



Functional characterization of GNAS mutations found in patients with pseudohypoparathyroidism type Ic defines a new subgroup of pseudohypoparathyroidism affecting selectively $Gs\alpha$ -receptor interaction

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Functional characterization of GNAS mutations found in patients with pseudohypoparathyroidism type Ic defines a new subgroup of pseudohypoparathyroidism affecting selectively Gsa-receptor interaction

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1 **Functional characterization of *GNAS* mutations found in patients with**
2 **pseudohypoparathyroidism type Ic defines a new subgroup of**
3 **pseudohypoparathyroidism affecting selectively *Gsa*-receptor interaction**
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5 Abbreviation title: Functional analysis of *GNAS* mutations in PHPIc
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Abbreviations: Pseudohypoparathyroidism (PHP), Pseudohypoparathyroidism type Ia (PHPIa), α -subunit of the stimulatory G protein ($G\alpha$), Pseudohypoparathyroidism type Ic (PHPIc), Albright hereditary osteodystrophy (AHO), mouse $G\alpha$ -null fibroblast-like cell line ($Gnas^{E2-/E2-}$), G protein-coupled receptor (GPCR), Pseudopseudohypoparathyroidism (PPHP), Pseudohypoparathyroidism type Ib (PHPIb), guanosine 5'-[γ -thio]triphosphate ($GTP\gamma s$), human TSH receptor (TSHR), human PTH receptor (PTHR), Cholera toxin (CTX), Isoproterenol (Iso), human [Y^{34}]PTH(1-34)amide (hPTH),

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Abstract

Objective: Pseudohypoparathyroidism type Ia (PHPIa) is caused by *GNAS* mutations leading to deficiency of the α -subunit of stimulatory G proteins ($G\alpha$) that mediate signal transduction of G protein-coupled receptors via cAMP. PHP type Ic (PHPIc) and PHPIa share clinical features of Albright hereditary osteodystrophy (AHO); however, *in-vitro* activity of solubilized $G\alpha$ protein is normal in PHPIc but reduced in PHPIa. **Methods:** We screened 32 patients classified as PHPIc for *GNAS* mutations and identified three mutations (p.E392K, p.E392X, p.L388R) in four unrelated families. These and one novel mutation associated with PHPIa (p.L388P) were introduced into a pcDNA3.1(-) expression vector encoding $G\alpha$ wild-type and expressed in a $G\alpha$ -null cell line ($Gnas^{E2-/E2-}$). To investigate receptor-mediated cAMP accumulation, we stimulated the endogenous expressed β_2 -adrenergic receptor, or the co-expressed PTH- or TSH receptors, and measured the synthesized cAMP by RIA. The results were compared to receptor-independent cholera toxin-induced cAMP accumulation. **Results:** Each of the mutants associated with PHPIc significantly reduced or completely disrupted receptor-mediated activation, but displayed normal receptor-independent activation. In contrast, PHPIa associated p.L388P disrupted both receptor-mediated activation and receptor-independent activation. **Conclusions:** We present a new subgroup of PHP that is caused by $G\alpha$ deficiency and selectively affects receptor-coupling functions of $G\alpha$.

Key Words: Pseudohypoparathyroidism type Ia, Pseudohypoparathyroidism type Ic, Albright hereditary osteodystrophy, $G\alpha$ protein activity, $Gnas^{E2-/E2}$

76 **Introduction**

77 The term pseudohypoparathyroidism (PHP) describes a group of related rare disorders
 78 characterized by end organ-resistance to PTH and other peptide hormones that mediate their
 79 actions through G protein-coupled receptors (GPCRs) via cAMP/ protein kinase A. Based on
 80 clinical and laboratory findings, and on the result of Gs α protein activity *in-vitro*, PHP has
 81 been divided into different subgroups. PHP type Ia (PHPIa, MIM: 103580) is commonly
 82 caused by heterozygous, maternally inherited inactivating mutations involving those exons of
 83 the *GNAS* locus (MIM: 610540) that encode the α -subunit of the stimulatory G protein (Gs α).
 84 These mutations lead to Gs α deficiency. Because there are some imprinted tissues where this
 85 signaling protein is derived only or predominantly from the maternal allele, including
 86 proximal renal tubules, thyroid, ovaries, and pituitary, affected individuals develop PTH-
 87 resistance leading to hypocalcemia and hyperphosphatemia, as well as resistance towards
 88 TSH and, sometimes, other peptide hormones and ligands. Due to haploinsufficiency of Gs α
 89 in non-imprinted tissues, patients affected by PHPIa develop additional features of Albright
 90 hereditary osteodystrophy (AHO), which include round face, short stature, brachymetacarpia,
 91 ectopic ossification, and mental retardation. Patients also sometimes present with obesity.
 92 Paternally inherited *GNAS* mutations lead to pseudo-PHP (PPHP, MIM: 612463)
 93 characterized by some features of AHO in the absence of hormone resistance (reviewed in
 94 Weinstein et al., 2002; Bastepe and Jüppner, 2005; Bastepe, 2008). Both, PHPIa and PPHP,
 95 show diminished Gs α protein activity as determined by an *in vitro* assay using solubilized
 96 Gs α from readily accessible cells such as red blood cells or fibroblasts and non-hydrolyzable
 97 guanosine 5'-[γ -thio]triphosphate (GTP γ s) (Levine et al., 1980, 1988). Gs α protein activity
 98 measurement has often been described as the first diagnostic step, followed by a molecular
 99 genetic analysis of *GNAS*.

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2 100 PHP type Ib (PHPIb, MIM: 603233) is associated with the loss of methylation at one or more
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4 101 maternally methylated regions within *GNAS* and can be caused by heterozygous, maternally
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6 102 inherited deletions up-stream of or within the *GNAS* locus or can occur sporadically (Jüppner
7
8 103 et al., 1998, 2006; Bastepe et al., 2001). As a result, *Gsα* expression is reduced in a few
9
10 104 tissues including the renal proximal tubules and, in some cases, the thyroid, leading to PTH-
11
12 105 resistant hypocalcemia and hyperphosphatemia and occasionally elevated TSH levels.
13
14 106 Typically, PHPIb patients lack AHO features and present with normal *Gsα* activity (Bastepe,
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16 107 2008). Recently, patients with PHP and AHO features have been described in association
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18 108 with methylation changes of *GNAS* (de Nanclares et al., 2007; Mantovani et al., 2010), which
19
20 109 are not included in the current classification of the disorders.
21
22 110 Pseudohypoparathyroidism type Ic (PHPIc, MIM: 612462) describes individuals who
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24 111 develop the same clinical and laboratory abnormalities as patients with PHPIa, including
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26 112 AHO and peptide hormone resistance, but in contrast to PHPIa, *in-vitro* assessment of *Gsα*
27
28 113 protein activity reveals no abnormality (table 1) (Weinstein, 1998). It has therefore been
29
30 114 postulated that PHPIc may not be caused by a functional impairment of the *Gsα* protein, but
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32 115 by another component of the cAMP-dependent signaling pathway, such as adenylyl cyclase,
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34 116 inhibitory G proteins, or phosphodiesterases (Farfel et al., 1981; Lania et al., 2001; Aldred,
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36 117 2006; Mantovani and Spada, 2006).
37
38 118 We screened a cohort of patients classified as PHPIc (based on clinical and laboratory data
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40 119 and the result of the *in-vitro* assay) and found mutations in the *GNAS* gene in a subgroup. We
41
42 120 then performed functional analysis of three naturally occurring mutations located in the
43
44 121 extreme C-terminus of *Gsα*. By analyzing the receptor-mediated activation of the *Gsα*-
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46 122 mutants in a mouse *Gsα*-null fibroblast-like cell line (*Gnas*^{E2-/E2-}) and comparing the results
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48 123 to receptor-independent activation, we demonstrate that these mutations selectively affect
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124 receptor-coupling but not adenylyl cyclase activating functions of $G\alpha$, while the *PHPIa*
125 associated mutation affects both.

127 Patients and methods

128 We screened 32 patients with PHP, AHO and normal measured $G\alpha$ protein activity for
129 mutations in exons 1-13 of *GNAS* including exon/intron boundaries. Here, we only describe
130 the phenotype and history of patients in whom we found *GNAS* mutations. The clinical and
131 laboratory data are summarized in table 2

132 **Patient A:** The male patient was delivered at term after an uneventful pregnancy by
133 Caesarian section. During birth he suffered from asphyxia, which was initially thought to be
134 the cause of a delay of his speech and psychomotoric development. At the age of 12 years, he
135 was hospitalized because of facial nerve palsy. He showed characteristic AHO features,
136 including round face, shortening of the 4th and 5th metacarpals and mental retardation with an
137 IQ of 40 (assessed by HAWIK). His mother had only mild signs of AHO, including short
138 stature (154 cm, <3rd centile), and brachymetacarpia of the 4th metacarpals, but no other
139 AHO features, and no evidence for hormonal resistance, consistent with the diagnosis of
140 PPHP. The patient does not have siblings and the father is healthy.

141 **Patient B:** The female patient was born at term after an uneventful pregnancy as the only
142 child of their parents. AHO and PHP were diagnosed at the age of 5.5 years when she
143 presented at the endocrine clinic with round face, brachymetacarpia of the 3rd, 4th, and 5th
144 metacarpals and obesity (22.5 kg, BMI 19.6 kg/ m², >97th centile). Her mother presented with
145 brachymetacarpia but no other AHO features and PTH and TSH levels were in the normal
146 range, consistent with the diagnosis of PPHP. The father did not present any AHO signs.

147 **Patient C:** The patient is a female, born at 38 weeks of gestation as the only child of their
148 parents. Diagnosis of AHO and PHP was suspected because of neonatal hypothyroidism. At

1
2
3 149 11 months of age she had a round face, her length was 65.5 cm (<3rd centile), she had already
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5 150 developed brachymetacarpia affecting the 4th and 5th phalanges and her weight was 7.8 kg
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7 151 (BMI 18,4 kg/m², 90th centile). Her mother was of short stature (142 cm, <3rd centile) and had
8
9 152 also brachymetacarpia, but no hormonal resistances.

10 153 **Patient D1:** The patient is a male twin born at term. He came for endocrinological
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12 154 assessment at the age of 13 years because of recurrent hypocalcemia, and presented with
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14 155 round face and brachymetacarpia of the 4th and 5th phalanges.

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16 156 **Patient D2:** The patient is the twin sister of D1, with an uneventful medical history until the
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18 157 age of 13 years. At this time, her weight was slightly elevated (55 kg, BMI 26.5 kg/m², 97th
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20 158 centile) and she presented with round face and brachymetacarpia. The mother of both
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22 159 children had a round face, was of short stature (152 cm, <3rd percentile), and demonstrated
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24 160 brachymetacarpia of the 4th and 5th metacarpals and metatarsals, but no hormonal alterations
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26 161 or further AHO signs. The father of both children is healthy.

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28 162 **Patient E:** The female patient was born after uneventful pregnancy. She was hospitalized at
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30 163 the age of 4 years when she presented with hypocalcemic seizures. At this time she
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32 164 demonstrated a round face, brachymetacarpia of the 5th metacarpals, and obesity (20.7 kg,
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34 165 >97th centile), but no other signs of AHO. Her mother also presented with short 5th
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36 166 metacarpals, however, PTH and TSH levels were in the normal range. The patient does not
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38 167 have siblings and the father does not show abnormalities.

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40 168 Patients B, D1 and D2 have in part already been described by de Sanctis (de Sanctis et al.,
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42 169 2003).

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44 170 Laboratory investigations at the time of diagnosis revealed for all patients elevated PTH and
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46 171 TSH levels. Vitamin D deficiency had been excluded before by measuring 25-OH-vitamin D
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48 172 levels within the reference range. The *in-vitro* Gsa activity was normal, except for patient E,
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50 173 (activity was reduced to 71%), when compared to healthy controls. All subjects or their

174 guardians gave informed consent to the study. Studies were approved by the ethical
175 committee of the University of Lübeck as part of the funded project on AHO (see
176 acknowledgment).

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178 Gsα protein activity and mutation analysis

179 The activity of Gsα protein from erythrocyte membranes of patients was investigated in
180 heparinized blood samples as described earlier (Levine et al., 1980; Ahrens et al., 2001).
181 Briefly, after solubilization the Gsα protein from patient derived erythrocyte membranes was
182 incubated with GTPγS. We added adenylyl cyclase from turkey red cell membranes and
183 measured the generated cAMP in the presence of ATP by RIA (Immuno Biological
184 Laboratories, Hamburg, Germany). Results obtained in triplicate were expressed as percent of
185 the mean of healthy controls (normal range: 85–115 %). For molecular genetic analysis we
186 isolated genomic DNA derived from peripheral leukocytes by standard procedures (Qiaquick
187 DNA kit, Qiagen, Hilden, Germany). *GNAS* exon 1-13, (RefSeq NM_000516.4) including all
188 intron/exon boundaries were amplified in 11 fragments by PCR (primer sequences available
189 upon request). PCR-amplified DNA was sequenced by direct cycle sequencing using the
190 BigDye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) and
191 an ABI 3130 capillary sequencer (Applied Biosystems, Foster City, CA).

192

193 Site-directed mutagenesis of expression plasmids

194 We introduced the detected mutations p.E392K, p.E392X, p.L388R and p.L388P into a
195 pcDNA3.1(-) vector encoding wild-type rat Gsα (which is identical to human Gsα), in which
196 a shortened hemagglutinine epitope-tag (DVPDYA) had been introduced into exon 3 by
197 PCR-based mutagenesis (Bastepe et al., 2002). The first PCR amplification was carried out
198 by using a sense primer in exon 4 and an antisense primer comprising the respective

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2 199 mutation. A second PCR was performed using a sense primer containing the mutation and an
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4 200 antisense primer (Bgh-as) located downstream of Gsα. PCR products were used as a template
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6 201 in a third PCR using the exon 4-sense and the Bgh-antisense primer. PCR products were
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8 202 amplified in a Mastercycler Gradient (Eppendorf, Hamburg, Germany) using the following
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10 203 cycling protocol: denaturation for 30 s at 98 C, followed by 29 cycles denaturation (10 s at 98
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12 204 C), annealing (30 s at 64 C), and elongation (45 s at 72 C) and 1 final cycle (10 min at 72 C).
13
14 205 The reaction mix contained 100 ng vector DNA, 1x Phusion-buffer HF, 2.5 U Phusion High-
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16 206 Fidelity DNA-Polymerase (New England Biolabs) and 200 μM dNTPs (Fermentas, St. Leon-
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18 207 Rot, Germany). The amplicon including the respective mutation was double digested with
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20 208 *AfeI* and *HindIII* (New England Biolabs, Beverley, CA) and subcloned into the original Gsα
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22 209 expression vector. All clones were verified by nucleotide sequence analysis including the
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24 210 entire inserts up to cloning borders.
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28 212 Cell culture, transfection, and stimulation
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30 213 For transfection experiments, we used Gnas^{E2-/E2-} cells, a clonal murine cell line in which
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32 214 *Gnas* exon 2 had been disrupted and thus does not express endogenous Gsα or XLas (Bastepe
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34 215 et al., 2002). Cells were seeded into 24-well plates at a density of 60-80 %. After 24 h, the
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36 216 cells were transfected with 0.2 μg/well of plasmid DNA encoding Gsα using Effectene
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38 217 (Qiagen, Valencia, CA). For cotransfection experiments with plasmids encoding the human
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40 218 TSH receptor (TSHR) or the human PTH receptor (PTHR), the total amount of DNA per well
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42 219 was 0.4 μg, which was kept constant by addition of empty vector. Cells were cultured at 37 C
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44 220 for 72 h with daily exchanges of DMEM-F12-medium containing 10 % fetal bovine serum
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46 221 followed by stimulation with different agonists. For determination of cholera toxin (CTX)-
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48 222 induced cAMP formation, cells were treated at 37 C for 2 h with 1 μg/ml CTX (Sigma-
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50 223 Aldrich Corp., St. Louis, MO). Stimulation with Isoproterenol (Sigma-Aldrich Corp., St.

224 Louis, MO), human [Y^{34}]PTH(1-34)amide (PTH) (Massachusetts General Hospital,
225 Biopolymer Core Facility, Boston, USA) and human TSH (Sigma-Aldrich Corp., St. Louis,
226 MO) were carried out in the presence of F12-DMEM containing HEPES-NaOH, 1 mg/ml
227 BSA, and 2 mM isobutylmethylxanthine (Sigma-Aldrich Corp., St. Louis, MO). Treatment
228 was followed by 15 min incubation at 37 C in a water bath. After incubation cells were lysed
229 by 50 nM HCl and cAMP accumulation in each well was determined by RIA as previously
230 described (Bastepe et al., 2002). All transfection experiments in this study were repeated at
231 least three times.

233 Western blot analysis

234 For Western blot analysis, 15 μ g of cellular protein were loaded onto a 12 % polyacrylamide
235 gel. Electrophoresis was carried out at 100 V for 120 min in a Mini-Protean 3 chamber
236 (BioRad, Munich, Germany). Proteins were transferred to nitrocellulose membranes (BA85,
237 Schleicher & Schüll, Dassel, Germany) at 100 V for 80 min using a Mini Trans-Blot cell
238 (BioRad, Munich, Germany) and using a blotting buffer containing 16.5 mM Tris, 150 mM
239 glycine and 20 % (V/V) methanol. Non-specific binding sites were blocked by immersing the
240 membranes overnight at 4 C in 5% non-fat milk (Becton-Dickinson, Franklin Lakes, USA)
241 dissolved in PBS/ Tween buffer (containing 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4 ,
242 2 mM KH_2PO_4 , 0.1 % Tween 20, pH 7.4). The blocked membranes were incubated with an
243 HA-antibody (Y11: sc805, Santa Cruz Biotechnology) diluted 1:100 in blocking buffer for 1
244 h at room temperature. The membrane was rinsed 4 times for 15 min in PBS/Tween buffer
245 and incubated with an anti-rabbit IgG peroxidase conjugate at a dilution of 1:1000 (Sigma,
246 Taufkirchen, Germany) for 1 h. After rinsing the membrane as above, protein bands were
247 visualized using the Western Lightning Chemiluminescence Reagent Plus substrate (Perkin

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2 248 Elmer, Boston, MA) and the Fusion SL detection system (Vilber Lourmat, Eberhardzell,
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4 249 Germany).
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8 251 Statistical analysis and structural analysis
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10 252 Student's *t* test for two independent samples was used to determine significance of observed
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12 253 differences of the cAMP levels after CTX-mediated stimulation of Gsα-388R (corresponding
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14 254 to the p.L388R mutant of Gsα) and Gsα-388P and after Isoproterenol-mediated stimulation of
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16 255 Gsα-392K and Gsα-392X. GraphPad 4.0 was used for determination of the EC₅₀. For the
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18 256 structural analysis the crystal structure of Gsα in complex with GTPγ-s (Sunahara et al.,
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20 257 1997) has been used. The structural representations were generated using the RIBBONS
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22 258 software (Kraulis, 1991).
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26 260 **Results**
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28 261 In 5 patients with PHPIc from 4 unrelated families our analysis of *GNAS* (RefSeq
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30 262 NM_000516.4) revealed 3 different heterozygous mutations in exon 13 affecting the two
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32 263 residues 388 and 392 in the carboxy-terminal portion of Gsα. In addition, we found one
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34 264 further mutation also affecting the residue 388, but associated with PHPIa.
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36 265 In patient A and his mother, a single nucleotide exchange in codon 388 (c.1163T>G) was
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38 266 identified, leading to an arginine instead of a leucine (p.L388R). This mutation has not been
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40 267 described so far. A missense mutation in codon 392 (c.1174G>A) was identified in patient C
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42 268 and her mother, resulting in a substitution of glutamic acid by lysine (p.E392K). In patient B,
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44 269 D1, and D2 and their mothers we confirmed the heterozygous nucleotide change
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46 270 (c.1174G>T) at codon 392 in concordance to the previously results in these patients (de
47
48 271 Sanctis et al., 2003) resulting in a stop codon (p.E392X). In patient E and her mother
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50 272 (PHPIa), we also found a novel heterozygous change at codon 388 (c.1163T>C) resulting in

proline instead of a leucine (p.L388P). [Nucleotide numbering reflects cDNA numbering system with +1 corresponding to the A of the ATG translation initiation codon in the reference sequence \(NM_000516.4\), according to the journal guidelines \(www.hgvs.org/mutnomen\).](#)

The analysis of the remaining 27 patients with PHP1c did not reveal mutations in the Gs α encoding region of *GNAS*. All variants were not detected in 100 normal individuals and all involve highly conserved amino acids (table 3). The mutations are summarized in table 2. The mutations were included in the [Leiden Open Variation Database \(http://www.lovd.nl/GNAS\)](#), according to the nomenclature of the HGVS site. In this report, however, the commonly used nomenclature of Kosaza et al. (1988) has been used.

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First we studied the ability of the missense mutants Gs α -388R and Gs α -392K (corresponding to p.L388R and p.E392K encoded in the Gs α -encoding vector) to be stimulated by the β_2 -adrenergic receptor expressed endogenously in our Gnas^{E2-/E2-} fibroblast-like cells. Receptor mediated cAMP accumulation in response to 10⁻⁵M Isoproterenol was measured after transient expression of mutant or wild-type Gs α . The results were compared to CTX-induced cAMP accumulation, which stimulates adenylyl cyclase by ADP-ribosylation independent of receptor activation (CTX-stimulated wild-type Gs α was set as maximal stimulation of 100%). After Isoproterenol stimulation Gs α -388R failed to induce any increase in intracellular cAMP (mean 4.5 %, SEM $\pm$ 0.3), compared to the basal cAMP level (mean 3.6 % of max, SEM $\pm$ 1.1), despite nearly normal CTX-induced cAMP levels (mean 81.1 % of max, SEM $\pm$ 8.2). In contrast, Gs α -392K was activated by Isoproterenol, but maximal cAMP levels were only about 50% of wild-type (mean 43.6% of max, SEM $\pm$ 3), whereas the CTX-induced cAMP accumulation was comparable to that of the wild-type (mean 105.6 %, SEM $\pm$ 8.3) (figure 1a). Non-transfected cells or cells transfected with the empty pcDNA3.1(-) vector, which were

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2 298 used as negative controls, failed to show an increase in basal, CTX- and Isoproterenol-
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4 299 stimulated cAMP levels (data not shown).
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6 300 To determine whether cAMP accumulation of the wild-type and the mutants is dependent on
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8 301 agonist levels, we stimulated the cells with concentrations of Isoproterenol ranging from 10⁻⁴
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10 302 to 10⁻⁸ M. Isoproterenol treatment increased cAMP formation in a dose-dependent manner in
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12 303 cells transiently expressing the wild-type Gsα (EC₅₀=10^{-5.895} M; 95 % confidence interval 10⁻
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14 304 ^{5.979} to 10^{-5.811}). Consistent with our previous experiments, Isoproterenol failed completely to
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16 305 increase cAMP generation in cells transiently expressing Gsα-388R (mean 5.8 %, SEM
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18 306 ±0.2). Gsα-392K led to a significant increase in cAMP formation in response to agonist
19
20 307 treatment with EC₅₀ values (EC₅₀=10^{-5.995} M; 95 % confidence interval 10^{-6.108} to 10^{-5.883})
21
22 308 similar to wild type Gsα; however, maximal response after stimulation with 10⁻⁴ M was
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24 309 reduced to about 50 % (mean 49.8 %, SEM ±4.4) (figure 1b).
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28 311 Since all patients presented with PTH resistance, experiments with cells transiently
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30 312 expressing both Gsα-mutants/wild-type and the PTHR were performed. Stimulation with 10⁻⁸
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32 313 M PTH revealed a similar pattern for Gsα-388R and Gsα-392K to that observed after
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34 314 stimulation of the endogenous β₂-receptor. Gsα-388R did not show any response after agonist
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36 315 stimulation (mean 16.73 % of max, SEM ±0.33) and Gsα-392K led to a diminished response
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38 316 to about 60 % (mean 60.5 % of max, SEM ±1.86) compared to the wild-type (mean 100 %, SEM ±3.31). These results were confirmed by dose dependent agonist stimulation (figure 1c).
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40 317
41 318 The EC₅₀ for PTH-induced cAMP accumulation through Gsα-392K (10^{-9.307} M) was similar
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43 319 as for wild-type Gsα (10^{-9.102} M).
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45 320
46 321 Furthermore, we cotransfected cDNA encoding the human TSHR and the vectors containing
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48 322 the Gsα-mutants, and stimulated the cells with 10 mIU/ml TSH. Again, the Gsα-388R mutant
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diminished response. Transfection with the empty vector, or the TSHR alone used as negative controls did not elevate the cAMP levels after stimulation with TSH (results not shown).

To investigate whether our cell model can discriminate different functional effects of mutations leading to the PHPIa or the PHPIc-subtype even if located in the same residue, we transfected the $Gnas^{E2-/E2-}$ cells with $G\alpha$ -388P associated with PHPIa. We compared the results to those of $G\alpha$ -388R. As $G\alpha$ -388R (mean 4.2 %, SEM ± 1.2), $G\alpha$ -388P could not be stimulated by Isoproterenol (mean 3.4 %, SEM ± 1). However, in concordance to the reduced $G\alpha$ protein activity in erythrocyte membranes leading to the diagnosis of PHPIa in this patient, the CTX-induced stimulation by $G\alpha$ -388P was reduced to nearly 60 % (mean 55.98 % of max, SEM ± 2.95), compared to the wild-type (mean 100 %, SEM ± 3.9). In contrast, the CTX-induced stimulation of $G\alpha$ -388R was similar to the wild type (mean 92.48 %, SEM ± 7) (figure 2a).

We also investigated the nonsense mutation $G\alpha$ -392X found in our patients B, D1 and D2 and compared the results to those of the missense mutant $G\alpha$ -392K affecting the same residue. In contrast to our findings in $G\alpha$ -392K (32.19 % of max, SEM ± 0.14), we could not demonstrate any significant stimulation via the β_2 -receptor in $G\alpha$ -392X (mean 7.32 % of max, SEM ± 0.36) (figure 2b).

The CTX-induced cAMP accumulation of the PHPIc associated mutants $G\alpha$ -388R, $G\alpha$ -392K, and $G\alpha$ -392X did not show significant differences to the wild-type, but the PHPIa associated mutant $G\alpha$ -388P led to diminished CTX-induced cAMP synthesis. To rule out different expression levels between $G\alpha$ -388P and the wild-type $G\alpha$, we established immunoblot analysis using hemagglutinine-tag specific antibody and demonstrated

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2 347 equivalent expression of all mutants except a slightly reduced expression of Gsα-392X
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4 348 (figure 2c).
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8 350 **Discussion**
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10 351 Historically, the term PHPIc has been used for a constellation including PHP, AHO signs and
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12 352 a normal Gsα activity measured *in-vitro*. Therefore, PHPIc is thought to be caused by
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14 353 impairment of another component of the cAMP-dependent pathway than Gsα (Aldred, 2006;
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16 354 Mantovani and Spada, 2006). In our study we demonstrate in a subset of patients with the
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18 355 former diagnosis PHPIc inherited maternal inactivating mutations in the Gsα encoding exons
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20 356 of *GNAS*. Furthermore, we prove impaired interaction between different GPCRs and the
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22 357 mutated Gsα forms leading to deficient Gsα signalling. Since these mutations lead, in contrast
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24 358 to classical PHPIc, to Gsα deficiency and affect, in contrast to PHPIa, selectively Gsα-
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26 359 receptor coupling functions, this constitutes a new subgroup of PHP based on distinct
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28 360 molecular dysfunction of Gsα.
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32 362 Two further patients harboring *GNAS* mutations with similar functional deficits have been
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34 363 described. The nonsense mutation p.Y391X was identified in a PHPIc patient (Linglart et al.,
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36 364 2002) and molecular investigation of this Gsα-mutant led to the proposal that PHPIc
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38 365 represents a subgroup of PHPIa in which the *GNAS* mutations affect receptor-coupling
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40 366 (Linglart et al., 2006; Bastepe, 2008). The second described patient (harboring the mutation
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42 367 p.R385H) also demonstrated impaired receptor-coupling without disturbing downstream Gsα
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44 368 signaling. However, since the assay used for diagnosis did involve GPCR-Gsα coupling the
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46 369 patient was termed as having PHPIa (Schwindinger et al, 1994).
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All *GNAS* mutations found in our patients are located in the $\alpha 5$ -helix concerning the extreme carboxyl-terminus of $G\alpha$ (which is highly conserved between different species, [see table 3](#)), whereas usually PHPIa-associated mutations are distributed throughout the gene (www.hgmd.cf.ac.uk). The results from a variety of studies implicate that this region contains the major sites for interaction between $G\alpha$ and GPCRs (Sullivan et al., 1987; Masters et al., 1988; Spiegel et al., 1990; Pantoloni et al., 1993; Rasenick et al., 1994; Hamm et al., 1998; Linglart et al., 2006; Zang et al., 2006). Consistent with these results, our data demonstrate that natural mutations located at the carboxy-terminal end of $G\alpha$ can disrupt or strongly impair the ability for receptor-coupling despite normal CTX- or GTP γ S induced adenylyl cyclase activity.

The $G\alpha$ -388R-mutant showed a complete loss of receptor-mediated stimulation of the β_2 -adrenergic receptor, the PTHR, and the TSHR, indicating a crucial role of this residue for receptor-coupling. We analyzed the three dimensional structure of *GNAS* that has been described previously (Sunahara et al., 1997) to examine the putative effects of the mutation. The hydrophobic side chain of L388 of the $\alpha 5$ -helix is part of the accessible surface of the molecule and thus directly involved in contact of $G\alpha$ to the GPCR. The p.L388R will introduce a positive charge at this position and thus interfere with receptor binding. The mutation p.L388P, associated with PHPIa, also leads to a reduced response to receptor-independent activation. Normally, the backbone amide group of L388 forms a hydrogen bond with the carbonyl group of Q384. Since proline is, in contrast to arginine, not able to form such a helix stabilizing hydrogen bond, the p.L388P mutation will lead to destabilization of the $\alpha 5$ -helix and thereby may disturb the whole tertiary structure of the molecule and thus reduce not only the receptor-dependent, but also the receptor-independent stimulation.

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3 395 The missense mutant Gsα-392K demonstrated a residual activity after receptor-coupled
4 396 stimulation with Isoproterenol, while in contrast the nonsense mutant Gsα-392X led to a
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6 397 complete loss of receptor-mediated activation through β₂-receptor stimulation. These results
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8 398 seem to be reflected even by the severity of PTH and TSH resistance found in the patients
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10 399 (see table 2). This strongly suggests that the last three amino acid residues of Gsα are
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12 400 essential for receptor-coupling and the results are in agreement with the findings of Linglart,
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14 401 who demonstrated a complete loss of the β₂-adrenergic receptor-mediated activation by the
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16 402 p.Y391X-mutant that lacks the last four residues of Gsα (Linglart et al., 2006). In addition,
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18 403 the loss of the last three amino acids may lead to instability of the protein, as suggested by
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20 404 slightly reduced expression of Gsα-392X in our Western blotting analysis (figure 2c).
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24 406 Although, we identified *GNAS* mutations in a subset of designated PHPIc patients, the
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26 407 ethiopathogenesis of the remaining 27 cases remains to be elucidated. In the literature, two
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28 408 PHPIc patients have been described in whom the disease is caused by epigenetic changes
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30 409 involving the *GNAS* locus (de Nanclares et al., 2007). However, several patients with the
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32 410 diagnosis of PHPIc neither show epigenetic nor molecular genetic changes in *GNAS*,
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34 411 demonstrating that PHPIc may be caused by a variety of pathogenetic mechanisms.
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38 413 In summary, our molecular genetic and functional data prove impaired Gsα function by
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40 414 naturally occurring mutations at the Gsα encoding exons of *GNAS* as one cause of patients
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42 415 formerly diagnosed with PHPIc. However, since these mutations concern selectively Gsα-
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44 416 receptor coupling functions and show fundamental differences from those mutations
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46 417 associated with PHPIa, we regard this as a new subgroup of PHP. On the basis of our
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50 419 Nanclares et al., 2007; Mantovani et al., 2010), a new classification of *GNAS* related

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disorders should be proposed [in the future](#), based not only on clinical and laboratory alterations, but also on molecular genetic, epigenetic, and functional changes.

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Figure legends:

Figure 1) Stimulation of PHPIc associated missense mutants Gsα-388R and Gsα-392K,

Panel a) shows cAMP accumulation of both transfected missense mutants after receptor mediated stimulation of the endogenous expressed β₂-receptor due to Isoproterenol compared to receptor-independent stimulation due to CTX. The mutation p.L388R results in a loss of receptor-mediated stimulation, despite normal receptor-independent activation, compared to the wild-type. The Gsα-392K mutant demonstrates a diminished activity after receptor-mediated stimulation, although the receptor-independent stimulation is comparable to that of the Gsα-wild-type. % max: maximal response after stimulation of the Gsα-wild type with CTX. Panel b) Different concentrations of Isoproterenol (Iso) ranging from 10⁻⁸ to 10⁻⁴ M lead to a dose dependent stimulation of the β₂ receptor in the wild-type and the Gsα-392K mutant. The Gsα-388R mutant failed to induce intracellular cAMP synthesis. Panel c) Similar results are shown after cotransfection with the PTHR: incubation with various concentrations of human PTH ranging from 10⁻¹¹ to 10⁻⁷ M lead to a dose dependent response for the wild-type, and the Gsα-392K mutant, whereas the Gsα-388R-mutant does not show any response.

Figure 2) PHPIc and PHPIa associated Gsα-mutants can be distinguished by in-vitro

stimulation tests. These experiments demonstrate that the cell model may be appropriate to reflect differences in receptor-independent activation between PHPIc and PHPIa and between the effects of missense and nonsense mutations: Panel a) In contrast to Gsα-388R, the Gsα-388P mutant found in a patient with PHPIa leads to a clearly diminished CTX induced cAMP synthesis that was significantly different from the corresponding cAMP levels in cells expressing Gsα-388R (P<0.001). Panel b) After receptor-mediated stimulation the Gsα-392K mutant revealed a residual activity that was absent in the nonsense mutant Gsα-392X

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($P < 0.001$), **Panel c**) Comparison of Gs α -protein levels in non-transfected (n.t.) and transfected Gnas^{E2-/E2-} cells by immunoblot analysis. Gs α -wild-type (WT), Gs α -388R (388R), Gs α -388P (388P), Gs α -392K (392K), and Gs α -392X (392X). The protein levels of the mutants were similar to those of the wild-type.

Figure 3) Ribbon representation of Gs α in complex with GTP- γ S. Left: The C-terminal helix ($\alpha 5$) is colored in light blue, the helix $\alpha 4$ and the β -strand $\beta 6$ in green and the GTP- γ S molecule is depicted as a ball and stick representation. C denotes the C-terminus. **Right:** Close-up of the C-terminal region. The side chain of L388 is depicted and the hydrogen bond between the amide group of L388 and the carbonyl group of Q384 is shown in red (dashed line).

	PHPIa	PPHP	PHPIb	PHPIc
AHO features	yes	yes	rarely	yes
PTH resistance	yes	no	yes	yes
GNAS defects	mutations in exons 1-13	mutations in exons 1-13	epigenetic changes at the <i>GNAS</i> locus e.g. loss of methylation	not known, one mutation in exon 13 (Linglart et al., 2002)
In-vitro Gsa protein activity *	diminished	diminished	normal	normal
Transmission	maternal	paternal	maternal	maternal

Table 1) Subtypes of PHP and differences in phenotype, molecular genetic defects and *in-vitro* Gsa protein activity. The classification of PHP is based on the presence or absence of AHO features and the result of the Gsa protein activity carried out on isolated Gsa from erythrocyte membranes derived from patients. The result of the Gsa protein activity assay is the only certain difference between PHPIc and PHPIa, which is diminished in PHPIa and normal in PHPIc.

*normal range: 85-115%

Patient	A	B	C	D1	D2	E
Age at diagnosis, sex	12 years, male	5.5 years, female	11 month, female	13 years, male	13 years, female	4 years, female
Clinical features	rf, sst, bm, mr, o	rf, bm, o	rf, sst, bm	rf, bm	rf, bm, o	rf, bm, o
AHO Mother	sst, bm	bm	sst, bm	rf, sst, bm	rf, sst, bm	bm
PTH* (pg/ml; normal: 10-65)	416	133	138	643	544	286
Calcium (mmol/l; normal: 2.1-2.6)	0.84	1.1	normal	1.5	1.9	1.7
Phosphorous (mmol/l; normal: 1.09-2) age-dependent	3	2.7	2.3	3.3	2.3	3.1
TSH (mU/l; normal: 0.5-4)	9.3	6	4.7	5.9	7.4	4.5
fT3 (pg/ml; normal: 3.3-6.7)	normal	normal	normal	normal	normal	normal
fT4 (pg/ml; normal: 0.9-1.6)	normal	normal	normal	normal	normal	normal
Gsa activity in % of healthy controls (normal: 85-115)	108	102	108	100	105	71
Mutation**	c.1163T>G p.L388R	c.1174G>T p.E392X	c.1174G>A p.E392K	c.1174G>T p.E392X	c.1174G>T p.E392X	c.1163T>C p.L388P

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Table 2) Phenotypic, laboratory results, *in-vitro* Gsa protein activity, and results of mutational analysis of our patients.

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Legend: rf: round face, sst: short stature, bm: brachymetacarpia, mr: mental retardation, o: obesity. Values in brackets define the normal range.

* all laboratory results at the time of initial diagnosis

**RefSeq: NM_000516.4

Nucleotide numbering reflects cDNA numbering system with +1 corresponding to the A of the ATG translation initiation codon in the reference sequence, according to the journal guidelines (www.hgvs.org/mutnomen).

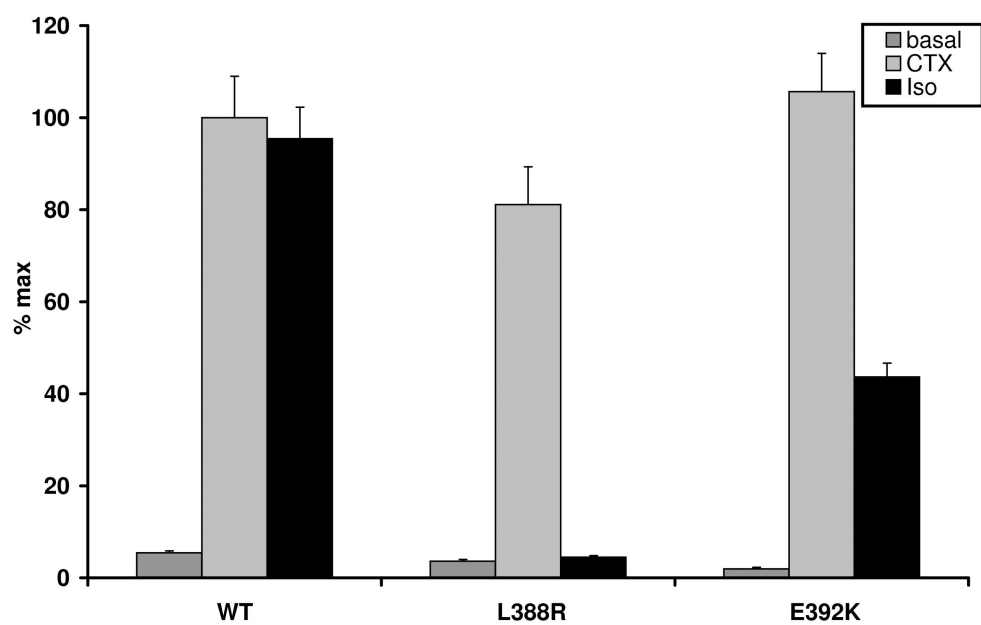
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Drosophila melanogaster	DTENIKRVFNDCRDIIQRMHLRQYELL
Xenopus laevis	DTENIRRVFNDCRDIIQRMHLRQYELL
Mus musculus	DTENIRRVFNDCRDIIQRMHLRQYELL
Macaca mulatta	DTENIRRVFNDCRDIIQRMHLRQYELL
Homo sapiens	DTENIRRVFNDCRDIIQRMHLRQYELL

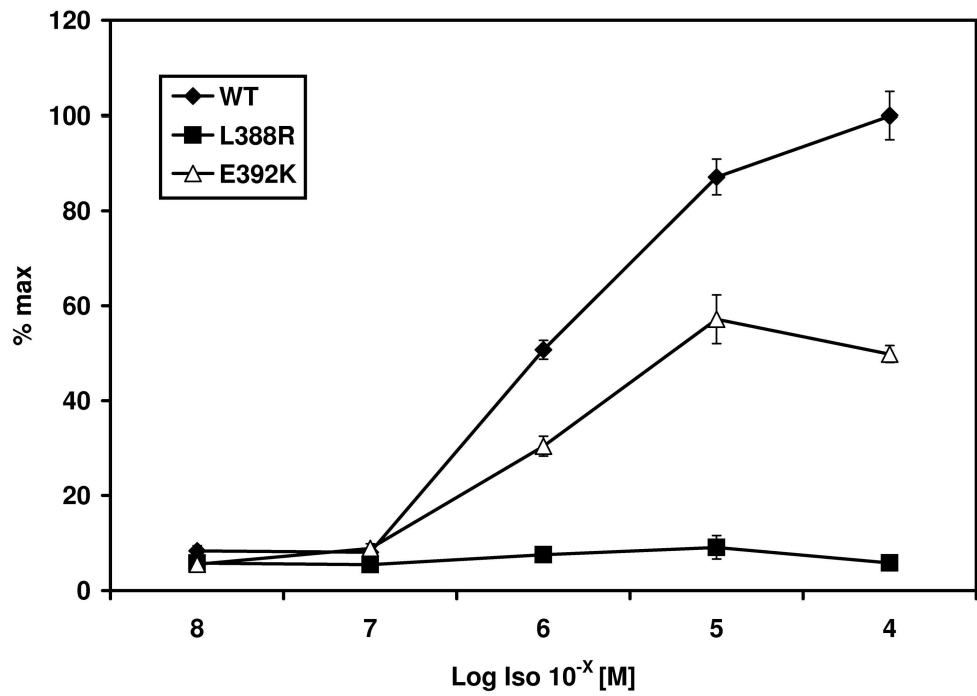
Table 3) Sequence alignment of the $\alpha 5$ helix of *Gsa* in different species. The C-terminus of

Gsa is highly conserved. Amino acid residues 388 and 392 are framed.

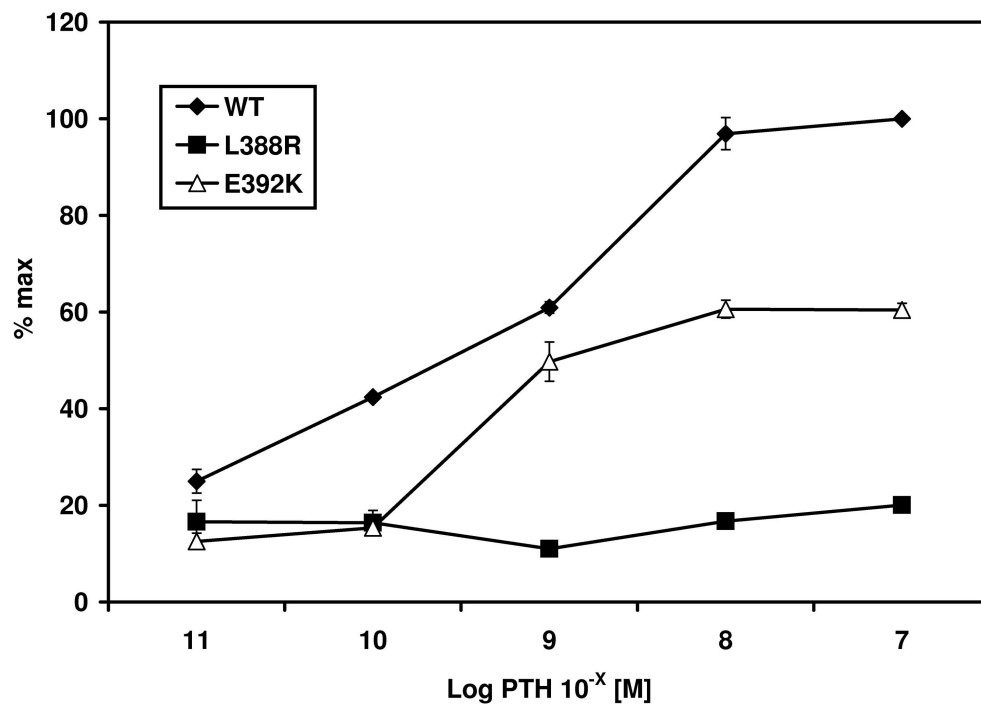
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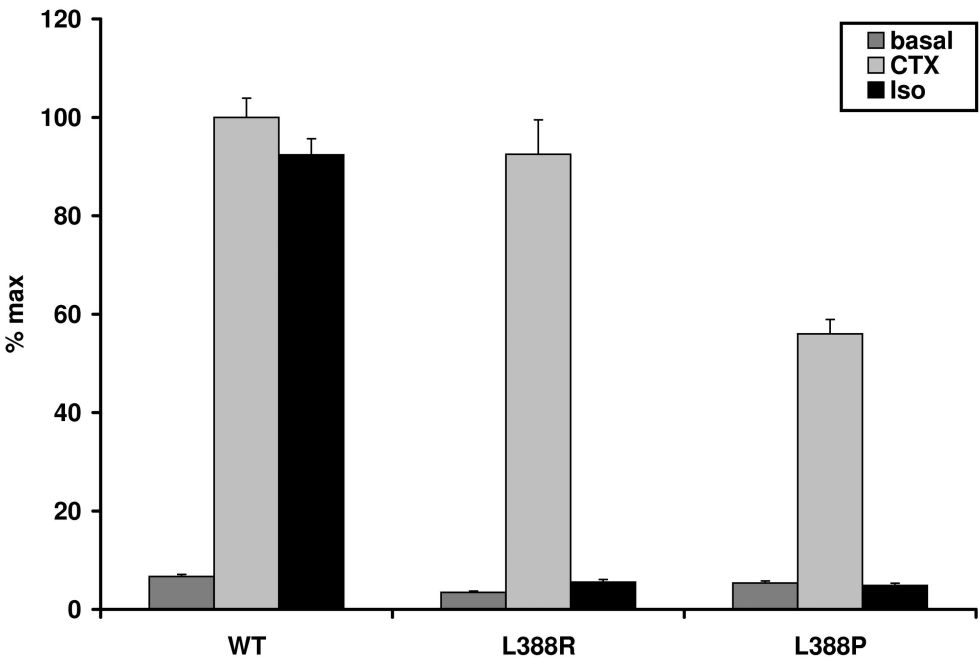
781x500mm (96 x 96 DPI)



781x545mm (96 x 96 DPI)

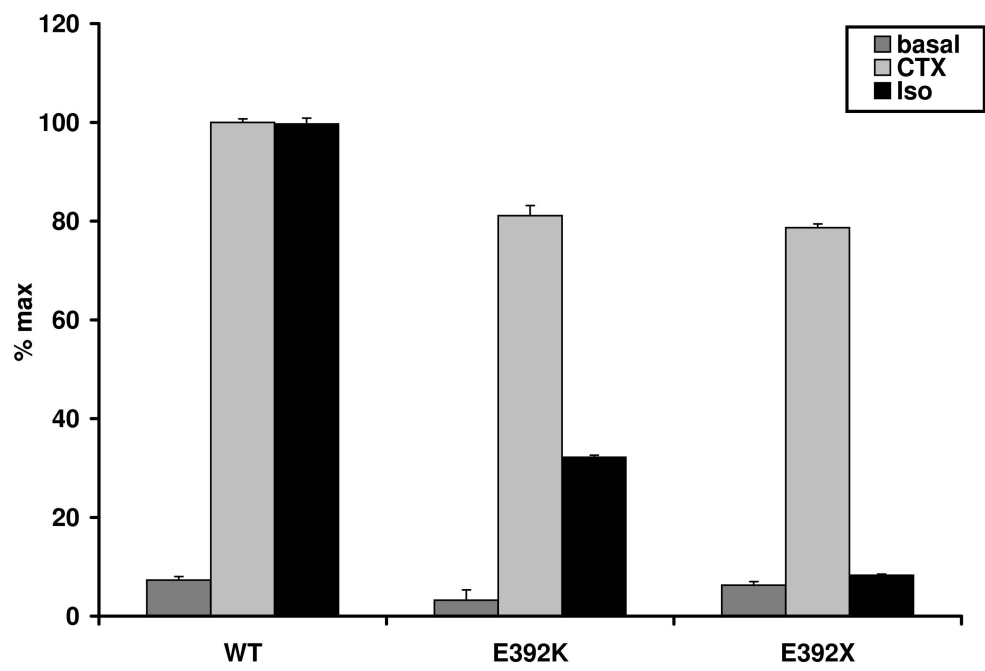


781x555mm (96 x 96 DPI)



781x527mm (96 x 96 DPI)

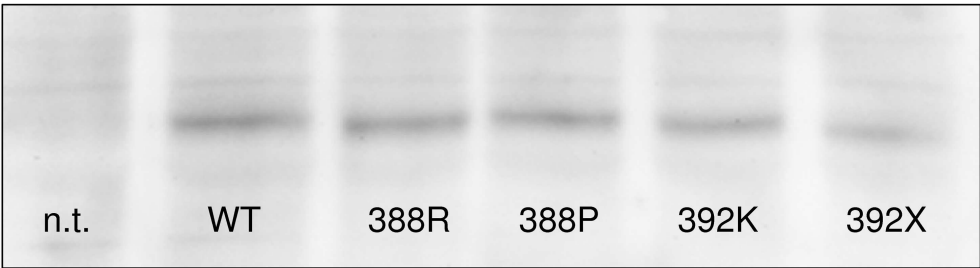
Review



781x543mm (96 x 96 DPI)

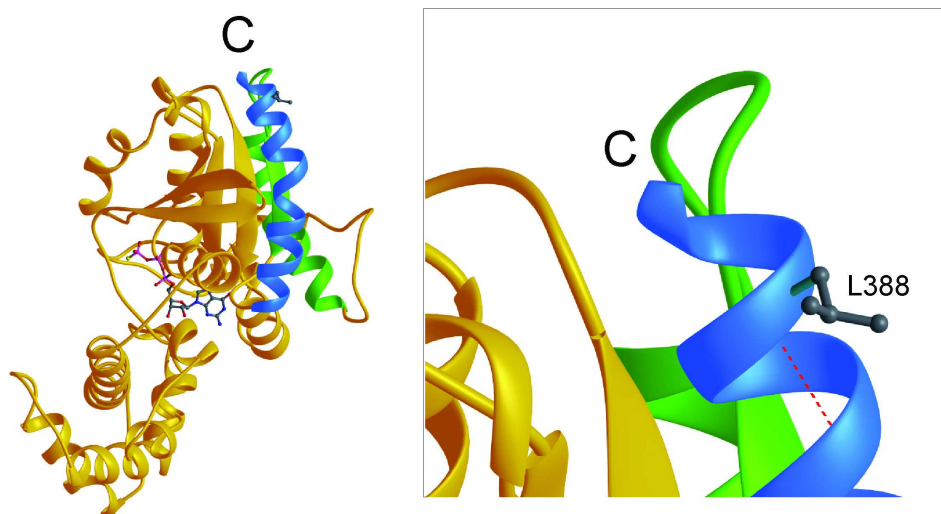
Review

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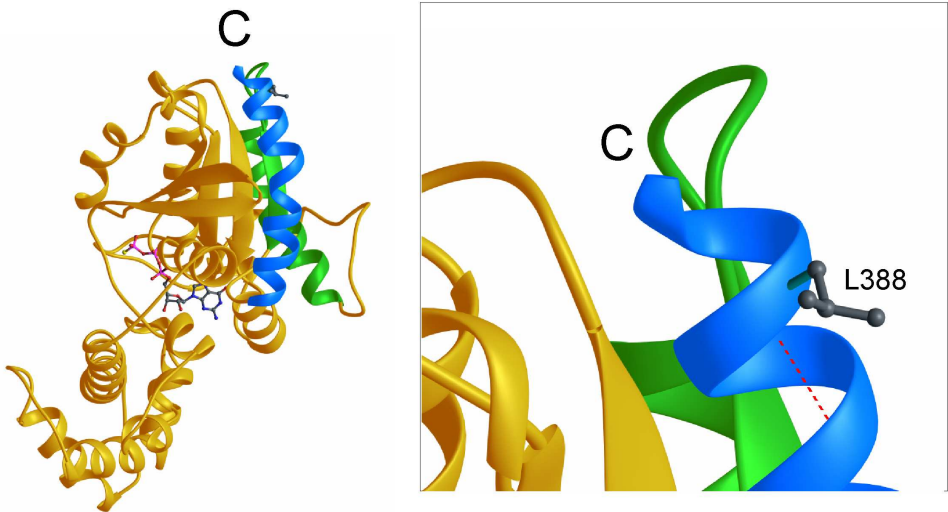


125x36mm (600 x 600 DPI)

For Peer Review



199x105mm (300 x 300 DPI)



199x105mm (300 x 300 DPI)

Peer Review