The human G protein β4 subunit: gene structure, expression, Gγ and effector interaction[1]

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Abstract The aim of this study was the characterization of the human Gβ4 subunit of heterotrimeric G proteins. Human Gβ4 is widely expressed. Its gene is located on chromosome 3 with a genomic structure indistinguishable from that of the genes of Gβ1 to Gβ3, but entirely different from Gβ5. In vitro translation co-precipitation analyses revealed that Gβ4 can form stable dimers with Gγ1, Gγ2, Gγ3, Gγ4, Gγ5, Gγ7, Gγ10, Gγ11, Gγ12, and Gγ13, dimers which were also able to stimulate phospholipase β2.

Key words: G protein; Signal transduction; Phospholipase C; WD domain

1. Introduction

Heterotrimeric G proteins, which are composed of Gα, Gβ and Gγ subunits, are key components for signal transduction from an activated heptahelical receptor to effector systems including enzymes (e.g. adenylyl cyclases, phospholipases C (PLC)), adapter proteins and ion channels (for review [1]). The Gα subunit is a GTPase that interacts in the GDP-bound state with Gβγ dimers. In the human genome 16 genes for Gα, five genes for Gβ, and 12 genes for Gγ subunits have been identified [2]. The proteins encoded by these genes give rise to the formation of more than 1000 different G protein heterotrimers and may thus determine receptor and effector specificity [1]. However, available evidence suggests that not all theoretically possible heterotrimers do exist or are of functional relevance in vivo [3]. The five known Gβ isoforms can be divided into the subfamily of Gβ1–Gβ4 proteins which share high homology (~80–90%) and the Gβ5/Gβ5L protein which is only ~50% homologous to the other Gβ proteins and which exhibits distinct biochemical properties [4]. All Gβ subunits belong to the superfamily of propeller proteins and are made up of seven regular WD domains (referring to a recurrent Trp-Asp motif among others) which form a toroid-like structure [5]. At the N-terminus an α-helical extension of 20 amino acids forms a coiled-coil domain [5]. Each of the WD domains is composed of four anti-parallel β-sheets, which are referred to as a–d, with the a strand located in the center of the tunnel and the d strand at the outside surface of the protein [5]. Beside the WD motif at the end of strand c, there is a highly conserved aspartate in the loop connecting strands b and c and a glycine-histidine motif following the d strand [5].

Gβ1 and Gβ2, which were cloned first, and Gβ5, which differs considerably from the other Gβ proteins, have been intensively characterized [1–3]. Gβ3 and its splice variant Gβ3s have attracted considerable pathophysiological and pharmacogenetic interest [6,7]. However, information on the biochemical and pharmacological properties of Gβ4 is scarce. Recently, the cDNA of the human Gβ4 subunit has been published and its properties have been analyzed with special focus on the activation of N-type Ca^{2+} channels [9]. Here, we report an in-depth analysis of the Gβ4 gene (*GNB4*) structure; we also determined the dimerization of Gβ4 with different Gγ subunits and their ability to activate PLCβ2.

2. Materials and methods

RNA, prepared from different tissues using the RNAeasy kit (Qiagen, Hilden, Germany), was in vitro transcribed as detailed [10]. Human, rat, and mouse Gβ4 cDNAs were amplified by reverse transcription polymerase chain reaction (RT-PCR) using *Pfu* polymerase (Promega, Madison, WI, USA). Amplicons were A-tailed, cloned into the pGEM-T easy vector (Promega) and sequenced. The same procedure was used to clone and sequence the various Gγ subunits (except Gγ2, Gγ3, Gγ5, Gγ7 and Gβ1 which were kind gifts of Dr. Lohse, Würzburg, Germany). All G protein subunits used were subcloned into the pcDNA3.1$^+$ expression vector (Invitrogen). For control purposes, transcripts of $Gα_{16}$, a G protein confined to hematopoietic cells, was amplified by RT-PCR with oligonucleotide primers encompassing the entire open reading frame (for sequences see the Table in the Supplementary material in the Web version).

For analysis of the exon–intron boundaries genomic DNA was purified from whole blood using the QiAMP blood kit and exons were amplified using primers located in the adjacent introns and sequenced.

To study Gβ/Gγ interactions co-precipitation analyses were performed with in vitro translated hemagglutinin (HA)-tagged Gγ subunits as devised by E. Neer [11]. Gγ subunits were tagged at the N-terminus with the HA epitope by PCR using the above generated vectors as templates with modified sense primers carrying the sequence for the HA epitope, an improved Kozak sequence and suitable restriction sites. The resulting PCR amplicons were ligated into pcDNA3.1$^+$ vector and sequenced. The primer sequences for all PCRs are detailed in the Table in the Supplementary material in the Web version.

Gβ and HA-Gγ subunits were in vitro translated with the TNT quick-coupled transcription/translation system (Promega) driven by

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the T7 promoter of the pcDNA3.1$^+$ vector. In brief, 1 μg vector DNA was mixed with 40 μl reticulocyte lysate and 0.8 MBq [^{35}S]L-methionine (Hartmann Analytic, Braunschweig, Germany), adjusted to a total of 50 μl with H_2O and incubated at 30°C for 90 min. Aliquots of all in vitro translated Gβ and Gγ subunits were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and the translation efficacy was determined by phosphoimaging. In vitro translation mix of each HA-tagged Gγ (15 to 32 μl, depending on the translation efficacy) was mixed with 20 μl of in vitro translated Gβ1 or Gβ4 and incubated for 90 min at 37°C. Subsequently, 20 μl protein A agarose (Santa Cruz Biotechnologies, Heidelberg, Germany) was added to these reaction mixes and pelleted by centrifugation. The supernatants were transferred into new reaction tubes and 10 μl (4 μg) of a monoclonal anti-HA antibody (clone 12CA5, Roche Mannheim) was added and incubated for 90 min at 4°C under constant shaking. Thereafter, 40 μl of protein A agarose was added and incubated for 60 min at 4°C. The reaction mixes were centrifuged, the supernatants were removed and the agarose pellets washed four times in 500 μl ice-cold RIPA buffer. Finally, the agarose pellets were washed in 400 μl ice-cold 50 mM Tris–HCl buffer (pH 7.5), resuspended in 70 μl of a modified SDS sample buffer (use of *m*-cresol as dye to allow for separation of Gγ subunits), incubated at 95°C for 5 min and separated on 15% SDS–PAGE. Gels were vacuum-dried, exposed to phospho-screens and the radioactive bands were visualized with a PhosphoImager.

Activation of PLCβ2 by Gβγ subunits was studied essentially as described [12]. The PLCβ2 clone was a kind gift of Dr. M.I. Simon, Pasadena, CA, USA. It was subcloned into the pcDNA3.1$^+$ vector. COS-7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum, 100 U/ml penicillin and 100 μg/ml streptomycin (Invitrogen). 6×10^4 cells were plated onto each well of a 24 well cell culture dish. After 24 h cells were transfected using the Effectene transfection reagent (Qiagen) according to the manufacturer's instructions. Onto each well 0.07 μg vector DNA was applied containing equal amounts of PLCβ2, Gβ and Gγ (non-modified wild-type) vector. In controls without PLCβ2 or G protein subunits the total amount of transfected DNA was kept constant by addition of empty pcDNA3.1$^+$ vector DNA. The next day, cells were labelled with 0.5 ml inositol-free DMEM containing 3.5 μCi *myo*-[^{3}H]inositol for another 24 h. Thereafter, the label medium was removed and the cells were incubated in 0.5 ml inositol-free DMEM containing 10 mM LiCl for 1 h at 37°C. Cells were lysed and water-soluble inositol phosphates were separated by anion exchange chromatography as described [12]. COS-7 cells transfected with Gγ subunits but no Gβ subunits served as controls and Gβγ-mediated stimulation of PLCβ2 was defined as the percent ratio of water-soluble inositol phosphates produced in the presence of overexpressed Gβγs versus the inositol phosphates formed in cells overexpressing the respective Gγ. All experiments were performed in triplicate and the results shown in Fig. 4 are means ± S.E.M. from three or four independent experiments. Gβγ-mediated activation of PLCβ2 was assumed if this ratio exceeded the 100% value (Student's *t*-test; $P<0.05$). Expression of the transfected Gβ subunits was verified by Western blot analysis using a anti-Gβ1 and anti-Gβ4 antibodies according to the supplier's instructions (Santa Cruz Biotechnology, antibodies sc-379 and sc-382). Likewise, expression of HA-Gγ subunits upon transfection was controlled with the anti-HA antibody.

3. Results and discussion

Based on the published mouse Gβ4 sequence we performed BLAST analyses in public data bases and identified the EST clone AK001890 from human placenta as a potential human Gβ4 clone. Using oligonucleotide primers derived from this sequence and starting with RNA from human B lymphoblasts and brain tissues we amplified the cDNA of the Gβ4 open reading frame and short adjacent 5′- and 3′-untranslated sequences (Fig. 1). The sequences from four independent clones confirmed the sequence of the EST clone (AK001890) and were identical to the one published recently (AF300648 [9]). However, in two independent Gβ4 cDNA clones from different individuals we identified single nucleotide polymorphisms

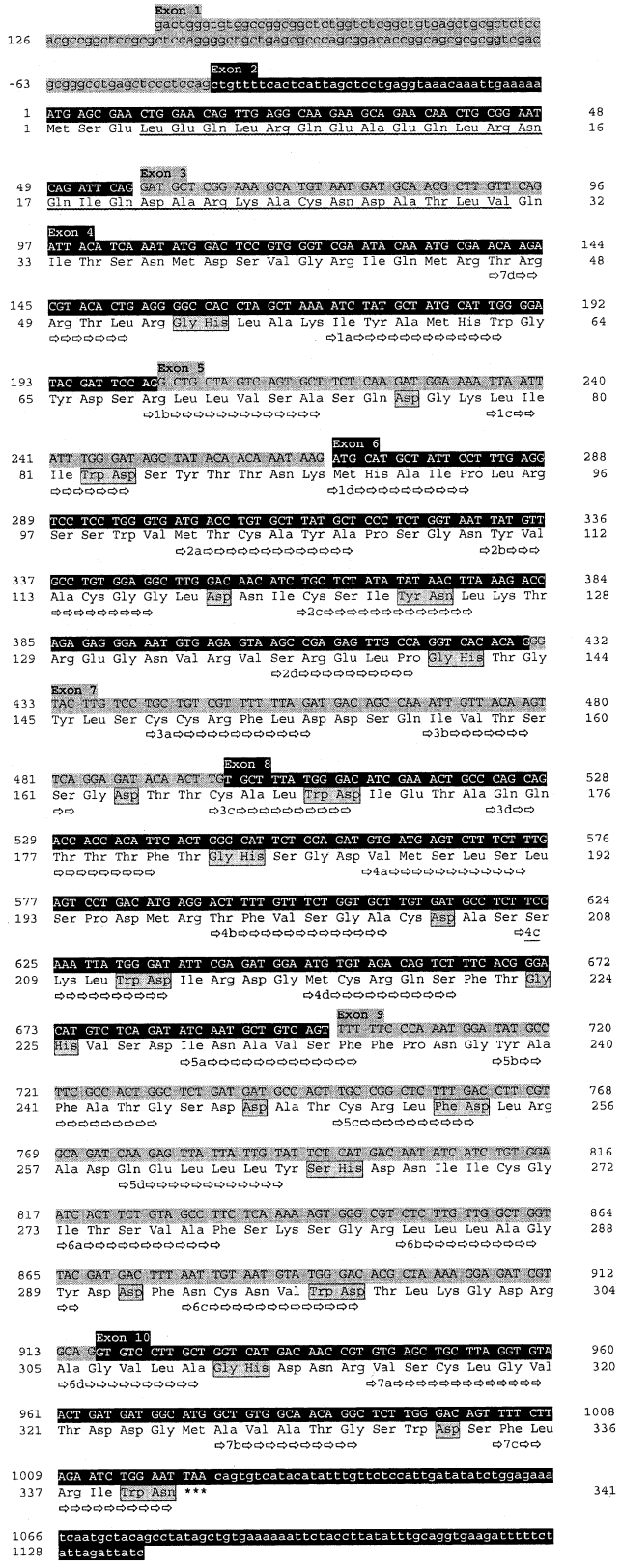

Fig. 1. Nucleotide and amino acid sequence of human Gβ4. Underscored is the N-terminal coiled-coil region. Arrows refer to the β-strands of the WD domains (numbered in lower case). Conserved motifs of WD domains (GH, WD motifs, aspartate between strands c and d) are marked in gray. The exons of Gβ4 are alternately indicated in gray and black in the cDNA sequence.

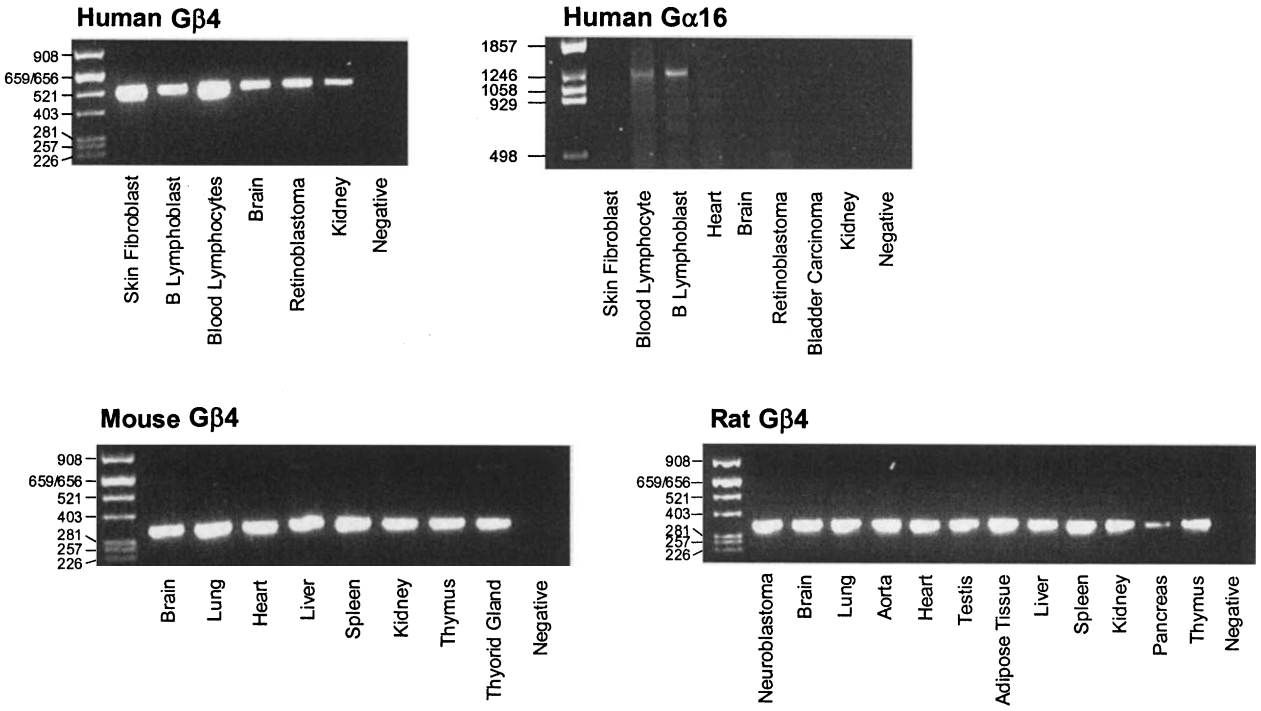

Fig. 2. Gβ4 transcript expression in different tissues of man, mouse, and rat demonstrated by RT-PCR. A control RT-PCR was performed to demonstrate $G\alpha_{16}$ transcripts. In contrast to Gβ4, $G\alpha_{16}$ transcripts were confined to hematopoietic cells only, indicating that our RT-PCR conditions are suitable to demonstrate differential transcript expression.

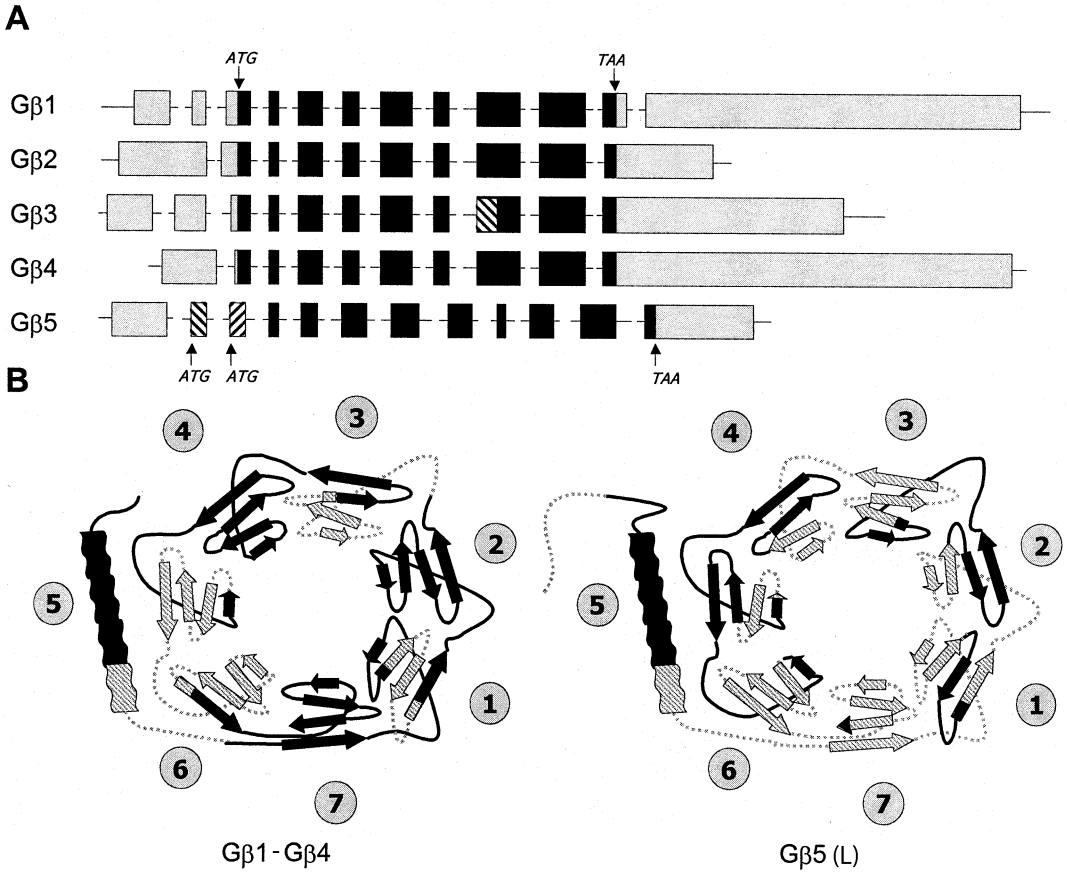

Fig. 3. Genomic organization of Gβ proteins. A: Exons are indicated by boxes, the open reading frame is shown in black. 'ATG' refers to the start codon. Hatched bars refer to alternatively spliced exons observed in Gβ3 and Gβ5. Schematic drawing, intron lengths not to scale. B: Schematic drawing of the seven-blade propeller structure of Gβ proteins according to [5]. Arrows indicate β-sheets. Exons are represented alternately by black solid and gray hatched boxes and black solid and gray interrupted lines.

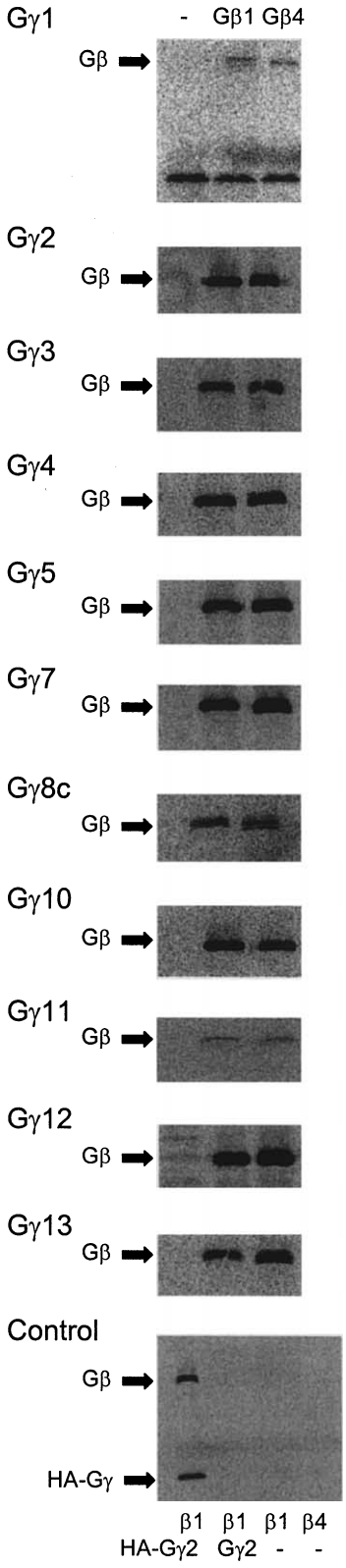

Fig. 4. Co-precipitation assays of in vitro translated Gβ and Gγ subunits. Gβ1, Gβ4 and HA-Gγs were in vitro translated separately, mixed and after formation of the Gβγ dimer immunoprecipitated. Control experiments (bottom) show that a Gβ precipitate is observed only in the presence of HA-Gγ.

(SNPs). One SNP involved a C117T exchange (number refers to the translation start codon ATG = 1) another a T186C exchange. Both SNPs do not affect the amino acid composition of the human Gβ4 and their biological significance – if any – remains to be determined.

In parallel, we generated a mouse Gβ4 clone which exhibited several nucleotide exchanges compared to the original Gβ4 sequence (M87286). Two variations resulted in amino acid exchanges (D132N; A140P; Figure in the Supplementary material in the Web version). We verified our sequence in independent clones and we obtained a rat Gβ4 clone. All of these Gβ4 clones have a proline at position 140 and an asparagine at position 132. The latter is located in the loop between strands c and d at the outer face of the protein and it is highly conserved in Gβ1–Gβ3 [5]. It is tempting to speculate that the weak biological activities observed with the M87286 clone are caused by these probable sequence errors [13,14].

The human Gβ4 protein shares the highest homologies with Gβ1 and Gβ2. The amino acid and nucleotide sequences of the human Gβ4 and an annotation of its structural elements derived from the comparison with the crystallized Gβ1 [5] are shown in Fig. 1. Two groups have analyzed the expression of mouse [8] and human [9] Gβ4 by RT-PCR and obtained diverging results. Whereas von Weizsäcker reported a ubiquitous expression of Gβ4 with high levels in brain, heart and spleen, Ruiz-Velasco observed a more restricted Gβ4 expression with low levels of Gβ4 transcripts in brain, heart and spleen. Our data, obtained from human, rat and mouse tissues and cultured cells, favor a wide if not ubiquitous tissue distribution (Fig. 2). A definite confirmation by Western blot analysis is needed.

Next, we performed genomic BLAST analyses and identified the clone NT005950 as a potential genomic clone of Gβ4. Based on this sequence we designed oligonucleotide primers adjacent to the putative exons, amplified them, and determined the genomic structure. The *GNB4* gene is located on chromosome 3 and consists of 10 exons and nine introns (Fig. 1). We have previously characterized human *GNB3* [15], and the genomic sequences of human *GNB1*, *GNB2*, and *GNB5* are obtainable by BLAST analyses in the human genome project database (AL109917, AC009488 and AC010674). With the exception of the 5′- and 3′-non-translated regions, Gβ1–Gβ4 exhibit an identical genomic organization (Fig. 3). Gβ5, the least homologous Gβ subunit (~53%), has a completely different genomic structure. This is in accordance with the notion that Gβ1–Gβ4 and Gβ5 belong to different ancient Gβ families [2]. In Fig. 3B the genomic organizations of the Gβ1–Gβ4 proteins and the Gβ5 protein are superimposed on their presumed three-dimensional structures as derived from crystallization studies [5]. Of note, there is no close correlation between exon structures and protein motifs, e.g. WD domains or propeller blades. Again, the totally different genomic organization of Gβ5 is evident.

Next, we focused on the biochemical properties of Gβ4 and investigated its ability to dimerize with Gγ proteins. Available evidence suggests that Gβγ dimers (except Gβ5) are stable upon dimerization and that Gβγ diversity may determine receptor and effector specificity [1,3]. With Gβ2 and Gβ3 not all theoretically possible Gβγ dimers are formed [3]. Several assays exist to test for Gβγ association. One possibility is to investigate the activation of Gβγ-dependent effector systems upon overexpression of Gβγ subunits. However, if the effector

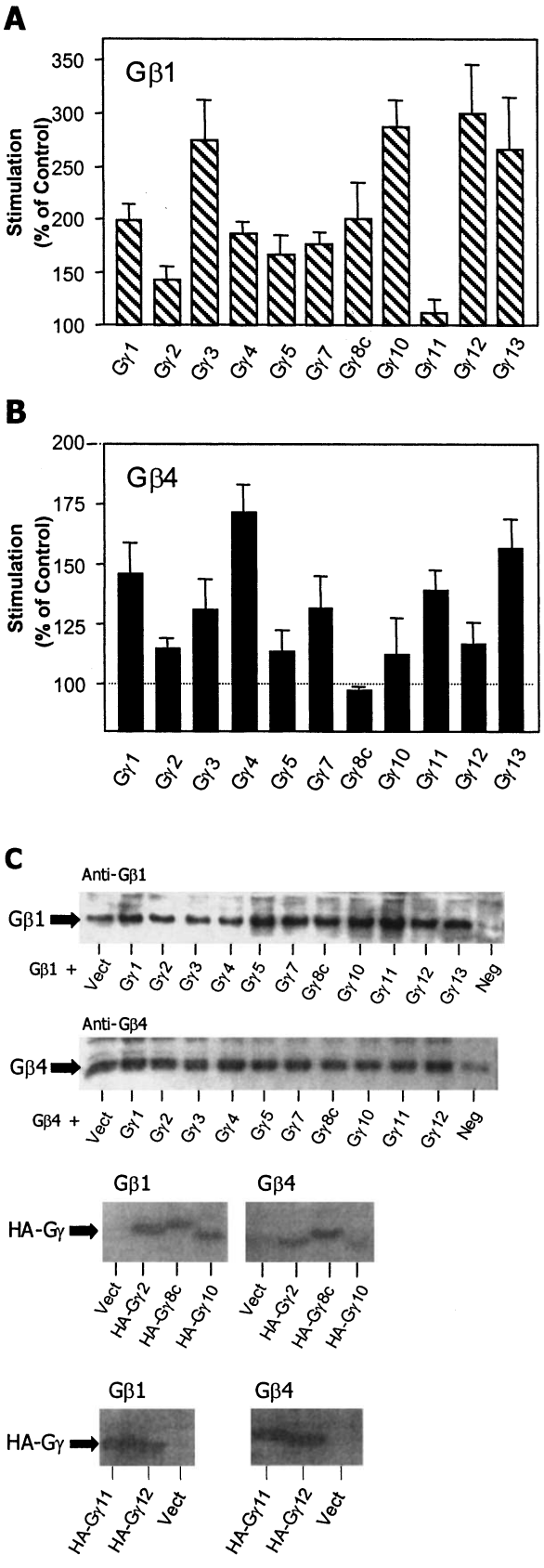

Fig. 5. Activation of PLCβ2 by Gβ1γ and Gβ4γ. PLCβ2 and the respective Gβs and Gγs were transfected into COS-7 cells and the Gβγ-mediated activation of PLCβ2 was quantified. Means ± S.E.M. from at least three different experiments for Gβ1 (A) and Gβ4 (B) with measurements made in triplicate. Results were normalized for the activation of PLCβ2 upon expression of Gγ only. C: Upper two panels: Western blot analyses demonstrating similar expression levels of Gβ1 and Gβ4 with each of the investigated Gγ subunits. Lower two panels: Western blot analyses for different HA-Gγ subunits expressed in the presence of Gβ1 or Gβ4. The results indicate that the absent PLCβ2 stimulation by Gβ1γ11 and Gβ4γ8c is not attributable to an altered expression of the respective Gγ subunits. ←

system is not activated, it is impossible to differentiate absent Gβγ dimerization from failure of the respective dimer to activate the effector. Therefore, we performed co-precipitation assays as described [11]. Gβs and HA-tagged Gγs were in vitro translated separately in the presence of [^{35}S]methionine. Thereafter, labelled Gβs and Gγs were mixed and incubated. In the presence of reticulocyte lysate which contains chaperonins, Gβs fold correctly, interact with Gγs and can dimerize [3,11]. These dimers can then be precipitated with an anti-HA antibody. Co-precipitation of a Gβ with a HA-Gγ indicates their mutual interaction. Here, we analyzed the interaction of Gβ4 – and as a control Gβ1 – with all known mammalian Gγ proteins except the highly specialized $G\gamma_{olf}$. As shown in Fig. 4 (bottom), Gβ1, Gβ4 and wild-type Gγs were not recognized by the anti-HA antibody. Precipitation of a Gβ protein was only observed in the presence of a HA-modified Gγ subunit. Our data indicate that both Gβ1 and Gβ4 form dimers with all known Gγ proteins although the interactions with Gγ1 and Gγ11 were quantitatively weaker. By yeast two-hybrid screen Yan et al. analyzed dimerization of some Gβγs [16]. In their assay, Gβ4 interacted with the tested Gγ2, Gγ3, Gγ4, Gγ5, and Gγ7, but not with Gγ1 [16], which was also the weakest interaction in our experiments.

Next, we investigated whether these Gβγ dimers activate a Gβγ-dependent effector system, in this case PLCβ2. Despite comparable protein levels upon transfection of Gβ1 and Gβ4, as determined by Western blot analysis (Fig. 5C, upper two panels), dimers with Gβ4 were generally weaker in activating PLCβ2 than dimers with Gβ1. Overexpression of most Gβγ dimers resulted in a significant although sometimes modest (e.g. Gβ4γ2, Gβ4γ5, Gβ4γ10, Gβ4γ12) activation of PLCβ2. However, the dimers Gβ1γ11 and Gβ4γ8c did not stimulate PLCβ2, although the respective Gβ and Gγ subunits could easily be detected by Western blotting (Fig. 5C, lower two panels). These results are interesting since the dimers Gβ4γ8c and Gβ1γ11 could be co-precipitated in the in vitro translation assays and may indicate Gβγ specificity to activate PLCβ2. On the other hand, Gβ1γ1, Gβ4γ1 and Gβ4γ11, for which weaker interactions were observed in the co-precipitation assays, did activate PLCβ2. The most active Gβ4γ dimer to stimulate PLCβ2 was Gβ4γ4. Similarly, Gβ4γ4 was found to be most effective in inhibiting N-type Ca^{2+} channels and activating GIRK potassium channels [9,17]. Beside Gβ4γ4 these latter studies examined only Gβ4γ2 and Gβ4γ3, which were also biologically active.

Taken together, our data show that the human Gβ4 is widely expressed and is able to dimerize with almost all Gγ subunits. Available evidence suggests that Gβ4 modulates important effector systems, i.e. N-type Ca^{2+} channels, GIRK potassium channels and phospholipase isoforms.

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