Molecular basis of glutathione reductase deficiency in human blood cells

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Hereditary glutathione reductase (GR) deficiency was found in only 2 cases when testing more than 15 000 blood samples. We have investigated the blood cells of 2 patients (1a and 1b) in a previously described family suffering from favism and cataract and of a novel patient (2) presenting with severe neonatal jaundice. Red blood cells and leukocytes of the patients in family 1 did not contain any GR activity, and the GR protein was undetectable by Western blotting. Owing to a 2246-bp deletion in the patients' DNA, translated GR is expected to lack almost the complete dimerization domain, which results in unstable and inactive enzyme. The red blood cells from patient 2 did not exhibit GR activity either, but the patient's leukocytes contained some residual activity that correlated with a weak protein expression. Patient 2 was found to be a compound heterozygote, with a premature stop codon on one allele and a substitution of glycine 330, a highly conserved residue in the superfamily of NAD(P)H-dependent disulfide reductases, into alanine on the other allele. Studies on recombinant GR G330A revealed a drastically impaired thermostability of the protein. This is the first identification of mutations in the GR gene causing clinical GR deficiency. (Blood. 2007;109:3560-3566)

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Introduction

The regulation of the cytosolic redox milieu is essential for cell survival. Glutathione reductase (GR [EC 1.8.1.7] catalyzing the reaction GSSG + NADPH + H$^+$ → 2 GSH + NADP$^+$) is a key enzyme in this system, because it maintains millimolar concentrations of reduced glutathione (GSH), rendering GSH the most abundant reductant in human cells.

GR is a homodimeric flavoprotein with a subunit M_r of 52.4 kDa. Its 2 identical redox active sites are formed by residues from both subunits, implying that monomeric GR is not active (Figure 1).[2,3] Human GR is encoded by a single gene (*GSR,* GeneID: 2936, MIM 138300), located on chromosome 8p21.1 and consisting of 13 exons spanning 50 kb (Figure 2A).[4] The gene contains 2 in-frame start codons that are thought to initiate the synthesis of mitochondrial and cytosolic GR.[5]

Since the holoenzyme consists of apoglutathione reductase (apoGR) and FAD as a prosthetic group, the lack or reduction of GR activity can have 2 causes. First, due to inherited mutations, the GR protein can be absent or exhibit low catalytic activity. Second, acquired FAD deficiency due to low amounts of riboflavin (vitamin B$_2$) in the diet (or failure to convert it sufficiently to FAD) may result in inactive apoGR. In the latter case, GR activity can be restored by riboflavin administration in vivo or FAD addition in vitro. Whereas inherited glutathione reductase deficiency is rare, FAD deficiency is common in malnourished populations. The clinical symptoms of GR deficiency include reduced lifespan of red blood cells (RBCs), cataract, and favism.

In 1976, we reported the first familial deficiency of glutathione reductase in human blood cells.[6] The activity could not be restored by in vivo riboflavin administration or in vitro FAD addition, which strongly suggests a mutation in the gene encoding glutathione reductase *(GSR).* We now describe the genetic defect underlying GR deficiency in this family. We also report a novel case of hereditary GR deficiency, in which the patient is a compound heterozygote for a nonsense and a missense point mutation. The enzymatic activity and protein expression in red blood cells and leukocytes were determined, and the enzymatic properties and stability of the recombinant missense mutant GR found in the second family were studied in detail.

Patients, materials, and methods

Case histories

Family 1. Patient 1a is a 54-year-old woman whose clinical history has been described before.[6] She was the index patient in a family with glutathione reductase deficiency who presented with a hemolytic crisis after eating fava beans. The patient, as well as her older brother and younger sister, had undetectable glutathione reductase activity in their red blood cells and strongly diminished GR activity in their leukocytes.[7] The parents (first cousins) had about half-normal GR activity in their RBCs.

At present, all 3 patients are in good health. Patient 1a suffered from bilateral cataract. At the age of 32 years, cataract extraction and intraocular lens implantation were performed with complete recovery of visual function. She frequently has migraine and uses paracetamol (acetaminophen) symptomatically. Except for varices of the lower limbs, she is in good health and without any further medication.
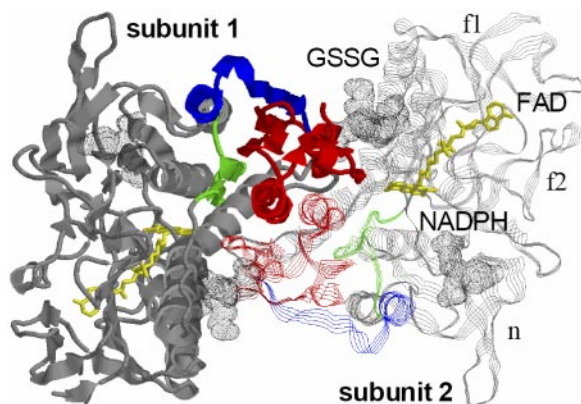
Figure 1. Structure of a human glutathione reductase dimer. The protein backbones of the 2 subunits are shown as ribbons. f1 indicates FAD domain 1; f2, FAD domain 2; and n, NADPH domain. The enzyme's FADs are shown in yellow. Each monomer is an essential part of both catalytic sites. The interface domain is highlighted in color: In the patients of family 1, only the green residues are identical with the wild-type enzyme. The blue residues are replaced by an aberrant sequence, and the red part is completely missing. The atoms of the substrates GSSG and NADPH are indicated as dotted spheres. Note that the nicotine amide ring— otherwise sandwich-like adjacent to the flavin ring—is missing here (1GRA). The structure was created with RasMol V 2.7.2.1.1 (Herbert J. Bernstein, www.bernstein-plus-sons.com/software/rasmol) based on the structural information provided by protein database file 1GRA.[1]

Her older brother (patient 1b), now 58 years old, also suffered from bilateral cataract, for which he was successfully treated surgically at the age of 25 years. Except for cystitis several years ago, for which he received antibiotics, he did not report serious infections. He suffers from

progressive hearing loss that resulted in deafness of one ear and the use of a hearing aid for the other. He has a good physical condition and does not use any medication.

The younger sister (patient 1c), now 48 years old, had bilateral cataract extraction and lens implantation at the age of 24 years. Thirty years ago she was diagnosed having hyperthyroidism. Treatment with radioactive iodine resulted in hypothyroidism for which she uses levothyroxine (175 µg daily). She had gynecologic problems, including a unilateral tubal occlusion that has been surgically corrected; she underwent several in vitro fertilization procedures without success and experienced one ectopic pregnancy, and finally, after a spontaneous pregnancy, a healthy girl was born (6 years old at present). All patients have eliminated fava beans from their diet and avoid exposure to drugs that can lead to hemolytic crises in individuals with glucose-6-phosphate (G6PD) deficiency.

Patient 2. Patient is a 2-year-old girl born as the fourth child of nonconsanguineous white parents of Dutch origin. Pregnancy and delivery were uneventful. On the seventh day after birth, patient 2 was presented to the pediatric department because of severe neonatal jaundice, which was first noticed at the age of 2 days. There was no history of fever, hypothermia, pallor, or other symptoms. She was breastfed without problems until the day before presentation. Upon examination, she appeared lethargic and hypotonic, without focal neurologic signs. The remainder of the neonatal examination was normal.

Laboratory investigations revealed severe unconjugated hyperbilirubinemia (maximum total plasma bilirubin concentration, 754 µM) in the presence of a normal hemoglobin concentration. The blood type was O Rhesus positive (mother: O Rhesus positive). The direct Coombs assay and tests for irregular antibodies were negative. Heinz bodies were not seen in the untreated RBCs. Remarkably, no signs of hemolysis were found (reticulocyte count, .016 [1.6%]; LDH level, 826 U/L [normal range, 200-1098 U/L]). Blood counts revealed less than 0.1×10^9 basophils/L,
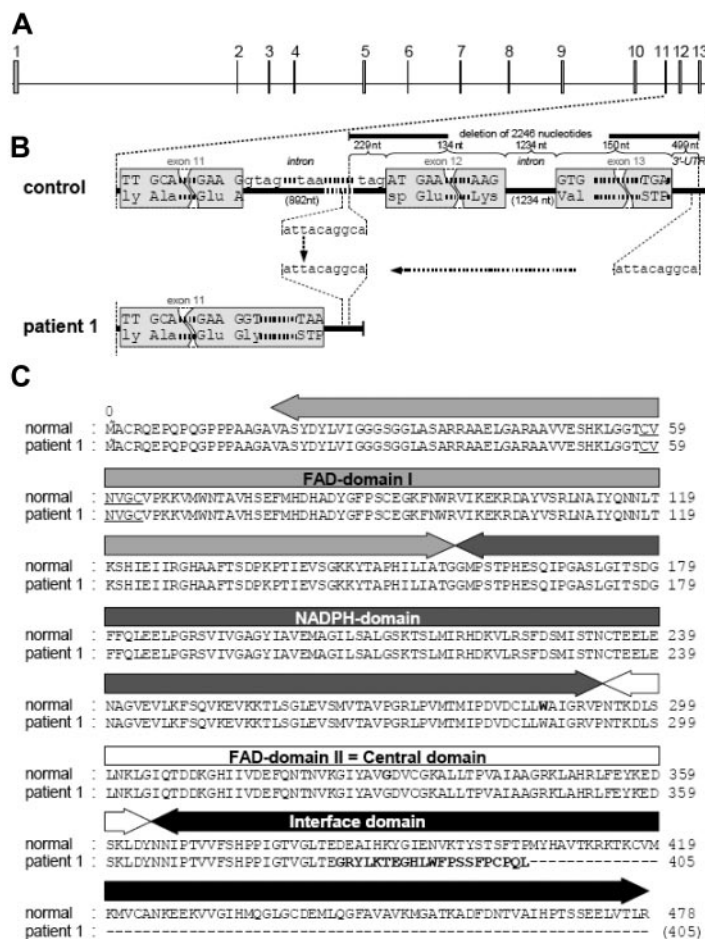
Figure 2. Genomic organization and protein sequence of human glutathione reductase. (A) Genomic organization (based on NCBI GenBank entry NC_000008.9 [30655977-30704985]) of the glutathione reductase gene *GSR* located at 8p21.1. Thirteen exons (shown as vertical bars) are separated by large introns. (B) Structure of the exons (boxes) and introns affected by the mutation found in patients 1a and 1b. The deletion of 2246 nucleotides removes not only exons 12 and 13 but also the appropriate signal required for proper splicing of the exon 11–intron 11 boundary, leading to a truncated protein with an aberrant sequence at its C-terminus. The deleted part is flanked on either side by an attacaggca sequence. One of these is part of the deletion. (C) Alignment of the normal (cytosolic) human GR protein sequence (NP_000628) and the mutated sequence found in patient 1. The redox-active site motif CVNVGC is underlined. The aberrant sequence resulting from the deletion of the 2246 nucleotides is highlighted in bold. The residues 287 (T) and 330 (G), which are affected in patient 2 (allele 1 Trp287Stop, allele 2 Gly330Ala), are highlighted in bold in the normal sequence. Indicated above the sequences is the respective protein domain.

0.6×10^9 eosinophils/L, 8.3×10^9 lymphocytes/L, 8.7×10^9 neutrophils/L, 2.7×10^9 monocytes/L, and 445×10^9 platelets/L. Liver enzymes (ALAT, ASAT, gamma-GT) and thyroid function tests were normal. Congenital infections (lues, toxoplasmosis, herpes simplex virus 1 [HSV1], HSV2, cytomegalovirus [CMV], rubella) and viral hepatitis (hepatitis A virus [HAV], HBV, HDV, HEV, Epstein-Barr virus [EBV]) were ruled out by appropriate tests. Viral cultures of feces were negative. Screens for inborn errors of metabolism included column chromatography of amino acids in plasma and urine, thin-layer chromatography of oligosaccharides in urine, and gas chromatography of nonvolatile organic acids, sugars, and alcohols in urine, the results of which were normal. Crigler-Najjar type I and type II disease and Gilbert syndrome were ruled out by routine sequence analysis of all exons and flanking intron sequences of the *B-UGT* gene. The patient was found to be heterozygous for 6 and 7 TA repeats in the promoter region of *B-UGT*. Homozygosity, but not heterozygosity, for 7 TA repeats has been found in some patients with Gilbert syndrome (personal written communication, October 20, 2004: Drs D. J. J. Halley and A. M. W. van den Ouweland, Department of Clinical Genetics, Erasmus MC, Rotterdam, the Netherlands). G6PD activity in erythrocytes was normal. GR activity in erythrocytes was almost absent before and only 1.0 IU/g Hb after treatment with riboflavin (5 mg/d for 3 weeks).

The girl was treated upon admission with an exchange transfusion and phototherapy for 48 hours, which resulted in a rapid decline of the total plasma bilirubin concentration to normal values within 5 days. Follow-up studies over the next year did not indicate any signs of hemolysis. Physical examination during control visits at the outpatient clinic revealed transient generalized muscle hypertonia and some irritability up to the age of 3 months. Magnetic resonance imaging of the brain showed multiple small areas of infarction in the vascular distribution of the right posterior cerebral artery (thalamus, subcortical white matter, and brain stem). The origin of these probably embolic infarctions remains unclear. The basal ganglia, invariably affected in kernicterus, appeared normal. Otoacoustic emissions and auditory brainstem-evoked potentials were normal. At the age of 18 months, her psychomotoric development and the neurologic examination were completely normal.

Methods

Heparanized venous blood was obtained from healthy donors and from GR-deficient patients and their family members after obtaining informed consent. The study was approved by the Sanquin Review Board in accordance with the standards of the Helsinki protocol.

Purification of RBCs and leukocytes, preparation of blood cell lysates, immunoblotting for GR protein expression, and DNA sequencing were performed according to standard methods, as briefly described in Document S1 and Table S1 (polymerase chain reaction [PCR] primer sequences), which are available on the *Blood* website; see the Supplemental Materials link at the top of the online article.

Cloning, expression, and purification of mutant glutathione reductase

GR of patient 1a. The truncated GR variant was amplified from cDNA (prepared from leukocytes of patient 1a) with the forward primer 5′ GAGAT*CATATG*GCCTGCAGGCAGGAGCCGCAGC 3′ (*Nhe*I site is shown in italics) and the reverse primer 5′ GAGAT*CAAGCTT*TTAAAGTT-GGGGGCAGGGGAAAG 3′ (*Hin*dIII site is shown in italics). After *Nhe*I/*Hin*dIII digestion, the gene was ligated into a *Nhe*I/*Hin*dIII-digested pET-28 vector and propagated in *E coli* BL21(DE3). The sequence of the insert was verified by sequence analysis. For protein expression, cells were grown overnight at 30°C in the presence of 50 µg/mL carbenicillin in Luria-Bertani (LB) medium. The culture was mixed with an equal volume of LB medium, and protein expression was induced by the addition of IPTG (final concentration, 0.1 mM). After 3 hours, cells were harvested by centrifugation, resuspended in PBS containing protease inhibitors (one tablet/50 mL buffer), and sonicated. The extract was loaded on a Nickel-sepharose (Qiagen, Valencia, CA) column, which was then washed with 2 column volumes of 10 mM imidazole in PBS. Truncated GR should have been released with more than 100 mM imidazole in PBS, but no protein was detected in the corresponding eluates.

GR of patient 2. The expression clone of the Gly330Ala GR variant (in *E coli* gor⁻ SG5 cells, which do not contain glutathione reductase of their own[8]), constructed by site-directed mutagenesis of a wild-type glutathione reductase clone, was a kind gift from Dr Rimma Iozef, Giessen University, Germany. The sequence of the insert was verified by sequence analysis. The mutant enzyme was purified according to Nordhoff et al.[9] Protein determination was based on an absorbance of 1.35 at 280 nm for a solution containing 1 mg/mL.

Enzyme activity measurements

Glutathione reductase activity was determined spectrophotometrically at 340 nm by monitoring the decrease in absorbance due to NADPH oxidation ($\epsilon_{340nm} = 6.22$ mM^{-1}cm^{-1}) in the presence of GSSG, as previously described.[6,7] One international enzyme unit (IU) of GR activity is defined as the GSSG-dependent oxidation of 1 µmol NADPH per minute at 25°C.

Activity of recombinant proteins. The concentrated enzyme solution was diluted to about 1 IU/mL with phosphate-based assay buffer containing 0.2 mg/mL bovine serum albumin. This solution (10 µL) was used for 1-mL assays. Kinetic measurements were carried out under the following assay conditions: 47 mM potassium phosphate, 200 mM KCl, 1 mM EDTA, 100 µM NADPH, and 1 mM GSSG at pH 6.9 and 25°C. For K_M determinations, NADPH was varied between 10 and 100 µM and GSSG between 20 and 1000 µM.

Activity in cell lysates. Packed red cells (6 µL) were added to 3 mL reaction buffer containing 48 mM Tris, 17 mM MgCl$_2$, 6.6 mM EDTA, 80 mM KCl, 1.3 mM GSSG, 100 µM NADPH, and 0.03% (wt/vol) saponin at pH 7.4. For the leukocyte assay, 2×10^6 cells were added to the same reaction buffer. Under V_{max} conditions as used for measuring GR activity in cell extracts, the Tris-buffered assay system and the phosphate-based system yield very similar results.

Effect of temperature on enzyme stability in the absence and presence of the substrates NADPH and GSSG. The $T_{50\%}$ value is defined as the incubation temperature that leads to 50% enzyme inactivation in 10 minutes. Incubation was conducted between 30°C and 90°C with samples containing 2.5 units enzyme in 47 mM potassium phosphate, 200 mM KCl, 1 mM EDTA, and 0.8 mg/mL bovine serum albumin at pH 6.9. NADPH (100 µM) or GSSG (1 mM) was added immediately before incubating the sample for 10 minutes at a given temperature. The activity was measured at 25°C immediately after the sample had been cooled for 30 seconds in ice water and had been cleared by centrifugation (1 minute at 13 000*g*). A control sample exposed to 30°C for 10 minutes served as the reference value for 100% activity.

Results

GR activity in blood cells of the patients

Hereditary GR deficiency is a rare condition.[6,10] In the family we described in 1976, the clinical symptoms were restricted to vulnerability of the red cells for oxidative stress (eg, hemolytic attack after eating fava beans) and cataract at early adulthood in all 3 homozygously affected members of this family.[6,7] These rather mild symptoms are not the reason for the low incidence of literature reports: Because GR is used in our red cell diagnostic department as a standard test for comparison with glucose-6-phosphate dehydrogenase, we have performed GR activity measurements in more than 15 000 different blood samples over the last 30 years, with only 2 deficiencies found.

Patients 1a and 1b. As previously reported, the GR activity in the RBCs of patients 1a and 1b was below the detection limit of our assay[6] (Table 1). For the leukocytes, we originally reported about 15% of normal activity.[6,7] However, in taking the basal NADPH oxidation rate in the absence of GSSG into account (14-63

Table 1. Glutathione reductase activity in blood cells of some members of family 1 and family 2

	GR activity in RBCs, IU/g hemoglobin	GR activity in leukocytes, IU/10¹¹ cells	Protein(s) encoded in the 2 *GSR* alleles
Patient 1a	< 0.4	< 5	Truncated and unfolded polypeptide with 405 amino acids
Patient 1b	< 0.4	< 5	Truncated and unfolded polypeptide with 405 amino acids
Father 2	1.1	140	Wild-type GR and the labile GR Gly330Ala mutant
Mother 2	1.6	116	Wild-type GR and the nonsense mutant ending at Leu286
Patient 2	< 0.4 (1.0)*	20	Labile GR Gly330Ala and the mutant ending at Leu286
100 healthy adults, range	2.7-5.8	118-164	Wild-type GR
100 healthy children younger than 3 mo, range	3.1-6.6	Not determined	Wild-type GR

*After riboflavin administration, the GR activity rose to 1.0 IU/g hemoglobin.

μmol/min per 10^{11} cells at 25°C), we conclude that no GR activity is present in the leukocytes of patients 1a and 1b (Table 1).

Patient 2. The RBCs from patient 2 did not display GR activity either. After riboflavin addition to her diet (5 mg/d for 3 weeks), the GR activity in her RBCs increased to 1.0 IU/g Hb, which is still far below the normal range of 3.1 to 6.6 IU/g Hb for children of her age. Both the father and the mother of patient 2 showed a significantly lower GR activity in the RBCs than control values (Table 1). The leukocytes from patient 2 contained a low but measurable GR activity. Both parents showed almost normal GR activity in their leukocytes.

GR protein expression in blood cells

We therefore checked the presence of the GR protein in RBCs and total leukocytes by immunoblotting.

Patient 1a. Figure 3 shows that neither RBCs nor leukocytes contained detectable amounts of GR protein.

Patient 2. Whereas GR protein was also absent in the RBCs of patient 2, the leukocytes still contained some GR protein, which correlates with the low GR activity in these cells. The RBCs and the leukocytes of the parents of patient 2 contained decreased amounts of GR protein. Remarkably, the long-living RBCs displayed no difference in GR protein content between the parents, whereas in the short-living leukocytes the father exhibited a higher GR protein level than the mother.

Identification of the patients' GR mutations

Patients 1a and 1b. PCR amplification of the exons plus intronic boundaries of *GSR,* the gene encoding human GR, from the
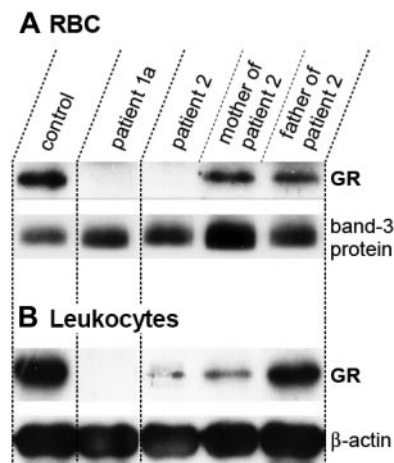

Figure 3. Western blot analysis of blood cell extracts. Immunoblot analysis of (A) erythrocyte and (B) total leukocyte lysates of healthy controls as well as of patient 1a, patient 2, and patient 2's parents. All samples were loaded on the same gel, allowing direct comparison. They are shown separately for clarification. Band-3 and β-actin served as loading controls. Note the faint GR staining in patient 2's leukocyte lysate.

genomic DNA of patient 1b yielded no products for exons 12 and 13. The reverse primer for exon 13 was chosen about 120 base pairs after the TGA stop codon in this exon. Also with cDNA, the PCR with a forward primer in exon 10 and a reverse primer in the coding region of exon 13 did not yield a product. However, with the same forward primer and a reverse primer in exon 11, we did obtain a PCR product, proving that cDNA from GR mRNA was present. This was found with cDNA generated with random hexamers as well as with cDNA generated with oligo-dT, indicating that the poly-A tail of the mRNA was intact. Indeed, when we amplified cDNA with the forward primer in exon 10 and a reverse primer annealing to the nucleotide sequence just prior to the poly-A sequence in the 3'-UTR (Table S1, Exon 13 rev2), we found a PCR product that was larger than the PCR product obtained from control cDNA. Sequencing revealed that the patient's cDNA contained 663 nucleotides of intron 11, followed by a sequence of the 3'-UTR starting 500 nucleotides downstream of the termination codon in exon 13 (Figure 2B). This result was confirmed with genomic DNA and appeared to represent a homozygous mutation. Sequencing of a gDNA PCR product obtained with a forward primer in exon 10 and the same reverse primer in exon 13 revealed a 2246-bp deletion in the patient's DNA, starting at nucleotide −229 in intron 11 and ending at nucleotide 500 in the 3'-UTR. The deletion was confirmed by Southern blot analysis after digestion with *Bam*HI and probing with a labeled *GSR* cDNA construct (not shown). This deletion is flanked on both sides by the same 10-bp sequence ATTACAGGCA, one of which was included in the deletion (Figure 2B). This deletion also removes the acceptor splice site of intron 11, which explains the inclusion of the remainder of the intron into the mRNA. This insertion predicts a partial translation of the former intron into 21 aberrant amino acids, until a UAA stop codon is encountered (Figure 2B). The same mutation was found in the DNA of patient 1a (not shown). A secondary structure prediction performed at http://bioinf.cs.ucl.ac.uk/psipred mainly proposed a coiled structure for these 21 amino acids. The deduced truncated and chimeric protein has a predicted molecular weight of 43.8 kDa. If expressed, this protein might be detected, since the polyclonal antibody was generated against full-length GR. However, upon reinspection of the Western blots, no band of the expected size was observed.

Patient 2. Patient was found to be a compound heterozygote. On one allele, a G861A substitution was identified, changing the TGG codon for Trp287 into a premature TGA stop codon in exon 9 (Figure 4A) (numbering refers to cDNA of the cytosolic form of GR). The other allele contained a G989C mutation in exon 10, changing the GGG codon for Gly330 into the GCG codon for alanine (Figure 4B). Gly330 is a highly conserved residue in the superfamily of NAD(P)H-dependent disulfide reductases. This residue is part of the FAD-binding motif described by Eggink et al.[11] Sequencing the DNA from the parents of patient 2 proved that the patient had inherited the nonsense mutation (Trp287Stop) from
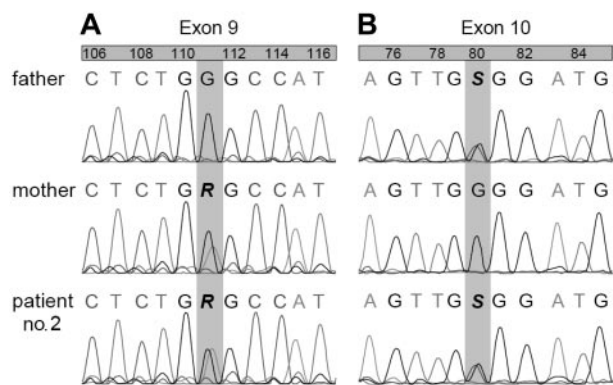
Figure 4. Sequence profile of family 2. The numbers on top refer to the position of the nucleotides in the respective exons in the cDNA of the cytosolic form of GR. (A) Exon 9, around 861G in *GSR*. (B) Exon 10, around 989G. R and S indicate the nucleotides that differ between the 2 alleles.

her mother and the missense mutation (Gly330Ala) from her father (Figure 4).

Recombinant expression and characterization of mutant protein species

Patients 1a and 1b. The truncated gene was cloned into a pET-28 vector, but we did not succeed in producing recombinant protein in *E coli.* The formation of insoluble aggregates or inclusion bodies was ruled out by Western blot analysis of *E coli* cell lysates.

Patient 2. The mutant protein GR G330A was expressed in GR-deficient *E coli*[11] and purified by affinity chromatography over 2′,5′-ADP-sepharose (Amersham Pharmacia Biotech, Piscataway, NJ).[12] The final yield was 7 mg mutant enzyme per liter of 2-YT medium. This is only 15% of the yield expected for the wild-type enzyme. GR G330A was found to be more than 97% pure by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS PAGE). The mutant enzyme had K_M values for NADPH and GSSG similar to wild-type GR (Table 2), and the NADPH oxidase activity of GR G330A was found to be as low as in the case of wild-type GR ($< 0.01\%$ of its GSSG reduction activity). However, the specific GSSG reduction activity of the mutant enzyme (90 IU/mg) was significantly lower compared with the wild-type enzyme (205 IU/mg) prepared in parallel. In comparison with the wild-type enzyme—which is stable as a holoenzyme with a half-life of more than 2 weeks—GR G330A has a tendency to release FAD. When stored at 4°C in FAD-free buffer, the mutant enzyme lost 20% of its enzyme activity in 48 hours. Storage at 25°C led to a loss of 50% of activity in 48 hours. The activity was fully restored by adding FAD (5 μM final concentration) to the GR assay mixture. This finding is consistent with the fact that Gly330 is part of the FAD-binding motif in disulfide reductases; any residue larger than glycine is expected to interfere with precise binding of FAD.[11] High FAD concentrations should be avoided, because this compound acts also as a noncompetitive inhibitor, the K_i value at 25°C being 130 μM both for the wild-type enzyme and for GR G330A.

Thermal instability of GR G330A

Compared with the enzyme from other sources, human GR is remarkably stable. We checked whether loss of enzyme activity and lower enzyme protein levels could be related to a lower stability of the mutant. Therefore, enzyme solutions with 2.5 IU GR/mL—representing physiological activities—were exposed to elevated temperatures for 10 minutes. The activity of the wild-type GR

dropped to 50% at 85°C. In the presence of 100 μM NADPH, 50% inactivation was already obtained after 10 minutes at 55°C (Table 2). In contrast, the corresponding $T_{50\%}$ values for GR G330A were found to be 60°C without NADPH and 33°C with NADPH, and thus far lower than for the wild-type enzyme. Under quasiphysiological conditions (50 μM NADPH, pH 7.3, and 37°C), the half-time of inactivation was found to be 16 minutes for the mutant enzyme and more than 100 hours for wild-type GR (Table 2). The degree of thermal inactivation depends on the enzyme concentration. When increasing the enzyme activity from 1 IU/mL to 20 IU/mL in the presence of 100 μM NADPH, the residual activity of GR G330A exposed to 37°C for 10 minutes increased from 23% to 82%.

Discussion

The complete absence of GR activity in blood cells of patients 1a and 1b can be well explained by the deletion of the segment Asp385-Arg478, encoding almost the entire dimerization or interface domain (Figures 1-2). The 43.8-kDa truncated protein was not detected on Western blot with a polyclonal antibody raised against the entire GR (Figure 3). Since we also did not detect any expression of truncated GR in *E coli,* it is likely that, upon misfolding, the expressed truncated GR is immediately degraded in human and in *E coli* cells.[12] Apparently, dimerization is also required to obtain a stable, properly folded protein. This is consistent with the observation that dimerization is a prerequisite for proper folding of the domains within the GR subunits.[13] Dimerization is initiated by pair formation of helix 11 and helix 11′. In the truncated 43.8-kDa mutant, the residues 439-454 and 439′-454′, which form these helices, are missing (Figure 1).

We hypothesize that the deletion in *GSR* has originally been caused by mismatched DNA duplication or DNA repair in germline cells or during early embryogenesis, because an identical stretch of 10 base pairs is located on each side of the deletion. Since the parents of patients 1a and 1b are first cousins, the deletion is probably present on both alleles in the patients. The father is no longer alive, so this notion cannot be confirmed directly.

The symptoms of the patients in family 1, favism and cataract, can be linked to increased risk of oxidative stress owing to absence of GR activity in the affected cells. Fava beans contain a high amount of the glycosides vicine and convicine. When ingested, the sugar moieties are removed by hydrolysis, resulting in the formation of divicine and isouramil, respectively. These 2 components undergo redox recycling, which is maintained by molecular oxygen as the oxidant and GSH as the reductant. If GSH is not regenerated at a sufficient rate, depletion of GSH will occur, finally resulting in a hemolytic crisis.[14,15] Cataract formation is probably the result of (primarily UV-induced) oxidative damage in the eye lens, again

Table 2. Properties of GR G330A compared with wild-type GR

Property	GR G330A	Wild-type GR
Specific activity, IU/mg	90	205
K_M for NADPH, μM	6.5	7.5
K_M for GSSG, μM	85	65
NADPH oxidase activity vs GR activity, %	< 0.01	< 0.01
Reversible release of FAD	Yes	No
$T_{1/2}$ at 37°C*	16 min	> 100 h
$T_{50\%}$ without substrate, °C†	60	85
$T_{50\%}$ with 100 μM NADPH, °C†	33	55

*Starting with 1 IU GR activity per mL in the presence of 50 μM NADPH at pH 7.3.
†Starting with 2.5 IU GR activity per mL at pH 6.9.

owing to insufficient protection against oxidation and insufficient reduction of oxidized proteins. In the lens fiber cells, de novo protein synthesis is impossible. Moreover, these cells are not renewed during the life of an individual, thus rendering them extremely dependent on intrinsic protection against oxidation.[16]

In the second family, described for the first time in this report, GR deficiency was found in a newborn with severe unconjugated hyperbilirubinemia, which could not be explained by other known causes. Remarkably, there was no evidence of enhanced hemolysis; this is reminiscent of neonatal hyperbilirubinemia due to G6PD deficiency, a condition often not associated with increased hemolysis.[17] Although bilirubin levels reached extremely high values, no kernicterus or other sequelae, such as damage to the cochlea, developed. The relation between GR deficiency and hyperbilirubinemia is not clear. Possibly, bilirubin conjugation to glutathione plays a role in the first few days after birth, when conjugation of bilirubin to glucuronic acid is still low, or perhaps a deficiency in glutathione conjugation of other orthobiotics must be compensated by glucuronidation, which then depresses further the already low hepatic bilirubin glucuronidation activity. It should be noted that the patients of family 1 did not remember any case of neonatal hyperbilirubinemia in their family history.

Although the RBCs of patient 2 did not show any GR activity or GR protein expression, the leukocytes contained some residual activity, which correlates with a weak GR protein expression on Western blot. This is understandable in the light of the long lifespan of RBCs and their lack of protein synthesis. In contrast, most leukocytes have a much shorter survival time and are still capable of synthesizing new proteins. The parents of patient 2 displayed GR activity in both cell types, although significantly less than in control cells. This suggested that the nonconsanguineous parents are heterozygous for a GR mutation, and that the patient inherited both mutant alleles. Indeed, sequencing revealed that the patient was a compound heterozygote. As the RBCs of both parents contained about half of the normal GR activity, this indicates a gene-dosage effect. Indeed, a 50% higher GR activity has been reported for trisomy 8.[18]

Similar to the mutation resulting in a C-terminal deletion in patient 1, truncation of GR at Trp287 in patient 2 is not expected to yield an active enzyme or properly folded protein, since the folding-initiating helix 11 formed by residues 439 to 454 is missing.[13] In contrast, the mutation of a glycine into an alanine seems not to be a drastic mutation. However, Gly330 is part of the TxxxxIYIIGD motif, a conserved region in FAD-binding enzymes.[13] This sequence forms a β-sheet that turns at the aspartate moiety. The conserved glycine ensures a proper turn of the C^α chain and creates enough space to accommodate the pyrophosphate group of FAD. The conserved aspartate forms hydrogen bonds with the hydroxyl groups of the FAD ribose moiety.[2] It could be envisaged that a Gly→Ala substitution disturbs proper FAD binding in GR G330A, thereby affecting catalysis and/or protein stability. Our studies on recombinant GR G330A indeed showed a lower activity and stability (Table 2). The notion of disturbed FAD binding is supported by the apparent increase in GR activity in the patient's RBCs after supplementation of her diet with riboflavin (Table 1). Furthermore, the recombinant enzyme mutant has a tendency to reversibly release FAD, which is not the case for wild-type GR. This means that the mutant enzyme needs higher concentrations of FAD for saturating the apoenzyme, required for proper folding, activity, and stability of GR.

GR in oxidized form (E_{ox}) is rather stable (Table 2). However, in the cytosol, under reducing conditions, NADPH causes reduction of the enzyme's disulfide, leading to EH_2, which binds NADPH rather tightly.[19] It has been found that this $EH_2 \cdot$ NADPH complex of various disulfide reductases has a labile structure.[20] Wild-type human GR is sufficiently stable when complexed with NADPH, but this is not the case for the mutant GR G330A. For this protein, the half-life is only 16 minutes at quasiphysiological conditions in the presence of 50 to 100 μM NADPH. This effect probably explains the phenotype of the mutant—the low expression yield as well as the short lifespan in vivo, which leads to a rapid decrease of GR activity in erythrocytes. Preliminary results indicate that reversible temperature inactivation is associated with a modified FAD spectrum, reflecting modified FAD binding. This will be addressed in a future paper reporting the crystal structure and the spectroscopic properties of human GR G333A.

In the light of the findings described in "GR activity in blood cells of the patients" and "GR protein expression in blood cells," it is possible to speculate on the relation between GR activity and GR protein in the cells of the parents of patient 2. The mother expresses only wild-type GR subunits from one allele and truncated, probably unstable mRNA and/or GR protein subunits from the other allele. The father expresses both wild-type GR and GR G330A mRNA. Theoretically, the father's mixed pool of GR subunits would result in formation of 50% heterodimers between wild-type GR and GR G330A subunits. Whereas GR G330A homodimers are less active and very unstable (as indicated by the thermostability assays of the recombinant protein), the heterodimers may still display activity, as inferred from the father's GR activity in his leukocytes. On the basis of the Western blot, this heterodimer is quite stable. In the long-living RBCs, the difference in GR protein expression is not detectable. It is tempting to speculate that in the RBCs of the father only some wt GR is left from the initial mixed GR pool. This might be concluded from the lower GR activity in the father's RBCs compared with those from the mother.

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Authorship

Contribution: N.M.K. performed research, collected and analyzed data, and wrote the paper; R.Z. collected patient material, performed research, and analyzed data; M.B. performed sequence analysis and analyzed data; G.M. performed sequence analysis; H.V. performed patient RBC analysis; N.B. treated one patient and collected data; C.L. treated one patient, collected data, and wrote a clinical report; K.M.D. interviewed 3 patients and wrote a clinical report; K.B. provided recombinant mutant protein and did enzymatic analysis of the proteins; R.H.S. contributed polyclonal antibodies and performed biochemical studies on wild-type and mutant proteins; S.G. analyzed data, created figures, and wrote part of the paper; D.R. supervised research, analyzed data, and wrote the paper.

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