

PTX-sensitive G proteins, their preparation and use

Description

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The present invention relates to novel human G proteins, in particular $\beta 3$ subunits of G proteins, a process for their preparation and their use in diagnosis and therapy.

- 10 Heterotrimeric guanine nucleotide-binding proteins (G proteins) have an outstanding importance in intracellular signal transduction. They mediate the relaying of extracellular signals after stimulation of hormone receptors and other receptors which undergo a conformational change after receptor activation. This
15 leads to activation of G proteins which may subsequently activate or inhibit intracellular effectors (eg. ion channels, enzymes). Heterotrimeric G proteins consist of three subunits, the α , β and γ subunits. To date, several different α subunits, 5 β subunits and about 12 γ subunits have been detected by biochemical and
20 molecular biological methods (Birnbaumer, L. and Birnbaumer, M. Signaltransduction by G proteins: 1994 edition. J.Recept.Res. 15:213-252, 1995; Offermanns, S. and Schultz, G. Complex information processing by the transmembrane signaling system involving G proteins. Naunyn Schmiedeberg's Arch.Pharmacol. 350:329-338, 1994;
25 Nürnberg, B., Gudermann, T., and Schultz, G. Receptors and G proteins as primary components of transmembrane signal transduction. Part 2. G proteins: structure and function. J.Mol.Med. 73:123-132, 1995; Neer, E.J. Heterotrimeric G proteins: Organizers of Transmembrane Signals. Cell 80:249-257, 1995;
30 Rens-Domiano, S. and Hamm, H.E. Structural and functional relationships of heterotrimeric G-proteins. FASEB J. 9:1059-1066, 1995).

- Receptor-mediated activation of certain α subunits can be inhibited by pretreatment with pertussis toxin (PTX). These include,
35 in particular, the α isoforms $\alpha i1$, $\alpha i2$ and $\alpha i3$, and various αo subunits. G Proteins of these types are also referred to as PTX-sensitive G proteins.

- 40 $\beta \gamma$ Subunits carry out essential functions in G protein activation and in the modulation of intracellular reactions. All G protein β subunits disclosed to date have high degrees of homology at the level of the nucleotide sequence and at the level of the amino-acid sequence. Moreover these similarities are found not only
45 within the human β subunits ($\beta 1$, $\beta 2$, $\beta 3$) but also in comparison with β subunits of other species, for example fruit fly or yeast.

X-Ray structural analyses have been able to determine those amino acids in α , β and γ subunits which are in contact with one another and are necessary for ordered formation of the heterotrimer.

- 5 All G protein β subunits disclosed to date belong to the WD repeat proteins. The N terminus of the β subunit interacts predominantly with γ subunits, and the C terminus is involved in the interaction with receptors.
- 10 β Subunits form what are called propeller structures. The β propellers of the G β subunits consist of 7 β propeller blades, each propeller blade consisting of 4 amino-acid regions in antiparallel arrangement. The seven-fold symmetry of the β propeller can be detected at the level of the amino-acid
- 15 sequence, which comprises 7 WD repeats. A WD repeat motif comprises about 40 amino acids and has a number of conserved amino acids, including Trp-Asp dipeptide sequences. This WD motif frequently terminates the WD repeat (Fig. 1).
- 20 It has now been found, surprisingly, that G protein β_3 subunits which consist only of 6 instead of the 7 WD repeat motifs otherwise described occur, for example, in hypertensives. The cellular activatability of PTX-sensitive G proteins is increased in these hypertensives compared with normotensives.
- 25 Molecular analysis revealed a novel amino-acid sequence for the β_3 subunit in these hypertensives, being shorter by 41 amino acids than the known sequence. The sequence is depicted in SEQ ID NO:2. Formally, it is derived from the known human β_3 subunit by
- 30 deletion of amino acids 167-207.

The corresponding DNA sequence coding therefor is described in SEQ ID NO:1.

- 35 The reason for the occurrence of the shortened G β_3 subunit in hypertensives is presumably an alternative splicing of the relevant gene. At the DNA level, there is an intron exactly in front of the putative splicing site. The intron starts behind nucleotide 497 in the open reading site (numbering as in SEQ ID
- 40 NO:1).

- It was also possible by PCR on genomic DNA to detect an intron starting at about nucleotide 620. The shortened form apparently comes about through deletion of a complete exon. The invention
- 45 furthermore relates to a process for preparing shortened forms of

human G β 3 subunits as mentioned above by expression of a nucleic acid sequence coding therefor in a host organism.

The recombinant expression preferably takes place by preparing a gene construct which, besides the coding nucleic acid sequence, also comprises other signal and regulatory sequences such as promoters, terminators, ribosome binding sites, polyadenylation sites and the like. The general procedure for recombinant expression of a gene is familiar to the skilled worker.

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The invention furthermore relates to the use of the nucleic acid sequences according to the invention for producing drugs for gene therapy. Introduction of these nucleic acid sequences in direct form or after preparation of an appropriate gene vector into patients' cells is able to achieve an increased activatability of G proteins therein.

This is desirable in a number of disorders in which there is dysregulation associated with G protein.

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Diseases associated with G protein dysregulation mean those disorders in which G protein is involved in signal transduction and does not perform its function in a physiological manner.

25 These disorders include cardiovascular disorders, metabolic disturbances and immunological disorders.

Cardiovascular disorders which should be mentioned are: hypertension, pregnancy hypertension (gestosis, hypertension in pregnancy), coronary heart disease, localized and/or generalized atherosclerosis, stenoses of blood vessels, restenosis after revascularizing interventions on vessels (eg. PTCA with and without stent implantation), proneness to stroke, thrombophilia and increased platelet aggregation.

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Metabolic disturbances which should be mentioned are: metabolic syndrome, insulin resistance and hyperinsulinemia, type II diabetes mellitus, diabetic complications (eg. nephropathy, neuropathy, retinopathy, etc.), lipid metabolism disturbances, disturbances of central chemoreception (CO₂ tolerance, acidosis tolerance, sudden infant death (SIDS)).

Immunological disorders which should be mentioned are: impaired strength of the body's immune response (formation of immunoglobulins, aggressiveness of T cells and NK cells), impaired general tendency to proliferation, including wound-healing capacity, tendency to tumor development and

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proliferation including metastatic potential of malignantly transformed cells, duration of the latency period after HIV infection until the disorder becomes clinically apparent, Kaposi sarcoma, tendency to cirrhosis of the liver, transplant tolerance and transplant rejection.

The invention furthermore relates to the use of the nucleic acid sequences according to the invention for the diagnosis of disorders, in particular including determination of the risk of suffering from a disease associated with G protein dysregulation.

Besides determining the risk of certain disorders, it is also possible to make general physiological data and statements through the use according to the invention, for example on central chemoreception, CO₂ tolerance, acidosis tolerance, risk of sudden infant death (SIDS), fitness for certain types of sport.

The invention furthermore relates to the use of nucleic acid sequences which are complementary to the nucleic acid sequences coding for the shortened form of the G β 3 subunit. Sequences of this type can be used as antisense constructs for the treatment or prevention of disorders associated with G protein dysregulation.

The invention furthermore relates to a method for determining a relative risk of suffering from diseases associated with G protein dysregulation for a subject by comparing the gene sequence for human G protein β 3 subunit of the subject with the gene sequence SEQ ID NO: 1 and, in the event that it coincides with SEQ ID NO: 1, assigning an increased risk to the subject.

In the method according to the invention for determining the relative risk, body material containing the subject's genetic information is taken from the subject. This is achieved, as a rule, by isolating the nucleic acid from a blood sample.

The structure of the gene for the G protein β 3 subunit is determined from the subject's isolated nucleic acid and compared with the sequence indicated in SEQ ID NO:1.

The structure of the gene can be determined by sequencing the nucleic acid. This can take place either directly from the genomic DNA or after amplification of the nucleic acid, for example by the PCR technique.

The structure of the gene can take place at the mRNA or cDNA level.

Determination is preferably by sequencing after PCR amplification of the cDNA. The primers suitable for the PCR reaction [sic] can easily be deduced by the skilled worker from the sequences depicted in SEQ ID NO:1. The procedure for this is advantageously such that in each case a primer binding strand and complementary strand in front of and behind the deletion site is chosen.

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However, the comparison of genes can also be carried out by other methods, for example by selective hybridization or by appropriate mapping using restriction enzymes.

15 The diagnostic methods described above can also be carried out at the protein level. For example, the proteins according to the invention can be used to produce specific antibodies to recognize the shortened form of the G β 3 subunit. Antibodies of this type can then be used to carry out, where appropriate, by conventional
20 ELISA methods, protein chemical investigations in addition or alternatively to the genetic investigations.

The invention furthermore relates to the production of transgenic animals which harbor the genetic modification described above
25 (shortening of the G β 3 subunit). Transgenic animals of this type are of great importance in particular as animal models for investigating and treating the diseases described above. Processes for producing transgenic animals are generally known to the skilled worker.

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The following Examples serve to illustrate the invention further.

Experimental part

35 1. Functional results on G protein activation in essential hypertension

A detailed characterization was carried out of the activation of G proteins from cells of normotensive subjects and hypertensive
40 patients. For this purpose, the stimulated incorporation of radiolabeled [35 S]GTP γ S was quantified by the method described by Wieland et al. (Wieland, T., Liedel, K., Kaldenberg-Stasch, S., Meyer zu Heringdorf, D., Schmidt, M., and Jakobs, K.H. Analysis of receptor-G protein interactions in permeabilized cells.
45 Naunyn-Schmiedeberg's Arch.Pharmacol. 351:329-336, 1995). G Proteins were activated initially by stimulating cells which had been permeabilized with digitonin using the peptide mastoparan 7.

This peptide simulates the configuration of an activated G protein-coupled receptor so that it can be used to induce direct G protein activation independent of receptors (Ross, E.M. and Higashijima, T. Regulation of G protein activation by mastoparan and other cationic peptides. *Methods Enzymol.* 237:27-38, 1994). The binding of GTP γ S induced by MAS-7 is completely PTX-sensitive so that it can be used to quantify the activation of heterotrimeric G proteins of the G_i type. Fig. 2 shows the concentration-dependence of the G protein activation induced by mastiparan 7 (MAS-7) in the normotensive (NT) and hypertensive (HT) state. MAS-7 induces strong [35 S]GTP γ S binding on HT cells with an EC₅₀ of about 5 μ M (Fig. 2). Maximum binding is reached at about 25 to 50 μ M MAS-7. In contrast to this, the concentration required for the same [35 S]GTP γ S binding to NT cells are [sic] 10 times higher (Fig. 2). These data demonstrate that activation of PTX-sensitive G proteins in HT cells requires distinctly less activated receptor than that in NT cells.

Fig. 3 shows the time course of the binding, stimulated by mastoparan 7, of [35 S]GTP γ S to cell lines from normotensives (NT) and hypertensives (HT).

The binding of [35 S]GTP γ S to hypertensive cells is distinctly speeded up (rate constants 0.005 s⁻¹ in the normotensive versus 0.01 s⁻¹ in the hypertensive state).

Fig. 4 shows the GDP-dependence of binding, stimulated by mastoparan 7, of [35 S]GTP γ S to isolated cell membranes from normotensives and hypertensives. It is evident that the maximum stimulated binding of [35 S]GTP γ S to membranes from hypertensives occurs at lower concentrations of GDP (about 0.2 μ mol/l), where a concentration of 1 μ mol/l GDP is required for the same effect in normotensives.

In a similar way, the maximum binding, stimulated by mastoparan 7, of [35 S]GTP γ S to membrane preparations from hypertensive cells a low concentration of free Mg²⁺ (about 0.01 mmol/l), while a free Mg²⁺ concentration of 0.1 mmol/l is necessary for the same maximum binding of [35 S]GTP γ S to membranes from normotensive cells (Fig. 5).

Experiments were subsequently carried out on the reconstitution of the increased activatability of G proteins from hypertensive cells. For this purpose, the photoreceptor rhodopsin, and the G protein α subunit transducin (α_t) from the bovine eye were purified (Phillips, W.J., Wong, S.C., and Cerione, R.A.

Rhodopsin/transducin interactions. II. Influence of the transducin- $\beta\gamma$ subunit complex on the coupling of the transducin- α subunit to rhodopsin. J.Biol.Chem. 267:17040-17046, 1992). In addition, G proteins were extracted from membranes from normotensive and hypertensive cells by addition of cholate (Mitchell, J., Northup, J.K., and Schimmer, B.P. Defective guanyl nucleotidebinding protein $\beta\gamma$ subunits in a forskolin-resistant mutant of the Y1 adrenocortical cell line. Proc.Natl.Acad.Sci.U.S.A. 89(19):8933-8937, 1992). The specific binding, induced by rhodopsin (= receptor), of [35 S]GTP γ S to α -transducin (αt) was measured, and the effect of cholate extracts from membranes of normotensive and hypertensive cells on this binding was investigated. The protein concentration in the cholate extracts was identical in all the experiments.

Fig. 6 shows the effect of cholate extracts from normotensive and hypertensive cells on the binding, stimulated by rhodopsin, of [35 S]GTP γ S, to αt .

It is clear in this case that cholate extracts from hypertensive cells promote the rhodopsin-catalyzed binding of [35 S]GTP γ S to αt distinctly more than on mediation by cholate extracts from normotensives.

The experiments which are shown permit the following conclusions to be drawn:

- There is increased activatability of G proteins in hypertensive cells. This is more efficient by comparison with G protein activation in normotensive cells in that hypertensive cells require distinctly less activated receptor and, in addition, the kinetics of G protein activation are distinctly speeded up (Figs. 2 and 3).
- Moreover, maximum G protein activation requires distinctly lower concentrations of free GDP and free Mg^{2+} in the case of hypertensive cells than in normotensive cells. This leads to the conclusion that the protein interactions of α , β and γ subunits take place distinctly more efficiently in hypertensive cells than in normotensive cells (Figs. 4 and 5).
- The rhodopsin-catalyzed binding of [35 S]GTP γ S to αt is enhanced distinctly more by cholate extracts from hypertensive cells than by cholate extracts from normotensive cells. This proves that the enhanced G protein activation in

the case of hypertensive cells can be reconstituted in an in vitro system (Fig. 6). Moreover, it is possible unambiguously to conclude from these findings that the underlying alteration in hypertensive cells is to be found in the $\beta\gamma$ subunits of heterotrimeric G proteins. In said reconstitution system there is no activation of the α subunits still present in the cholera extracts in the presence of αt . Moreover, the added photoreceptor rhodopsin specifically activates only the added αt but not the α subunits remaining endogenously in cholera extracts.

The novel protein consists of 299 amino acids. Compared with the human $G\beta 3$ subunit previously described there is a deletion in the region of the 4th WD repeat. However, because of the regularity of the sequence of certain amino acids, it can be predicted that the novel, short $G\beta 3$ protein will likewise form a proper propeller structure, but this new propeller would now consist of 6 propeller blades (Fig. 9), no longer of seven (Fig. 1).

Since both the N terminus and the C terminus of the novel, short $G\beta 3$ subunits are unchanged from the previously known $G\beta 3$ subunit, it is possible to predict unimpaired interaction with α and γ subunits of heterotrimeric G proteins and with coupling receptors. It is evident, in connection with the functional results described above, that the novel, short $G\beta 3$ subunit functionally mediates the increased activation of heterotrimeric G proteins observed in hypertensives.

The reason for the occurrence of the shortened $G\beta 3$ subunits in hypertensives is assumed to be an alternative splicing of the gene coding for human $G\beta 3$. In fact at the DNA level there is an intron exactly in front of the putative splicing site (Fig. 10).

The intron starts behind base 497 in the open reading frame when the A of the ATG start codon is defined as +1.

The intron limits and the branch site are shown in italics and underlined.

GGA CAC CAC GTG gtgaggctgaacattgctggtgctggggcttgggagtgggcccg
cctttccttaacagtcctccctccattttggcag TGC CTT GTG GGA

Since an intron can also be detected starting at about base 620 in the open reading frame by PCR on genomic DNA, the shortened form of human G β 3 described herein is evidently the result of an alternative splicing of the original G β 3, resulting in deletion
5 of a complete exon.

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SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:

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(ii) TITLE OF APPLICATION: PTX-sensitive G proteins, preparation and use

(iii) NUMBER OF SEQUENCES: 2

(iv) COMPUTER-READABLE FORM:

- (A) MEDIUM TYPE: Floppy disk
- (B) COMPUTER: IBM PC compatible
- (C) OPERATING SYSTEM: PC-DOS/MS-DOS
- (D) SOFTWARE: PatentIn Release #1.0, Version #1.25 (EPA)

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1394 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA to mRNA

(iii) HYPOTHETICAL: NO

(iii) [sic] ANTISENSE: NO

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: Homo sapiens

(ix) FEATURES:

- (A) NAME/KEY: CDS
- (B) LOCATION: 1..900

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

ATG GGG GAG ATG GAG CAA CTG CGT CAG GAA GCG GAG CAG CTC AAG AAG	48
Met Gly Glu Met Glu Gln Leu Arg Gln Glu Ala Glu Gln Leu Lys Lys	
1 5 10 15	
CAG ATT GCA GAT GCC AGG AAA GCC TGT GCT GAC GTT ACT CTG GCA GAG	96
Gln Ile Ala Asp Ala Arg Lys Ala Cys Ala Asp Val Thr Leu Ala Glu	
20 25 30	
CTG GTG TCT GGC CTA GAG GTG GTG GGA CGA GTC CAG ATG CGG ACG CGG	144
Leu Val Ser Gly Leu Glu Val Val Gly Arg Val Gln Met Arg Thr Arg	
35 40 45	
CGG ACG TTA AGG GGA CAC CTG GCC AAG ATT TAC GCC ATG CAC TGG GCC	192
Arg Thr Leu Arg Gly His Leu Ala Lys Ile Tyr Ala Met His Trp Ala	
50 55 60	

CATGGGACTG	TGCCTTTGGG	AGGCAGCATC	AGGGACACAG	GGGCAAAGAA	CTGCCCCATC	1097
TCCTCCCATG	GCCTTCCCTC	CCCACAGTCC	TCACAGCCTC	TCCCTTAATG	AGCAAGGACA	1157
ACCTGCCCCCT	CCCCAGCCCT	TTGCAGGCCC	AGCAGACTTG	AGTCTGAGGC	CCCAGGCCCT	1217
AGGATTCTCTC	CCCCAGAGCC	ACTACCTTTG	TCCAGGCCTG	GGTGGTATAG	GGCGTTTGGC	1277
CCTGTGACTA	TGGCTCTGGC	ACCACTAGGG	TCCTGGCCCT	CTTCTTATTC	ATGCTTTCTC	1337
CTTTTCTAC	CTTTTTTCT	CTCCTAAGAC	ACCTGCAATA	AAGTGTAGCA	CCCTGGT	1394

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 299 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

Met	Gly	Glu	Met	Glu	Gln	Leu	Arg	Gln	Glu	Ala	Glu	Gln	Leu	Lys	Lys	1	5	10	15
Gln	Ile	Ala	Asp	Ala	Arg	Lys	Ala	Cys	Ala	Asp	Val	Thr	Leu	Ala	Glu	20	25	30	
Leu	Val	Ser	Gly	Leu	Glu	Val	Val	Gly	Arg	Val	Gln	Met	Arg	Thr	Arg	35	40	45	
Arg	Thr	Leu	Arg	Gly	His	Leu	Ala	Lys	Ile	Tyr	Ala	Met	His	Trp	Ala	50	55	60	
Thr	Asp	Ser	Lys	Leu	Leu	Val	Ser	Ala	Ser	Gln	Asp	Gly	Lys	Leu	Ile	65	70	75	80
Val	Trp	Asp	Ser	Tyr	Thr	Thr	Asn	Lys	Val	His	Ala	Ile	Pro	Leu	Arg	85	90	95	
Ser	Ser	Trp	Val	Met	Thr	Cys	Ala	Tyr	Ala	Pro	Ser	Gly	Asn	Phe	Val	100	105	110	
Ala	Cys	Gly	Gly	Leu	Asp	Asn	Met	Cys	Ser	Ile	Tyr	Asn	Leu	Lys	Ser	115	120	125	
Arg	Glu	Gly	Asn	Val	Lys	Val	Ser	Arg	Glu	Leu	Ser	Ala	His	Thr	Gly	130	135	140	
Tyr	Leu	Ser	Cys	Cys	Arg	Phe	Leu	Asp	Asp	Asn	Asn	Ile	Val	Thr	Ser	145	150	155	160
Ser	Gly	Asp	Thr	Thr	Cys	Ala	Lys	Leu	Trp	Asp	Val	Arg	Glu	Gly	Thr	165	170	175	
Cys	Arg	Gln	Thr	Phe	Thr	Gly	His	Glu	Ser	Asp	Ile	Asn	Ala	Ile	Cys	180	185	190	
Phe	Phe	Pro	Asn	Gly	Glu	Ala	Ile	Cys	Thr	Gly	Ser	Asp	Asp	Ala	Ser	195	200	205	
Cys	Arg	Leu	Phe	Asp	Leu	Arg	Ala	Asp	Gln	Glu	Leu	Ile	Cys	Phe	Ser	210	215	220	
His	Glu	Ser	Ile	Ile	Cys	Gly	Ile	Thr	Ser	Val	Ala	Phe	Ser	Leu	Ser	225	230	235	240
Gly	Arg	Leu	Leu	Phe	Ala	Gly	Tyr	Asp	Asp	Phe	Asn	Cys	Asn	Val	Trp	245	250	255	
Asp	Ser	Met	Lys	Ser	Glu	Arg	Val	Gly	Ile	Leu	Ser	Gly	His	Asp	Asn	260	265	270	

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Arg Val Ser Cys Leu Gly Val Thr Ala Asp Gly Met Ala Val Ala Thr
275 280 285
Gly Ser Trp Asp Ser Phe Leu Lys Ile Trp Asn
290 295