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G α_{16} interacts with tetratricopeptide repeat 1 (TPR1) through its $\beta 3$ region to activate Ras independently of phospholipase C β signaling

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Abstract

Background: G protein-coupled receptors constitute the largest family of cell surface receptors in the mammalian genome. As the core of the G protein signal transduction machinery, the G α subunits are required to interact with multiple partners. The GTP-bound active state of many G α subunits can bind a multitude of effectors and regulatory proteins. Yet it remains unclear if the different proteins utilize distinct or common structural motifs on the G α subunit for binding. Using G α_{16} as a model, we asked if its recently discovered adaptor protein tetratricopeptide repeat 1 (TPR1) binds to the same region as its canonical effector, phospholipase C β (PLC β).

Results: We have examined the specificity of G α_{16} /TPR1 association by testing a series of chimeras between G α_{16} and G α_z . TPR1 co-immunoprecipitated with G α_{16} and more tightly with its constitutively active G α_{16} QL, but not G α_z . Progressive replacement of G α_{16} sequence with the corresponding residues of G α_z eventually identified a stretch of six amino acids in the $\beta 3$ region of G α_{16} which are responsible for TPR1 interaction and the subsequent Ras activation. Insertion of these six residues into G α_z allowed productive TPR1-interaction. Since the $\beta 3$ region only minimally contributes to interact with PLC β , several chimeras exhibited differential abilities to stimulate PLC β and Ras. The ability of the chimeras to activate downstream transcription factors such as signal transducer and activator of transcription 3 and nuclear factor κ B appeared to be associated with PLC β signaling.

Conclusions: Our results suggest that G α_{16} can signal through TPR1/Ras and PLC β simultaneously and independently. The $\beta 3$ region of G α_{16} is essential for interaction with TPR1 and the subsequent activation of Ras, but has relatively minor influence on the PLC β interaction. G α_{16} may utilize different structural domains to bind TPR1 and PLC β .

Background

Heterotrimeric guanine nucleotide-binding proteins (G proteins) are multifaceted signaling modules that relay extracellular signals detected by G protein-coupled receptors (GPCRs) to intracellular effector [1-3]. At the core of the G protein signal transduction machinery is the G α subunit, a GTPase which acts as a timer to limit the activation signal. In the classical G protein activation cycle, the G α subunit needs to associate with the G $\beta\gamma$ dimer, the GPCR, and effectors separately or simultaneously at different stages of the cycle. A variety of

accessory proteins are now known to modulate the fidelity of the G protein signal. They include regulators of G protein signaling (RGS) [4], activators of G protein signaling (AGS) [5], and adaptor proteins such as tetratricopeptide repeat 1 (TPR1) [6]. These additional components allow for rapid inactivation or receptor-independent activation of the G α subunit, as well as signal diversification. The large number of different types of binding partners for the G α subunit requires optimal utilization of structural domains that are available for protein-protein interactions. Given that G α subunits are typically less than 50 kDa in size and are attached to the inner leaflet of the plasma membrane, the binding surfaces available for interaction are limited. Nature has partially resolved this constraint by generating different

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conformations of the $G\alpha$ subunit through the binding and hydrolysis of GTP.

The resolution of the crystal structures of several $G\alpha$ subunits in their GDP- or GTP-bound states [7,8] and as complexes with the $G\beta\gamma$ dimer [9-11] or other interacting proteins [12-17] have provided valuable insight into the molecular mechanisms of G protein signal transduction. Structurally, the $G\alpha$ subunit can be broadly divided into the GTP hydrolase (GTPase) and helical domains (Figure 1) with the former harboring the GTP-binding pocket [18,19]. Several regions (Switch I-IV) spreading across the GTPase and helical domains exhibit profound conformational changes when the $G\alpha$ subunit shifts between the GDP- and GTP-bound states [20,21]. Changes in the switch regions provide the molecular basis of G protein activation and effector regulation. In the GTP-bound active state, the $G\alpha$ subunit releases the $G\beta\gamma$ dimer and thus allows effectors to bind to the newly exposed surfaces such as the Switch II region [20,21]. This simplistic view, however, cannot accommodate the increasing numbers of $G\alpha$ -interacting proteins. The activated $G\alpha$ subunit is a preferred partner for multiple effectors, adaptors, and RGS proteins. A central question is whether an activated $G\alpha$ subunit can concurrently regulate multiple signaling pathways by simultaneously binding to different partners in much the same way as an inactive $G\alpha$ subunit forms a complex with the $G\beta\gamma$ dimer and the receptor.

Among the different subfamilies of $G\alpha$ subunits, members of the $G\alpha_q$ subfamily have the capacity to activate phospholipase C β (PLC β) [22,23] as well as interact with the guanine nucleotide exchange factor p63Rho-GEF [24,25], G protein-coupled receptor kinase GRK2 [26-28], adaptor proteins such as TPR1 [6], and several RGS proteins [29-32]. These molecules bind to overlapping as well as distinct regions on $G\alpha_q$ (Figure 1). It is often assumed that the primary signal generated by G_q -coupled receptors is the formation of inositol trisphosphates (IP $_3$) by PLC β , and that the regulation of downstream kinases and transcription factors are consequential to the production of IP $_3$ and the subsequent Ca $^{2+}$ mobilization. However, recent studies suggest that $G\alpha$ subunits can concurrently regulate multiple signaling pathways. The ability of $G\alpha_{16}$, a member of the $G\alpha_q$ subfamily, to interact with the adaptor protein TPR1 [6] has raised some interesting scenarios. $G\alpha_{16}$ is primarily expressed in hematopoietic cells and it can regulate multiple signaling pathways [25,33]. Interestingly, TPR1 can directly interact with Ras especially when the latter is activated [6] and it appears to link $G\alpha_{16}$ to Ras activation [34]. Ras is a small GTPase which acts as a molecular switch for linking various cell surface receptors to intracellular signaling pathways, resulting in cell proliferation and differentiation [35].

We have recently demonstrated that constitutively active $G\alpha_{16}$ ($G\alpha_{16}QL$) induces the phosphorylations of transcription factors, such as signal transducer and activator of transcription 3 (STAT3) and nuclear factor κB (NF κB), through PLC β and Ras/Raf-1/MEK/ERK signaling cascades in human embryonic kidney 293 (HEK 293) cells [36,37]. However, it is not known if the binding of TPR1 to $G\alpha_{16}$ affects PLC β signaling and whether TPR1 and PLC β utilize the same docking site on $G\alpha_{16}$. Although $G\alpha_{16}$ has been shown to interact with the C-terminus of TPR1 [6], the structural requirement for $G\alpha_{16}$ to interact with TPR1 has yet to be defined. In the present study, we examined the structural domain of $G\alpha_{16}$ for interacting with TPR1 and assessed whether the same domain is responsible for regulating PLC β .

Methods

Materials

The human cDNAs of $G\alpha_{16}$, $G\alpha_{16}QL$, $G\alpha_z$ and $G\alpha_zQL$ were obtained from Missouri S&T cDNA Resource Center. C25 and C44 cDNAs were previously constructed and characterized [38]. Cell culture reagents, including LipofectamineTM and PlusTM reagents, and AccuPrimeTM Pfx SuperMix were purchased from Invitrogen (Carlsbad, CA). Anti- $G\alpha_{16}$ (N-terminus) and anti- $G\alpha_z$ (C-terminus) were obtained from Gramsch Laboratories (Schwabhausen, Germany). Anti- $G\alpha_{16}$ (C-terminus) was purchased from Torrey Pines Biolabs (East Orange, NJ). Anti- $G\alpha_z$ (N-terminus) and PLC β antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Unbound and affinity gel-conjugated anti-FLAG antibody were from Sigma-Aldrich (St. Louis, MO). Other antibodies were purchased from Cell Signaling Technology (Danvers, MA). Protein G-agarose and protein cross-linking agent dithiobis[succinimidylpropionate] were from Pierce Biotechnology (Rockford, IL). ECL kit was from GE Healthcare Bio-Sciences (Piscataway, NJ). Ras activation kit was a product of Millipore (Billerica, MA).

Construction of chimeras

$G\alpha$ chimeras were constructed from the cDNAs encoding the human $G\alpha_{16}$ and $G\alpha_z$ by using PCR techniques. The N-terminal 102, 155, 188, 200, 210, 246, 266 and 295 residues of $G\alpha_{16}$ were substituted by the corresponding regions of $G\alpha_z$ to generate N102, N155, N188, N200, N210, N246, N266 and N295 chimeras, respectively. Primer pairs were designed to cover the overlapping regions in forward and reversed directions. For each construct, the 5' fragment was generated with the reversed and T7 primers, whereas the 3' fragment was made with the forward and SP6 primer. The two half-products were then annealed together to generate a full-length fragment by another round of PCR using T7 and SP6 primers. Mirror-images of these constructs were

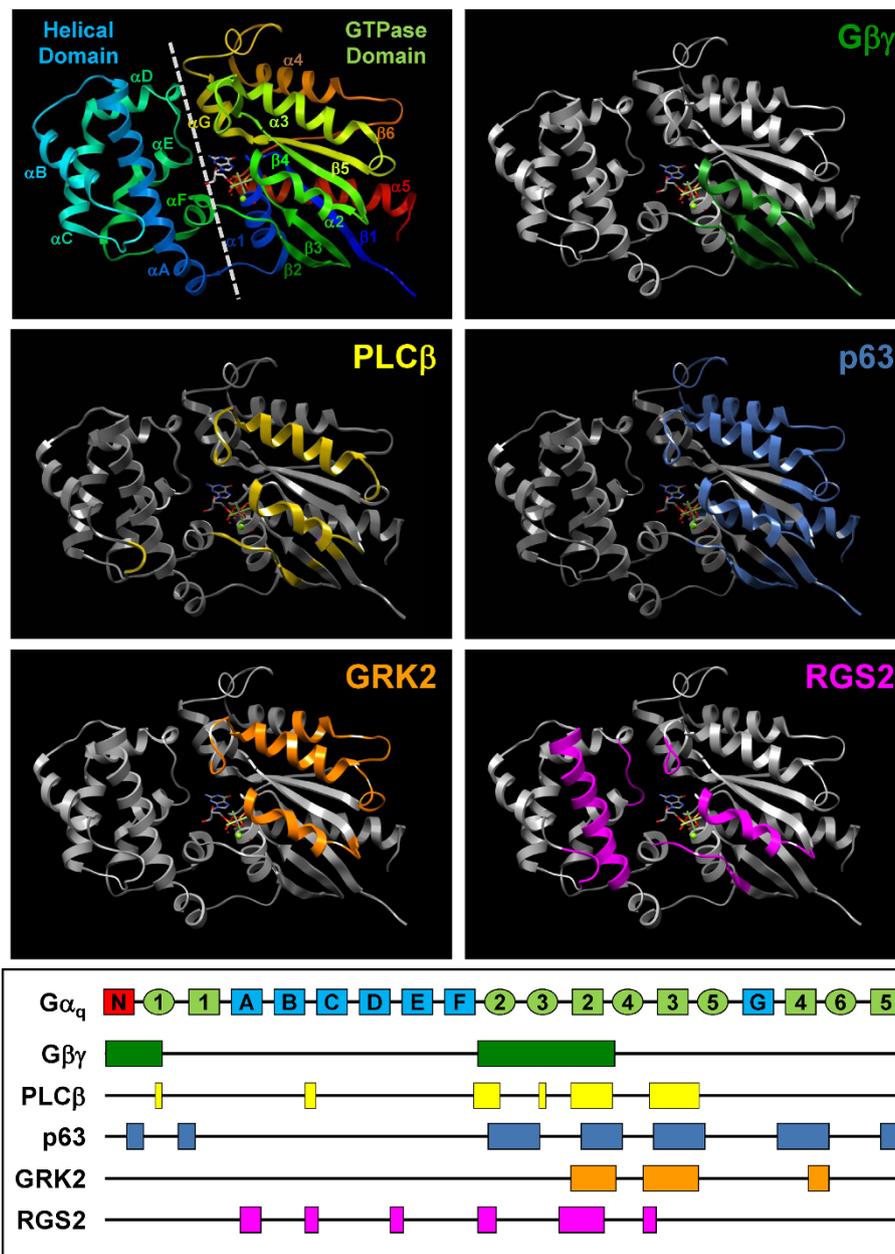


Figure 1 Structural representations of the functional domains of $G\alpha_q$. The crystal structure of $G\alpha_q$ (based on the complex with p63RhoGEF and RhoA, PDB ID: 2rgnA) is depicted with the different functional domains highlighted. The top left panel is the $G\alpha_q$ structure in rainbow colors (blue to red as from N- to C-terminus). Nomenclature of α helices and β strands is according to the first resolved $G\alpha$ crystal structure [19]. The dotted line divides the structure into two parts, known as the helical and GTPase domains. The other five structures are shown in the same orientation as the top left panel, with the putative interacting domains for $G\beta\gamma$ (green), PLC β (yellow), p63RhoGEF (p63, light blue), GRK2 (orange) and RGS2 (magenta) highlighted. Mapping of the various interacting domains are based on resolved crystal structures (for $G\beta\gamma$, PLC β , p63RhoGEF and GRK2) or structural alignment with other $G\alpha$ subunit complex structures (for RGS2 in complex with $G\alpha_{13}$). The bottom panel is a simplified linear representation of $G\alpha_q$ with the secondary structures belonging to helical (light blue) and GTPase domains (light green) highlighted; α -helices and β -strands are depicted as rectangles and ovals, respectively. The N-terminal helix colored in red remains unresolved in the crystal structure of $G\alpha_q$. The corresponding functional domains of the five interacting partners as shown in the molecular models above the schematic are indicated with the same color scheme.

generated analogously and were named C164, C174, C186, C219 and C272 chimeras. Primers for chimera construction are listed in Table 1. PCR was carried out using AccuPrime™ Pfx SuperMix (30 cycles each with 94°C for 60 s, 58°C for 60 s and 72°C for 90 s). The N200-C164 and N188-C164 chimeras were constructed using C164 as the initial template for the 3' half-products and those primers designed for N200 and N188, respectively. Likewise, zβ3 and zβ2β3 were constructed using the C174 and C164 primers with N210 as the initial templates. All chimeras were checked by restriction mapping and then subcloned into pcDNA3 at *Hind* III and *Xho* I sites. The constructs were fully sequenced by dideoxynucleotide sequencing to confirm the identities.

Cell culture and co-immunoprecipitation experiments

HEK 293 cells were obtained from the American Type Culture Collection (CRL-1573, Rockville, MD). They

were maintained in Eagle's minimum essential medium at 5% CO₂, 37°C with 10% fetal bovine serum (FBS), 50 units/ml penicillin and 50 µg/ml streptomycin. HEK 293 cells were grown to 80% confluency in 100-mm tissue culture plates and then co-transfected with 800 ng Gα and 800 ng FLAG-TPR1 cDNAs using 15 µl Plus™ and Lipofectamine™ reagents in Opti-MEM™. FBS was replenished 3 h after transfection. Cross-linking was performed one day after transfection. Transfected cells were washed with PBS twice and then treated with 0.5 mM dithiobis[succinimidylpropionate] in PBS for 15 min at room temperature. Cells were then washed again with PBS and maintained in quenching solution (50 mM glycine in PBS, pH 7.4) for 5 min. Subsequently, cells were lysed in ice-cold RIPA buffer (25 mM HEPES at pH 7.4, 0.1% SDS, 1% Igepal CA-630, 0.5% sodium deoxycholate, 1 mM dithiothreitol, 200 µM Na₃VO₄, 4 µg/ml aprotinin, 100 µM phenylmethylsulfonyl fluoride, and 2 µg/ml leupeptin). Cell lysates were gently rocked with an anti-

Table 1 Primer sequences for constructing chimeras between Gα₁₆ and Gα_z.

Construct	Primers: Antisense Primer Sense Primer	Size (aa)
N102	5'-GGGCCTGCTGAATGG GATCTGAGGGCGGC -3' 3'-CCCGGACGACTTACC CTAGGACACCCGCCG -5'	365
N155	5'-GGCTGAATCGAGCAG GTGGTACTCGCTGGA -3' 3'-CCGACTTAGCTCGTC CACCATGAGCGACCT -5'	367
N188	5'-GTTGATGCCAGTGGT CATGTCCCGGGAGCG -3' 3'-CAACTACGGTCACCA GTACAGGGCCCTCGC -5'	367
N200	5'-CCGCAGGTTGGTTTT CTTGAAGGTGAACTT -3' 3'-GGCGTCCAACCAAAA GAACCTCCACTTGAA -5'	367
N210	5'-CTCTGACTTCTGGCC CCCCACGTCCACCAT -3' 3'-GAGACTGAAGACCGG GGGGTGCAGGTGGTA -5'	367
N210QL	5'-CTCTGACTTCTGGCC CCCCACGTCCACCAT -3' 3'-GAGACTGAAGTCCCG GGGGTGCAGGTGGTA -5'	367
N246	5'-CATGCGGTTCTCCTG GTTATCCTCGTAGAG -3' 3'-GTACGCCAAGAGGAC CAATAGGAGCATCTC -5'	367
N266	5'-GGATGTGCTTTTGAA CCAGTTGTTGTCGA -3' 3'-CCTACACGAAAAC TTGGTCAACAACAACGT -5'	367
N295	5'-GCCCTGGAACTGGG AAAGCAGATGGTGAG -3' 3'-CGGGACCTTTGACCC TTTCGTCTACCACTC -5'	367
C164	5'- CTCTGACCTCGCCCCCGACGTCCACGAT -3' 3'- GAGCAYGGAGACGGGGGGCTGCAGGTGCTA -5'	362
C164QL	5'- CTCTGACCTCAGCCCCCGACGTCCACGAT -3' 3'- GAGCATGGAGTCGGGGGGCTGCAGGTGCTA -5'	362
C174	5'- CTTGAAGGTGAGCTCCTGCACGGAGAAGCA -3' 3'- GAACCTCCACTCGAGGACGTGCCTCTTCGT -5'	362
C186	5'- CACAATGCCCGTGGTGGGCATGCGGCTGCG -3' 3'- GTGTTACGGGCACCA CCCGTACGCCGACGC-5'	362
C219	5'- CGCGTTGTCTCCAGTGGGAATCCCGCCG -3' 3'- GCGCAACAGGTCCAC CACCTTAAGGGCGGC-5'	362
C272	5'- CTTGATTTGCGGCGGTGGTCCCTGCTTCTG -3' 3'- GAACTAAAGCGCCGC CACCAGGACGAAGAC-5'	362

Bold and italic nucleotides denote the Gα₁₆-derived sequences.

$G\alpha_{16}$, anti- $G\alpha_z$ or anti-PLC β antiserum at 4°C overnight, and then incubated in 30 μ l protein G-agarose (50% slurry) at 4°C for 2 h. Alternatively, the cell lysates were incubated in 30 μ l anti-FLAG affinity agarose gel (50% slurry) at 4°C overnight. Immunoprecipitates were washed with ice-cold RIPA buffer (400 μ l) for four times, resuspended in 50 μ l RIPA buffer and 10 μ l 6 \times sample buffer and then boiled for 5 min. $G\alpha_{16}$, $G\alpha_z$, PLC β and FLAG-TPR1 proteins in the immunoprecipitates were analyzed by Western blots.

Ras activation assay

HEK 293 cells were co-transfected with 800 ng $G\alpha$, 800 ng FLAG-TPR1 and 400 ng Ras cDNAs. After 1 day, transfectants were serum-deprived for 4 h. Cells were then washed twice with ice-cold PBS and lysed with the 1 \times Mg²⁺ lysis buffer (MLB: 25 mM HEPES at pH 7.5, 150 mM NaCl, 1% Igepal CA-630, 10 mM MgCl₂, 1 mM EDTA, 2% glycerol, 200 μ M Na₃VO₄, 4 μ g/ml aprotinin and 2 μ g/ml leupeptin). Cell lysates were pre-cleaned with Protein G-agarose and activated Ras was immunoprecipitated with 20 μ l Raf-1 Ras-binding domain-conjugated agarose (Millipore) for 45 min and followed by three washes of 400 μ l ice-cold MLB. Immunoprecipitates was finally reconstituted in 50 μ l MLB and 10 μ l 6 \times sample buffer and resolved in SDS-PAGE for detecting Ras using specific antibody.

Assays for phosphorylated ERK, IKK and STAT3

HEK 293 cells were seeded on a 6-well plate at 4.5 \times 10⁵ cells/well 1 day prior to transfection. Transfection was performed with 200 ng $G\alpha$ and 200 ng FLAG-TPR1 cDNAs using 4 μ l PlusTM and LipofectamineTM reagents in Opti-MEMTM. The transfectants were serum-deprived overnight one day after transfection. Cells were treated with pertussis toxin (PTX; 100 ng/ml for 16 h) and N⁶-cyclohexyladenosine (CHA; 10 μ M for 15 min) where appropriate, lysed, and then assayed for phosphorylation statuses of ERK, STAT3, and IKK as described previously [36,37].

Inositol trisphosphate accumulation assay

HEK 293 cells were seeded on a 12-well plate at 2 \times 10⁵ cells/well one day prior to transfection. Cells were transfected with 300 ng $G\alpha$ with or without 200 ng type 1 adenosine receptor (A₁R) cDNAs using 2 μ l LipofectamineTM 2000 reagent in Opti-MEMTM containing 5% FBS. Transfectants were labeled with 2.5 μ Ci/ml *myo*-[³H]inositol and subsequently assayed for CHA-induced [³H]IP₃ formation as described previously [39].

NF κ B-driven and STAT3-driven luciferase assays

For STAT3-driven luciferase assay [40], HEK 293 cells were seeded on a 96-well microplate at 10,000 cells/well

one day before transfection. Cells were transfected with 10 ng $G\alpha$ and 100 ng pSTAT3-luc luciferase reporter using 0.2 μ l PlusTM and LipofectamineTM reagents in Opti-MEMTM and FBS was replenished after 3 h. When A₁R-induced signals were analyzed, 10 ng receptor cDNA was included in the transfection. For NF κ B-driven luciferase assay, HEK 293 cells stably transfected with pNF κ B-luc luciferase reporter were seeded on a 96-well microplate at 15,000 cells/well. The setup and transfection were as described previously [36]. One day after transfection, transfectants were serum-deprived for 4 h and PTX (100 ng/ml) was added where necessary. Cells were challenged with or without 10 μ M CHA for 16 h before measuring the luciferase activity as reported previously [36].

Western blotting analysis

Protein samples were resolved on 12% SDS-PAGE and transferred to nitrocellulose membrane (GE Osmonics). Resolved proteins were detected by their specific primary antibodies and horseradish peroxidase-conjugated secondary antisera. The immunoblots were visualized by chemiluminescence with the ECL kit from GE Healthcare Bio-Sciences, and the images detected in X-ray films were quantified by densitometric scanning using the Eagle Eye II gel documentation system (Stratagene, La Jolla, CA, USA).

Molecular modeling of $G\alpha$ subunits

$G\alpha_q$ in the complex with p63RhoGEF and RhoA (PDB ID: 2rgnA) [13] was employed for the illustration of functional domains of $G\alpha_q$, and for creating a molecular model of $G\alpha_{16}$ by homologous modeling using SWISS-MODEL [41], which allowed manual adjustments to the alignment of the sequences of $G\alpha_{16}$ with $G\alpha_q$ in order to accommodate the extraordinarily long α 4/ β 6 loop of $G\alpha_{16}$. Molecular models of $G\alpha_z$ with or without the mutations at β 2/ β 3 loop (¹⁹⁴ELTFKM \rightarrow KTNLRI) were generated using 3D-JIGSAW [42] based on the crystal structure of $G\alpha_{i1}$ bound to AlF₄⁻ and GDP (PDB ID: 2h1bA) [43] as selected by the default automatic mode. 3D-JIGSAW-generated models showed greater dynamics in the loop structures and allowed for the exploration of potential conformational variations caused by the mutations. Visualization of various structures was accomplished using UCSF Chimera [44].

Results

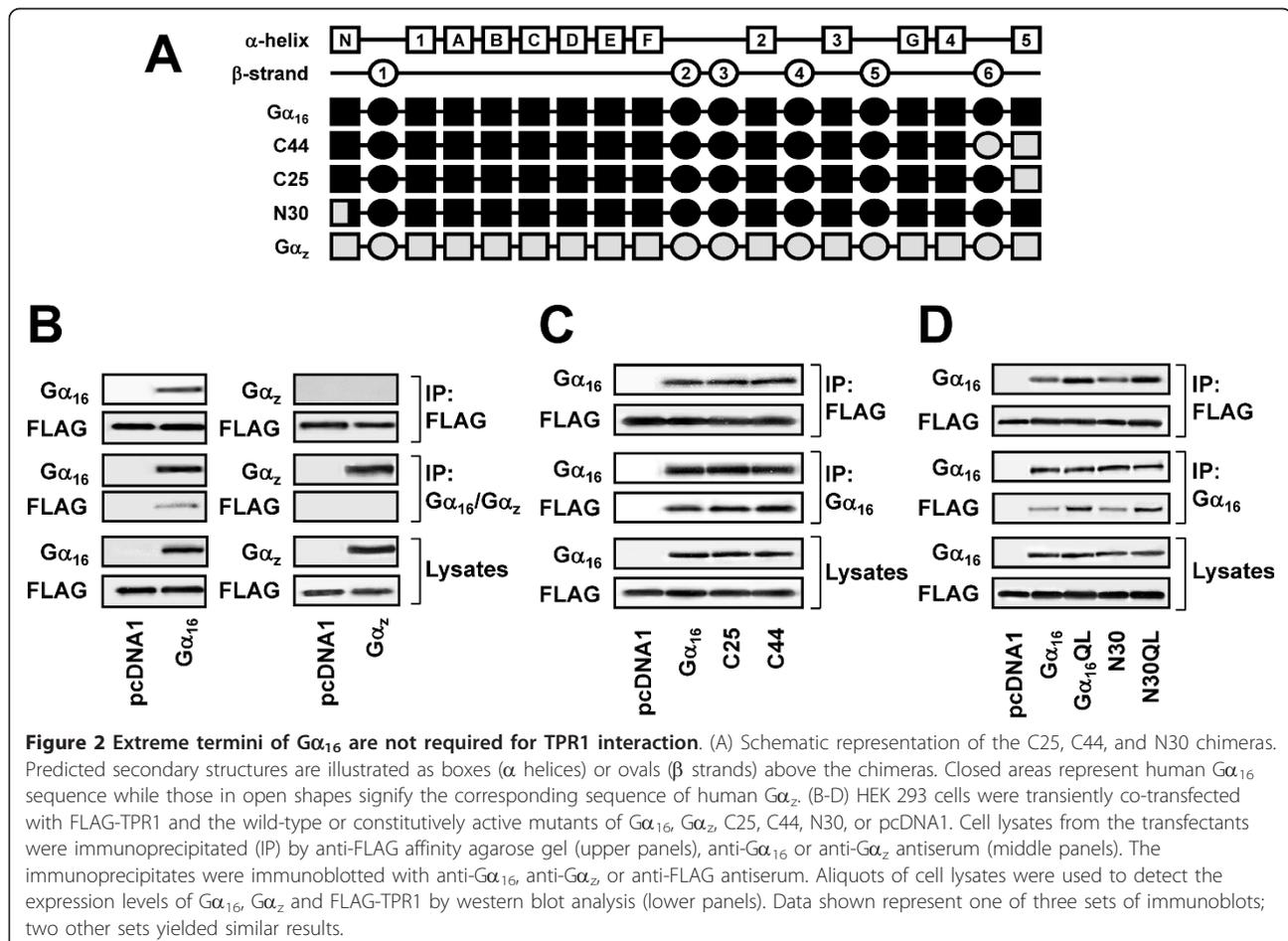
Extreme termini of $G\alpha_{16}$ are not involved in TPR1 interaction

Unlike the receptor-interacting domain which is composed of five distinct structures [45-47], no discrete localization for effector interaction is generally applicable to the $G\alpha$ subunits, probably because the different

signaling pathways entail a diverse spectrum of effector molecules. The $G\alpha_{16}$ regions responsible for effector interaction have not been mapped, but there is evidence to suggest that the Switch III and the $\alpha 3$ helix may participate in the binding of p63RhoGEF [48] and that the PLC β -interacting domain on $G\alpha_q$ mainly encompasses the $\alpha 2$ - $\beta 4$ - $\alpha 3$ - $\beta 5$ regions [49]. The involvement of $\beta 2$ strand, $\alpha 2$ and $\alpha 3$ helices in the $G\alpha_q$ /PLC β complex formation has been revealed clearly in the very recently resolved crystal structure [50] which also confirmed that PLC β can serve as a GAP for $G\alpha_q$ [51]. In order to identify the structural domains on $G\alpha_{16}$ that interact with TPR1, $G\alpha_{16}$ sequences were progressively replaced by those of $G\alpha_z$ because the latter does not recognize TPR1 (Figure 2B). Chimeras composed of $G\alpha_{16}$ and $G\alpha_z$ residues were preferred because they are structurally viable [38]. A series of chimeras was made by swapping discrete regions between $G\alpha_{16}$ and $G\alpha_z$. Construction of the chimeras was guided by the predicted tertiary structure of the $G\alpha$ subunits as well as by our previous experience in determining the receptor and effector interacting domains of $G\alpha_{16}$ and $G\alpha_z$ [38,52-55]. For

chimeras with substitutions at either the N- or C-terminus, they were named with a single letter (N or C) followed by the numbers of amino acids of $G\alpha_z$ that substituted the corresponding regions of $G\alpha_{16}$. The two chimeras containing either $\beta 2$ or $\beta 2$ - $\beta 3$ strands of $G\alpha_z$ were named with the a letter "z" followed by " $\beta 2$ " or " $\beta 2\beta 3$ ", respectively, in order to distinguish them from the descriptions of specific β strand structures.

We first examined two chimeras of $G\alpha_{16}$ containing either 25 or 44 amino acids of $G\alpha_z$ at the C-terminus; representing changes in the $\alpha 5$ helix alone and $\alpha 4/\beta 6$ loop plus the $\alpha 5$ helix, respectively. These two chimeras, named C25 and C44 respectively (Figure 2A), have been previously constructed and characterized (equivalent to the 16z25 and 16z44 of [38], respectively). Both chimeras exhibit enhanced coupling to G_i -coupled receptors and possess the ability to stimulate PLC β . HEK 293 cells were co-transfected with FLAG-TPR1 in combination with pcDNA1, $G\alpha_{16}$, C25 or C44. As illustrated in Figure 2C (upper panels), $G\alpha_{16}$, C25 and C44 were co-immunoprecipitated with the anti-FLAG affinity gel with similar levels of FLAG-TPR1. Moreover, FLAG-



TPR1 was coimmunoprecipitated along with $G\alpha_{16}$, C25 or C44 (Figure 2C, middle panels). $G\alpha_{16}$, C25, C44 and FLAG-TPR1 were expressed at detectable and comparable levels in the transfectants (Figure 2C, lower panels). Since both chimeras retained the ability to interact with FLAG-TPR1, it implies that the C-terminal $\beta 6$ and $\alpha 5$ regions of $G\alpha_{16}$ are not required for the interaction with TPR1.

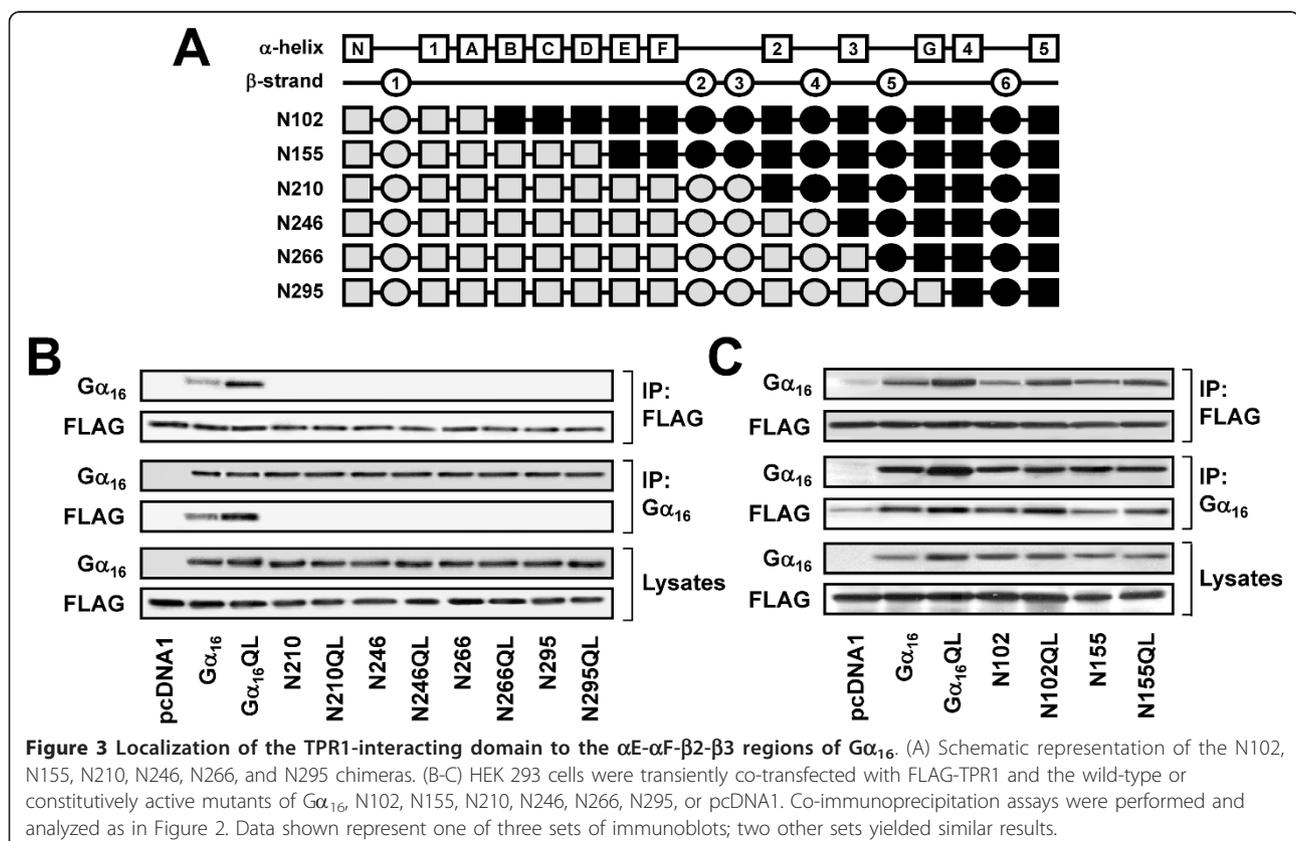
The N-terminus of $G\alpha$ subunits participates in membrane attachment, $G\beta\gamma$ binding as well as receptor recognition. Another previously constructed chimera, N30 [38], was employed to test the possible involvement of the N-terminus of $G\alpha_{16}$ in TPR1-binding. The αN helix (first 30 amino acids) of N30 is composed of $G\alpha_z$ sequence (Figure 2A). As shown in Figure 2D (upper panels), both $G\alpha_{16}$ and N30 co-immunoprecipitated with FLAG-TPR1 and similar levels of FLAG-immunoreactivity were observed. Similarly, FLAG-TPR1 co-immunoprecipitated with $G\alpha_{16}$ and N30 and the levels of these $G\alpha$ subunits in the immunoprecipitates were essentially the same (Figure 2D, middle panels). Like $G\alpha_{16}QL$, constitutively active N30QL was more readily associated with TPR1 (Figure 2D, upper and middle panels) and this effect was not due to variations in expression levels (Figure 2D, lower panels). Given that N30 still possessed the ability to interact with FLAG-

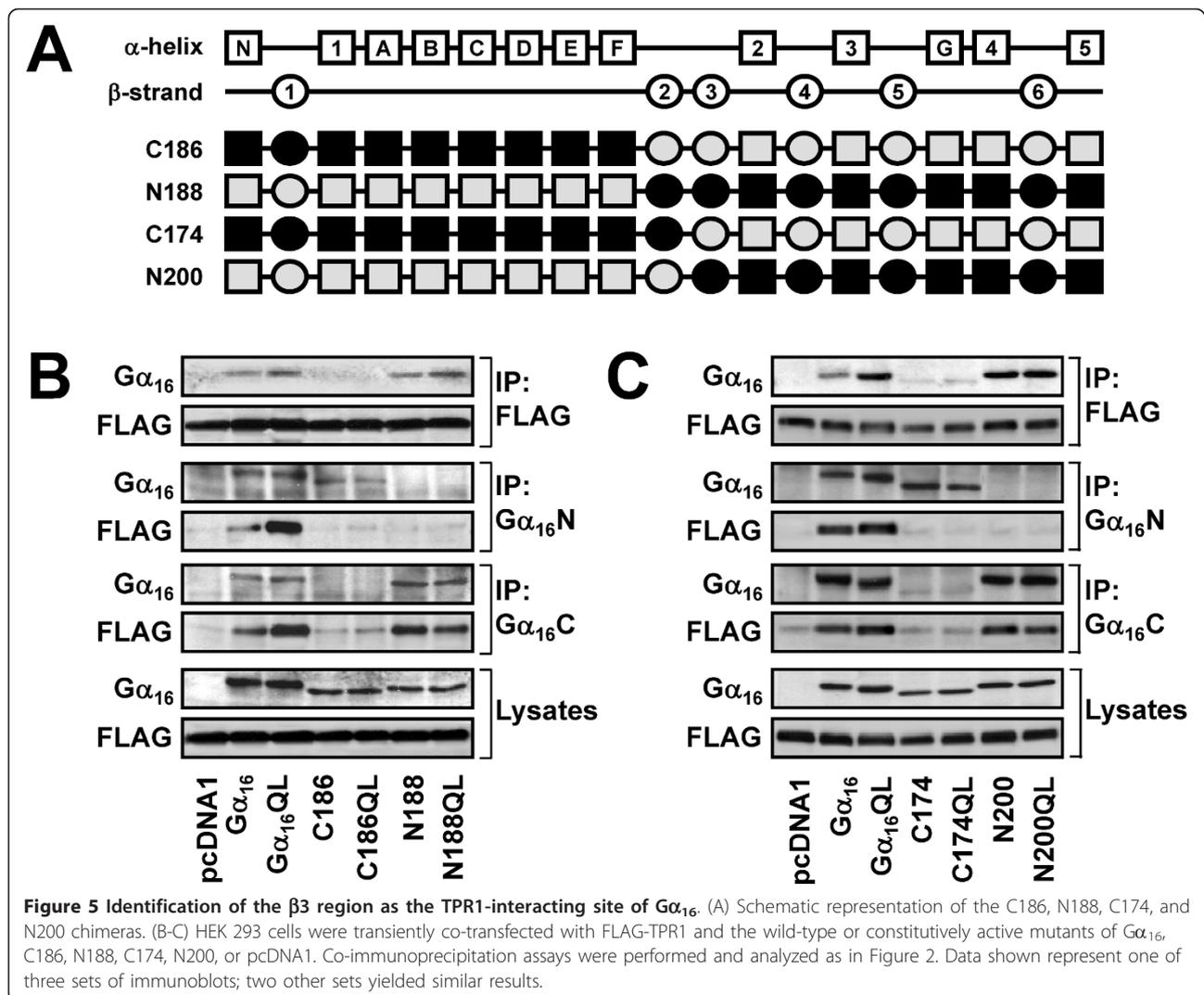
TPR1, our results suggest that the region which is critical for direct or indirect binding of TPR1 may lie between the $\beta 1$ strand and $\alpha 4$ helix of $G\alpha_{16}$.

The $\beta 3$ region of $G\alpha_{16}$ interacts with TPR1

To further examine the structural domain of $G\alpha_{16}$ for TPR1 interaction, additional chimeras were constructed by replacing different regions of $G\alpha_{16}$ with those of $G\alpha_z$. Four different chimeras named N210, N246, N266 and N295 were thus constructed (Figure 3A) by replacing a $G\alpha_z$ backbone with $G\alpha_{16}$ sequences starting from the $\alpha 2$, $\alpha 3$, $\beta 5$, and $\alpha 4$ regions, respectively. However, none of the four $G\alpha_{16}$ chimeras could be pulled down by anti-FLAG affinity gel (Figure 3B, upper panels). Similarly, FLAG-TPR1 could not be co-immunoprecipitated by anti- $G\alpha_{16}$ antiserum in chimera-expressing cells (Figure 3B, middle panels) despite detectable expression levels of FLAG-TPR1 and the chimeras in the cell lysates (Figure 3B, lower panels). In control experiments, both $G\alpha_{16}$ and $G\alpha_{16}QL$ were co-immunoprecipitated with FLAG-TPR1 (Figure 3B). These findings demonstrate that the TPR1-interacting domain must reside between residues 30 and 210 that represent the regions from $\beta 1$ to $\beta 3$ of $G\alpha_{16}$.

Two more chimeras were constructed to map the TPR1-interacting domain within the first 30-210





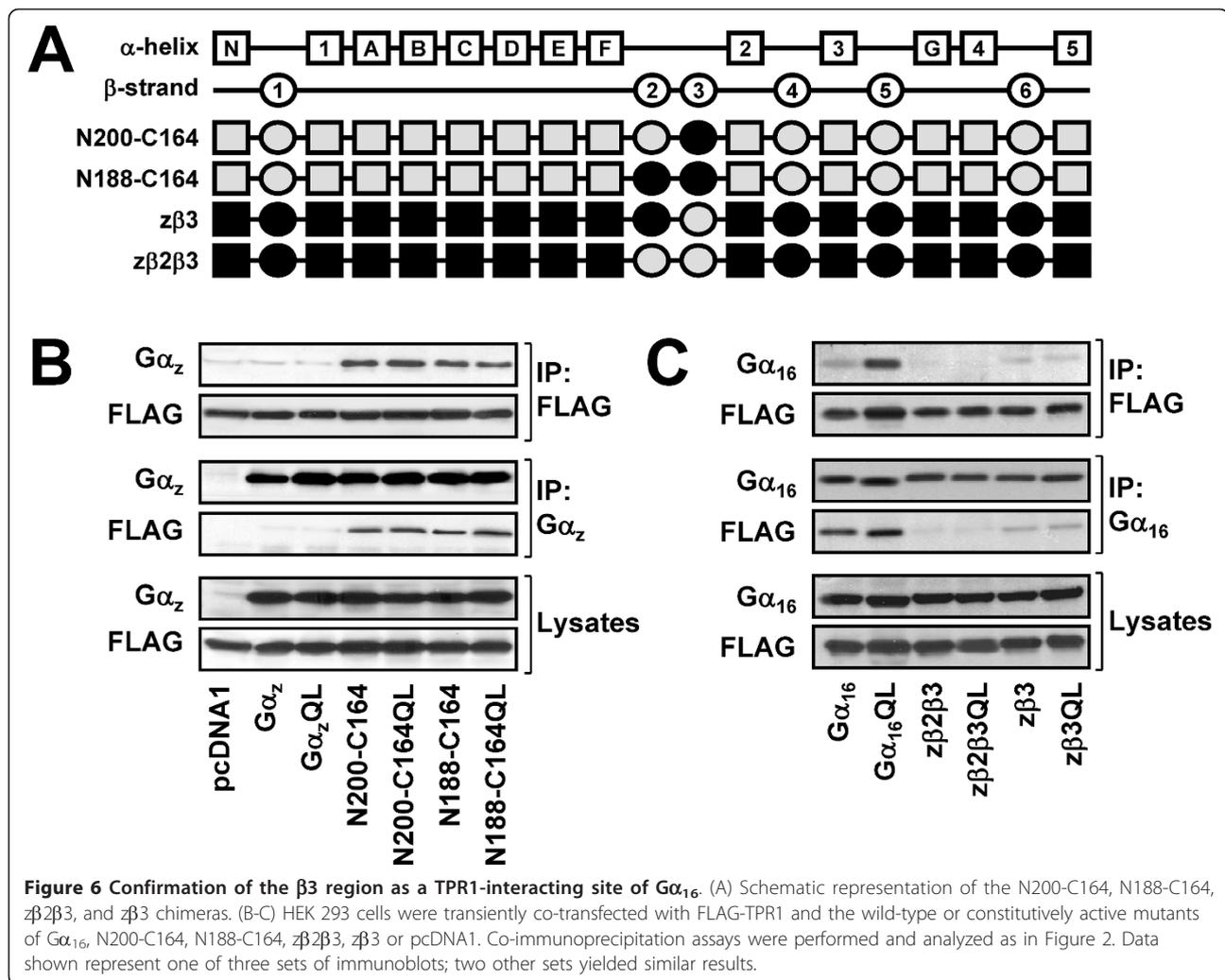
C174QL were ineffective (Figure 5C). Reverse co-immunoprecipitations with anti- $G\alpha_{16}$ antisera confirmed the ability of TPR1 to associate with N200 and N200QL.

In order to validate the importance of the $\beta 3$ region of $G\alpha_{16}$ for TPR1 interaction, N188-C164 and N200-C164 were constructed as shown in Figure 6A. N188-C164 is primarily a $G\alpha_z$ backbone with the $\beta 2$ and $\beta 3$ regions from $G\alpha_{16}$, while N200-C164 is essentially $G\alpha_z$ with the $\beta 3$ region made up of the $G\alpha_{16}$ sequence. Co-immunoprecipitation experiments using anti-FLAG or anti- $G\alpha_z$ antisera showed that both chimeras and their respective constitutively active mutants interacted with FLAG-TPR1 (Figure 6B). Association of N200-C164 with FLAG-TPR1 suggests that the $\beta 3$ region of $G\alpha_{16}$ alone is sufficient to confer upon $G\alpha_z$ the ability to interact with TPR1. On the other hand, replacement of the $\beta 3$ region of $G\alpha_{16}$ with the cognate sequence of $G\alpha_z$ is expected to disrupt $G\alpha_{16}$ /TPR1 interaction. Two additional chimeras, named $\beta 3$ and $\beta 2\beta 3$, with either the $\beta 3$ or $\beta 2$ - $\beta 3$ regions of $G\alpha_z$

inserted into a $G\alpha_{16}$ backbone (Figure 6A) were constructed to test this hypothesis. $\beta 2\beta 3$ and $\beta 2\beta 3$ QL failed to co-immunoprecipitate with TPR1 (Figure 6C), thus demonstrating the importance of the $\beta 2$ and $\beta 3$ regions of $G\alpha_{16}$ for interaction with TPR1. However, very weak but detectable associations of $\beta 3$ and $\beta 3$ QL with FLAG-TPR1 were observed in co-immunoprecipitation assays (Figure 6C). These results confirm the crucial role of the $\beta 3$ region for the $G\alpha_{16}$ /TPR1 interaction, and further suggest that the $\beta 2$ region may facilitate the actions of the $\beta 3$ strand.

Activation of Ras via the association of TPR1 with $G\alpha_{16}$ and its chimeras

Association of TPR1 with Ras may provide a more direct link for $G\alpha_{16}$ to activate the ERK cascade instead of going through the PLC β /PKC pathway. If the $\beta 3$ region of $G\alpha_{16}$ is essential for functional interaction with TPR1 and Ras, chimeras containing this region

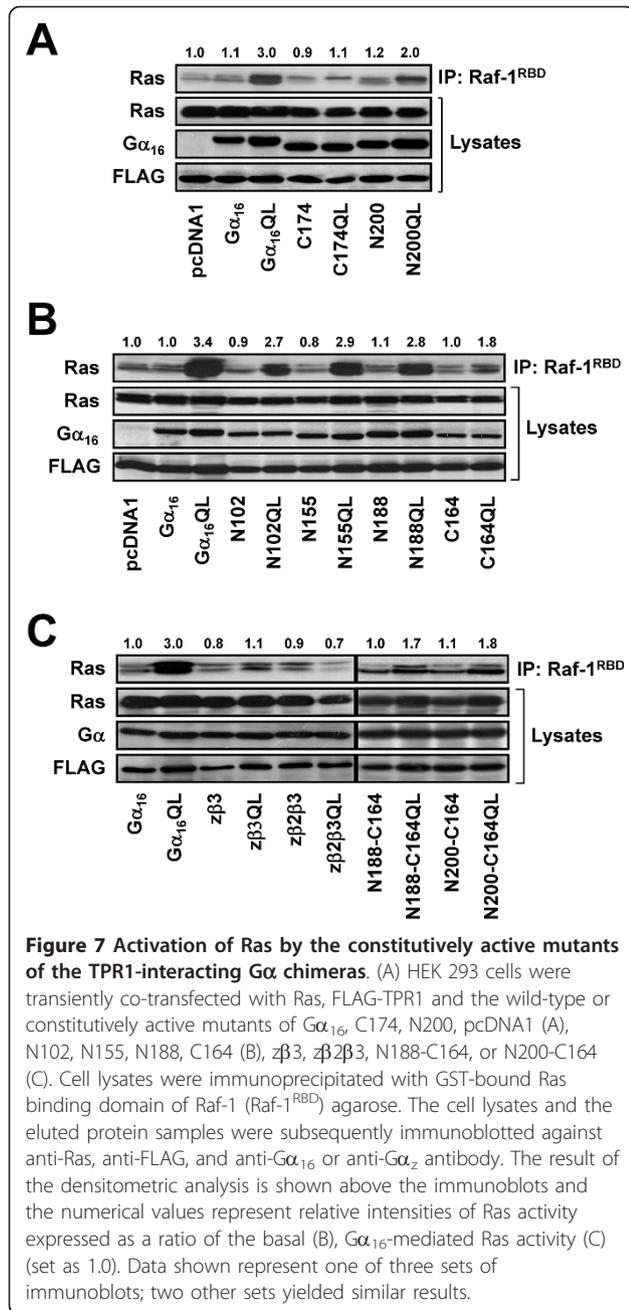


should facilitate the activation of Ras while those lacking this region ought to be inactive. As predicted, transfectants expressing N200QL (carrying the β 3 region of $G\alpha_{16}$) exhibited elevated Ras activity whereas C174QL (β 3 region from $G\alpha_z$) did not activate Ras (Figure 7A). To extend this study, other TPR1-interacting chimeras were evaluated (Figure 7B). No significant Ras activation was detected when wild-type N102, N155, N188 and C164 were overexpressed. Upon the introduction of the QL mutants of these chimeras, all chimeras induced detectable Ras activation as compared to their corresponding wild-type counterparts (Figure 7B). Likewise, N188-C164 and N200-C164 should be capable of activating Ras because they possess the β 3 region of $G\alpha_{16}$. Indeed, the constitutive active mutants of these chimeras activated Ras (Figure 7C). Conversely, z β 2 β 3 and z β 3 (the mirror images of N188-C164 and N200-C164, respectively) did not stimulate the Ras activity (Figure 7C). These results indicate that chimeras containing the $G\alpha_{16}$ -specific β 3 region possess the ability to activate

Ras, and such activity is dependent on the GTP-bound conformation of the chimeras.

$G\alpha_{16}$ QL-induced Ras activation is independent of PLC β signaling

The PLC β -interacting domain of $G\alpha_q$ was initially mapped to residues 217-276 [49], corresponding to the α 2- β 4- α 3- β 5 regions of $G\alpha_{16}$. Recent resolution of the crystal structure of $G\alpha_q$ -PLC β has refined the PLC β -interaction surface on $G\alpha_q$ to encompass mainly β 2 strand, α 2 and α 3 helices [50]. Since the putative PLC β -interacting domain of $G\alpha_{16}$ is in proximity of the TPR1-interacting β 3 region, we asked if activation of Ras and PLC β can occur independently. The chimeras were transiently expressed in the absence or presence of the G_i -coupled A $_1$ R and then assayed for IP formation with or without 10 μ M CHA. Predictably, CHA-induced IP formation was observed with transfectants co-expressing $G\alpha_{16}$, N30, C25, or C44 (Table 2). Chimeras with parts of the PLC β -interacting regions of $G\alpha_{16}$ replaced by



cognate $G\alpha_z$ sequences should exhibit impaired ability to regulate PLC β . Such chimeras include N246, N266, N295, C272, C219, C186, C174, C164, N188-C164, and N200-C164. Indeed, all ten chimeras failed to stimulate PLC β in response to CHA (Table 2), although CHA was capable of inhibiting cAMP formation in the transfectants (data not shown). The putative PLC β -interacting region of $G\alpha_{16}$ is intact in N102, N155, N188, N200, N210, $\beta 2\beta 3$, and $\beta 3$, and hence these chimeras are expected to support A₁R-mediated IP formation. However, only transfectants harboring N188, $\beta 2\beta 3$ or $\beta 3$

responded to CHA with a significant increase in IP formation (Table 2). The lack of response to CHA challenge may be attributed to impairment in receptor/G protein recognition. To exclude such a possibility, we tested the ability of the constitutively active mutants of the chimeras to stimulate PLC β . Those mutant chimeras with the PLC β -interacting region disrupted or replaced by $G\alpha_z$ residues did not exhibit any stimulation of PLC β , while $G\alpha_{16}$ QL and the previously characterized mutants such as C44 efficiently induced IP formation in the transfectants (Table 2). Among the mutant chimeras with the $\alpha 2$ - $\beta 4$ - $\alpha 3$ - $\beta 5$ region intact, only N188QL, N200QL, $\beta 2\beta 3$ QL and $\beta 3$ QL constitutively stimulated the PLC β activity (Table 2). The constitutive activity of N200QL suggests that the inability of N200 to mediate A₁R-induced stimulation of PLC β may be attributed to defective recognition of receptor. With the exception of N102, N155, and N210, the PLC β -stimulating abilities of the chimeras were in general agreement with the predicted presence of the putative PLC β -interacting region.

To confirm that the PLC β -interacting regions of $G\alpha_{16}$ -interaction are distinct from that of TPR1, two series of $G\alpha_{16}$ chimeras were tested. The first series contained chimeras harboring the putative PLC β -interacting domains, including N188, N200, and N210. HEK 293 cells were cotransfected with PLC β_2 and $G\alpha_{16}$ or a chimera and then subjected to co-immunoprecipitation using antisera against either $G\alpha_{16}$ or PLC β_2 . Interaction between PLC β_2 and $G\alpha_{16}$ was clearly evident, with $G\alpha_{16}$ QL generating a stronger interaction with PLC β_2 (Figure 8A). Chimeras N188, N200, and N210 were all capable of being co-immunoprecipitated with PLC β_2 . Another set of chimeras including C164, C174, and C186 (the mirror images of the first series) was similarly tested (Figure 8B). Due to the replacement of the putative PLC β -interacting regions with sequences of $G\alpha_z$, these chimeras were expected not to interact with PLC β_2 . Indeed, even with successful expression of the chimeras and PLC β_2 , no PLC β_2 interaction was detectable for these chimeras (Figure 8B). These results support the notion that the $\beta 3$ region of $G\alpha_{16}$ is dispensable for the interaction with PLC β (e.g. C164).

Since several chimeras can apparently activate Ras through TPR1 despite their inability to stimulate PLC β , they represent useful tools in delineating complex signaling networks such as those for the regulation of STAT3 and NF κ B. If these chimeras can induce the phosphorylation and activation of STAT3 and NF κ B, then it would imply that PLC β activity is not essential. Conversely, a lack of activity on STAT3 and NF κ B by these chimeras would indicate that PLC β action is required. Transfectants co-expressing the chimeras and A₁R were stimulated with CHA, and the phosphorylation of STAT3 and inhibitor of κ B kinase (IKK)

Table 2 Ability of G $\alpha_{16/z}$ chimeras to stimulate IP $_3$ production in HEK 293 cells.

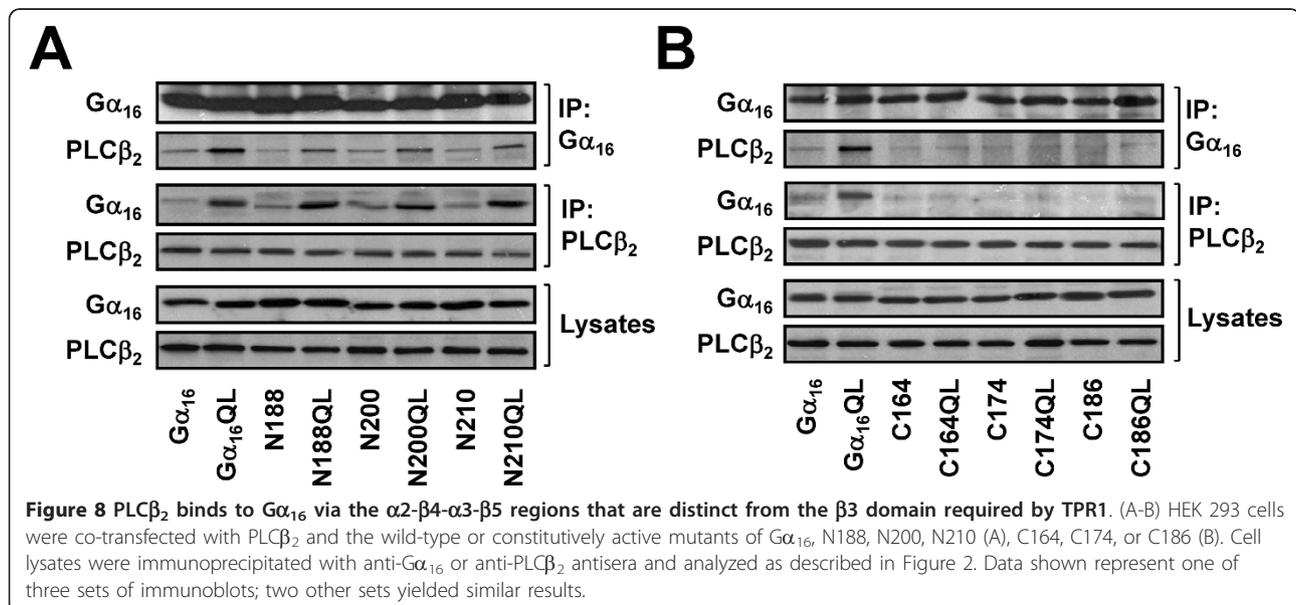
Construct	Intact PLC β Domain	A $_1$ R-induced IP accumulation			QL-induced IP accumulation
		Basal	10 μ M CHA	Fold Stimulation	Fold Stimulation
pcDNA1	N/A	10 \pm 3	11 \pm 2	1.1 \pm 0.2	N/A
G α_{16}	Yes	23 \pm 5	237 \pm 13	10.0 \pm 0.6*	7.8 \pm 0.4[#]
N30	Yes	23 \pm 4	81 \pm 10	3.5 \pm 0.7*	6.5 \pm 0.4[#]
N102	Yes	17 \pm 4	13 \pm 4	0.8 \pm 0.2	1.1 \pm 0.1
N155	Yes	13 \pm 3	10 \pm 1	0.8 \pm 0.1	1.0 \pm 0.1
N188	Yes	19 \pm 4	155 \pm 13	8.2 \pm 0.3*	4.2 \pm 0.2[#]
N200	Yes	11 \pm 3	15 \pm 2	1.3 \pm 0.1	2.0 \pm 0.2[#]
N210	Yes	12 \pm 3	10 \pm 2	0.8 \pm 0.2	1.3 \pm 0.1
N246	No	10 \pm 2	10 \pm 1	1.0 \pm 0.1	0.8 \pm 0.0
N266	No	27 \pm 3	17 \pm 2	0.6 \pm 0.3	1.2 \pm 0.2
N295	No	9 \pm 1	9 \pm 1	1.0 \pm 0.1	0.9 \pm 0.2
C25	Yes	15 \pm 3	92 \pm 7	6.1 \pm 0.5*	4.1^a
C44	Yes	78 \pm 11	183 \pm 15	2.3 \pm 0.2*	2.6 \pm 0.1[#]
C164	No	9 \pm 2	8 \pm 3	0.9 \pm 0.3	1.3 \pm 0.2
C174	No	13 \pm 3	14 \pm 9	1.1 \pm 0.1	1.4 \pm 0.2
C186	No	4 \pm 1	6 \pm 2	1.5 \pm 0.5	1.4 \pm 0.2
C219	No	7 \pm 1	9 \pm 2	1.3 \pm 0.3	1.2 \pm 0.1
C272	No	8 \pm 1	9 \pm 1	1.1 \pm 0.1	1.2 \pm 0.1
β 3	Yes	21 \pm 5	51 \pm 2	3.0 \pm 0.4*	4.3 \pm 0.4[#]
β 3	Yes	14 \pm 1	79 \pm 5	5.6 \pm 0.3*	6.9 \pm 0.4[#]
N200-C164	No	15 \pm 1	19 \pm 1	1.3 \pm 0.1	0.9 \pm 0.2
N188-C164	No	13 \pm 0	14 \pm 1	1.1 \pm 0.0	1.0 \pm 0.2
G α_z	No	10 \pm 1	9 \pm 3	0.9 \pm 0.3	1.1 \pm 0.1

HEK 293 cells were co-transfected with A $_1$ R and pcDNA1 or the indicated G α subunit. Transfectants were labeled with [3 H]inositol (2.5 μ Ci/ml) in DMEM containing 5% FBS overnight. IP $_3$ formations were examined in the absence (basal) or presence of 10 μ M CHA for 60 min. Fold stimulations were calculated as the ratios of CHA-induced to basal IP $_3$ accumulations or QL-induced to wild-type IP $_3$ accumulations. Data represent the mean \pm S.D. of triplicate determinations of a single representative experiment, n = 3; significant responses are shown in bold and italic. Constructs containing an intact putative PLC β binding domain (α 2- β 4- α 3- β 5 regions) of G α_{16} are marked with a "Yes".

* CHA-stimulated IP $_3$ production is significantly greater than basal (DMSO vehicle); paired *t*-test, *p* \leq 0.05.

[#] QL-stimulated IP $_3$ production is significantly greater than the corresponding wild-type activity; paired *t*-test, *p* \leq 0.05.

^a QL-induced fold stimulation of 16z25 was extrapolated from [38].



monitored by western blot analysis using phosphoprotein-specific antibodies. As shown in Table 3, chimeras with dual TPR1/Ras and PLC β activating capabilities effectively mediated CHA-induced STAT3 (at Tyr⁷⁰⁵ and Ser⁷²⁷) and IKK phosphorylations, with magnitudes similar to those of G α_{16} . These chimeras included N30, N188, C25, C44, z β 2 β 3, and z β 3. N200 failed to mediate the CHA-induced STAT3 phosphorylation but supported the IKK phosphorylation (Table 3). TPR1-interacting chimeras which lack PLC β activity (N102, N155, C164, N188-C164, and N200-C164) were unable to support CHA-induced phosphorylation of STAT3 and IKK (Table 3). Because ERK is required for G α_{16} -mediated STAT3 and NF κ B activation [36,37], we further tested the ability of the chimeras to mediate ERK phosphorylation. The profile of ERK phosphorylation mediated by the chimeras closely resembled those for STAT3 and IKK (Table 3). Chimeras lacking both PLC β and TPR1/Ras activities, including N210, N246, N266, N295, C174, C186, C219, and C272, did not support any of the

CHA-induced responses, whereas N30, N188, N200, C25, C44, z β 2 β 3, and z β 3 mediated CHA-induced ERK phosphorylation (Table 3).

Finally, we employed luciferase reporter gene assays to demonstrate transcriptional regulation of STAT3 and NF κ B by those chimeras that possess dual TPR1/Ras and PLC β activating capabilities. In agreement with our previous studies [36,37], CHA induced STAT3- and NF κ B-driven luciferase activities in transfectants co-expressing G α_{16} but not G α_z (Table 4). N30, N188, N200, C25, C44, and z β 3 chimeras all supported CHA-induced STAT3- and NF κ B-driven luciferase activities, whereas no transcriptional activation was observed in transfectants co-expressing chimeras that failed to mediate STAT3, IKK, and ERK phosphorylations (Table 4). Collectively, these studies demonstrate that dual TPR1/Ras and PLC β activating capabilities of G α_{16} may be essential for its regulation of complex signaling networks such as those for the activation of STAT3 and NF κ B.

Table 3 G $\alpha_{16/z}$ chimera-mediated phosphorylations of STAT3 (both Tyr⁷⁰⁵ and Ser⁷²⁷), IKK, and ERK in HEK 293 cells.

Construct	TPR1/Ras Interaction	PLC β Activity	Fold Stimulation of Protein Phosphorylation			
			P-Tyr ⁷⁰⁵ STAT3	P-Ser ⁷²⁷ STAT3	P-IKK	P-ERK
pcDNA1	N/A	N/A	1.0 \pm 0.0	1.0 \pm 0.0	1.2 \pm 0.1	1.1 \pm 0.1
G α_{16}	Yes	Yes	2.3 \pm 0.3*	2.0 \pm 0.1*	2.4 \pm 0.3*	2.6 \pm 0.1*
N30	Yes	Yes	1.7 \pm 0.1*	1.7 \pm 0.2*	1.7 \pm 0.2*	1.9 \pm 0.1*
N102	Yes	No	1.2 \pm 0.1	1.1 \pm 0.1	1.2 \pm 0.0	1.3 \pm 0.1
N155	Yes	No	1.1 \pm 0.0	1.1 \pm 0.1	1.2 \pm 0.0	1.5 \pm 0.0
N188	Yes	Yes	1.7 \pm 0.0*	1.4 \pm 0.1*	1.9 \pm 0.2*	2.2 \pm 0.1*
N200	Yes	Yes ^a	0.9 \pm 0.0	1.1 \pm 0.1	1.6 \pm 0.2*	1.9 \pm 0.1*
N210	No	No	0.9 \pm 0.1	0.9 \pm 0.0	1.0 \pm 0.0	1.1 \pm 0.0
N246	No	No	1.1 \pm 0.1	1.1 \pm 0.1	1.0 \pm 0.0	1.1 \pm 0.1
N266	No	No	1.4 \pm 0.3	1.1 \pm 0.0	1.2 \pm 0.2	1.3 \pm 0.1
N295	No	No	1.0 \pm 0.1	0.9 \pm 0.0	1.0 \pm 0.1	1.1 \pm 0.0
C25	Yes	Yes	1.7 \pm 0.1*	1.5 \pm 0.1*	1.6 \pm 0.1*	2.2 \pm 0.0*
C44	Yes	Yes	1.6 \pm 0.0*	1.4 \pm 0.0*	1.5 \pm 0.1*	1.8 \pm 0.0*
C164	Yes	No	0.9 \pm 0.1	1.0 \pm 0.1	1.0 \pm 0.0	1.3 \pm 0.0
C174	No	No	1.1 \pm 0.1	0.9 \pm 0.0	0.6 \pm 0.1	1.2 \pm 0.2
C186	No	No	0.9 \pm 0.1	1.1 \pm 0.2	1.0 \pm 0.1	1.3 \pm 0.1
C219	No	No	1.1 \pm 0.1	1.1 \pm 0.2	1.3 \pm 0.2	1.2 \pm 0.1
C272	No	No	1.0 \pm 0.1	0.9 \pm 0.1	1.1 \pm 0.1	0.8 \pm 0.0
z β 2 β 3	No	Yes	1.6 \pm 0.1*	1.5 \pm 0.1*	1.5 \pm 0.1*	2.2 \pm 0.2*
z β 3	No ^b	Yes	2.1 \pm 0.2*	1.9 \pm 0.1*	2.0 \pm 0.2*	2.7 \pm 0.1*
N200-C164	Yes	No	1.0 \pm 0.1	1.1 \pm 0.1	0.9 \pm 0.1	1.2 \pm 0.0
N188-C164	Yes	No	1.0 \pm 0.0	0.9 \pm 0.1	1.0 \pm 0.1	1.2 \pm 0.0
G α_z	No	No	1.0 \pm 0.0	0.9 \pm 0.0	1.1 \pm 0.0	1.1 \pm 0.2

HEK 293 cells were co-transfected with adenosine A₁R and pcDNA1 or the indicated G α subunit. Transfectants were serum starved overnight in the presence of 100 ng/mL of PTX and then challenged with 10 μ M CHA for 15 min. Cell lysates were then resolved and immunoblotted against various specific antibodies. Results are expressed as fold stimulation over the basal (DMSO vehicle), n = 3; significant responses are shown in bold and italic. Constructs shown to co-immunoprecipitate with FLAG-TPR1 or stimulate PLC β activity (Table 2) are marked with a "Yes".

* CHA-stimulated phosphorylation of the target protein is significantly greater than the basal; paired t-test, p \leq 0.05.

^a significant PLC β activation observed with the QL mutant only (see Table 2).

^b Very weak association with TPR1 was detected in co-immunoprecipitation assays (Figure 9).

Table 4 $G\alpha_{16/z}$ chimera mediated STAT3-driven and $NF\kappa B$ -driven luciferase activities.

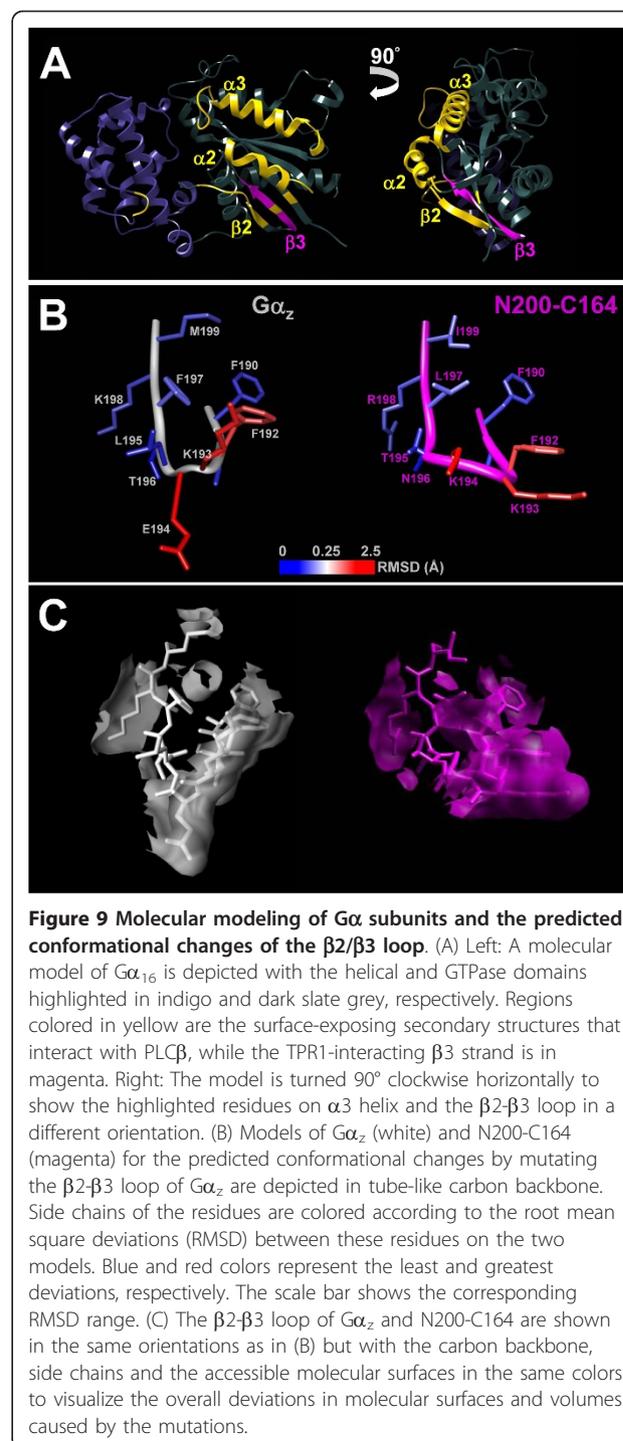
Construct	STAT3-driven Luciferase Fold Stimulation	$NF\kappa B$ -driven Luciferase Fold Stimulation
pcDNA1	1.0 ± 0.0	1.1 ± 0.1
$G\alpha_{16}$	2.4 ± 0.1*	3.0 ± 0.2*
N30	1.7 ± 0.2*	2.1 ± 0.2*
N102	0.9 ± 0.1	1.3 ± 0.1
N155	1.0 ± 0.0	1.3 ± 0.0
N188	1.9 ± 0.2*	1.9 ± 0.2*
N200	1.5 ± 0.0*	1.7 ± 0.1*
N210	1.2 ± 0.1	1.2 ± 0.1
N246	0.9 ± 0.1	1.2 ± 0.1
N266	1.1 ± 0.1	1.2 ± 0.1
N295	1.2 ± 0.2	1.1 ± 0.1
C25	1.8 ± 0.1*	2.0 ± 0.1*
C44	1.6 ± 0.0*	1.9 ± 0.2*
C164	1.2 ± 0.0	1.3 ± 0.2
C174	1.2 ± 0.1	1.2 ± 0.1
C186	1.2 ± 0.0	1.3 ± 0.1
C219	1.3 ± 0.2	1.2 ± 0.1
C272	1.1 ± 0.1	1.2 ± 0.1
$\beta 3$	1.7 ± 0.2*	2.4 ± 0.0*
N200-C164	1.2 ± 0.1	1.0 ± 0.1
N188-C164	1.2 ± 0.1	1.3 ± 0.0
$G\alpha_z$	1.1 ± 0.0	1.2 ± 0.0

HEK 293 cells were co-transfected with adenosine A_1R , pSTAT3-luc/p $NF\kappa B$ -luc, and pcDNA1 or the indicated $G\alpha$ subunit. Transfectants were serum starved for 4 h in the presence of 100 ng/mL of PTX and then challenged with 10 μM CHA overnight. Cell lysates were analyzed for luciferase activity. Data represent the mean \pm S.D. of triplicate determinations, $n = 3$; significant stimulations are shown in bold and italic. * CHA-stimulated protein phosphorylation is significantly greater than the basal; paired t -test, $p \leq 0.05$.

Discussion

Protein-protein interactions are central to the functions of $G\alpha$ subunits and each $G\alpha$ subunit has to coordinate such interactions in a timely manner according to its guanine nucleotide binding state. Once activated, the GTP-bound $G\alpha$ subunit has a limited time-span to regulate downstream effectors before its intrinsic GTPase activity turns it back to the inactive GDP-bound state, a process which can occur rapidly in the presence of RGS proteins. Hence, simultaneous regulation of multiple signaling pathways by activated $G\alpha_{16}$ is desirable but this entails the deployment of different binding surfaces. Although Ras can be activated indirectly by PLC β signaling, our results provide a structural basis for $G\alpha_{16}$ to stimulate Ras through interaction with TPR1 instead of PLC β . Moreover, functional analyses of $G\alpha_{16/z}$ chimeras reveal that dual stimulation of TPR1/Ras and PLC β may be essential for $G\alpha_{16}$ to activate downstream transcription factors such as STAT3 and $NF\kappa B$.

The extensive array of $G\alpha_{16/z}$ chimeras has enabled us to pinpoint the TPR1-interacting domain to the $\beta 3$ region (and also possibly $\beta 2$ as well) of $G\alpha_{16}$. The $\beta 2$ - $\beta 3$ region lies between the Switch I (mainly $\beta 2$ strand) and II (mainly $\alpha 2$ helix) regions [56] and is accessible for protein-protein interaction (Figure 9A). Since activation of the $G\alpha$ subunit alters the conformation of several



switch regions drastically, proteins like PLC β that bind to the Switch regions will interact with the GTP-bound active state much effectively than the GDP-bound basal state of the $G\alpha$ subunit (Figure 8). However, the differences of the interactions between TPR1 and $G\alpha_{16}$ WT or $G\alpha_{16}$ QL are comparably smaller, and TPR1 can obviously bind to $G\alpha_{16}$ WT. Such observation implies that TPR1 may bind to $G\alpha_{16}$ at regions with relatively less drastic conformational changes. Our results suggested that the β 3 strand of $G\alpha_{16}$ alone appears to be sufficient for the interaction with TPR1. The mere incorporation of the β 3 region of $G\alpha_{16}$ confers upon $G\alpha_z$ the ability to bind TPR1 (N200-C164 chimera). N200-C164 has the amino acid sequence ²⁰¹KTNLRIVDVG from $G\alpha_{16}$ inserted into a $G\alpha_z$ backbone, but only the first six residues are different from the corresponding $G\alpha_z$ sequence (¹⁹⁴ELTFKMVDVG). Based on the crystal structure of $G\alpha_{i1}$ [43], molecular modeling of $G\alpha_z$ reveals that Glu¹⁹⁴ is located at the "hook-shaped" β 2- β 3 turn (Figure 9B), which is often composed of two oppositely charged residues flanked by two interacting hydrophobic residues except $G\alpha_{16}$, with a threonine following the Lys²⁰¹. The predicted β 2- β 3 turn of N200-C164 showed a more widened conformation (Figure 9B-C), which presumably forms a characteristic microdomain on $G\alpha_{16}$ for TPR1 interaction. The fact that the chimera z β 3 exhibited residual binding to TPR1 (Figure 6C) suggested that other regions like the β 2 strand may also participate in the binding of TPR1 because its removal in the z β 2 β 3 chimera can further suppress TPR1 binding as compared to z β 3 (Figure 6C). Since several chimeras failed to generate a response in all of the functional assays, these chimeras might not be able to adopt the active conformation properly and it remains possible that additional residues other than the β 3 region may bind to TPR1.

Although the chimeras used in the present study were not tailored for the mapping of the PLC β -interacting domain of $G\alpha_{16}$, they nonetheless proved useful in locating the overall site for the binding of PLC β . As in the case of $G\alpha_q$ [49,50], the regions comprising of β 2, β 3, α 2, and α 3 are likely to form the PLC β -interacting surface of $G\alpha_{16}$ (Figure 1) because chimeras with one or more of these regions disrupted all failed to stimulate IP formation (Table 2). A more interesting observation is that chimeras including N102, N155, N200, and N210 could not activate PLC β at all, and only the constitutively active form of N200 could stimulate PLC β weakly, whereas the chimera N188 recapitulates the PLC β -activating capability. N102 and N155 contained all the putative PLC β -interacting domains and yet could not activate PLC β , albeit both interacted with TPR1 and activated Ras. One plausible explanation is that both chimeras have a hybrid helical domain (α A- α G; Figure

3A) which may affect its structural and functional integrity. Indeed, all $G\alpha_{16/z}$ chimeras that can stimulate PLC β activity (e.g., C44 and N188) contain a contiguous helical core (α A- α F helices) in addition to the putative PLC β -interacting domains. An early study of $G\alpha_s$ suggested that its helical domain could function as an internal GAP for the GTPase domain [57], and the intramolecular interaction between the GTPase and helical domains was proven to be essential for guanine nucleotide binding and receptor-mediated activation [58,59]. Furthermore, the α C- α D loop of $G\alpha$ subunits has been recognized as Switch IV which shows significant conformational changes in different guanine nucleotide-binding states and could reduce the nucleotide exchange rate when mutated [60]. It is therefore possible that a hybrid helical domain may impede the activation of PLC β by the $G\alpha_{16/z}$ chimeras. The sudden regain of PLC β -activating property of N188 which contains a helical domain completely derived from $G\alpha_z$ also implies that structural integrity of the helical domain is critical for PLC β activation. As revealed in the very recent crystal structure of $G\alpha_q$ -PLC β 3 [50], the β 2 and β 3 strands of $G\alpha_q$ interact extensively with the C2 domain of PLC β 3, and these two strands also heavily contribute to the overall scaffold of the GTPase domain. Replacement of one or both of them, as in the N200 and N210 chimeras, might disrupt PLC β interaction severely. Nonetheless, N200 can successfully form a heterotrimer with FLAG-tagged $G\beta\gamma$ dimer in co-immunoprecipitation experiment (unpublished data), excluding the possibility of improper folding of the chimera.

A molecular model of $G\alpha_{16}$ is constructed by homologous modeling based on the crystal structure of $G\alpha_q$ to visualize the potential interacting surfaces for PLC β and TPR1. Except for the unusually long α 4- β 6 loop and differences in the N-terminal helix (not shown in the model), $G\alpha_{16}$ basically fit very well to the structure of $G\alpha_q$. Assuming that $G\alpha_{16}$ utilizes domains similar to those of $G\alpha_q$ for the binding of PLC β [49], it is entirely feasible for $G\alpha_{16}$ to simultaneously regulate PLC β and TPR1. As shown in the right panel of Figure 9A, the critical PLC β -interacting residues are clustered mostly on the left hand side of the GTPase domain of $G\alpha_{16}$ (yellow-colored), while the putative TPR1-interacting β 3 region is located in the lower quadrant of the right hand side without severe overlap with the PLC β -interacting region. Preliminary co-immunoprecipitation studies indeed suggest the existence of $G\alpha_{16}$ /PLC β and PLC β /TPR1 complexes (unpublished data). Further studies will be required to confirm if a $G\alpha_{16}$ /PLC β /TPR1 complex truly exists. TPR motif-containing adaptors such as Rap-syn are known to cluster signaling molecules for the efficient propagation of signals [61]. It is conceivable that TPR1 may serve a similar function in G protein

pathways. Its lack of association with $G\alpha_{13}$, $G\alpha_{t1}$ [6] and $G\alpha_z$ (Figure 2B) suggests that TPR1 can selectively link G protein signals to Ras-dependent pathways. For those that can interact with TPR1, it remains to be determined if binding to TPR1 confers upon them the same repertoire of signaling capabilities.

The distinct locations for PLC β - and TPR1-interacting regions infer that $G\alpha_{16}$ can regulate them independently. Indeed, we have demonstrated that some chimeras (e.g., N102, N155, C164, N188-C164, and N200-C164) can bind TPR1 (Figures 3, 4, and 6) and induce Ras activation (Figure 7) even though they lack the ability to stimulate PLC β (Table 2). This raises an interesting possibility that, depending on the composition of the signaling modules within a cell, activation of $G\alpha_{16}$ may differentially regulate TPR1/Ras and PLC β signaling pathways. Both Ras and PLC β activities are apparently required for the regulation of STAT3 and NF κ B [36,37], but it is not clear if the two components are arranged in parallel or in series. Given that the activation of STAT3 and NF κ B could only be detected with chimeras possessing the ability to activate both Ras and PLC β , it would seem that the two pathways are independently required for the regulation of STAT3 and NF κ B. The need for multiple input signals increases signaling fidelity and specificity as well as ensuring a stringent control of transcription.

The possibility of $G\alpha_{16}$ to bind PLC β and TPR1 simultaneously raises some interesting questions regarding the fidelity of G protein signals. One concern is whether the two signaling pathways can be regulated independently. Stimulation of PLC β by GPCR can be mediated via members of the $G\alpha_q$ subfamily [62,63] or through the G $\beta\gamma$ dimer [64,65], with the latter restricted primarily to some isoforms of PLC β . Like $G\alpha_{16}$, both $G\alpha_q$ [6] and $G\alpha_{14}$ (unpublished data) can interact with TPR1. Since many ligands that act on G_q -coupled receptors are mitogenic [10,66], linkage through $G\alpha_q$ /TPR1 provides a means for the efficient stimulation of the Ras/Raf/MEK/ERK cascade for cell proliferation. Given that both PLC β and TPR1 are ubiquitously expressed, the ability of $G\alpha_{16}$ as well as other $G\alpha_q$ subfamily members to selectively activate one of the two pathways may have to rely on alternative means of signal segregation, such as spatial orientation [67], formation of macromolecular signaling complexes [68], and compartmentalization of signaling components [69]. Attachment of the $G\alpha_q$ subunit to the lipid bilayer [70], as well as its targeting to plasma membrane microdomains and to intracellular organelles [71], have been shown to affect $G\alpha_q$ signaling. It should also be noted that the presence of p63RhoGEF can affect the binding of PLC β and TPR1 to $G\alpha_{16}$ and differentially inhibit their signaling [25]. The p63RhoGEF-interacting domain on $G\alpha_{16}$ has yet to

be elucidated, but is expected to encompass the α 2- β 4- α 3- β 5 regions and the C-terminal α 5 helix based on the crystal structure of $G\alpha_q$ -p63RhoGEF-RhoA complex (Figure 1) [13]. In this regard, the $G\alpha_{16/z}$ chimeras represent useful tools to confirm such a prediction.

Conclusions

This study provided evidence for the importance of the β 3 strand of $G\alpha_{16}$ for the interaction with TPR1 and subsequent activation of Ras, but the β 3 strand appears to be dispensable for PLC β interaction. The integrities of both helical and GTPase domains are essential for PLC β activation. $G\alpha_{16}$ can signal through TPR1/Ras and PLC β simultaneously and independently to regulate transcriptional events involving STAT3 and NF κ B by utilizing different structural domains to bind TPR1 and PLC β . Overall, $G\alpha_{16}$ is able to interact with multiple molecular partners to convey different streams of signal transduction.

List of abbreviations used

A₁R: type 1 adenosine receptor; AGS: activator of G protein signaling; CHA: N²-cyclohexyladenosine; FBS: fetal bovine serum; G protein: heterotrimeric guanine nucleotide-binding proteins; GPCR: G protein-coupled receptor; GTPase: GTP hydrolase; IKK: inhibitor of κ B kinase; IP₃: inositol trisphosphates; NF κ B: nuclear factor κ B; PLC β : phospholipase C β ; PTX: pertussis toxin; RGS: regulator of G protein signaling; STAT3: signal transducer and activator of transcription 3; TPR1: tetrapeptide repeat 1

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Authors' contributions

AMFL and RKHL performed most biological experiments. EXG assisted in experiments. MKCH performed homology modeling. AMFL, MKCH and YHW conceived and designed the experiments and wrote the manuscript. RDY participated in the design of the study. All authors read and approved the final manuscript.

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