβγ binding region in the C-terminal domain of GIRK1. (A) Schematic diagram of GST fusion protein constructs of the C-terminal domain of GIRK1 and Gβγ binding activities for these constructs. (+) Indicates maximal binding activity; (↓) denotes reduced binding activity. (B) Localization of Gβγ binding region. GST fusion proteins containing fragments of the C-terminal domain of GIRK1 or the control GST-K_V1.2C or GST alone (200 nM) were incubated with purified bovine brain Gβγ (40 nM) and processed for coprecipitation by glutathione-Sepharose beads. Filter was probed with antibodies against Gβ. The immunoreactivity for Gβ is indicated by the arrow. Similar results were observed in 3 other experiments. (C) Partial inhibition of the binding of Gβγ to GST-$GKC_{340-462}$ but not the binding to GST-$GKC_{180-374}$ by peptide GC. Binding of Gβγ to GST-$GKC_{340-462}$ or GST-$GKC_{180-374}$ was examined in the presence or absence of 100 μM peptide GC. Peptide GC contains amino acids 434–462 of GIRK1. A similar result was observed in another experiment.

homomeric GIRK2 or GIRK4 channels [12,13,16–19] suggest that both GIRK2 and GIRK4 contain Gβγ binding site. Given that the region of A.A. 318–374 of GIRK1 is highly conserved in GIRK1–4 (Fig. 2A), we wonder whether the C-terminal domain of GIRK2–4 would also interact with Gβγ. Indeed, GST fusion proteins containing the C-terminal domains of GIRK2 (GST-G2C), GIRK3 (GST-G3C), and GIRK4 (GST-G4C) all bound Gβγ (Fig. 2B). The Gβγ binding activity of GST-G2C, GST-G3C or GST-G4C is equivalent to that of GST-$GKC_{180-374}$, but is less than that of GST-$GKC_{180-462}$. The control fusion protein containing the C-terminal domain of IRK1, GST-IKC, did not show significant binding to Gβγ. The greater Gβγ binding activity of GST-$GKC_{180-462}$ could be accounted for by the finding that both the regions of A.A. 318–374 and A.A. 390–462 of the C-terminal domain of GIRK1 contribute to Gβγ binding activity.

3.3. Gβγ binding site in the N-terminal domain of GIRK1–4

The N-terminal domain of GIRK1 (GKN_{1-86}) binds Gβγ with a lower affinity as compared to the C-terminal domain [25]. To further isolate the region which contains the Gβγ binding activity of the N-terminal domain of GIRK1, we tested GST-GKN_{1-33} and GST-GKN_{34-86} for their abilities to bind Gβγ. The fusion protein GST-GKN_{34-86} bound Gβγ, but not as well as the full-length GST-GKN_{1-86} did. In contrast, GST-GKN_{1-33} did not demonstrate significant binding to Gβγ (Fig. 3). As the A.A. sequence in GST-GKN_{34-86} of GIRK1 is conserved in GIRK1–4 (Fig. 4A), we examined the interaction between Gβγ and the N-terminal domain of GIRK2 (GST-G2N), GIRK3 (GST-G3N) or GIRK4 (GST-G4N). GST-G2N, GST-G3N and GST-G4N bound Gβγ as well as GST-GKN_{1-86}, while the control N-terminal domain of IRK1 (GST-IKN) did not bind Gβγ (Fig. 4B). Thus, the N-terminal domains of GIRK1–4 bound Gβγ.

3.4. The N-terminal and C-terminal domains of GIRKs interact with each other

Our finding of Gβγ binding to both the N- and C-terminal

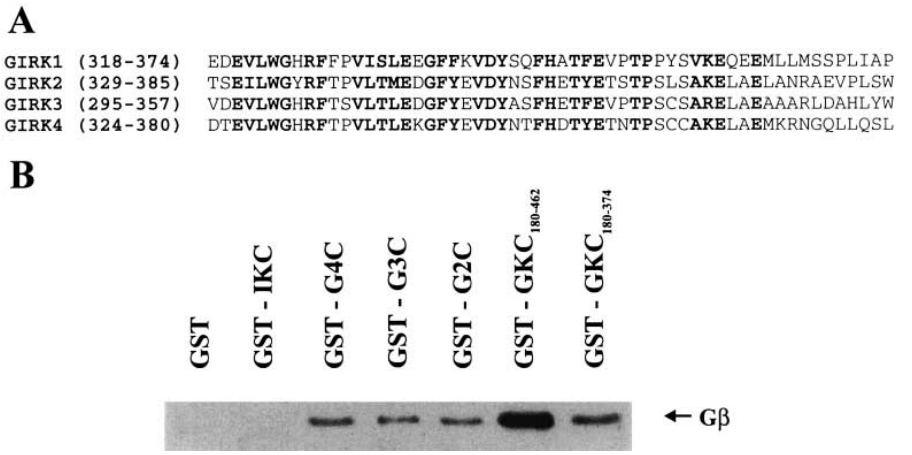

Fig. 2. Binding of Gβγ to C-terminal domains of GIRK2–4. (A) Sequence comparison between amino acid 318–374 of GIRK1 and homologous regions of GIRK2, 3 and 4. The number in parentheses ( ) indicates amino acids for each channel shown for comparison. Amino acids are shown in single-letter code. Letter in bold indicates identity or similarity of amino acids among GIRK1–4. (B) Co-precipitation of Gβγ with C-terminal domains of GIRK1–4. GST fusion protein containing C-terminal domain of GIRK1 (GST-$GKC_{180-462}$; GST-$GKC_{180-374}$), GIRK2 (GST-G2C), GIRK3 (GST-G3C) or GIRK4 (GST-G4C) (200 nM) as indicated were incubated with Gβγ (40 nM) and processed for co-precipitation as described in Fig. 1 and Section 2. Filter was probed with antibodies against Gβ. The immunoreactivity for Gβ is indicated by the arrow. Similar results were observed in 2 other experiments.

domains of the GIRKs raises the possibility that the two domains may interact with each other to form a stronger binding site for Gβγ. We therefore looked for interaction between GST-GKN and GST-GKC using the coprecipitation assay. A GST-GKC-H6 fusion protein was constructed by adding a hexahistidine affinity tag to the C-terminus of GST-$GKC_{180-462}$. A control GST-H6 was also constructed. By incubating a solution containing 1 μM GST-GKN and 200 nM of either GST-GKC-H6 or GST-H6 with nickel affinity beads, we found that GST-GKN coprecipitated with

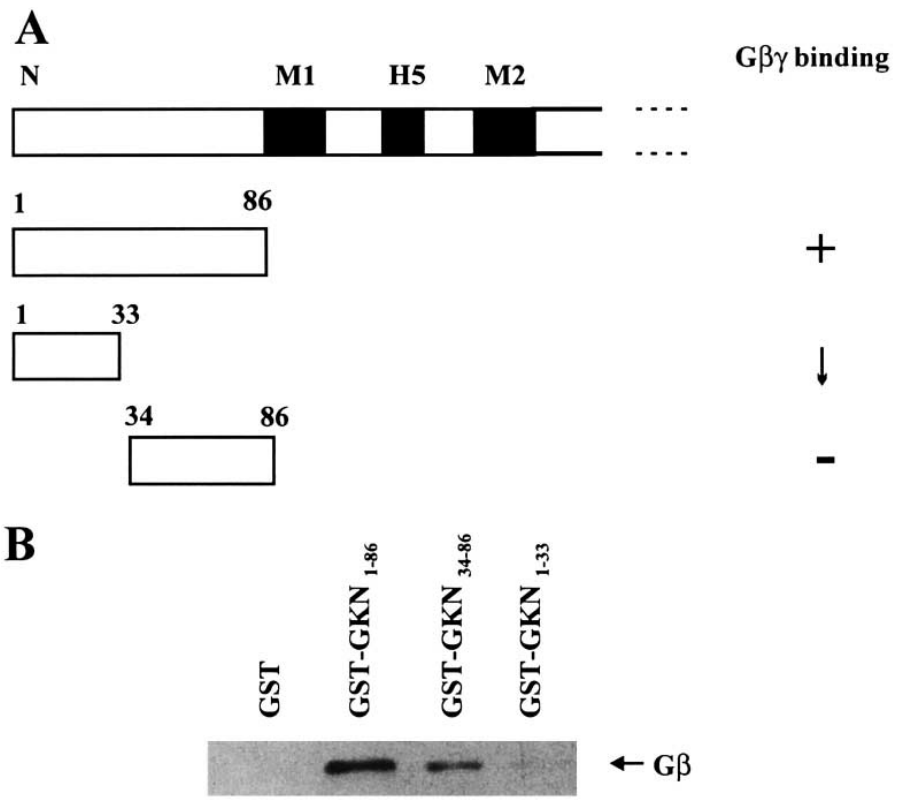

Fig. 3. Gβγ binding region in the N-terminal domain of GIRK1. (A) Schematic diagram of GST fusion protein constructs of the N-terminal domain of GIRK1 and Gβγ binding activities for these constructs. (+) Indicates maximal activity; (↓) denotes reduced activity; (–) indicates no detectable activity. (B) Localization of Gβγ binding region. GST fusion proteins containing fragments of N-terminal domain of GIRK1 as indicated (200 nM) or the control GST alone were incubated with Gβγ (40 nM) and processed for coprecipitation. Nitrocellulose filter was probed with antibodies against Gβ. The immunoreactivity for Gβ is indicated by the arrow. Similar results were observed in 2 other experiments.

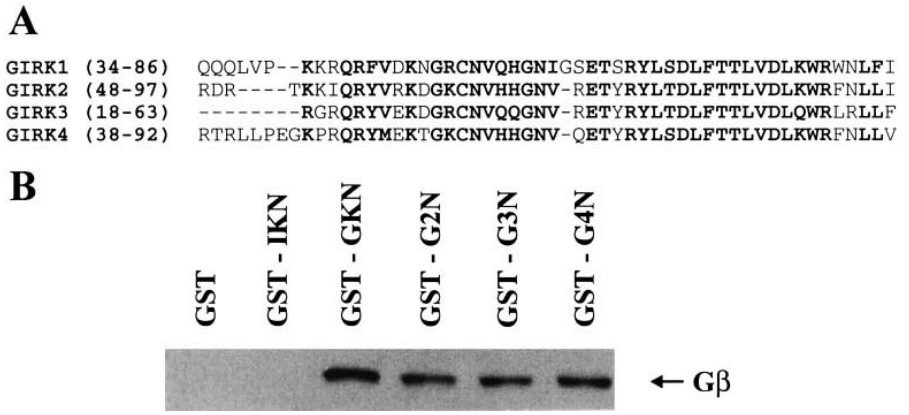

Fig. 4. Binding of Gβγ to N-terminal domains of GIRK2–4. (A) Sequence comparison between amino acids 34–86 of GIRK1 and homologous regions of GIRK2–4. The number in parentheses ( ) indicates amino acids for each channel shown for comparison. Amino acids are shown in single-letter code. Letter in bold indicates identity or similarity of amino acids among GIRK1–4. (B) Co-precipitation of Gβγ with N-terminal domains of GIRK1–4. GST fusion proteins containing fragments of the N-terminal domain of GIRK1 (GST-GKN), GIRK2 (GST-G2N), GIRK3 (GST-G3N) or GIRK4 (GST-G4N) (200 nM) as indicated were incubated with Gβγ (40 nM) and processed for co-precipitation. Filter was probed with antibodies against Gβ. The immunoreactivity for Gβ is indicated by the arrow. Similar results were observed in 3 other experiments.

GST-GKC-H6, but not with GST-H6 (Fig. 5A), indicating a specific protein-protein interaction between the N- and C-terminal domains of GIRK1. The fusion protein GST-G4N also coprecipitated specifically with GST-GKC-H6 (5B), indicating that the C-terminal domain of GIRK1 also interacts with the N-terminal domain of GIRK4, consistent with the previous reports that GIRK1 and GIRK4 form heteromultimer in the heart [13].

To see if the interaction of the N-terminal domain with the C-terminal domain of the channel alters the binding of Gβγ to the channel fragments, we used nickel affinity beads to precipitate GST-GKC-H6, which was present at 50, 100 or 200 nM together with GST, GST-GKN or GST-G4N and with or without Gβγ. GST-GKN or GST-G4N coprecipitated with GST-GKC-H6 in a dose-dependent manner using nickel affinity beads (not shown). In the presence of GST, GST-GKN or GST-G4N, Gβγ coprecipitated with increasing concentrations of GST-GKC-H6 in a dose-dependent manner (Fig. 6). However, the amount of Gβγ coprecipitating with 100 nM GST-GKC-H6 in the presence of 1 μM GST-GKN or GST-G4N was equivalent to that with 200 nM GST-GKC-H6 in the presence of 1 μM control GST. This increase in the binding of Gβγ to GST-GKC-H6 is more than an additive effect of the binding of Gβγ to GST-GKC-H6 and that of Gβγ to GST-GKN or GST-G4N. The N-terminal domain has a lower affinity for binding Gβγ [25]. Given that at most one tenth of the GST-GKN or GST-G4N coprecipitated with GST-GKC-H6 using nickel affinity beads under the experimental condi-

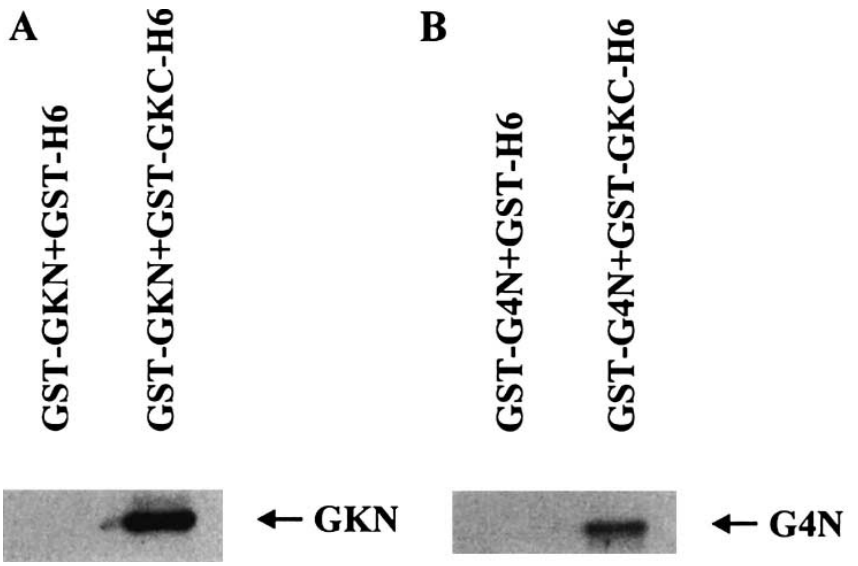

Fig. 5. Interaction between the C-terminal domain of GIRK1 and the N-terminal domain of GIRK1 (A) or GIRK4 (B). (A) GST-GKN (1 μM) was incubated with GST-GKC-H6 or the control GST-H6 (200 nM) and processed for coprecipitation by nickel affinity beads. Nitrocellulose membrane was probed with antibodies against the GST-GKN. The immunoreactivity for GST-GKN is indicated by arrow. Similar results were obtained in 2 other experiments. (B) GST-G4N (1 μM) was incubated with GST-GKC-H6 or the control GST-H6 (200 nM) and processed for coprecipitation by nickel affinity beads. Filter was probed with antibodies against GST-G4N. The immunoreactivity for GST-G4N is indicated by the arrow. Similar results were observed in 2 other experiments.

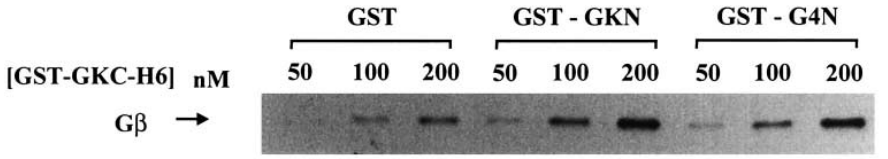

Fig. 6. Enhanced binding of Gβγ to GST-GKC-H6 in the presence of GST-GKN or GST-G4N. GST-GKC-H6 (50, 100 or 200 nM) were incubated with Gβγ (40 nM) in the presence of 1 μM GST (left panel), GST-GKN (middle panel) or GST-G4N (right panel) and processed for coprecipitation by nickel affinity beads. Filter was probed with antibodies against Gβ. The immunoreactivity for Gβ is indicated by the arrow. A similar result was observed in another experiment.

tions (see Section 2), the amount of Gβγ predicted to be associated with GST-GKN or GST-G4N in the precipitate would be negligible (< 5% of the observed level). Thus, the observed ~2-fold increase in Gβγ binding to GST-GKC-H6 in the presence of GST-GKN or GST-G4N indicates a synergistic effect. This potentiation of Gβγ binding due to the interaction of the N- and C-terminal domains could arise from the formation of a stronger Gβγ binding site that is contributed by both N- and C-terminal domains. Alternatively, it could reflect an allosteric effect between the N- and C-terminal domains. Further studies are necessary to determine whether this synergistic effect occurs in the heteromeric channels in the heart and, if it does, whether this interaction takes place between the N- and C-terminal domains of the same or adjacent subunits.

4. Discussion

By studying coprecipitation of Gβγ with a series of fusion proteins containing fragments of GIRK channel subunits, we have found that all four mammalian GIRKs contain Gβγ binding regions and further identified regions of GIRKs that are important for Gβγ binding. Based upon the findings that fusion proteins GST-$GKC_{180-462}$, GST-$GKC_{318-462}$, GST-$GKC_{180-374;\ 390-462}$, GST-$GKC_{180-374}$ and GST-$GKC_{340-462}$ all bound Gβγ and the binding activities for GST-$GKC_{180-462}$, GST-$GKC_{318-462}$ and GST-$GKC_{180-374;\ 390-462}$ were equal but greater than the activity for GST-$GKC_{340-462}$ or GST-$GKC_{180-374}$, we conclude that both the fragments of A.A. 318–374 and A.A. 390–462 contribute to the Gβγ binding activity in the C-terminal domain of GIRK1 (Fig. 1). The existence of Gβγ binding site(s) in the fragment of A.A. 318–374 is evident from the finding that both GST-$GKC_{180-374}$ and GST-$GKC_{318-462}$ bound Gβγ. Addition of amino acids 390–462 to the fragment of A.A. 318–374 (as in GST-$GKC_{180-374;\ 390-462}$ or GST-$GKC_{318-462}$) markedly enhanced the Gβγ binding activity of the fusion protein, either by providing an additional Gβγ binding site or by increasing the stability of the fusion protein. The finding that a peptide corresponding to A.A. 434–462 of GIRK1 partially inhibited the binding of Gβγ to GST-$GKC_{340-462}$ suggests that the fragment of A.A. 390–462 also contains binding site(s) for Gβγ. Nevertheless, as GST-$GKC_{390-462}$ did not show detectable Gβγ binding, it remains possible that A.A. 390–462 contributes to the Gβγ binding by stabilizing the conformation of the fusion protein.

With respect to the N-terminal domain, we found that amino acids 34–86 of GIRK1 bound Gβγ, but its binding activity is lower compared to that of the full-length GKN_{1-86}. The region of A.A. 1–33 may contribute to the maximal Gβγ binding activity of the N-terminal domain through providing an additional binding site or via stabilizing the conformation of the N-terminal domain. The finding that the N-terminal domains of GIRK2–4 (which have significant sequence homology to GIRK1 in the region of A.A. 34–86) bound Gβγ as well as GST-GKN_{1-86} is consistent with the observation that the region of A.A. 34–86 of GIRK1 binds Gβγ. Our previous finding of partial inhibition of the binding between GKN_{1-86} and Gβγ by a peptide (GN) derived from A.A. 1–38 of the N-terminal domain of GIRK1 [25], however, indicates that A.A. 1–38 may have weak interaction with Gβγ. Alternatively, GN peptide may interact with the N-terminal domain and thereby interfere with the binding of Gβγ to GST-GKN_{1-86}.

We found that all 4 GIRK isoforms exhibit Gβγ binding activity on both the N- and C-terminal domains of the channel proteins. G protein-gated inward-rectifying K^+ channels can be comprised of identical or similar GIRK subunits. The heteromultimers of GIRK1 and GIRK4, and GIRK1 and GIRK2 correspond to some of the cardiac muscarinic G protein-gated inward-rectifying K^+ channels, I_{KACh} [13] and the G protein-gated K^+ channel in the brain [16,20], respectively. While the homomultimers of GIRK2 or GIRK4 are functional G protein-gated K^+ channels [13,16,18,19], the homomultimers of GIRK1 or GIRK3 do not give rise to K^+ current [13,16,21]. Our results indicate that the inability of the homomultimers of GIRK1 or GIRK3 to produce K^+ current is not due to a lack of Gβγ binding domain.

Using fusion proteins containing fragments of channel proteins, we found that the Gβγ binding activities for the N-terminal domain of GIRK1–4 are roughly equal and that for the C-terminal domain of GIRK1 is greater than the binding activity for the C-terminal domain of GIRK2, 3 or 4. Binding of Gβγ to both the N- and C-terminal domains of GIRK1 is important for channel activation [25,29,31]. It thus seems likely that binding of Gβγ to both the N- and C-terminal domains of GIRK2, 3 or 4 is also important for channel activation, as the Gβγ binding regions of GIRK2–4 are homologous to regions of A.A. 34–86 and A.A. 318–374 of GIRK1. The relative contribution of each region to the overall binding of Gβγ to the full-length polypeptide or the native multimeric channel, however, is unknown. A previous study using the full-length GIRK1 and GIRK4 proteins expressed in Sf 9 cells reported that the Gβγ binding activity for GIRK4 is slightly greater than that for GIRK1 [32]. Since we do not know how the individual Gβγ binding region on the N- or the C-terminal domain folds together to form the Gβγ binding site in the full-length protein, it is difficult to compare the results of the two studies. Nevertheless, our investigation

clearly demonstrates that residues that are important for Gβγ binding are distributed on both the N- and C-termini of the channel.

It is possible that the Gβγ binding regions on the N- and C-terminal cytoplasmic domains are interrelated. The two cytoplasmic domains interact with each other, and this interaction potentiates the binding of Gβγ to the channel. This finding of synergistic action between the N- and C-terminal domains in the activation of channel by Gβγ is supported by a recent report by Tucker et al. [31]. In that study, they made chimera channels containing the transmembrane domain of the G protein-insensitive subunit $K_{ir}4.1$ and the N-terminal, the C-terminal or both domains of GIRK1, and examined the role of these cytoplasmic domains in activation of channel by Gβγ. They found that K^+ current through chimeric channels containing the N- or the C-terminal domain of GIRK1 was stimulated by Gβγ by ~1.5- or ~2-fold, respectively; K^+ current through chimeric channels containing both the N- and C-terminal domains of GIRK1, in contrast, was maximally stimulated by Gβγ by >7-fold. Our results of a synergistic effect between the N- and C-terminal domains for binding Gβγ reinforce this physiological finding using chimeric channels.

In addition to the G protein-gated inwardly rectifying K^+ channels, Gβγ regulates many other effectors including phospholipase C-β, phospholipase A_2, type 2 adenylate cyclase, β-adrenergic receptor kinase (βARK), and Ca^{2+} channel [38]. The Gβγ binding sites for βARK [33] and phosducin [39] have been mapped. The consensus sequence, if any, for binding Gβγ is still elusive. Having found that synthetic peptide derived from A.A. 956–982 of adenylate cyclase type 2 inhibits Gβγ activation of cyclase, phospholipase C-β, βARK and GIRK K^+ channel, Chen et al. [36] proposed a consensus sequence for Gβγ binding. Our result using the fusion protein with deletion of A.A. 375–389 does not support the idea that residues Asn-X-X-Glu-Arg at A.A. 378–382 are crucial for GIRK1 binding with Gβγ. It is possible that other Gβγ binding regions of GIRK1, such as the N-terminal domain, may be more homologous to the region of A.A. 956–982 of adenylate cyclase type 2 [40].

Recently, the 3-dimensional crystal structures of the βγ dimer of the G protein [41] and the αβγ trimer [42,43] have been solved. The β subunit consists of a seven-blade β-propeller and is partially encircled by the γ subunit. The interaction between the α subunit and the βγ dimer alters the conformation of the α but not of the βγ subunits. Future studies on the structure of the βγ-effector co-crystals will undoubtedly provide invaluable insights into the interaction between Gβγ and its effectors.

Acknowledgements: We thank Mei Yu for excellent technical assistance, Drs. A. Gilman and S. Mumby for Gβ antibodies, Dr. P. Casey for purified bovine brain Gβγ, and Dr. P. Slesinger for discussion on the project. C.L.H. was supported by an AHA postdoctoral fellowship. Y.N.J. and L.Y.J. are Howard Hughes Medical Institute Investigators.

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