Identification and characterization of G β 3s2, a novel splice variant of the G-protein β 3 subunit

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The T-allele of a polymorphism (C825T) in the gene for the G-protein β 3 subunit (*GNB3*) is associated with cardiovascular and metabolic disorders, distinct cellular features and altered drug responses. The molecular mechanisms that give rise to this complex phenotype have been linked to the occurrence of G β 3s, a splice variant of *GNB3*. G β 3s is predominantly expressed in cells with the 825T-allele. In the present study we describe the identification and characterization of an additional G β 3 splice variant referred to as G β 3s2. Its mRNA is expressed in heart, blood cells and tumour tissue, and its expression is also tightly associated with the *GNB3* 825T-allele. G β 3s2 is generated by alternative splicing using non-canonical splice sites. G β subunits belong to the family of propeller proteins and consist of seven regular propeller blades. Transcripts for G β 3s2 are lacking

129 bp of the coding sequence of the wild-type G β 3 protein. Thus the predicted structure consists of only six propeller blades, which resembles the structure of G β 3s. Co-immunoprecipitation analyses indicated that G β 3s2 dimerizes with different G γ subunits, e.g. G γ 5, G γ 8_c and G γ 12. In Sf9 insect cells, expression of G β 3s2 together with G γ 12 enhances receptor-stimulated activation of G α_{i2} . Expression of G β 3s2 in mammalian cells activated the mitogen-activated protein kinase cascade. Together, these results suggest that G β 3s2 is a biologically active G β variant which may play a role in the manifestation of the complex phenotype associated with the 825T-allele.

Key words: GNB3, G-protein β subunit, hypertension, pharmacogenetics, polymorphism, signal transduction.

INTRODUCTION

Heterotrimeric G-proteins are key mediators for signal transduction via heptahelical receptors, the largest mammalian receptor family (reviewed in [1]). G β proteins form dimeric complexes with G γ subunits which interact with G α subunits to generate a heterotrimeric G-protein. Agonist-mediated receptor activation causes an exchange of GDP for GTP at the G α subunit which then switches to an active conformation. Activated G α *GTP is no longer able to bind G β γ subunits. Free G β γ dimers act as scaffolding and coordinating proteins which allow the binding of effector proteins and their subsequent activation. These effector systems include adenylyl cyclases, phospholipase C- β (PLC β) isoforms, the mitogen-activated protein (MAP)-kinase cascade, ion channels and adaptor proteins, to name but a few [1]. G α subunits possess an intrinsic GTPase activity which mediates the hydrolysis of GTP and initiates the reassembly of the heterotrimeric complex [1,2].

At present, 16 genes for G α subunits, 5 genes for G β proteins and 12 G γ genes have been identified in the human genome [3]. Alternative splicing is a frequent mechanism to increase protein complexity, and 30–50% of all genes are estimated to be subject to alternative splicing [4–6]. Splice variants have been identified for the G-proteins G α_s , G α_o and G β 5 [3]. We detected the splice variant G β 3s, which is associated with the T-allele of a single nucleotide polymorphism (SNP; C825T) in *GNB3*, the gene for the G β 3 subunit [7]. The 825T-allele of this polymorphism is part of a haplotype consisting of seven polymorphisms which are distributed along the entire *GNB3* [8].

G β subunits, including G β 3, belong to the family of propeller proteins and consist of seven regular WD domains, which are defined by conserved amino acid motifs including tryptophan (W) and aspartate (D). G β 3s, the splice variant associated with the 825T-haplotype lacks the equivalent of one WD domain [7]. However, several non-G β WD proteins with only six WD domains have been identified (Conserved Domain Data Base of the National Center for Biotechnology Information at <http://www.ncbi.nlm.nih.gov/Structure>, and [9]). This suggests that G β 3s may actually form a regular propeller structure. Evidence from transient expression experiments suggests that G β 3s is a biologically active protein which actually confers an enhanced signal transduction [7,10].

The *GNB3* C825T polymorphism has been the subject of many clinical and pharmacological investigations. For example, the 825T-allele has been shown to be associated with essential hypertension [7,11–14], stroke [15], left ventricular hypertrophy [16,17], orthostatic hypotension [18], alterations in body weight regulation [19–21] and transplant survival [22]. However, negative results from association studies have also been published [23–26], which may relate in part to ethnic effects. Interestingly, *GNB3* haplotypes in Africans and Asians differ from those in Caucasians [8] and further epidemiological studies are required to unravel their effects on disease susceptibilities.

Physiological and cell biological investigations support the notion that the *GNB3* 825T-allele status affects various body functions including control of systemic, renal and coronary blood flow [27–29], atrial potassium currents [30], lipolysis [31] and immune responses [10,32,33]. Other studies indicate the

Abbreviations used: AcNPV, *Autographa californica* nucleopolyhedrosis virus; G α , G β , G γ , α -, β - and γ - subunits of G-proteins; *GNB3*, G-protein β 3 subunit gene; HA, influenza haemagglutinin epitope tag; HEK-TSA, human embryonic kidney cells expressing the large T-antigen of SV40 virus; HIS, hexa-histidine epitope tag; MAP-kinase, mitogen-activated protein kinase; MBP, myelin basic protein; M₂ mAChR, M₂ muscarinic acetylcholine receptor; PLC β , phospholipase C- β ; RT-PCR, reverse transcriptase-PCR; SNP, single nucleotide polymorphism.

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value of the *GNB3* C825T polymorphism as a pharmacogenetic marker to predict responses to antihypertensive and anti-depressant drugs [34,35].

In contrast with this considerable amount of data from association studies, information on the immediate biochemical and molecular mechanisms which may cause the complex phenotype associated with the 825T-allele is scarce. In the present study we have described the identification of a novel alternatively spliced *GNB3* transcript, referred to as *Gβ3s2*. The aim of the present investigation was to characterize this novel *Gβ3* variant. Our results indicate that the occurrence of *Gβ3s2* is tightly linked to the 825T-allele status and that this protein is biologically active. Taken together, these results add further to the complexity of mechanisms that may ultimately participate in the phenotypes described above.

EXPERIMENTAL

Reverse transcriptase (RT)-PCR, cloning and sequencing

Total RNA was prepared from different tissues and cell lines using the RNeasy kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. RNA was transcribed *in vitro* using Moloney-murine-leukaemia virus reverse transcriptase (Superscript®; Life Technologies, Karlsruhe, Germany). Total RNA (3 µg) was incubated at 70 °C for 5 min, quick-chilled and transcribed *in vitro* in 60 µl of first-strand buffer (Life Technologies) containing 1.2 µg of oligo p(dT)₁₀ (Roche, Mannheim, Germany), 1 mM each of dATP, dCTP, dGTP and dTTP, 30 units RNasin (MBI Fermentas, St. Leon-Rot, Germany), 10 mM dithiothreitol and 600 units of reverse transcriptase. The reaction mixture was incubated at 37 °C for 1 h and, thereafter, heated to 80 °C for 5 min to inactivate the enzyme.

Routine PCRs were performed with 5 µl of the reverse transcription reaction mixture in a final volume of 50 µl with 1.0 units of *Taq* polymerase and the standard PCR buffer (MBI Fermentas). Oligonucleotide primers were routinely used at 0.4 µM each, and 0.2 mM of each dNTP was added. A first denaturation step of 95 °C for 5 min was followed by 35 cycles at 94 °C for 1 min, at 60 °C for 45 s and at 72 °C for 1 min. The reaction was completed by a final extension step at 72 °C for 7 min. For sequencing PCR amplicons were ligated into pGEM-T easy cloning vector (Promega, Heidelberg, Germany). For generation of expression vectors *Taq* polymerase was substituted by *Pfu* polymerase (Promega) and used according to the manufacturer's instructions. DNA fragments were purified using the Qiaquick PCR purification kit (Qiagen). All vector constructs used were verified by DNA sequencing. The sequences of the primers used for amplification of *Gβ3/Gβ3s2* are 5'-GAGGGG-ACCTGCCGTCAGACTTTCAGTGGC-3' (P2-1), 5'-TGTTCACTGCCTTCCACTTCC-3' (P2-2), 5'-CGTAGCCAGCGAATAGTAGGCGGCC-3' (P1-2) and 5'-CGATGGGGGAGATGGAGCAACTGCGTCAG-3' (P1-1). Vector inserts and PCR fragments were sequenced at the sequencing facility of Essen University Hospital.

Gβ-Gγ interaction assay

To study *Gβ*-*Gγ* interaction, co-precipitation analyses of *in vivo* expressed *Gβ* and *Gγ* subunits were performed as described previously [36,37]. The *Gγ* subunits *Gγ5*, *Gγ8_c* and *Gγ12* were epitope-tagged with the influenza virus haemagglutinin (HA) nonapeptide antigen. Constructs were generated by PCR with modified sense primers carrying the sequence for the HA-epitope, an improved Kozak sequence and suitable restriction sites. Likewise, *Gβ1*, *Gβ2* and the *Gβ3* variants were epitope-tagged

with a hexa-histidine (His-) antigen. Templates for construction of the epitope-tagged vectors were kindly provided by Dr U. Quitterer and Dr M. Lohse (Department of Pharmacology, University Hospital, Würzburg, Germany; *Gβ1*, *Gγ5*), Dr O. C. Ong and Dr B. K. Fung (Department of Molecular and Medical Pharmacology, Jules Stein Eye Institute, University of California at Los Angeles, CA, U.S.A.; *Gγ8_c*), Dr T. Asano (Department of Biochemistry, Institute for Developmental Research, Aichi Human Service Center, Kasugai, Aichi, Japan; *Gγ12*) and Dr J. C. Garrison (Department of Pharmacology, University of Virginia, Charlottesville, VA, U.S.A.; *Gβ2*). Plasmids for the *Gβ3* variants were generated by RT-PCR as described above. PCR amplicons of wild-type and epitope-tagged constructs were ligated into pcDNA3.1⁺ (Invitrogen, Breda, The Netherlands) and sequenced. Expression vectors encoding the unmodified proteins were used for control experiments.

Human embryonic kidney cells stably transfected with the large T-antigen of the SV40 virus (HEK-TSA) were grown on 6-cm diameter plates (approx. 3 × 10⁵ cells) and transfected with pcDNA3.1⁺ expression vectors encoding the modified *Gβ*s and *Gγ*s (2 µg of total vector DNA per dish) using Effectene® transfection reagent (Qiagen) according to the manufacturer's instructions. After transfection (24 h), HEK-TSA cells were metabolically labelled for 5 h with 300 µCi of [³⁵S]methionine labelling reagent/dish (Hartmann Analytics, Braunschweig, Germany). Thereafter, cell lysates were prepared and immunoprecipitated with an anti-HA antibody (clone 12CA5; Roche, Mannheim, Germany) as described previously [36,37]. Briefly, lysates were pre-cleared with 40 µl of Protein-A-agarose (Santa Cruz Biotechnology, Heidelberg, Germany) for 45 min. The resulting supernatants were incubated with 15 µl of the anti-HA antibody for 16 h at 4 °C. Immune complexes were precipitated (80 µl of Protein-A-agarose), washed four times, size fractionated by SDS/PAGE (15% gels) and visualized by PhosphorImage analysis (Molecular Dynamics, Krefeld, Germany).

GTPγS binding to digitonin-permeabilized Sf9 insect cells

Sf9 cells were infected with recombinant baculoviruses encoding different *Gβ* subunits together with the M₂ muscarinic acetylcholine receptor (M₂ mAChR), *Gα₁₂* and *Gγ12*. A multiplicity of infection value of 3 was used for each virus. For control purposes recombinant viruses were substituted by the wild-type *Autographa californica* nucleopolyhedrosis virus (AcNPV). Two days after infection, cells were harvested and the carbachol-stimulated [³⁵S]GTPγS binding to digitonin-permeabilized Sf9 cells was quantified as described previously [7]. Recombinant baculoviruses were generated using the transposition-based Bac-to-Bac® system (Life Technologies). Shuttle vector constructs (pFastBac) were verified by sequencing. The templates for the M₂ mAChR and *Gα₁₂* viruses were kind gifts from Dr C. J. van Koppen (Department of Pharmacology, University Hospital, Essen, Germany) and Dr M. I. Simon (Division of Biology, California Institute of Technology, Pasadena, CA, U.S.A.). Expression of *Gβ* subunits was analysed by Western blotting with an anti-*Gβ_{common}* antibody which recognizes a C-terminal epitope conserved in all mammalian *Gβ* subunits except *Gβ5* (Santa Cruz Biotechnology). Preparation of plasma membranes, protein fractionation by SDS/PAGE and immunostaining were performed as described previously [7].

Activation of PLCβ2

Activation of PLCβ2 by overexpressed *Gβγ* dimers was analysed upon transfection of COS-7 cells with different *Gβ* and *Gγ* subunits essentially as described previously [38]. The PLCβ2

clone was a gift from Dr M. I. Simon. It was subcloned into the pcDNA3.1⁺ vector. COS-7 cells (6×10^4) were plated on to each well of a 24-well cell-culture dish. After 24 h cells were transfected using the Effectene[®] transfection reagent according to the manufacturer's instructions. A total of 0.07 μ g of vector DNA, containing equal amounts of PLC β 2, G β and G γ vectors, was applied on to each well. In controls without PLC β 2 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 of inositol-free Dulbecco's modified Eagle's medium (Life Technologies) containing 3.5 μ Ci of *myo*-[³H]inositol (NEN, Bad Homburg, Germany) for another 24 h. Thereafter, the label medium was removed and the cells were incubated in 0.5 ml of inositol-free DMEM medium 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 previously [38]. G $\beta\gamma$ -mediated stimulation of PLC β 2 was defined as the percentage ratio of water-soluble inositol phosphates produced in the presence of overexpressed G $\beta\gamma$ s compared with the inositol phosphates formed in cells overexpressing PLC β 2 together with the respective G γ , but no G β . All experiments were performed as triplicates.

MAP-kinase assay

Activation of an epitope-tagged p44^{HA-erk1} MAP-kinase was determined using bovine myelin basic protein (MBP; Sigma, Deisenhofen, Germany) as substrate. The epitope-tagged MAP-kinase construct was kindly given by Dr S. Meloche (Institut de Recherches Cliniques de Montreal, Montreal, Canada). HEK-TSA cells were transfected with the MAP-kinase construct and G-protein subunits, as described above. After transfection (24 h), cells were serum starved for 24 h. Thereafter, cells were lysed in 200 μ l of ice-cold RIPA lysis buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 0.5% sodium deoxycholate, 1% Nonidet P-40, 0.1% SDS, 10 mM NaF, 10 mM sodium pyrophosphate and 0.1 mM phenylmethylsulphonyl fluoride) and clarified by centrifugation (15 min at 14000 g). Supernatants were incubated with 6.5 μ g of anti-HA antibody (12CA5 clone, Roche) and Protein-A-agarose. Immune complexes were washed twice with ice-cold RIPA lysis buffer and twice with kinase buffer (20 mM Hepes, pH 7.4, 10 mM MgCl₂ and 1 mM dithiothreitol). Phosphorylation of MBP was performed at 25 °C for 30 min in 40 μ l of kinase buffer containing 250 μ g/ml MBP and 20 μ M [γ -³²P]ATP (4 μ Ci/tube; Hartmann Analytics). Reactions were terminated by addition of 40 μ l of 2 $\times$ Laemmli sample buffer, and ³²P-labelled MBP was resolved by SDS/PAGE and quantified by PhosphoImage analysis. The G β 4 plasmid used was generated by PCR from rat brain cDNA, sequenced and subcloned into pcDNA3.1⁺.

RESULTS

Detection and sequence of G β 3s2 transcripts

Previously, we have identified the *GNB3* splice variant G β 3s [7]. *GNB3* is located on chromosome 12, comprises approx. 7 kb and consists of 10 exons and 9 introns [40]. G β 3s is generated by alternative splicing through the use of an alternative splice acceptor site within exon 9. Thus a 123 bp fragment at the 5' end of exon 9 is deleted in G β 3s transcripts [7]. For experiments aiming to quantify the total G β 3 transcripts in human B lymphoblasts, we used oligonucleotide primers which are located in the 3' part of exon 9 (P2-1) and in exon 10 (P1-2; Figure 1B). These primers encompass identical amplicons in G β 3 and G β 3s. Surprisingly, we observed the occurrence of a smaller band in addition to the expected 224 bp amplicon. Interestingly, this

Figure 1 Detection and sequence of G β 3s2 transcripts

(A) G β 3-specific transcripts were amplified by RT-PCR with RNA from human B-lymphoblasts using oligonucleotide primers P2-1 and P1-2. B-lymphoblasts were derived from blood lymphocytes by Epstein-Barr virus immortalization. The expected G β 3 fragment size is 224 bp. Genotype refers to the C825T SNP. Arrows at the left-hand side refer to known bands of the marker (M; plasmid pBR322 digested with *Alu*I). (B) cDNA sequence of G β 3. Upper case letters represent the coding region and lower case letters the 3' non-translated region. Vertical double arrows indicate exon boundaries. Horizontal arrow heads indicate localizations and directions of oligonucleotide primers. The deletion of G β 3s is shaded grey; the deletion observed in G β 3s2 transcripts is indicated in black. The G β 3s2 deletion includes one of two flanking CTTC motifs (indicated in grey). Therefore, an exact localization of the G β 3s2 deletion is not possible. Vertical arrow marks the position of the C825T SNP.

second band of 95 bp occurred in close association with the 825T-allele status (Figure 1A). We cloned and sequenced this smaller band and observed that it comprised the expected G β 3 cDNA fragment, but contained a deletion of 129 bp. In subsequent analyses we discovered 14 independent clones from different individuals and various tissues that all contained the identical deletion. This deletion was also observed in PCR products obtained with primers P2-1 and P2-2 (Figure 1B).

Next, we conducted RT-PCR studies with oligonucleotide primers which flank the entire coding region of G β 3 (primers P1-1 and P2-2). Again, we observed the expected wild-type band and additional shorter fragments. These shorter fragments were cloned, analysed by PCR using primers P2-1 and P2-2, and sequenced. In addition to G β 3s transcripts (see also [7]), we detected 18 independent full-length transcripts with the novel

Figure 2 Structure of *GNB3* and processing of *Gβ3* transcripts

Boxes indicate positions and sizes of exons which are alternately shaded in dark grey and light grey. ATG and TGA indicate start and stop codons respectively, with numbers referring to the transcription start nucleotide as 1. C825T refers to the localization of this SNP in *GNB3*. Horizontal stripes indicate the fragment of exon 9 which is deleted in *Gβ3s*. The respective deletion of *Gβ3s2* within exon 10 is marked by a squared pattern. Horizontal arrows mark the position and directions of the oligonucleotide primers used for RT-PCR experiments.

Alternative	5'-Site	3'-Site	Processed Sequence	5'-3'-Intron Boundaries
1	GTTT-CTTCCCAA	---TGGC-CTTCTCC	GTTTCTTCTCC	CT - GC
2	GTTTC-TTCCCAA	---TGGCC-TTCTCC	GTTTCCTTCTCC	TT - CC
3	GTTTCT-TCCCAA	---TGGCCT-TCTCC	GTTTCTTCTCC	TC - CT
4	GTTTCTT-CCCAA	---TGGCCTT-CTCC	GTTTCTTCTCC	CC - TT
5	GTTTCTTC-CCCAA	---TGGCCTTC-TCC	GTTTCTTCTCC	CC - TC

Figure 3 Potential splice sites for processing of *Gβ3s2* transcripts

It is impossible to determine whether the upstream or the downstream CTTC motif in *Gβ3s2* is retained. Therefore, several combinations of non-canonical splice donor and acceptor sites can potentially contribute to the alternative splicing of *Gβ3s2*. It is not known which of these alternatives is actually used. All combinations result in an identical *Gβ3s2* cDNA, however, the intron boundaries are different.

129 bp deletion (15 from carriers of the TC-/TT-genotypes; 3 from carriers of the CC-genotype). Because of the obvious similarity to *Gβ3s*, we referred to this novel transcript as '*Gβ3s2*'. Figure 1(B) shows the partial cDNA sequence of *Gβ3* with the deletions of *Gβ3s* and *Gβ3s2*, exon boundaries and localizations of oligonucleotide primers.

Processing of *Gβ3s2* transcripts

Figure 2 shows the structure of *GNB3* and the proposed processing of *Gβ3*, *Gβ3s* and *Gβ3s2* transcripts. Interestingly, the deletion in *Gβ3s2* includes the C825T SNP. Since one of two flanking CTTC motifs is retained in *Gβ3s2* transcripts (Figure 1B), it is not possible to define the exact locus at which the processing of the pre-mRNA actually occurs. Comparing the sequence of the *Gβ3s2* transcript with that of the wild-type transcript and the genomic sequence, it is evident that five different theoretical modes for processing the pre-mRNA are

possible which will all result in the same *Gβ3s2* transcript (Figure 3). Thus in *Gβ3s2* a minimum 2 and a maximum 6 nucleotides are retained from the 5' part of exon 10 which are followed by 148–152 nucleotides from the 3' portion of this exon (Figure 1B). None of these five potential splice sites includes canonical splice acceptor and donor motifs ('GT-AG rule').

Splicing at non-canonical donor and acceptor sites is a rare event. Based on data from more than 43 000 introns compiled in the Splice Site DataBase (<http://genomic.sanger.ac.uk/spldb/SpliceDB.html> and [41]), only 0.76 % of all introns are estimated to be processed at non-canonical sites. In the case of *Gβ3s2*, processing may formally occur at CT-GC, TT-CC, TC-CT, CC-TT or CC-TC intron sites (5'-3'; Figure 3). Several of these motifs have been implicated in pre-mRNA processing (Splice Site DataBase). Examples include the human Fcγ receptor IIA (cDNA and genomic accession numbers are M90721 and HG_0006494 respectively), the rat homeobox gene PEM (U52034, RDG0027714), the human interleukin 15 gene (X91233,

Figure 4 Genotype-dependent expression of G β 3s2-specific transcripts

Occurrence of G β 3s2-specific transcripts in various tissues from different individuals. G β 3-specific transcripts were amplified by RT-PCR with primers P2-1 and P2-2. Heart muscle tissue was obtained from atria (ten different individuals). Neutrophils (six different individuals) were prepared from peripheral blood by dextran sedimentation and density gradient centrifugation. Peripheral blood lymphocytes were prepared by density gradient centrifugation, partially enriched for T-lymphocytes by affinity chromatography and stimulated with interleukin-2. Human bladder carcinoma tissue was obtained from surgical specimens of 20 different patients. Representative bands were excised and sequenced to verify their identity. Arrows indicate positions of G β 3 and G β 3s2. V, positive control using G β 3s2 vector DNA. M and N indicate nucleotide size marker and negative controls respectively.

HG+0014129) and the mouse growth hormone gene (U34362, RDG0013338).

We repeated the PCR experiments using genomic DNA to exclude a processed G β 3s2-specific pseudogene as source for the observed PCR fragments. However, the only PCR fragments observed under these conditions (primers P2-1/P2-2) were large (> 2 kb) and resembled most closely the respective *GNB3* fragment including introns 9 and 10. Furthermore, all RNA preparations used were treated with RNase-free DNase and PCR studies using such treated RNA specimens without reverse transcription were always negative.

Expression of G β 3s2

Subsequently, we analysed the genotype-dependent expression of G β 3s2 transcripts in various tissues and cell types by RT-PCR (Figure 4). We detected G β 3- and G β 3s2-specific transcripts in all examined tissues including heart, brain (results not shown),

blood cells and bladder carcinoma. Furthermore, we observed a close association between G β 3s2 expression and T-allele status. However, faint G β 3s2-specific bands were also detected in cells with the C-allele, which indicates that the predominant expression of G β 3s2 associated with the T-allele is a quantitative, but not a qualitative feature of this polymorphism or haplotype. Identity of PCR fragments was repeatedly confirmed by sequencing.

At present, there exists no G β 3s2-specific antibody. Available antibodies recognize either an epitope directly upstream of the G β propeller or a C-terminal sequence. Western blot analyses have already demonstrated the occurrence of short bands of approx. 32 kDa in cells from carriers with a T-allele in addition to the wild-type G β 3 protein [7]. In retrospect, it is not possible to unequivocally attribute these bands to G β 3s (calculated molecular mass, 32.8 kDa), G β 3s2 (calculated molecular mass, 32.6 kDa) or to both G β 3 variants.

Structure of G β 3s2

Next, we analysed the structural consequences of the deletion in G β 3s2. Similar to G β 3s, this deletion of 129 bp in G β 3s2 does not affect the open-reading frame in the remaining 3'-part of the transcript. The three-dimensional structure of G β 1 has been resolved by crystallization analysis and revealed a propeller-like structure comprising seven blades that are regularly arranged around a central pore [42]. This geometric structure has also been referred to as 'torus' [43]. Each blade consists of four anti-parallel β -sheets termed 'a' to 'd' from the innermost to the outermost strand. The d-strands connect subsequent propeller blades and form most of the accessible surface of the protein. In principle, each blade can be attributed to the equivalent of one WD domain (the original definition based on linear sequence analyses in the absence of structural data begins with a d-strand followed by strands a–c of the next blade and terminates with the WD motif at the end of strand c). The G β torus is closed by a 'velcro' mechanism between a first N-terminal d-strand which contacts strands a to c formed by the C-terminus of the protein. In addition to this propeller domain of G β 1 there is an N-terminal coiled-coil extension which interacts with G γ subunits [42].

Amino-acid determinants of WD-domains, which are essential for the formation of the propeller structure, are highly conserved in all G β proteins [43]. The mammalian G β -protein isoforms G β 1 to G β 4 are identical in more than 80% of their amino-acid composition [3]. Therefore, it is safe to assume that the structure of wild-type G β 3 will strongly resemble that of G β 1 [43]. Based on this structure the deletion in G β 3s2 will affect strands b–d of blade 5 and strand a of blade 6 (nomenclature of propeller blades according to [43]). Figure 5 shows the G β 3s2 deletion superimposed on the schematic structure of G β proteins. It is evident that this deletion corresponds exactly to the equivalent of one WD domain. Thus the structure of G β 3s2 is similar to G β 3s which also lacks the equivalent of one WD domain [7]. In the case of G β 3s, parts of blades 3 and 4 are affected by the respective deletion (Figure 5).

At present, more than a 100 different proteins are known which contain between 4 and 16 WD motifs (Conserved Domain Data Base of the National Center for Biotechnology Information). At least 16 different proteins with six WD domains have been registered in this database. For one of these proteins, Sec13, functional data exist which suggest a highly condensed structure compatible with a six-blade propeller [44].

Hence, there is good evidence to assume that G β 3s and G β 3s2 may also form a six-blade propeller. Figure 5 shows this hypothetical structure.

Figure 5 Proposed structures of Gβ3, Gβ3s and Gβ3s2

The deletion of Gβ3s2 is superimposed on the three-dimensional schematic structure of Gβ3. For comparison, the deletion in Gβ3s is also depicted. The lower panel displays the proposed six propeller blade structure of Gβ3s and Gβ3s2. Note that different propeller blades are retained in Gβ3s and Gβ3s2. The nomenclature of the propeller blades is as described previously [43].

Biological activity of Gβ3s2

Despite the major deletion in Gβ3s, overexpression studies yielded evidence for its biological activity [7,10]. Hence, we investigated whether Gβ3s2 also shares typical characteristics of Gβ proteins. Such features of Gβ proteins include the ability to modulate receptor-stimulated Gα activation and to regulate effector systems [43]. For both activities Gβ proteins must form stable dimers with Gγ subunits.

In the first series of experiments, we investigated whether Gβ3s2 is able to form dimeric complexes with Gγ subunits. Folding of Gβ proteins requires Gγ subunits and chaperonins [43]. From the 12 known human Gγ proteins we chose Gγ5, Gγ12 and Gγ8_c for this analysis. Gγ5 has been shown to interact with Gβ3 and Gβ3s in previous experiments [7]. Gγ12 is related to Gγ5 and both are widely expressed. Gγ8_c co-localizes with Gβ3 in cone cells of the retina, and the dimer Gβ3γ8_c has been implicated in signal transduction of colour vision [45,46]. Furthermore, Gγ8_c differs from Gγ5 and Gγ12 in its lipid modifi-

Figure 6 Gβ–Gγ interaction analysis

HEK-TSA cells were co-transfected with tagged Gβ and Gγ subunits as indicated. Cells were metabolically labelled with [³⁵S]methionine, and 2 days after transfection lysates were prepared and immunoprecipitated with an anti-HA antibody. Precipitates were then size fractionated by SDS/PAGE and visualized by Phosphorimaging. In the absence of transfected HA–Gγ no precipitation was observed (compare with lane 1 of the HA–Gγ8_c gel). HA–Gγ also interacted with endogenous Gβ proteins (e.g. weak bands in lanes of Gβ3s and Gβ3s2 co-migrating with wild-type Gβ subunits). Transfected Gβ subunits carried a His-tag modification as an additional means to differentiate them from endogenous Gβ proteins. Arrows indicate positions of Gβ and Gγ proteins.

cation (farnesylation versus geranyl-geranylation) which is required for attachment of the Gβγ complex to the cell membrane.

Gβ–Gγ interactions were analysed by co-precipitation studies with epitope-tagged Gγ subunits. Since we included wild-type Gβ3 and Gβ1 in these experiments, it was necessary to modify Gβ1 and Gβ3 with an N-terminal His-tag to allow their discrimination from endogenous Gβ proteins. For comparability Gβ3s and Gβ3s2 were also modified with this tag. Evidence from others and own control experiments (activation of PLCβ2 by His–Gβ1/HA–Gγ5; results not shown) suggest that such N-terminal modifications of Gβ and Gγ subunits do not apparently interfere with their ability to dimerize and bind to interacting

Figure 7 Activation of heterotrimeric G proteins in the presence of Gβ3s2

Sf9 insect cells were infected with recombinant baculoviruses encoding the M₂ mAChR, Gα_{i2}, Gγ12 and different Gβ3 variants. All infections were performed at a multiplicity of infection of 3 for each vector. In all experiments a constant amount of virus was used and required the supplementation with wild-type AcNPV as indicated. (A) Western blot analysis of expressed Gβ3 variants in plasma membranes of Sf9 insect cells. Immunostaining was performed with an anti-Gβ_{common} antibody. (B) Carbachol-stimulated activation of M₂ mAChR in the presence of Gβ3s. At 2 days after infection, Sf9 cells were harvested and the carbachol-stimulated (100 μM) [³⁵S]GTPγS binding to digitonin-permeabilized Sf9 cells was determined. All measurements were performed as quadruplicates. The increase in bound GTPγS after 10 min incubation time is shown. *Significantly different from other samples without Gβ3s2 at $P < 0.05$ (Student's *t* test). Representative results from a total of three experiments with qualitatively identical results. (C) Effect of Gβ3 variants on receptor-stimulated GTPγS binding. *Significantly different ($P < 0.05$; Student's *t* test) from Sf9 cells which were infected with the wild-type AcNPV virus instead of a Gβ-virus (first column). Representative experiment of three others with qualitatively identical results.

proteins [36,37]. Figure 6 shows typical results from these experiments. Gβ and Gγ subunits were transiently expressed in HEK-TSA cells which were metabolically labelled with [³⁵S]-methionine. Cells were lysed and the co-precipitation of Gβ subunits was analysed upon immunoprecipitation with an anti-HA antibody. In the absence of expressed HA-Gγ proteins no Gβ subunits were detectable in immunoprecipitates (Figure 6, experiment for Gγ8_c). Co-expression of Gβs with HA-Gγ subunits resulted in co-precipitation of all Gβ subunits examined, including Gβ3s and Gβ3s2. Interestingly, in the case of Gγ8_c the

amount of precipitated Gβ3s and Gβ3s2 equalled that of Gβ1 and Gβ3. Gβ3s and Gβ3s2 also dimerized with Gγ5 and Gγ12, although this interaction was apparently weaker, which may indicate a certain Gβ-Gγ specificity of Gβ variants. Similar results were obtained when His-tagged Gβ3 variants were substituted by their wild-type proteins (results not shown).

Having demonstrated a stable interaction between Gβ3s2 and Gγ subunits, we investigated whether such Gβ3s2γ dimers are able to interact with Gα subunits and modulate receptor-stimulated Gα activation. For this, we analysed the receptor-stimulated binding of GTPγS, a stable GTP analogue to Gα subunits in permeabilized Sf9 insect cells. Similar experiments have demonstrated the biological activity of Gβ3s [7]. Infection of Sf9 cells with recombinant baculoviruses for Gβ3, Gβ3s and Gβ3s2 resulted in the generation of proteins of the expected sizes (Figure 7A). Stimulation of wild-type insect cells (not infected with the recombinant M₂ mAChR virus) with carbachol (100 μM), a specific agonist for mAChRs, resulted in a small but insignificant increase in GTPγS binding (Figure 7B). Sole infection of Sf9 cells with the M₂ mAChR resulted in a small but significant stimulation of GTPγS binding ($23 \pm 3\%$; $n = 3$ independent experiments), which was further increased upon successive supplementation with Gα_{i2}, Gγ12 and, finally, Gβ3s2. Carbachol-stimulated GTPγS binding amounted to $76 \pm 4\%$ ($n = 3$ independent experiments; measurements performed as quadruplicates) in the presence of Gα_{i2}, Gγ12 and Gβ3s2. These results suggest that the M₂ mAChR may interact with endogenous G-proteins from insect cells. However, stimulation was most efficient in the presence of a mammalian heterotrimer, in this case with Gβ3s2. Based on these results, we compared the carbachol-stimulated GTPγS binding in the presence of Gα_{i2}, Gγ12 and Gβ3, Gβ3s and Gβ3s2 (Figure 7C). Again, we observed an increase in GTPγS binding upon stimulation with carbachol in cells expressing the M₂ mAChR together with Gα_{i2} and Gγ12. However, receptor-stimulated GTPγS binding significantly increased in cells which co-expressed one of the Gβ3 variants (Figure 7C).

Subsequently, we investigated whether Gβγ-containing Gβ3s2 can activate Gβγ-dependent effector systems. An important effector regulated by Gβγ complexes is PLCβ2. Upon overexpression in COS-7 cells of Gβ1 together with Gγ5 or Gγ12, we measured 2–5-fold increases in inositol phosphate generation (results not shown). Overexpression of Gβ3 together with Gγ12 resulted in a 2-fold increase in inositol phosphate generation. We observed only a modest increase in inositol phosphates (approx. 20%) in cells expressing Gβ3γ5. However, in COS-7 cells expressing Gγ5 or Gγ12 together with Gβ3s or Gβ3s2, inositol phosphate generation was not different from control cells (results from three independent experiments with measurements performed as triplicates). Western blot analyses revealed that the amounts of overexpressed Gβ1, Gβ3, Gβ3s and Gβ3s2 were similar. Hence, Gβ3s and Gβ3s2 do not activate PLCβ2, at least when complexes with Gγ5 and Gγ12 are considered (results not shown).

Finally, we examined another effector system regulated by Gβγ dimers, the activation of the MAP-kinase cascade. This signalling pathway is an important step in the control of cell proliferation. Gβγ dimers route the signal from activated receptors which couple to pertussis toxin-sensitive G-proteins to this cascade. The exact pathways are complex and not fully understood, but involve adaptor proteins like Shc and Grb2, Src kinases, transactivation processes to receptor tyrosine kinases and proteins involved in receptor desensitization (reviewed in [47]). Free Gβγ dimers generated by overexpression have been demonstrated to activate this cascade, ultimately quantified by

Figure 8 MAP-kinase activation upon overexpression of G β subunits in HEK-TSA cells

(A) A HA-tagged MAP-kinase construct (p44^{erk-1}) was overexpressed together with different G β isoforms in HEK-TSA cells. For controls the amount of vector DNA was kept constant by supplementation with empty pcDNA3.1⁺ vector. Two days after transfection, the HA-tagged MAP-kinase was immunoprecipitated and its activity determined by kinase assays *in vitro* with the substrate MBP. Representative results from three independent experiments. (B) Densitometric analysis of MBP phosphorylation from three independent experiments. Shown is the percentage increase in MBP phosphorylation above vector control (taken as 100%). *Significantly different from vector control at $P < 0.05$ (Student's *t* test).

an increase in the activity of the kinase [48]. We expressed an epitope-tagged MAP-kinase construct (p44^{HA-erk1}) in HEK-TSA cells and examined the activation of the encoded protein upon immunoprecipitation by its ability to phosphorylate the substrate MBP as described previously [39]. Western blot controls confirmed that the expression of the kinase construct was identical in all cells upon transfection. Likewise, there was no difference in the amounts of expressed G β 1, G β 3, G β 3s, G β 3s2 and G β 4 in transfected HEK-TSA cells. Overexpression of G β proteins without G γ subunits sufficed to increase MBP phosphorylation (Figure 8A). This is best explained by the dimerization of overexpressed G β s with endogenous G γ s which increase the amount of free G $\beta\gamma$ complexes. Interestingly, MBP phosphorylation was markedly increased in cells overexpressing G β 3s or G β 3s2. Co-expression of G γ 5 or G γ 12 with G β 3s or G β 3s2 did not further increase MAP kinase activity (results not shown). Densitometric analysis of three independent experiments indicated an increase in MBP phosphorylation by $66 \pm 11\%$, $33 \pm 8\%$, $182 \pm 24\%$, $107 \pm 13\%$ and $42 \pm 7\%$ above vector control for G β 1, G β 3, G β 3s, G β 3s2 and G β 4 respectively (Figure 8B). Thus activation of the MAP kinase cascade was significantly ($P < 0.05$) enhanced in cells expressing G β 3s or G β 3s2 compared with the wild-type protein.

DISCUSSION

We have identified and characterized a novel G β 3 variant which appears to be widely expressed. Another G β 3 variant, G β 3s, has already been implicated in the enhanced signal transduction observed in cells from carriers of the *GNB3* 825T-allele. Now,

our present data unravel an additional complexity that raises several important questions.

Expression of G β 3s2 occurs in tight association with the 825T-allele. G β 3s2 transcripts have been detected in all tissues investigated so far, although cells with the 825C-allele express only minor amounts of this transcript. Unfortunately, available antibodies do not distinguish between G β 3s2 and the formerly described variant G β 3s. Previous studies have demonstrated the expression of a short G β 3 variant by Western blotting in association with the 825T-allele [7]. Hence, there is good evidence for the existence of (a) short G β 3 variant(s). Clarification of their identities will require the generation of G β 3s- and G β 3s2-specific antibodies.

The mechanisms that contribute to the splice mechanisms leading to the generation of G β 3, G β 3s and G β 3s2 are not clear, as the regulation of splicing itself is not well understood. The *GNB3* C825T polymorphism and other polymorphisms associated with this SNP do not directly affect canonical splice sites or associated motifs (e.g. the lariat-binding region), which could explain the processing to G β 3s2 (and G β 3s) in a conventional manner. The C825T SNP is located within 6–10 nucleotides from the acceptor site for the generation of G β 3s2. Thus it may directly influence the splice machinery. On the other hand, one has to consider the complex haplotype comprising seven polymorphic sites along the entire *GNB3* pre-mRNA, which may have a major impact on pre-mRNA folding, as already shown by modelling studies [8]. Thus G β 3s2 is a member of a highly exclusive group of transcripts comprising only 0.6% of all spliced products that use non-canonical sites [41].

Next, we have to ask how G β 3s and G β 3s2 may affect signal transduction and ultimately contribute to the complex phenotype associated with the 825T-allele. One straight-forward explanation is a direct effect of G β 3s2 or G β 3s on receptor-mediated G α activation. We propose that G β 3s and G β 3s2 fold into six-blade propeller proteins despite their major deletions. Recently Tup1, a transcriptional repressor protein from yeast has been crystallized [49]. This protein consists of seven WD domains, but shares no other sequence homologies with G β proteins. Each of its seven WD motifs fold into a propeller blade structure [49]. This confirms the principal conclusions from the crystallization of G β 1, the first member of the WD domain protein family for which the structure has been solved [42]. Furthermore, available evidence suggests that the six-WD-domain-containing protein Sec13 forms a highly condensed structure compatible with a six blade propeller [44]. Thus it appears plausible that G β 3s and G β 3s2 will also fold into a six-blade propeller-like structure. Our data also suggest that G β 3s and G β 3s2 are able to form stable immunoprecipitable G $\beta\gamma$ dimers, a major characteristic of G β proteins. Overexpression of G β 3s and G β 3s2, as shown in the present study and in previous studies [7, 10], enhances receptor-mediated GTP γ S binding, a measure for G α GDP/GTP exchange. Furthermore, overexpression of G β 3s results in enhanced cellular responses as shown for chemotaxis [10]. Therefore, it is attractive to speculate, that G β 3s γ or G β 3s2 γ dimers may actually interact with G α proteins and for instance, destabilize G α -GDP binding, which could facilitate receptor-mediated G α -GDP/GTP exchange. The (increased) activation of the MAP-kinase cascade by G β 3s and G β 3s2 is the first argument for the ability of free G β 3s γ and G β 3s2 γ dimers to interact with effector systems. At present, the molecular mechanisms that mediate the increase in MAP-kinase activity in the presence of G β 3s and G β 3s2 are not clear and require an in-depth investigation of the involved signaling pathways. Nevertheless, the activation of the MAP-kinase cascade by G β 3s and G β 3s2 could offer one explanation for the enhanced pro-

liferation of cells which were used for the discovery of the C825T SNP [7,50].

On the other hand, the lack of effect of G β 3s and G β 3s2 on PLC β 2 in contrast with G β 1 and G β 3 may suggest a certain effector specificity potentially related to the deletions in G β 3s and G β 3s2. The various propeller blades of a given G β subunit differ considerably in their amino acid sequence of their d-strands, i.e. their outside accessible parts. However, these d-strand sequences are highly conserved between different G β subunits (i.e. G β 1 to G β 5) and different species [37]. In other words, blade 2 of G β 3 will differ considerably from blade 7, but shares a high homology with blade 2 of G β 1, G β 2, G β 4 and G β 5. Therefore, different blades may interact specifically with different effector proteins [37,51]. Based on this hypothesis, it is likely that the deletions in G β 3s and G β 3s2 may have different effects on their ability to interact with effector proteins, including phospholipases, adenylyl cyclases, K⁺- and Ca²⁺-channels, receptor kinases and many more.

Despite these intriguing possibilities, cautionary notes are necessary regarding the roles of G β 3s and G β 3s2 for the expression of the complex phenotype associated with the 825T-allele. The data presented here on the biological activities of G β 3s and G β 3s2 result from overexpression studies which may have limited relevance for physiological situations *in vivo*. Thus we should not leave altered expression of wild-type G β 3 protein out of consideration. Generation of G β 3s and G β 3s2 transcripts occurs at the expense of regular G β 3 transcripts. In our first publication on G β 3s we quantified small and regular transcripts by counting clones obtained from PCR cloning [7]. The prime aim of this experiment was to demonstrate the quantitative association between the 825T-allele and the occurrence of short G β 3 transcripts. In retrospect, it becomes obvious that this experiment classified as short transcripts both G β 3s and G β 3s2. Thus the main conclusion remains true, that the occurrence of short G β 3 variants is associated with the 825T-allele. However, alternative aspects of this experiment have not yet been discussed. Assuming identical rates of transcription for the different genotypes, generation of short G β 3 transcripts will result in reduced amounts of 'wild-type' G β 3 transcripts in carriers of the TC and TT genotypes. Based on these published data [7], we estimated that carriers of the TC- and TT-genotype generate only 77 % and 65 % of regular G β 3 transcripts compared with carriers of the CC genotype. The verification of such minor differences requires highly sensitive transcript and protein quantification techniques and large samples of specimens from human tissues. In addition, these clone-counting studies [7] confirm the observation from the present work that short G β 3 variants amount to a small fraction of total G β 3 only. Although our data from overexpression studies support the notion that G β 3s and G β 3s2 are biologically active, we presently do not know whether the sole presence of short G β 3 variants suffices for the generation of the GNB3 825T phenotype *in vivo*. Alternatively, the altered balances in G β 3 wild-type and variant proteins may account for the observed variations in signal transduction. To test these hypotheses, we have to reconstitute such 'minor' changes in the balance of G-proteins subunits, which is not an easy task. Nevertheless, for G α_s , another G-protein subunit, available evidence suggests that a reduction by less than 50 % exerts major impacts on signal transduction [2].

Taken together we have identified an additional G β 3 variant that is expressed predominantly in cells with the 825T-allele. Further experiments are required to characterize its molecular functions and to delineate how the generation of such protein variants affects the delicate balance of G-protein subunits. The investigation of these issues may aid in understanding the

mechanisms which give rise to the complex phenotype associated with the GNB3 825T-allele.

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