A Novel Mutation Found in the 3′ Domain of NADH-Cytochrome B5 Reductase in an African-American Family With Type I Congenital Methemoglobinemia

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Congenital methemoglobinemia caused by an erythrocytic deficiency of cytochrome b5 reductase (b5R; type I) in African-American individuals was first reported by this laboratory. The rarity of this observation is possibly due to the difficulty detecting cyanosis that is masked by naturally occurring dark skin pigment. Since previous biochemical studies on the African-American family with variant enzyme b5R-Shreveport showed enzyme instability, we focused on molecular analysis of its transcript. The transcript size was the same as that of a normal control. The nucleotide sequence of both normal and variant transcripts were examined by directly sequencing reverse transcriptase-polymerase chain reaction (RT-PCR) product. The propositus was found to be homozygous for a G to A transition at codon 212 in exon 8, changing a glutamate to a lysine (E212K). In addition, a C to G transversion was found at codon 116 in exon 5, changing a threonine to a serine (T116S). Using allele-specific PCR, we determined that E212K was found only in the propositus and her heterozygous mother. Furthermore, E212K is predicted to disrupt an α-helix peptide structure of b5R, suggesting that this is likely the disease-causing mutation. In contrast, T116S was found to be a high-frequency polymorphism specific for the African-American population. The E212K mutation is uniquely present in the 3′ end of the b5R gene (exon 8), which differs from those b5R mutations found among Japanese subjects (exons 3 and 5) and in an Italian subject (exon 4) and, thus, further contributes to our understanding of the structure/function relationship of this housekeeping enzyme.

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CONGENITAL methemoglobinemia due to a deficiency of NADH-cytochrome b5 reductase (b5R, EC 1.6.2.2) is an autosomal recessive disorder (RCM; McKusick no. 250800).[1] b5R is a member of a flavoenzyme family of dehydrogenases-electron transferases[2] that participates in the transfer of electrons from the NADH generated by glyceraldehyde 3-phosphate dehydrogenase to cytochrome b5.[3,4] The b5R gene is 31 kb in length, has nine exons and eight introns,[5] and has been localized to chromosome 22q13-qter.[6]

There are two clinical forms of congenital methemoglobinemia caused by b5R deficiency. Type I b5R deficiency is characterized by cyanosis due to methemoglobinemia and an isolated deficiency of b5R in erythrocytes.[7] The erythrocytic soluble isoform of b5R consists of 275 hydrophilic amino acids[8,9] and is involved in methemoglobin reduction.[10,11] Erythrocyte-limited b5R deficiency can be explained by demonstration of protein instability since mature erythrocytes do not have protein synthesizing machinery and, thus, there is no continuous protein synthesis in contrast to other cell types. Type II b5R deficiency is characterized by a deficiency of the enzyme in all cell types, methemoglobinemia, and mental retardation.[12] The membrane-associated isoform of b5R is found in the outer mitochondrial membrane and the endoplasmic reticulum[13,14] and consists of the same 275 amino acid residues as the erythrocytic form and also has 25 N-terminal hydrophobic amino acid residues.[15,16]

Populations with endemic erythrocytic b5R deficiency have been reported in Athabaskan Alaskans, Navajo Indians, and Puerto Ricans.[17] Two deficient variant b5Rs (type I) referred to as b5R-Birmingham and b5R-Shreveport were characterized in two unrelated African-American families in our laboratory, an observation previously unreported in African-Americans possibly because of the difficulty detecting cyanosis that is masked by naturally occurring dark skin pigment.[18] Although the populations with endemic methemoglobinemia may have a darker complexion than the average white person, the average African-American has more pigmentation, which may result in possible underreporting of methemoglobinemia in the African-American population.

In this study, we identified a novel mutation at codon 212 in exon 8 (E212K) of b5R-Shreveport in both alleles of the propositus and one allele of her heterozygous mother but not in other methemoglobinemic subjects or other normal relatives. We also provide evidence that this is not a polymorphism but is a mutation that is likely causative of the disease phenotype.

MATERIALS AND METHODS

Case report and family pedigree. The pedigree for the family with b5R-Shreveport is shown in Fig 1. To our knowledge there is no consanguinity. Studies were performed on the propositus, II.2, previously reported as having congenital methemoglobinemia type I.[18] In brief, she was a healthy 14-year-old African-American who was found to have a "chocolate brown" color of her blood during labor in Shreveport, LA. Laboratory studies[18] showed an elevated methemoglobin level measured by the method of Evelyn and Malloy[19] and a decreased b5R activity measured using the NADH-ferricyanide reductase activity.[20] The propositus' (II.2) enzyme activity falls close to the heterozygous range. Differentiating between methemoglobinemic and heterozygous individuals is based on an increased methemoglobin level and not solely on decreased enzyme activity. We do not have an adequate explanation for the observed discrepancy between methemoglobinemia and higher-than-expected enzyme activity. However, enzyme activity as measured in vitro under

Individual	II.1	III.1	II.2	II.3	II.4	II.5	I.1	I.2	Normal Range
b5R Activity (IU/gHb)	Not Studied	11.35	5.98	12.92	8.03	19.21	8.03	Not Studied	11.51-26.91
MetHb (% total Hb)	Not Studied	0	21.5	0	0	0	0	Not Studied	<1
E212K (G→A)	Not Studied	Not Studied	A/A	G/G	Not Studied	Not Studied	G/A	Not Studied	G/G
T116S (C→G)	Not Studied	Not Studied	G/G	C/C	Not Studied	Not Studied	C/G	Not Studied	C/C

Fig 1. Pedigree and summary table for the family with b5R-Shreveport. Individual II.2 is the propositus affected with congenital methemoglobinemia due to an erythrocytic deficiency of b5R (type I). Each family member's affected, unaffected, or carrier state was determined in a previous study[18] based on clinical symptoms and biochemical data. For the present study, EBV lines were available only from individuals I.1, II.2, and II.3. The enzyme activity was measured in erythrocytes in international units expressed per gram hemoglobin (IU/gHb) (the propositus' [II.2] value represents a mean enzyme activity from five measurements), and the methemoglobin (metHb) is expressed as a percentage of the total hemoglobin.[18] The normal range for each assay is provided. The presence of wild-type and mutant alleles for the E212K mutation and the T116S polymorphism are shown. G is the normal and A is the mutant allele in the E212K mutation while C is the normal and G is the mutant allele in the T116S polymorphism.

highly artificial conditions does not necessarily correlate with the in vivo enzyme activity in the cells where known and unknown activators, inhibitors, and other factors may be present. It is possible that leukocyte contamination may explain this discrepancy; however, leukocytes have been removed from the hemolysate by column filtration.[21] The propositus' (II.2) partially purified erythrocyte b5R was found to be heat labile and a decreased amount of antigen (b5R) in erythrocyte lysate was seen on a Western blot. Her nucleated peripheral blood cells and Epstein-Barr virus (EBV)-transformed lymphocytes had normal or only slightly decreased b5R activity. Her methemoglobinemia was previously unsuspected and was found only when her blood sample was inspected directly.

Preparation of cells as a source of b5R transcript. EBV-transformed lymphocytes were previously prepared on the propositus (II.2), her mother (I.1), brother (II.3), and an unrelated normal control.[18] The frozen aliquots of the cell lines were resuspended and grown at 37°C with 5% CO_2 in RPMI-1640 medium supplemented with 10% fetal bovine serum, 1% penicillin-streptomycin, and 1% L-glutamine (Sigma, St Louis, MO).

RNA isolation and Northern blot analysis. RNA was prepared from EBV-transformed lymphocytes of the propositus (II.2), her mother (I.1), brother (II.3), and an unrelated normal control using acid guanidinium thiocyanate-phenol-chloroform extraction.[22] These RNA samples were separated by gel electrophoresis and transferred to a nylon membrane (GeneScreen Plus; DuPont/NEN, Wilmington, DE) that was hybridized according to standard Northern blot procedure.[23] A plasmid containing a 1.8-kb EcoRI fragment of human

liver b5R cDNA, pCb5R141 (kindly provided by Yoshiyuki Sakaki, Kyushu University, Japan) was used as a probe.[24] The membrane was rehybridized for the β-actin transcript using a 281-bp polymerase chain reaction (PCR) product derived from a pHFβ-A_1 template (kindly provided by Larry Kedes, Stanford University, Palo Alto, CA) as a probe.[25] Both probes were radiolabeled with [α-^{32}P]dCTP (3,000 Ci/mmol; Amersham, Amersham, UK) using nick translation (Boehringer Mannheim, Mannheim, Germany).[26] The radiolabeled membrane was analyzed by autoradiography and the signal intensity was also quantitated using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

cDNA synthesis. Using 2 μg total RNA isolated from EBV-transformed lymphocytes of the propositus (II.2), her mother (I.1), brother (II.3), and an unrelated normal control, cDNA synthesis was performed in a 20-μL reaction volume containing 1× First Strand buffer (50 mmol/L Tris-HCl [pH 8.3], 75 mmol/L KCl, 3 mmol/L $MgCl_2$), 5 mmol/L dithiothreitol (DTT), 0.75 mmol/L each dNTP (Boehringer Mannheim), 2 μg random hexanucleotide primer (Pharmacia, Uppsala, Sweden), 8 U RNasin (Promega, Madison, WI), and 200 U Superscript II M-MLV RNase H⁻ reverse transcriptase (GIBCO BRL, Gaithersburg, MD). The reaction was allowed to proceed for 75 minutes at 42°C followed by inactivation of the reverse transcriptase (RT) for 3 minutes at 65°C.

PCR. The entire b5R cDNA coding region starting with the ATG initiation site in exon 1 through the TGA stop site in exon 9 was amplified using 6 overlapping sets of gene specific primers with each primer pair generating approximately 200-bp PCR fragments
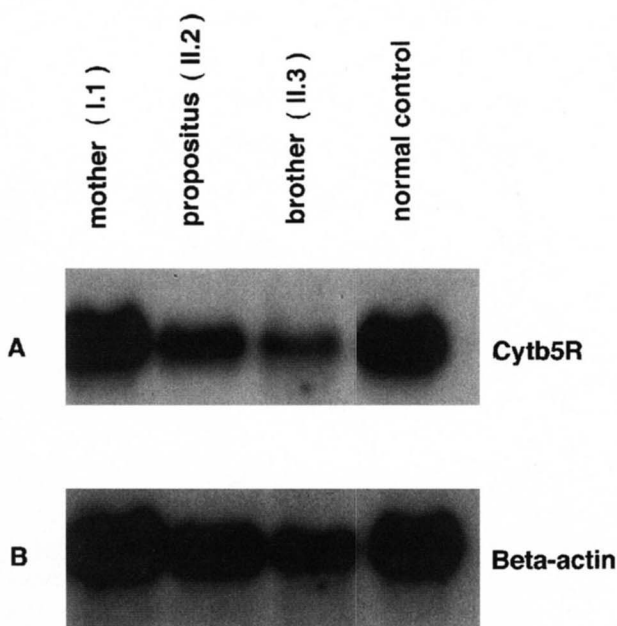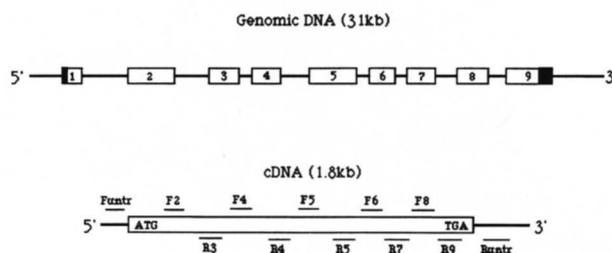
Fig 2. Northern blot hybridization. Lymphoblastoid cell-derived RNA from all available members of the family with b5R-Shreveport was analyzed. For subject designation see Fig 1. (A) The blot was probed with a ^{32}P-labeled portion of b5R. The same size transcript in all three family members as well as a normal control is shown. (B) The same membrane was reanalyzed with a ^{32}P-labeled β-actin fragment used as an internal control.

Fig 3. Schematic map of cytochrome b5 reductase gene. The relative sizes of the genomic DNA and cDNA are not drawn to scale. A schematic genomic map in the top panel represents the 9 exons and 8 introns of the b5R gene. The untranslated regions are shaded. The genomic DNA is 31 kb in length. The cDNA of b5R, seen in the bottom panel, is 1.8 kb in length. ATG is the initial codon in exon 1 and TGA (stop) is the last codon of exon 9. The approximate location of the six sets of overlapping primers (see Table 1) used to amplify the coding region by RT-PCR is shown.

(see Table 1 and Fig 3). Amplification was performed in a GeneAmp PCR System 6000 (Perkin-Elmer Cetus, Norwalk, CT) using a 50-μL reaction volume containing 1× PCR buffer (20 mmol/L Tris-HCl [pH 8.4], 50 mmol/L KCl), 2 mmol/L MgCl$_2$, 2 U *Taq* polymerase (GIBCO BRL), 0.2 mmol/L of each dNTP, and 10 pmol of each primer. The conditions for PCR were: an initial denaturation at 94°C for 3 minutes, 35 cycles of denaturation at 95°C for 20 seconds, annealing and elongation at 70°C for 40 seconds with an additional extension at 72°C for 6 minutes after the last cycle. PCR products were visualized by gel electrophoresis on a 1% agarose gel stained with ethidium bromide.

Direct nucleotide sequencing of amplified products. Preparation of the PCR products for sequencing consisted of treatment with a combination of exonuclease I and shrimp alkaline phosphatase to remove residual single-stranded primers, extraneous single-stranded DNA, and remaining dNTPs (Sequenase PCR Product Sequencing Kit; United States Biochemical, Cleveland, OH). The sequencing was performed by analyzing both the coding strand (using forward primers) and the complementary strand (using reverse primers). The reaction products were radiolabelled using [α-^{35}S]dATP (>1,000 Ci/mmol; Amersham), 1× Sequenase buffer, a 1:5 diluted 7-deaza-dGTP labeling mix, and Sequenase DNA polymerase and subsequently analyzed on a 6% denaturing polyacrylamide gel containing 7 mol/L urea and 1× glycerol tolerant gel buffer (United States Biochemical). Gels were fixed in a solution of 15% methanol/5% acetic acid for 15 minutes, and dried at 80°C for 40 minutes before overnight exposure on X-OMAT film (Eastman Kodak Co, Rochester, NY).

Allele-specific PCR (ASPCR). Any deviations from the published sequence found in the analysis of RT-PCR products from the propositus (II.2) were subsequently examined in all available family members, other methemoglobinemic patients, and normal controls using a rapid, nonisotopic screening method, allele-specific PCR, which selectively amplifies specific alleles and detects known, single-base substitutions. ASPCR was first described by Wu et al[27] in 1989, and more recently, was modified in our laboratory by Liu et al[28] to include two rounds of PCR with the first round amplifying both alleles yielding a high purity and concentration of DNA template using upstream and downstream oligonucleotide primers that flank the nucleotide substitution. In the second round of PCR amplification, two allele-specific oligonucleotide primers are used with a common downstream primer. One of the two upstream allele-specific primers is fully complementary to one of the alleles while the other upstream primer is mismatched at its 3′ end for the same allele; the reverse is true for the other allele. In the second round, only five cycles of PCR are performed so that the product generated by the mismatched primer is not visible. Two reactions are performed using sample cDNA with each reaction containing only one of the upstream allele-specific primers, while the downstream primer is common to both alleles. Thus, efficient amplification will occur only in the reaction with the perfectly complementary allele-specific primer and not in the reaction with the 3′ mismatch. Using this technique, we could clearly distinguish the normal and mutant alleles present in both heterozygous and homozygous combinations.

RESULTS

Since the previous description of the propositus with b5R-Shreveport,[18] the family has been restudied. The updated

Table 1. Primers for PCR Amplification of b5R cDNA

Amplified Region	Fragment Size (bp)	Primer	Sequence (5′ → 3′)
5′ untr +	220	Funtr5	GGCGGCGGCGACAGAGCGAG
Exon 1-3		R3	AAGCGGAAGCGCCGGGTGTCAT
Exon 2-4	198	F2	ATCAAGTACCCGCTGCGGCTCATC
		R4	CCACGAAGCCCTTGTCATCATCGCT
Exon 4-5	228	F4	CAGCACATCTACCTCTCGGCTCG
		R5	CCTGGTAGACCAGCAGCCCACT
Exon 5-7	221	F5	TCAGTACCTGGAGAGCATGCAG
		R7	GTGTGGTCATCAGGGTCCTTCA
Exon 6-9	246	F6	TGAAGTCTGTGGGCATGATCGC
		R9	TTCACGAAGCCCTGGCCGTAGT
Exon 8-9 +	244	F8	CTGCACGCTTCAAGCTCTGGTACAC
3′ untr		Runtr3	GGTGGCCGTGTGACCGGTGC

Abbreviations: untr, untranslated region; F, forward; R, reverse.
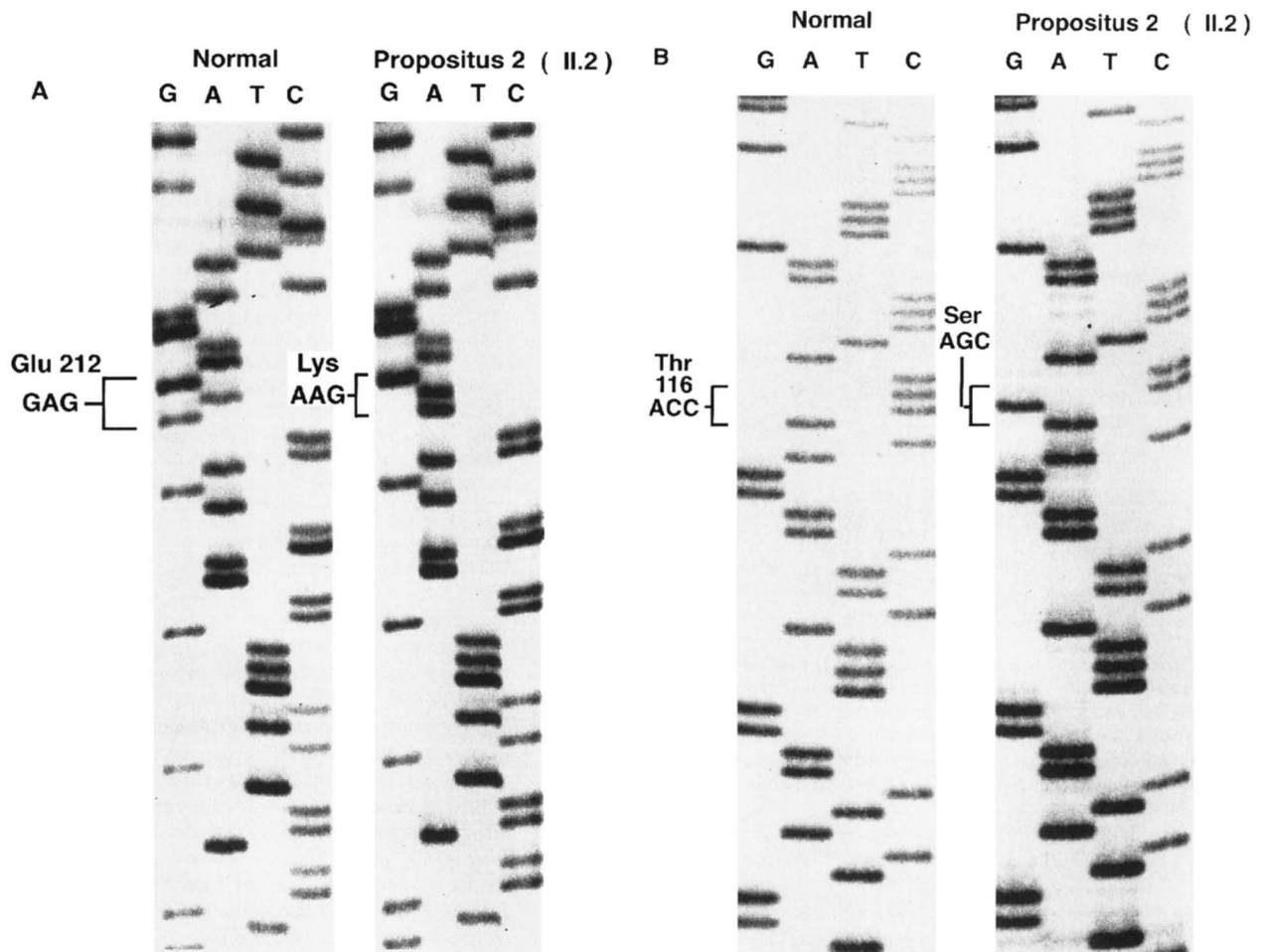
Fig 4. Nucleotide sequence analysis of the b5R gene of a normal and mutated allele. (A) b5R cDNA sequence from a normal individual compared with cDNA from the propositus with b5R-Shreveport (II.2). At the first position of codon 212 in exon 8 is a G to A transition that changes a glutamate to a lysine in both alleles of individual II.2. (B) A C to G transversion was found at the second position of codon 116 in exon 5 that changes a threonine to a serine in both alleles of individual II.2.

pedigree is shown in Fig 1, and for this study, the only available EBV-transformed lymphocytes were on the propositus (II.2), her heterozygous mother (I.1), and her brother (II.3) who has a normal b5R enzyme activity.
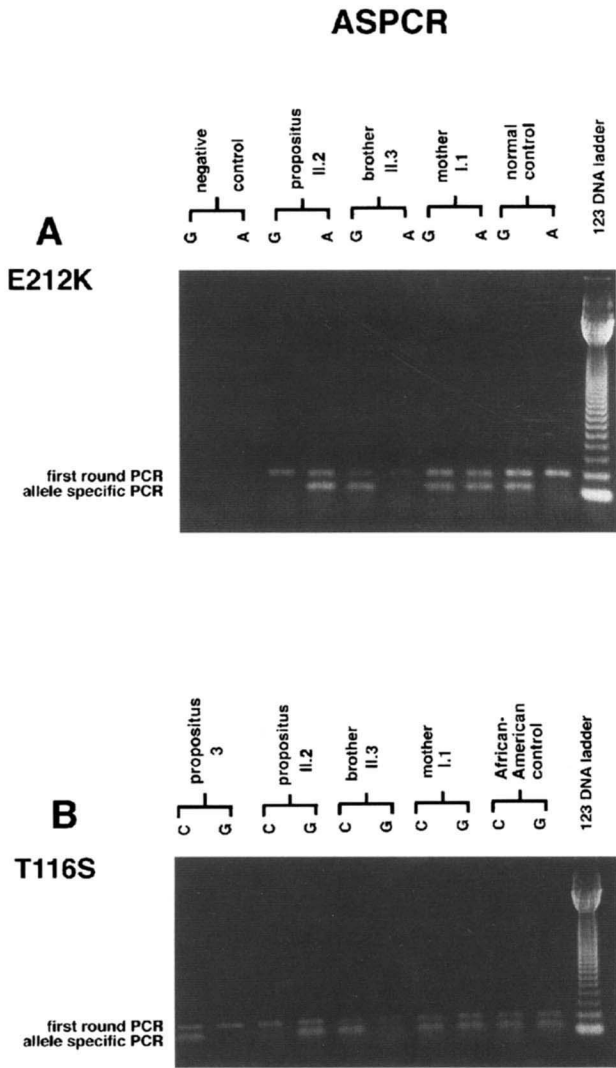
Analysis by Northern blot hybridization showed that the propositus (II.2), her mother (I.1), and brother (II.3) had the same size RNA transcript as an unrelated normal control using the EcoRI insert of pCb5R141 as the probe (Fig 2A). The hybridizing signal revealed a b5R transcript size of about 2 kb, similar to that reported by Tomatsu et al.[5] Because of a highly variable amount of RNA applied to the membrane as detected by both the b5R probe and the ubiquitously expressed β-actin probe (Fig 2B), minor quantitative differences of the b5R mRNAs could not be detected.

cDNA fragments created using 6 overlapping sets of gene specific primers (Fig 3 and Table 1) from the propositus (II.2) and an unrelated normal control were amplified and sequenced directly from PCR products. The propositus (II.2) was found to be homozygous for a G to A transition in exon 8 at codon 212 (Fig 4A). This nucleotide substitution causes

an amino acid change from a glutamate to a lysine (E212K). An additional C to G transversion was found at codon 116 in exon 5 (Fig 4B). Both of these nucleotide changes were confirmed by repeat analysis of newly prepared RT-PCR products.

Using ASPCR, we restudied cDNA from the propositus (II.2), her mother (I.1), and brother (II.3), 2 other unrelated methemoglobinemic patients and their 6 family members, as well as cDNA from unselected African-American individuals (56 chromosomes) and unselected white individuals (54 chromosomes). Only the propositus (II.2) and her mother (I.1) were found to have the G to A transition of E212K. The propositus (II.2) had only the mutant allele (A). Her mother (I.1), whose decreased enzyme activity was within the range expected for heterozygous individuals, had 1 copy each of both the mutant (A) and the normal (G) alleles, indicating that she is heterozygous for both alleles (Fig 5A and Table 2).

In contrast, analysis of the C to G transversion (T116S) showed that the propositus (II.2) had only the mutant allele

ASPCR

A

E212K

first round PCR
allele specific PCR

B

T116S

first round PCR
allele specific PCR

Fig 5. Allele-specific PCR analysis. cDNA from unselected populations of different ethnic background was used in this PCR-based rapid analysis that uses allele-specific primers (see Table 2). (A) Using primers specific for the E212K substitution (G → A, exon 8), the propositus (II.2) was confirmed to be homozygous for the A allele, her enzyme nondeficient brother (II.3) homozygous for the G allele, her mother who was heterozygous for enzyme deficiency (I.1) heterozygous for both alleles, and a normal control homozygous for the G allele. (B) Using primers specific for the T116S substitution (C → G, exon 5), the propositus with b5R-Shreveport (II.2) was determined to be homozygous for the G allele, her brother (II.3) homozygous for the C allele, her mother (I.1) heterozygous for both alleles. An unrelated white methemoglobinemic individual with type II b5R deficiency (propositus 3) was homozygous for the C allele, and an unrelated African-American control with normal b5R enzyme activity was heterozygous for both alleles.

(G), her mother (I.1) had 1 copy each of both the mutant (G) and the normal (C) alleles, other unrelated African-American methemoglobinemic patients, and African-American healthy controls who had normal enzyme activities were either heterozygous or homozygous for this substitution, as shown in Fig 5B. In preliminary studies, no white, Indo-Aryan, Arabic, or Asian individuals exam-

ined, to date, had this polymorphic transversion, but all had only the normal (C) allele.

DISCUSSION

The African-American family with b5R-Shreveport was studied previously in this laboratory, and because of the abnormal properties of the mutant enzyme, we concentrated our study on the transcript of the gene to find the molecular defect responsible for the disease phenotype. We found no gross alterations in the transcript by Northern blot analysis using lymphoblastoid-derived RNA. A guanine to adenine transition was found in both alleles of the propositus' (II.2) b5R transcript causing a change from glutamate to lysine at codon 212 in exon 8 (E212K). In addition, a cytosine to guanine transversion was found in both alleles of the propositus' (II.2) b5R transcript causing a change from threonine to serine at codon 116 in exon 5 (T116S). Neither of these changes has ever been reported in any methemoglobinemic subjects, in the African-American population, or in any other population.

No commercially available restriction endonucleases had cleavage sites at either of these positions. To determine if E212K and T116S were disease-causing mutations or polymorphisms, and to confirm these nucleotide changes, we used allele-specific PCR. We have screened all family members, including the propositus (II.2), her mother (I.1), and her brother (II.3), other unrelated methemoglobinemic patients and their families, and unselected nonmethemoglobinemic African-American and white individuals for these nucleotide substitutions.

This G to A transition at nucleotide 25126 in b5R genomic DNA (G/A 25126 leads to an amino acid substitution of E212K) was found in both alleles of the propositus (II.2) and in only one allele of her mother (I.1) in the b5R-Shreveport family, but it was not found in her nonaffected brother (II.3), or in other methemoglobinemic patients, their families, or normal controls, suggesting that this is indeed a disease-causing mutation. This 3′ domain mutation has never been reported and is unique for this family. Furthermore, no previously reported b5R mutations have been found in exon 8, but were found in exon 3 (R57Q),[29] exon 5(L148P),[29,30] and exon 4 (V105M).[31,32] To our knowledge, it is not clear that studies were performed on these Japanese and Italian mutations to distinguish them from polymorphisms and it is possible that healthy, nonmethemoglobinemic individuals carry these nucleotide substitutions. Our finding of a type I

Table 2. Primers for ASPCR Amplification of b5R cDNA

Nucleotide Substitution	Fragment Size (bp)	Primer	Sequence (5′ → 3′)
GAG → AAG E212K	145	212N	TGCTCTTTGCCAACCAGACCG
		R9	TTCACGAAGCCCTGGCCGTAGT
	147	212M	ACTGCTCTTTGCCAACCAGACCA
		R9	TTCACGAAGCCCTGGCCGTAGT
ACC → AGC T116S	131	116N	AATCAAGGTTTACTTCAAGGACAC
		R5	CCTGGTAGACCAGCAGCCCACT
	133	116M	AAAATCAAGGTTTACTTCAAGGACAG
		R5	CCTGGTAGACCAGCAGCCCACT

Abbreviations: N, normal allele; M, mutant allele; R, reverse.

mutation in the 3' end of the b5R gene (exon 8) contradicts the prediction that all type I mutations are found in the 5' end of the gene whereas all type II mutations are found in the 3' end of the gene.[33]

In this novel congenital methemoglobinemia type I mutation (E212K), glutamate, an acidic amino acid, is replaced with lysine, a larger basic amino acid. The glutamate at codon 212 is conserved in bovine, rat, and human b5R,[34] suggesting that it plays a significant role in the structure or function of this gene. Using cDNA-derived b5R peptide sequence, the secondary structure of this enzyme has been analyzed by the Chou and Fasman method[35] and codon 212 is predicted to form an α-helical structure. Replacement of glutamate with lysine, which has a side chain that readily forms cross-linking structures, likely disrupts the α-helical secondary structure of b5R. This disruption may play a causative role in the instability of the enzyme and in the resulting selective erythrocytic enzyme deficiency.

In contrast, the T116S substitution was found to occur with a high frequency (in excess of 20%) in normal as well as methemoglobinemic individuals in the African-American population, indicating that this is a frequent polymorphism that appears to be unique to individuals of African ancestry (Jenkins and Prchal, submitted). The combination of a polymorphism and a single pathogenic mutation resulting in disease phenotype has been reported in the prion protein. The genotypic basis for the difference between fatal familial insomnia (FFI) and a subtype of familial Creutzfeld-Jacob disease (178Asn CJD) lies in a polymorphism of the mutant prion protein gene: the mutation with a methionine at the polymorphic site results in FFI, the same mutation with a valine at the polymorphic site results in CJD.[36] In primary hyperoxaluria type 1 (PH1), a single mutation, in combination with a normally occurring polymorphism, appears to be responsible for the unprecedented peroxisome-to-mitochondrion mistargeting phenotype of the normally liver-specific peroxisomal enzyme alanine:glyoxylate aminotransferase.[37] Thus, it is possible but not certain that the E212K mutation alone is causative of the observed disease phenotype in this African-American family with b5R-Shreveport. However, although the frequent T116S African polymorphism appears to be innocuous on its own, it may interact with the E212K mutation, and this combination may cause or contribute to the disease phenotype.

REFERENCES

1. McKusick VA: Mendelian Inheritance in Man: Catalogs of Autosomal Dominant, Autosomal Recessive, and X-Linked Phenotypes (ed 12). Baltimore, MD, The Johns Hopkins Press, 1994

2. Karplus PA, Daniels MJ, Herriott JR: Atomic structure of ferrodoxin-NADP+ reductase: Prototype for a structurally novel flavoenzyme family. Science 251:60, 1991

3. Strittmatter P: The reaction sequence in electron transfer in the reduced nicotinamide adenine dinucleotide-cytochrome b5 reductase system. J Biol Chem 240:4481, 1965

4. Iyanagi T, Watanabe S, Anan KF: One-electron oxidation-reduction properties of hepatic NADH-cytochrome b5 reductase. Biochemistry 23:1418, 1984

5. Tomatsu S, Kobayashi Y, Fukumaki Y, Yubisui T, Orii T, Sakaki Y: The organization and the complete nucleotide sequence of the human NADH-cytochrome b5 reductase gene. Gene 80:353, 1989

6. Bull P, Shephard E, Povey S, Santisteban I, Phillips I: Cloning and chromosomal mapping of human cytochrome b5 reductase (DIA1). Ann Hum Genet 52:263, 1988

7. Scott EM, Griffith IV: The enzyme defect of hereditary methemoglobinemia: Diaphorase. Biochim Biophys Acta 34:584, 1959

8. Yubisui T, Miyata T, Iwanaga S, Tamura M, Takeshita M: Complete amino acid sequence of NADH-cytochrome b5 reductase purified from human erythrocytes. J Biochem (Tokyo) 99:407, 1986

9. Passon PG, Hultquist DE: Soluble cytochrome b5 reductase from human erythrocytes. Biochim Biophys Acta 275:62, 1972

10. Hultquist DE, Slaughter SR, Douglas RH, Sannes LJ, Sahagian GG: Erythrocyte cytochrome b5: Structure, role in methemoglobin reduction, and solubilization from endoplasmic reticulum. Prog Clin Biol Res 21:199, 1978

11. Hultquist DE, Passon PG: Catalysis of methaemoglobin reduction by erythrocyte cytochrome b5 and cytochrome b5 reductase. Nature 229:252, 1971

12. Leroux A, Junien C, Kaplan J-C, Bamberger J: Generalised deficiency of cytochrome b5 reductase in congenital methaemoglobinaemia with mental retardation. Nature 258:619, 1975

13. Borgese N, Pietrini G: Distribution of the integral membrane protein NADH-cytochrome b5 reductase in rat liver cells, studied with a quantitative radioimmunoblotting assay. Biochem J 239:393, 1986

14. Tamura M, Yubisui T, Takeshita M, Kawabata S, Miyata T, Iwanaga S: Structural comparison of bovine erythrocyte, brain and liver NADH-cytochrome b5 reductase by HPLC mapping. J Biochem 101:1147, 1987

15. Ozols J, Korza G, Heinemann FS, Hediger MA, Strittmatter P: Complete amino acid sequence of steer liver microsomal NADH-cytochrome b5 reductase. J Biol Chem 20:11953, 1985

16. Ozols J, Carr SA, Strittmatter P: Identification of the NH2-terminal blocking group of NADH-cytochrome b5 reductase as myristic acid and the complete amino acid sequence of the membrane-binding domain. J Biol Chem 259:13349, 1984

17. Schwartz JM, Reiss Al, Jaffe ER: Hereditary Methemoglobinemia with deficiency of NADH-cytochrome b5 reductase, in Stanburg JB, Wyngaarden JB, Fredrickson DS, Goldstein JL, Brown MS (eds): The Metabolic Basis of Inherited Disease (ed 5). New York, NY, McGraw-Hill, 1983, p 1654

18. Prchal JT, Borgese N, Moore MR, Moreno H, Hegesh E, Hall MK: Congenital methemoglobinemia due to methemoglobin reductase deficiency in two unrelated American black families. Am J Med 89:516, 1990

19. Evelyn KA, Malloy HT: Microdetermination of oxyhemoglobin, methemoglobin, and sulfhemoglobin in a single sample of blood. J Biol Chem 126:655, 1938

20. Board PG: NADH-ferricyanide reductase, a convenient approach to the evaluation of NADH-methemoglobin reductase in human erythrocytes. Clin Chim Acta 109:233, 1981

21. Beutler E: Red Cell Metabolism: A Manual of Biochemical Methods (ed 2). New York, NY, Grune & Stratton, 1975

22. Chomczynski P, Sacchi N: Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem 162:156, 1987

23. Sambrook J, Fritsch EF, Maniatis T: Molecular Cloning, A Laboratory Manual (ed 2). Cold Spring Harbor, NY, Cold Spring Harbor Laboratory, 1989, p 7.43

24. Yubisui T, Naitoh Y, Zenno S, Tamura M, Takeshita M, Sakaki Y: Molecular cloning of cDNAs of human liver and placenta NADH-cytochrome b5 reductase. Proc Natl Acad Sci USA 84:3609, 1987

25. Gunning P, Ponte P, Okayama H, Engel J, Blau H, Kedes L:

Isolation and characterization of full-length cDNA clones for human alpha-, beta-, and gamma-actin mRNAs: Skeletal but not cytoplasmic actins have an amino-terminal cysteine that is subsequently removed. Mol Cell Biol 3:787, 1983

26. Rigby PWJ, Dieckmann M, Rhodes C, Berg P: Labeling deoxyribonucleic acid to high specific activity in vitro by nick translation with DNA polymerase I. J Mol Biol 113:237, 1977

27. Wu DY, Ugozzoli L, Pal BK, Wallace RB: Allele-specific enzymatic amplification of beta-globin genomic DNA for diagnosis of sickle cell anemia. Proc Natl Acad Sci USA 86:2757, 1989

28. Liu Y, Luhovy M, Prchal JT: A simple genotype screening assay for X chromosome transcriptional polymorphisms: allele specific PCR (ASPCR). Blood 84:38a, 1994 (abstr, suppl 1)

29. Katsube T, Sakamoto N, Kobayashi Y, Seki R, Hirano M, Tanishima K, Tomoda A, Takazakura E, Yubisui T, Takeshita M, Sakaki Y, Fukumaki Y: Exonic point mutations in NADH-cytochrome b₅ reductase genes of homozygotes for hereditary methemoglobinemia, types I and III: Putative mechanisms of tissue-dependent enzyme deficiency. Am J Hum Genet 48:799, 1991

30. Nagai T, Shirabe K, Yubisui T, Takeshita M: Analysis of mutant NADH-cytochrome b₅ reductase: Apparent "type III" methemoglobinemia can be explained as type I with an unstable reductase. Blood 81:808, 1993

31. Borgese N, Pietrini G, Gaetani S: Concentration of NADH-cytochrome b₅ reductase in erythrocytes of normal and methemoglobinemic individuals measured with a quantitative radioimmunoblotting assay. J Clin Invest 80:1296, 1987

32. Shirabe K, Yubisui T, Borgese N, Tang C-Y, Hultquist DE, Takeshita M: Enzymatic instability of NADH-cytochrome b₅ reductase as a cause of hereditary methemoglobinemia type I (red cell type). J Biol Chem 267:20416, 1992

33. Mota Vieira L, Kaplan J-C, Kahn A, Leroux A: Four new mutations in the NADH-cytochrome b₅ reductase gene from patients with recessive congenital methemoglobinemia type II. Blood 85:2254, 1995

34. Pietrini G, Carrera P, Borgese N: Two transcripts encode rat cytochrome b5 reductase. Proc Natl Acad Sci USA 85:7246, 1988

35. Chou PY, Fasman GD: Empirical predictions of protein conformation. Annu Rev Biochem 47:251, 1978

36. Petersen RB, Goldfarb LG, Tabaton M, Brown P, Monari L, Cortelli P, Montagna P, Autilio-Gambetti L, Gajdusek DC, Lugaresi E, Gambetti P: A novel mechanism of phenotypic heterogeneity demonstrated by the effect of a polymorphism on a pathogenic mutation in the PRNP (prion protein gene). Mol Neurobiol 8:99, 1994

37. Danpure CJ, Jennings PR, Fryer P, Purdue PE, Allsop J: Primary hyperoxaluria type 1: Genotypic and phenotypic heterogeneity. J Inherit Metab Dis 17:487, 1994